ABSTRACT

SPER, RENAN BUENO. Towards the Development of an Immunodeficient Pig Model for Regenerative Medicine. (Under the direction of Dr. Jorge A. Piedrahita).

Severe combined immunodeficient mice models have been extensively used for the past decade not only to understand fundamental biological processes in the field of immunology but also to better understand the aspects of immune dynamics upon allogeneic and xenogeneic hematopoietic stem cell transplantation. A severe combined immunodeficient pig model is an attractive concept, due to its similarities to humans at multiple levels (genetic, anatomy, physiology). Here I describe the generation and characterization of two inter-related transgenic pig models. One model was developed for cell fate lineage and chromosome dynamics studies. This was done via fusion of the porcine Histone 2 B (pH2B) nucleosomal protein to eGFP and placing it under the transcriptional control of the \( \beta\text{-ACTIN} \) promoter. Transgenic pigs expressed pH2B-eGFP ubiquitously (somatic cells, stem cells, embryos) allowing chromosome dynamic visualization and the ability to track stem cell. The second model was generated to develop a several combined immunodeficient pig using the double knockout approach (DKO) previously used in mice. The \( RAG\text{-}2 \) and \( IL2RG \) genes were disrupted by homing endonucleases. \( RAG\text{-}\text{2}^{-/-} IL2RG^{-/-} \) pigs lacked peripheral blood mature B and T and NK lymphocytes and IgH and TCR-\( \beta \) gene rearrangement, while exhibiting a thymic and splenic CD3 + CD4 + CD8 - (CD4 single positive) population and splenic Nkp46 + cells.

To investigate the ability of \( RAG\text{-}\text{2}^{-/-} IL2RG^{-/-} \) pigs to sustain pH2B-eGFP allogeneic and human CD34 + xenogeneic engraftment, either allogeneic pH2B-eGFP labeled or human...
hematopoietic stem cells were transplanted into RAG-2\(^{-}\)/IL2RG\(^{-}\)/y pigs via in Utero Stem Cell Transplantation (IUHCT). IUHCT with allogeneic hematopoietic stem cells revealed T, B and NK cell lymphoid lineage reconstitution of thymus and spleen in neonatal RAG-2\(^{-}\)/IL2RG\(^{-}\)/y pigs. Flow cytometry analysis of allografted lymphoid tissue revealed a population of CD4 single positive (SP) cells co-expressing Nkp46.

Xenogeneic transplantation via IUHCT with human CD34 + cells into the RAG-2\(^{-}\)/IL2RG\(^{-}\)/y background resulted in robust human T cell engraftment, with thymus reaching up to 80% human chimerism. Significant human cells clearance from peripheral blood, spleen and bone marrow was observed between birth and day 7 of life. The thymus was the only lymphoid tissue capable of maintained human engraftment. Immunofluorescence analysis of allogeneic and xenogeneic lymphoid organs support up regulation of components of the humoral and innate system in xenografted tissues but not allografted tissues.

This thesis describes not only the development and characterization of two novel large animal models for regenerative medicine applications (cell tracking/severe combined immunodeficient), but also how they can be used in combination to address fundamental questions of transplantation immunology and identification of xenogeneic specific immune barriers.
Towards the Development of an Immunodeficient Pig Model for Regenerative Medicine.

by
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DEDICATION

“This dissertation is dedicated to my family, in special to my Grand Mother Ruth de Abreu, who passed away last year in Brazil while I was in the USA. Because of her financial support, I could achieve the education that I always dreamed off. “Thank you, grand mom.”

“I would like to also dedicate this work to my Mother Sandera Maria Bueno Sper and my Father Clinton de Abreu Sper, thank you so much for always believing in me, I would not have accomplished this without your guidance and parenting. From you I learned how to respect others and have empathy. I would also like to dedicate this Thesis to my sister Erica Wilcox, my brother-in-law Jonathan Wilcox, and my 3 adorable nieces, Bianca, Valentina and Antonella. Thank you for everything you have done for me. I love you all”
BIOGRAPHY

Renan Bueno Sper was born on February 16, 1984, in the city of Sao Sebastiao, Sao Paulo, Brazil. He grew up with his four family members, his father, Clinton de Abreu, his mother, Sandera Maria Bueno Sper, his sister, Erica Wilcox and Grand Mother, Ruth de Abreu. The early exposure to domestic animals lead him to pursue a Doctor in Veterinary Medicine degree from the Federal University of Uberlandia. During veterinary school, he found great satisfaction teaching and performing scientific research. Shortly after graduation, he worked as a research assistant for 1 year at Cornell University, leading him to attend Theriogenology internship and residency clinical program at NCSU. His endless curiosity and need for scientific discovery led him to pursue a Ph.D. program in Comparative Biomedical Science under the supervision of Dr. Jorge A. Piedrahita, allowing him to see nature and its form in a much smaller scale. Despite his clinical training, the Ph.D. program reinforced his long-desired dream of pursuing a carrier as a fulltime scientist. Thank you Dr. Jorge A. Piedrahita.
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CHAPTER 1: GENERATION OF A STABLE TRANSGENIC SWINE MODEL EXPRESSING A PORCINE HISTONE 2B-eGFP FUSION PROTEIN FOR CELL TRACKING AND CHROMOSOME DYNAMICS.


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Generation of a Stable Transgenic Swine Model Expressing a Porcine Histone 2B-eGFP Fusion Protein for Cell Tracking and Chromosome Dynamics Studies

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Abstract

Transgenic pigs have become an attractive research model in the field of translational research, regenerative medicine, and stem cell therapy due to their anatomic, genetic and physiological similarities with humans. The development of fluorescent proteins as molecular tags has allowed investigators to track cell migration and engraftment levels after transplantation. Here we describe the development of two transgenic pig models via SCNT expressing a fusion protein composed of eGFP and porcine Histone 2B (pH2B). This fusion protein is targeted to the nucleosomes resulting a nuclear/chromatin eGFP signal. The first model (I) was generated via random insertion of pH2B-eGFP driven by the CAG promoter (chicken beta actin promoter and rabbit Globin poly A; pCAG-pH2B-eGFP) and protected by human interferon-β matrix attachment regions (MARs). Despite the consistent, high, and ubiquitous expression of the fusion protein pH2B-eGFP in all tissues analyzed, two independently generated Model I transgenic lines developed neurodegenerative symptoms including Wallerian degeneration between 3–5 months of age, requiring euthanasia. A second transgenic model (II) was developed via CRISPR-Cas9 mediated homology-directed repair (HDR) of IRES-pH2B-eGFP into the endogenous β-actin (ACTB) locus. Model II transgenic animals showed ubiquitous expression of pH2B-eGFP on all tissues analyzed. Unlike the pCAG-pH2B-eGFP/MAR line, all Model II animals were healthy and multiple pregnancies have been established with progeny showing the expected Mendelian ratio for the transmission of the pH2B-eGFP. Expression of pH2B-eGFP was used to examine the timing of the maternal to zygotic transition after IVF, and to examine chromosome segregation of SCNT embryos. To our knowledge this is the first viable transgenic pig model with chromatin-associated
eGFP allowing both cell tracking and the study of chromatin dynamics in a large animal model.

Introduction

Pigs are an attractive research model in the field of surgical and procedure training, disease progression and pathology, translational research, and regenerative medicine/stem cell therapy, due to their anatomic, genetic, and physiological similarities with humans. In addition, the availability of the pig genome sequence, the development of somatic cells nuclear transfer (SCNT) and high efficiency genome editing tools such as Transcription activator-like effector nucleases (TALENs) and Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9), have allowed precise and efficient genetic engineering in the pig [1-5].

With available transgenic pig technology and the generation of more suitable biomedical research models for translational research, the need for more effective methods for in vivo and ex vivo cell tracking have increased. This is particularly true in the area of regenerative medicine. While cells can be loaded ex vivo with a range of dyes, these dyes disappear over time limiting their usefulness for long-term studies [6-8]. Therefore, the development of fluorescence proteins as molecular tags has allowed complex biochemical processes to be correlated with protein functionality in living cells [6]. In addition, genetic engineering of encoded biological fluorescent proteins have marked an evolution in the field of stem cell biology, allowing the development of cell-traceable systems and the ability to track the fate of adult stem cells for therapeutic purpose in biomedical models [9, 10]. Among these molecular tags, the most widely used one is the green fluorescent protein (GFP) from the jellyfish Aequorea victoria [6, 11]. The mutant variant, eGFP (enhanced), has been shown to be more stable and to fold correctly at 37°C, and can be fused with virtually any protein of interest allowing co-localization of eGFP and the protein of interest [6].

Based on this concept, transgenic mice, rats, rabbits and pigs expressing eGFP under a variety of conditions have demonstrated their usefulness in basic and translational research [2, 12, 13]. Furthermore, nuclear fluorescence tagging, by adding nuclear localization signals that translocate eGFP into the nucleus [14], or by fusing eGFP to proteins that bind chromatin, has allowed the tagging of the nucleus versus the cytoplasm and a better understanding of the complexity of cell cycle/division, chromosome abnormalities associated with cancer biology, and real-time chromosomal dynamics [15]. One of the approaches used to accomplish this is via fusion of eGFP to the histone 2B protein (H2B) [16, 17]. Histone H2B is a one of the main core proteins of the nucleosome and plays a key role in chromatin assembly [18]. By fusing eGFP to H2B it is possible to co-localize the eGFP to the chromosomes and observe their behavior in real time. While transgenic mice models expressing the fusion protein H2B-eGFP under different conditions have been described [19], to our knowledge the development of a stable transgenic pig expressing such fusion protein is currently lacking. Such model can be used as a tool in multiple fields of biomedical research including cell tracking after transplantation, cell fusion analysis, cell division kinetic analysis in vivo and in vitro [20], and chromatin remodeling in early embryos [21].

Here we describe two transgenic pig models expressing pH2B-eGFP. One developed using random insertion and a well-characterized, strong, ubiquitous promoter (pCAG) protected by Scaffold/Matrix attachment regions (MARs). MARs are DNA elements that are part of the nuclear matrix, and are known to reduce what is a known as “position effect variegation”
(PEV). PEV is characterized by heterogeneous expression (and in some cases silencing) of randomly integrated transgenes [22–24]. The use of MARs has been described in multiple species including swine [25, 26]. The second model generated via CRISPR-Cas9-mediated HDR insertion of pH2B-eGFP transgene in the ACTB locus, allowing its expression to be regulated by the ACTB promoter.

Material and Methods

Animal Welfare

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animals used in this study were obtained from a university-owned herd, and all animal procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University (Raleigh, NC). Animals were sacrificed by one of two methods, intravenous injection of sodium pentobarbital, or penetrating captive bolt euthanasia followed by jugular exsanguination. Both methods meet the recommended guidelines of the American Veterinary Medical Association for euthanasia in pigs. All surgeries were performed under isoflurane anesthesia, and a post-surgical regimen of bupivacaine, Banamine-S was administered to minimize pain.

Model Development

Model 1 (pCAG-pH2B-eGFP/MAR); non-targeted integration. Model 1 line was generated by random integration in the genome and used a strong, well-characterized promoter and MARs to control PEV of the pH2B-eGFP. To generate Model 1, three constructs were used (Fig. 1A and 1C), PGK-puro, human interferon-β MARs and pCAG-pH2B-eGFP-rabbit Globin poly A. The porcine H2B coding region (single exon) was amplified from the pig genome via PCR, sequenced, fused to eGFP and cloned into a pCAG expression vector (Clontech, catalog #60851). The 812 bp human interferon-β MAR was amplified and sequenced from pCpGfree-vitroNLacZ (InvivoGen, Catalog# pcppgn-lz) plasmid using the forward and reverse primers, 5′-CTGCCAGGAAACACTGCAATAGCTC-3′ and 5′-GGCAACGATGTATTACCTAGGC TTT-3′. Finally, PGK-puro was used for selection.

Two independent porcine fetal fibroblast (pFFs) lines isolated from two different fetuses were used for generation of Model 1. All pFFs lines were obtained by breeding Landrace X Yorkshire mix boars and sows. Male wild type pFFs (400,000 cells) were mixed with 2 μg linearized pCAG-pH2B-eGFP plasmid, 2 μg hIFNβ/MAR and 500 ng PGK-Puro plasmid, and electroporated at 490 V, 3 pulses of 1 ms duration using the BTX Electro-cell manipulator ECM2001. Electroporated cells were plated in alpha MEM supplemented with 15% FBS and penicillin/streptomycin. Twenty-four hours later, cells were placed on selection media containing 0.75 mg/ml puromycin. Following selection for 10 days, surviving colonies were expanded and briefly analyzed by epi-fluorescence microscopy to phenotypically evaluate colonies based on nuclear pH2B-eGFP expression. Colonies showing heterogeneous pH2B-eGFP expression were discarded. Colonies that showed homogeneous and ubiquitous nuclear pH2B-eGFP expression were further expanded and tested to confirm the presence of MARs before using for SCNT. Two independent Model 1 lines where established and studied separately. The presence of MARs adjacent the pH2B-eGFP was examined by PCR as shown in Fig 1A. Primers used were MAR forward 5′-ATG AGA TCT GGG GAA AAG ACT CA-3′ and MAR reverse 5′-CTTACTCAGGGCGTTGCCAATATTTTCGTC-3′, reverse pCAG 5′-ACTAGGGAACA TAGGCTAATATTGAC-3′ and forward rGBP(A) 5′-TGA CGCATCTGACTCTGGC-3′. All PCR reaction were performed under the following conditions 98°C for 1 min, 35 X (98°C for 10 s, 62°C for 10 s, 72°C for 60 s), 72°C for 1 min, hold at 4°C.
In order to identify the transgene integration site the Universal GenomeWalker 2.0 kit was used (Clontech, cat#636-405). Adapter-ligated libraries were generated with genomic DNA digested with four different blunt-end restriction endonucleases (Dral, EcoRV are showed) and CAG-pH2B-eGFP specific primers were designed to work in conjunction with adaptor primers (provided with the kit). A specific positive band from line 1 EcoRV digested/adaptor ligated library (1.4Kb) was obtained by PCR using a custom designed CAG reverse and adaptor primers. The band was gel extracted and submitted for DNA sequencing revealing 900 bp sequence with 100% homology to the pig genomic region identified that allowed mapping and confirmation by PCR of the insertion site (Fig. 2).

The insertion site for the additional Model I line was identified by whole genome sequencing by using a commercial service (Rapid Genomics, Gainesville). For library preparation the DNA sample was quantified using Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad) and normalized to 15 ng/ul. A total of 500 ng of DNA was physically fragmented to an average fragment size of 400 bp. An Illumina-compatible DNA library was constructed by end-repairing the sheared DNA, A-tailing and adapter ligation. The ligated libraries were PCR-amplified for 9 cycles. The product was sequenced on one lane of Illumina HiSeq X-Ten (Illumina, San Diego) with paired end reads of 150 bp each. Paired end raw reads were cleaned and trimmed with trim galore/0.4.1 (Babraham Bioinformatics—Trim Galore! Babraham Bioinformatics—Trim Galore! [http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/]. Reads where one pair mapped to the transgene and its mate pair mapped to the pig
genome were used to identify possible insertion sites. To identify these pairs, bowtie2 [27] was used to map the cleaned reads to the transgene sequences. The bowtie mapping results were filtered with bamsamba [28] for reads where only one mate mapped to the transgene. Blast [29] was used to map the mate of the read that hit the transgene to the pig genome (Genome assembly: Sus scrofa 10.2, http://useast.ensembl.org/Sus_scrofa/Info/Index).

Model II (ACTB-ires-pH2B-eGFP): homology directed repair (HDR). Model II was developed using CRISPR-Cas9 and HDR to drive integration of the pH2B-eGFP into one allele of the ACTB locus resulting in ubiquitous expression of the pH2B-eGFP. The CRISPR-Cas9 system consisted of the bicistronic expression vector px330 expressing Cas9 and sgRNA under U6 promoter (addgene, catalog #42230). An sgRNA target sequence to the 3' UTR of the ACTB locus was identified using ZIFit Targeter Version 4.2 (http://zifit.partners.org/ZIFIT/ChoiceMenu.aspx). This sequence was added to the plasmid using complimentary oligos, 5' - CACCGACGCGTGAAGCTGACAGCATTG - 3' and 5' - AAGACCTGGTGGTTGCTACGCCTCC - 3'.

For the generation of the IRES-pH2B-eGFP targeting construct, the internal ribosomal entry site (IRES) sequence from Encephalomyocarditis virus was amplified from pCAGIG plasmid (addgene, cat# 11159), and fused to pH2B-eGFP from the pCAG-pH2B-eGFP plasmid. Porcine ACTB homology arms of 1Kb were amplified from pig genomic DNA and cloned into the targeting vector flanking IRES-pH2B-eGFP (Fig 1B). The targeting vector did not contain the β-actin CRISPR-Cas9 target sequence. The strategy consisted of generating a chimeric mRNA of ACTB and IRES-pH2B-eGFP by inserting the transgene in the 3' untranslated region (UTR). The stop codon and the endogenous ACTB Poly(A) signal was kept intact.

For electrochemical transfection, 5x10⁵ wild type male pFFs were transfected using the Amaxa Nucleofector kit (U-23 settings) (Lonza,Verviers, Belgium). Transfection groups included pMAXGFP (500ng) as positive control, non-transfected pFFs as negative control,
and ACTB CRISPR-Cas9 (500ng) plus circular targeting vector (500ng) as the HDR group. Ninety-six hours post transfection cells were trypsinized, resuspended in fetal fibroblast culture media (alpha MEM supplemented with 15% FBS and penicillin/streptomycin) and Fluorescence Activated Cell Sorted (FACS). Wild type pFIs served as negative control and pFIs transfected with pMAXGFP plasmid as a positive control. Cells were briefly trypsinized, washed twice in PBS and sorted for GFP on a Beckman Coulter MoFlo Legacy system (sort pressure 25PSI using a 100 micron nozzle) using a 488nm laser for side-scattered light (SSC) and forward-scattered light (FSC), and a 530/40 filter to capture the eGFP emission. Positive cells from HDR group were harvested and plated at low density (100 cells/100mm petri dish) for single cell colony expansion. Five to seven days later, single cell colonies were picked and transferred to a 24-well plate.

To confirm the targeted insertion of IRES-pH2B-eGFP into the ACTB locus, primers were designed to amplify a 1.9kb 5’ boundary fragment and a 1.7kb 3’ boundary fragment. As shown in **Fig 1B**, primers were designed to specifically amplify IRES-pH2B-eGFP flanked by the correct location of the ACTB 3’UTR region. For 5’ boundary, primers were 5’ CCGAGGTCT CCTTTGGAACCTCTGCCG 3’ and 5’ CTGGTGAATACGCTGGAGGAGC 3’. PCR conditions were 98°C for 1 min, 35 X (98°C for 10 s, 66°C for 10 s, 72°C for 30 s), 72°C for 1 min, hold at 4°C. For the 3’ boundary primers were 5’ GTACAATCAACACGCGCCCGACTG 3’ and 5’ CTGACGTTGTTTCTGCAGCTGGCC 3’ under following condition 98°C for 1 min, 35 X (98°C for 10 s, 68°C for 10 s, 72°C for 30 s), 72°C for 1 min, hold at 4°C. For determining homozygous vs. heterozygous HDR, primers were designed to bind outside the areas of homology arms of ACTB, resulting in amplification of a 2.9kb amplicon for unmodified wild type allele. Primers were 5’ GTGAGGCTCCTTTGGAACCTCTGCCG 3’ and 5’ CTGACGTTGTTTCTGCAGCTGGCC 3’ under condition of 98°C for 1 min, 35 X (98°C for 10 s, 68°C for 10 s, 72°C for 45 s), 72°C for 1 min, hold at 4°C.

Somatic Cell Nuclear Transfer
Both models (I and II) were generated using the same SCNT protocol [30]. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. Oocytes from mixed commercial breed sows were collected from local slaughterhouses. Cumulus cells were removed from the oocyte by vortex for 5 min in 0.1% bovine testicular hyaluronidase. Oocytes were incubated in manipulation media (Ca-free NCSU-23 with 5% FBS) containing 5 μg/ml bisbenzimide and 7.5 μg/mL cytochalasin B for 5 min. Following the incubation period, oocytes were enucleated by removing the first polar body and metaphase II plate. Single cells expressing pH2B-eGFP were injected and fused to each enucleated oocyte. Fusion/Activation was induced by two DC pulses of 140V for 40 μsec in 280 mM mannitol, 0.001 mM CaCl₂, and 0.05 mM MgCl₂. After fusion/activation, oocytes were placed back in NCSU-23 medium with 0.4% BSA and cultured at 38.5°C, 5% CO₂ in a humidified atmosphere for less than an hour, before being surgically transferred into a synchronized recipient.

Histology and Immunohistochemistry (IHC)
To determine the usefulness of the pH2B-eGFP marker, frozen and paraffin embedded samples were examined for pH2B-eGFP expression after sectioning without use of anti-GFP antibody (direct GFP detection) as well as after using anti-GFP antibody at 1/400 dilution (Alexa Fluor 555 conjugated, rabbit polyclonal IgG (indirect detection, cat # A-31851, ThermoFisher). Five to 10 μm sections were obtained from both paraffin-embedded and OCT-embedded tissues. For paraffin embedded blocks samples were fixed in 10% neutral buffered formalin overnight, followed by fixation with 70% ethanol and paraffin embedding. For OCT-embedded
tissue blocks samples were fixed with 4% paraformaldehyde overnight followed by 30% sucrose solution (also overnight). Once fixed, samples were embedded in OCT and frozen in liquid nitrogen. The cryosectioned tissues slides were immunostained as described below. Slides were permeabilized with 0.25% Triton X-100 in PBST (PBS with 0.1% Tween 20) for 10 min. Slides were incubated for one hour at room temperature in 1% BSA in PBST, then with Alexa555 conjugated Anti-GFP (Invitrogen) overnight at 40°C. Next day, slides were washed three times with PBST and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) and visualized with TE2000 fluorescence microscope (Nikon, Melville, NY, USA). The same slides used for immunostaining and/or other slides from serial sections were subsequently used for hematoxylin and eosin (H&E) staining as previously described [31].

For natural eGFP fluorescence analysis, tissues from both Model I and Model II were fixed with 4% (wt/vol) paraformaldehyde (PFA) in PBS overnight, followed by 30% sucrose solution and OCT embedding. Cryosection from multiple organs were counterstained with 1 mg/mL of DAPI in Vectorshield mounting medium. Primary cultured stem cells were analyzed for eGFP expression without fixation. All samples were observed with a fluorescence microscope (LSM510 Meta, Zeiss, Thornwood, NY).

In addition, bone marrow mesenchymal stem cells (BM-MSCs), umbilical cord mesenchymal stem cells (UC-MSCs), neural stem cells (NSC), and chondrocytes were isolated from both models and examined for expression of pH2B-eGFP. For isolation of BM-MSCs, bone marrow was collected in PBS supplemented with 5% FBS and Streptomycin, Penicillin, and Amphotericin. The bone marrow was manually fragmented using sterile forceps, followed by manual agitation to release the cells and filtered using a 40μM filter. The cell suspension was gently laid on a Fycoel gradient and centrifuged at 400 x g for 40 minutes to enrich for mononuclear cells. Mononuclear cells were plated overnight on DMEM supplemented with 10% FBS and Streptomycin/Penicillin to enrich for adherent cells. The following day non-adherent cells were removed by washing with PBS 2x, and remaining adherent cells isolated by trypsinization. The cells were further characterized by RT-PCR to confirm expression of the MSC markers CD105, CD90 and lack of expression for CD34.

UC-MSCs were isolated as described previously [32]. Briefly, pig umbilical cords were aseptically collected from newborn piglets and umbilical arteries and vein removed. The remaining tissue, containing the Wharton jelly, was minced in DMEM media with Penicillin, Streptomycin and Amphotericin B (Corning, Inc. NY). The explants were transferred to six-well plates containing the above media along with 20% fetal bovine serum (FBS) and left undisturbed for 5-7 days to allow migration of cells from the explants. They were fed twice weekly and passaged as necessary (cells passaged at 80–90% confluence). The cells were further characterized by RT-PCR to confirm expression of the MSC markers CD105, CD90 and lack of expression for CD34.

The techniques used for cell isolation of pig fetal NPCs used in this study were adapted and modified from a protocol described previously for humans [33]. Fetal brains collected from D42 fetuses were enzymatically digested with collagenase type IV 0.5mg/ml (Worthington, Lakewood, NJ) and 0.05% trypsin (Corning, Inc., NY) for 20 minutes at 37°C, and the resulting cell suspension was washed, filtered through a 40 μM filter and plated in ultra low attachment plates (Corning, Inc. NY) for the development of neurospheres. The cell culture media consisted of Dulbecco’s modified Eagle’s medium/F-12 with high glucose (Corning, Inc. NY) containing L-glutamine (200 mM), B27 (100% by volume; StemCell Technologies, Vancouver, British Columbia, Canada), EGF (20 ng/ml), bFGF (40 ng/ml), platelet-derived growth factor-AB (20 ng/ml), and antibiotics. Neurospheres were visualized after 5 and 6 days and images
taken. The cells were further characterized by RT-PCR and shown positive for the neural stem cell markers GFAP, TUJ1 and negative for CD34.

To isolate primary chondrocytes costal cartilage was minced into fine fragments (less than 1 mm³), fragments washed in Dulbecco's phosphate buffered saline (DPBS; Cellgro, Manassas, VA) and digested with 1.2% type II collagenase (Worthington, Lakewood, NJ) at 37 °C for 6 hr. The cell suspension was filtered through a cell strainers (70 μm; BD Falcon, Franklin Lakes, NJ) to obtain single cells. Cells were washed three times in DPBS and re-suspended in DMEM containing 10% FBS. The number of total cells and viable cells were calculated by Trypan blue staining (Sigma, St. Louis, MO) and by counting with a Bright-Line™ Hemacytometer (Sigma; average cell yield: 470,000 cells per gram). Viable cells were plated into 6- and 24-well plastic culture dishes at a density of 15,000 cells/cm². Cells were cultured at 37 °C under 5% CO₂ conditions in DMEM (Corning, Inc. NY) with 10% FBS. Identity of chondrocytes was confirmed by expression of COL2 and ACAN.

pH2B-eGFP Expression After In Vitro Fertilization (IVF) and SCNT

To examine pH2B-eGFP expression in the early IVF embryos, frozen epididymal sperm from an pH2B-eGFP boar was used for IVF as previously described [34]. Matured oocytes with a first polar body were washed three times in manipulation media (Ca-free NCSU-23 with 5% FBS). Approximately 30 to 35 oocytes were transferred into 50 μl droplets of in vitro fertilization (IVF) medium covered with mineral oil that had been equilibrated at 38.5 °C in 5% CO₂ in air. A 0.1 μl frozen semen pellet was thawed at 38.5 °C in 10 μl of sperm-washing medium. After washing twice by centrifugation (1,900RPM at room temperature, 4 min), cryopreserved epididymal spermatozoa were resuspended with fertilization medium to a concentration of 1 × 10⁶ cells/ml. Fifty microliters of the sperm sample was added to the fertilization droplets containing the oocytes, giving a final sperm concentration of 0.5 × 10⁶ cells/ml. Oocytes were co-incubated with sperm for 4 to 6 h. After fertilization, oocytes were washed three times and cultured in 500 μl in NCSU-23 medium with 0.4% BSA in 4 well dishes at 38.5 °C in 5% CO₂ for 6 days. Expression of pH2B-eGFP was evaluated through preimplantation development, from fertilization until blastocyst stage.

For SCNT embryos, following fusion and activation, embryos were fixed with 4% (wt/vol) paraformaldehyde (PFA) in PBS for 30 min at room temperature, counterstained with 1 mg/ml of DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to stain DNA and mounted on glass slides to examine pH2B-eGFP expression. In addition, unfixed embryos were used for time-lapse imaging following SCNT activation and until the 2–4 cells stage.

For live imaging, IVF or SCNT embryos were transferred to drops of medium on a glass-bottomed dish (20 embryos per 50μl drop), placed in the incubator on the microscope stage and incubated at 38.5 °C under 5% CO₂ in air. The system has a Z motor and an auto X–Y stage. Images were acquired in two channels (light microscope, green fluorescence) using the auto shutter. Device control and image analysis were performed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). For time-lapse observations, images were taken over 50 h at 15 min intervals.

Expression Levels of pH2B-eGFP in Model I and Model II

Expression levels were compared using Western blotting, flow cytometry fluorescence intensity profiles, and image analysis of fluorescent intensities in two cell types (BM-MSC and chondrocytes). Cells previously isolated and frozen from two aged-matched animals were used for each Model I and Model II donors and a wild type control. Passage 3 cells were thawed, plated
(5 x 10^5/well) in 100mm dishes and grown to 100% confluence. Once confluent, total proteins were extracted and 20 μg of total protein separated on a 4–20% precast stain-free gel (Bio-Rad, cat#456–8094). Proteins were transferred to a PVDF membrane, and the membrane probed with rabbit IgG anti-GFP polyclonal antibody (1:5000; Abcam, cat#ab6556) at 5°C overnight, followed by incubation at room temperature for 1 hour with goat anti-rabbit IgG HRP-conjugated (1:10000; Abcam, cat#ab97051). The PVDF membrane was then washed and treated with ECL for detection and image capture. For β-Actin detection the membrane was stripped with 0.5M Tris HCl, 10% SDS and β-mercaptoethanol at 50°C for 45 minutes and checked to ensure loss of pH2B-eGFP signal. It was then probed with rabbit IgG anti-β-actin polyclonal antibody (1:5000, Abcam, cat#ab8227) at 5°C overnight, followed by incubation at room temperature for 1 hour with goat anti-rabbit IgG HRP-conjugated (1:10000; Abcam, cat#ab97051). PVDF was washed and treated with ECL for detection. The predicted size for pH2B-eGFP fusion protein was 41kDa, and β-actin 43kDa. Intensity profiles were obtained using Image Lab (Bio-Rad) and normalized to β-actin.

Model I and Model II eGFP fluorescence intensity was also compared using cultured BM-MSCs and chondrocytes from two animals per model. All cell lines used were in passage 3. BM-MSCs and chondrocytes cells were plated in triplicate 35mm wells (2 x 10^5/well) and grown to 100% confluence in pHF media. Five microscopic fields (40X) were randomly selected from each well, a gate/active region created to represent the cell nucleus corresponding to pH2B-eGFP signal, and GFP intensity was acquired for each cell nucleus using the active gate. The GFP intensity profile from 100 cells/field was measured with the active gate (500 cells per replication, 1500 per animal). GFP intensity analysis was performed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). The same strategy was applied to wild-type cells and gate used to establish background intensity, and values used as threshold. Statistical analysis comparison was performed using one-tailed student’s t-test with a significance value of p < 0.05. In addition, flow cytometry was used to compare fluorescence intensity between Model I and Model II BM-MSC cells. Prior to flow cytometry analysis, cells from control, Model I and Model II donors were thawed, washed twice with PBS, plated at an equal density (50.000 viable cells per 100mm plate) and allowed to growth to 80% confluence. Cells were then tripinized, washed twice with PBS, and analyzed by flow cytometry for eGFP mean fluorescence intensity using a LSR II system (488nm laser and 530/30 filter). At least 400.000 cells per sample were examined.

Results
Generation and Analysis of Model I Pigs
Two independent PFF lines, selected as described in material and methods, were used as nuclear donors for SCNT. Reconstructed SCNT embryos (181 and 126; Table) were transferred into two surrogate gilts generating thirteen piglets from one line and seven piglets from the other (Model I parental generation, P1). Pigs from both Model I lines developed neurological clinical signs between three to five months of age (hind limb lameness, generalized muscular tremor, ataxia, incoordination) and had to be humanely euthanized. To determine whether the effect was SCNT-related, cauda epididymal sperm were isolated from a Model I boar, frozen and used for in vitro fertilization (IVF) with wild type oocytes obtained from the slaughterhouse. A total of 212 zygotes obtained via IVF were transferred to a recipient sow, resulting in the birth of three piglets, with two piglets expressing pH2B-eGFP (Model I, F1 generation). Model I F1 piglets developed similar neurological clinical signs as the P1 generation animals and were humanely euthanized at five months of age for further necropsy evaluation.
While the two Model I lines were made independently, we examined the insertion sites to ensure that the effect seen was not due to an insertional inactivation. Using the Genome-Walker Universal Kit (Clontech, Mountainview, CA) a 900 bp flanking the insertion site was identified in one of the two Model I lines. Mapping of this region indicated that the transgene was located in intron 2 of the adhesion G protein-coupled receptor G6 gene (ADGRG6) located in chromosome 1 (http://useast.ensembl.org/Sus_scrofa/Location/View?db=core&id=125368173-25427711&d=l8Jw0z3VID7h62c-188938-483094307). In spite of multiple attempts, the GenomeWalker system did not yield any positive results with the second Model I line. To identify the insertion site in this line, the whole genome was sequenced as described previously. Results identified the overall organization of the insert as well as the genomic flanking region. The insertion site was located in an as yet unmapped region of the pig genome. This region is defined as GL896235 (Scaffold ChrUScaf4119, NW_003541011.1). As shown in Fig 2 the accuracy of both the GenomeWalker and the whole genome sequencing results were confirmed by PCR. For the Model I line 1, internal primers were designed to chromosome 1 (S'–AGCCGCTTATGGCGGGAGGTGAGAACTGAG–3') and MAR (S'–AGCTCAGAACACAACCCAGTTAGATATA–3') to amplify a 500 bp amplion. For Model I line 2 primers were designed to Scaffold GL896235 (S'–CUCCTCCTGAGGTGACAGTGGAG–3'), and a MAR primer was used as forward primer (S'–AGCTCAGAACACACGGACGTTAGATATA–3'), to amplify a 700 bp amplion. Additional sequence and mapping data is provided in S1 File. The results confirm that both lines are independent and support that the Model I phenotype observed is not due to the location of the transgene.

Generation and Analysis of Model II Pigs

To generate Model II transgenic pigs, pFFs were co-transfected with custom designed ACTB CRISPR-Cas9 and a pH2B-eGFP targeting vector (Fig 1B) and analyzed for targeted integration of the pH2B-eGFP into the ACTB locus (Fig 1D). GFP positive cells were isolated via FACS analysis revealing a HDR efficiency of 2.4% (S1 Fig). Sorted eGFP-positive cells were used to generate clonally derived colonies and analyzed for DNA targeted integration via PCR. Ten independent pFFs colonies were selected for PCR analysis, all being positive and heterozygous for IRES-pH2B-eGFP integration into the ACTB locus (Fig 1D). Positive cells from one colony were used for SCNT and reconstructed embryos transferred into surrogate gilts. Three transgenic boars (Model II parental generation) were generated from one recipient after transferring 119 embryos. Two Model II parental boars are currently over 26 months old and present no abnormalities. The third boar died prior to weaning of undetermined causes. Heterozygous Model II boars were used to artificially inseminate three wild type Landrace sows, resulting in three litters. Two litters were carried to term with a total of thirteen pH2B-eGFP positive and eight negative piglets, and one pregnancy was collected at day 42 for isolation of primary cells. Transgene transmission rate was 55.8%, in concordance with Mendel’s Law upon Chi Square test (p < 0.05). Unlike Model I animals, no deleterious phenotype has been detected at 28 months in any of the Model II P1 or F1 (12 months) transgenic animals.

pH2B-eGFP Expression From Model I and Model II Transgenic Pigs

To determine if pH2B-eGFP expression was ubiquitous and non-variagated, multiple tissues from Model I and Model II piglets (P1 and F1 generation) were collected and OCT embedded cryo-sections analyzed via fluorescence microscopy. Nucleus-specific eGFP expression was co-localized with DAPI staining in intestine, skeletal muscle, and skin (Fig 3). We further confirmed nucleus-specific eGFP expression in the heart, brain, lung, bladder and kidney (data not shown). Primary cultures of stem/progenitor cells were also established from various tissues of newborn
Fig 3. Ubiquitous pH2B-eGFP expression in Model I and Model II pigs. Fluorescence microscopy images from OCT frozen sections from intestines, skeletal muscle, and skin are shown for 40X magnification for bright field, DAPI, GFP and merged GFP+DAPI. Images indicate ubiquitous expression of nuclear GFP.

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piglets or D42 fetuses, including BM-MSCs, UC-MSCs, fetal neural stem cells (NSCs) and chondrocytes (Fig 4). Expression of pH2B-eGFP with intranuclear localization of GFP was
Fig 4. Expression of pH2B-eGFP in in vitro cultured adult stem/progenitor cells isolated from Model I and Model II pigs. Fluorescence microscopy (10X) showing nuclear specific expression of GFP detected from bone marrow mesenchymal stem cells (BM-MSCs), chondrocytes, umbilical cord mesenchymal stem cells (UC-MSCs) and neural stem cells (NSCs) derived from Model I and Model II pigs; pH2B-eGFP was ubiquitously expressed and nuclear localized.

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demonstrated in all cells types examined. In both Model I and Model II samples, eGFP expression was ubiquitous and localized to the nucleus. No variegated expression could be detected in either model. In addition, in pregnancies carrying pH2B-eGFP expressing fetuses, the fetal-maternal placental interphase (endometrium/placental) was easily distinguished by eGFP versus DAPI fluorescence microscopy evaluation at 42 days of gestation (Fig 5). To determine the effect of fixation on pH2B-eGFP detection, OCT and paraffin embedded samples were compared. As

Fig 5. pH2B-eGFP expression from Model II 42 day fetal chorionic membrane. Fluorescence microscopy of OCT embedded fetal-maternal chorionic interphase at 42 days of gestation, showing 10x magnification for bright field, DAPI, GFP and DAPI + GFP. pH2B-eGFP expression is confined to the fetal side of fetal membrane.

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shown in Fig 6, the fixation protocol used in paraffin embedded samples squelches the fluorescent pH2B-eGFP signal. However, the protein can still be detected by the use of anti-GFP antibodies.

To demonstrate the usefulness of the pH2B-eGFP to examine nuclear dynamics during mitosis, pH2B-eGFP pFFs were cultured in vitro and analyzed via live imaging fluorescence microscopy (Fig 7). We found that chromatin positioning through pH2B-eGFP microscopy visualization allowed staging of the cell cycle and a clear visualization of the metaphase plate using conventional (versus confocal) photomicroscopy.

pH2B-eGFP Expression From IVF and SCNT Embryos

Nuclear transfer and IVF was performed with Model I and II donors. The generated embryos were observed either using live-cell imaging or collected, fixed and stained with DAPI at various stages after SCNT or after IVF. For IVF generated embryos, the nuclear GFP signal could be detected starting at 68–69 hr after embryos were removed from the IVF drop (Fig 8), indicating transcription and translation of the pH2B-eGFP and modeling the maternal to zygotic transition in pigs (4 to 8 cells) [35]. For SCNT embryos, the pH2B-eGFP signal was evident from the moment the embryos were reconstructed (fusion) until the blastocysts stage, allowing visualization in real time of chromosome segregation during blastomere cleavage. Fig 9 shows representative images of normal and abnormal chromosome segregation and cleavage patterns after SCNT.

Model I versus II pH2B-eGFP Fluorescence Intensity

When examining the OTC frozen sections we noticed a subjective difference in the intensity of the pH2B-eGFP signal between Model I and II tissues. This was confirmed by measuring protein expression by Western analysis. As shown in Fig 10, BM-MSC from Model I animals had
Fig 7. Live imaging of mitosis of Model II (ACTB-IRES-ph2B-eGFP) porcine fetal fibroblasts. Time-lapse GFP fluorescence images of cultured Model II pPFS taken at 5 min intervals. Red arrows indicate cell progression from metaphase to cytokinesis (A through H). A cell progressing from prophase to telophase is shown by blue arrows (D through L). The clear and intense chromosome-associated signal allows visualization of chromosome movements in dividing cells in real time. It also allows a rapid assessment of cell cycle synchronization protocols.
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more than twice the level of pH2B-eGFP. In addition, two different cell types, BM-MSCs and chondrocytes were examined by fluorescent microscopy. Signal intensity measurements supported the Western results with both cell types showing a significant increase in pH2B-eGFP signal intensity in Model I compared to Model II (Fig 1B). Finally, this difference was confirmed by flow cytometry. Model I and Model II BM-MSCs were analyzed via flow cytometry for mean GFP fluorescence. Approximately 400,000 passage 2 BM-MSC from each phenotype were tested in triplicate, with wild type cells serving as negative control. Mean GFP fluorescence for Model I (pH2B-eGFP/PGK/MAR) and Model II (β actin IRES-ph2B-eGFP) were 151.8±10^3 and 87.6±10^3, respectively (Fig 10). All three methods yielded analogous results with a significant (p < 0.05) higher level of expression or signal intensity in Model I versus Model II samples/animals.

Wallerian Degeneration Accompanied by Muscle Fiber Atrophy in Model I Transgenic Pigs

As mentioned previously, all Model I P1 and F1 pigs developed neurological clinical signs with onset around three to five months of age. In all Model I animals, signs were consistent, with initial hind limb lameness, followed by hind limb ataxia and tremors, leading to progressive loss of hind muscle control, at which point animals were humanely euthanized. To determine the probable cause of the neurological symptoms, tissues from an affected F1 generation pig
Fig 8. Zygote genome activation in IVF embryos. Sperm from an H2B-GFP boar was used to in vitro fertilize wild-type oocytes and the appearance of the H2B-GFP signal detected using time lapse (every 5 min) photography. Both the oocyte and sperm are H2B-GFP negative at fertilization as expected. Nuclear GFP is observed only after transcription and translation from the sperm DNA, thus modeling the maternal to zygotic transition. A representative picture of spermatozoa is shown in (A) bright field, (B) GFP and (C) DAPI. Reactivated expression of H2B-GFP is shown by co-localization of (D) bright field, (E) GFP and (F) DAPI from late 4-cell stage embryo. Representative time-lapse images of reactivation of H2B-GFP during the 4-cell stage are shown at (G) 54.5, (H) 55.5, (I) 56 and (J) 56 hr post-fertilization in bright fields (top) and GFP (bottom).

euthanized at five months were submitted for pathological evaluation. Microscopic histopathology findings included mild to moderate multifocal Wallerian degeneration of multiple regions of the brain and spinal cord (Fig 11) as well as multifocal skeletal myofiber polyphasic degeneration and atrophy. None of these lesions were observed in age-matched Model II pigs or in wild type controls (Fig 11). In addition, macroscopic necropsy findings included generalized coagulopathy with fibrin deposition in abdominal cavity, pericardium sac and pulmonary thromboembolism. Minimal necrosis or inflammation was identified. In contrast to Model I, none of the Model II P1 or F1 animals have shown similar neurological signs at 28 months of age for the P1 generation and 12 months for the F1 generation.
Discussion

Transgenic pigs harboring and expressing green fluorescent proteins under different conditions have been described [46–48]. However, identification and quantification of engrafted donor cells after cell/tissue transplantation remains challenging due to strong auto-fluorescence, especially when GFP is expressed in the cytoplasm [49, 50]. In addition, the diversity of cell phenotype and shapes make it difficult to distinguish/count GFP-positive donor cells when utilizing automated systems. This difficulty can be overcome via nuclear GFP labeling, allowing easy and convenient cell tracking after stem cells/tissue transplantation studies.

Nuclear localization of GFP can be achieved by addition of a nuclear localization signal peptide [46] or by fusion of GFP with proteins of the nucleosome core such as histones (i.e. H2B). H2B-GFP expression in cell lines [15] or transgenic mouse models [17] have been described and shown to be of great value in the field of stem cell tracking, cancer biology and chromosome dynamic studies [42, 48].

Here we describe two pH2B-eGFP transgenic pig models expressing chromatin associated GFP. Model I made via random integration of pCAG-pH2B-eGFP and MARs, and Model II via CRISPR-Cas9-mediated integration of IRs2-pH2B-eGFP into the ACTB locus. The ACTB locus was chosen as it is known to be expressed in all tissues and has been used previously to drive exogenous genes [49, 50]. Another commonly used ubiquitously expressed locus is the ROSA26 locus. This has been widely used in mice and more recently in swine [51–54]. However, there have been reports that ROSA26 expression can vary widely in particular in certain cell types. For instance, Cutler and colleagues [51] reported that expression levels of lacZ from the ROSA26-lacZ reporter mouse changed drastically during remodeling of arteries. Of greater concern for transplantation studies is the discrepancy between ROSA26 locus expression and
other markers. Theise and colleagues [55] reported that after bone marrow transplantation of ROSA26-lacZ cells into irradiated mice, splenic engraftment was 90% when measured by Y-chromosome analysis but only 50% when measured by lacZ staining. This suggests that under certain conditions the ROSA26 locus will give inaccurate information of the degree of engraftment after transplantation.

Overall, our data demonstrates that both Model I and Model II expressed pH2B-eGFP in a ubiquitous manner in all cells examined. Consistent high-level expression in all nuclei and no
Fig 11. Wallerian degeneration from white matter of the Brain from Model I pig. H&E microscopic images (40X) of brainstem region (white matter) for six months old wild type pig (A), six months Model II pig (B) and five months old Model I pig (time when neurological signs were severe). Digestion chambers are indicated by black arrows, revealing loss of normal tissue architecture. Few vessels contain aggregates of red cells and fibrin. None of this abnormalities were present in wild type or Model II pigs.

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Evidence of position effect variegation were seen in either model. Moreover, the nuclear signal can be easily visualized without the use of antibodies in frozen OTC sections in all tissues tested (Figs 3 and 4). By combining GFP detection with DAPI staining, the number of transgenic nuclei in a given field of vision can be easily calculated. This greatly facilitates quantification of engrafted cells after transplantation, which can be more difficult to do when using cytoplasmic GFP due to auto fluorescence and the complex 3D structure of cytoplasmic membranes. In addition, if higher resolution tissue architecture is required, the pH2B-cGFP protein can be detected using widely available anti-GFP antibodies in more hardly fixed tissues where the endogenous GFP signal is lost (Fig 6). Similarly, the use of the pH2B-GFP marker allows clear differentiation between the maternal and the fetal interface during pregnancy and this facilitate studies examining the potential interactions and trafficking of cells between the two compartments in normal and diseased placentas (Fig 5).

Histone tagged fusion fluorescence protein has been suggested as a strategy to label chromosomes in living cells and animals and has been applied to the study of chromosome and cell cycle dynamics [66-69]. When performing live image fluorescence microscopy of Model I and Model II fetal fibroblasts, we could visualize chromatin position and dynamics through pH2B-cGFP signal during various stages of cell cycle division (Fig 7). This finding was in concordance with the previous work with mouse cells expressing H2B-GFP [16, 19]. Cell cycle progression and chromosome dynamics can be evaluated with this model for a variety of stem or somatic differentiated cells, supporting that the developed pig model will be useful in field of chromosome dynamics and cancer biology. We could also examine the maternal to zygotic transition (MTZ) after IVF by looking at the timing of appearance of chromatin-associated pH2B-cGFP and confirm previous observations that it occurs during the early stages of the 4-cell stage [61].

During SCNT the donor somatic nucleus undergoes a series of complex modifications characterized by reprogramming events initiated by maternal factors. Remodeling, not of the underlying genetic sequences that comprise the genome, but of the epigenome, is a key factor controlling differentiation and development at this stage. However, in most, if not all, mammalian species used for SCNT to date there is a large proportion of reconstructed embryos that are lost at the very early cleavage stages. Examinations of both centrosomes and microtubules have shown that there is a number of defects associated with abnormal chromosome segregation and abnormal cytokinetics [62-67]. In the case of the pig, these defects are exacerbated the longer the time period between fusion and activation [67]. However, in all of the manuscripts referenced above, examination of nucleosome dynamics and microtubules required fixation of the zygotes. Unlike cytoplasmic GFP, H2B-cGFP is embedded in the incoming donor cell chromatin as part of the nucleosomes and as such is protected from degradation. If no additional H2B-GFP is made, the signal intensity is lowered by half with each cell division. This property has been used to examine the proliferative state of certain stem cells in vivo [20] and allowed us to examine chromosome dynamics in SCNT embryos prior to the maternal to zygotic transition. While our IVF data indicates that newly synthesized pH2B-GFP is detectable by the 4-cells stage, there is still sufficient pH2B-GFP in the incoming donor cells to allow careful examination of chromosome segregation in early cleavage stage SCNT embryos (Fig 9). This allowed real time visualization of chromosome dynamics and rapid identification of multi-polar spindle formation (Fig 9). This provides a unique tool not only to better
understand the dynamics of chromosome segregation in SCNT embryos but equally important to rapidly evaluate potential treatments to reduce these defects that can affect as many as 50% of SCNT embryos [69].

However, while both Model I and II expressed pH2B-eGFP ubiquitously, all Model I transgenic pigs developed neurological clinical signs starting at 4–5 months of age. All animals were unresponsive to treatment (antibiotics and steroids). Post mortem analysis identified severe multifocal Wallerian degeneration of brain and spinal cord segments (Fig. 11) and generalized coagulopathy, with absence of necrosis or evidences of sepsis. The disseminated fibrin deposition in body cavities was likely related to the multifocal neuron degeneration, resulting in release of phospholipids in the blood stream, which has been associated with initiation of coagulation and coagulopathy disorders in human suffering from traumatic brain injury [68]. Wallerian degeneration is a process characterized by axonal degeneration due to nerve fiber discontinuity as a result of trauma/injury or impaired axonal transport [69, 70]. While this is by no means a comprehensive analysis of the complex neurological phenotype of Model I pigs, it does suggest that in two independent lines overexpression of pH2B-eGFP leads to axonal degeneration. We confirmed that the two lines have independent insertion sites suggesting this is not an insertional effect (Fig. 2). We also demonstrated, via multiple methods, that cells from Model I animals express approximately twice the level of pH2B-eGFP than Model II cells (Fig. 10). In addition, others have shown that in a direct comparison of the expression levels of the ACTB and the CAG promoter, the CAG promoter is approximately 10× more active that the ACTB promoter [71]. While our analysis only shows a 2× increase it is clear from our own data and previous reports that there are significant differences in expression between the two reporters. Interestingly, Brunetti and colleagues [71] generated transgenic pigs expressing cytoplasmic GFP using the similar method described here (pCAG promoter 3’ flanking MARs/SCNT) and did not report any abnormalities in the established lines. We can only speculate that is it the fusion of the GFP with the pH2B histone that is leading to the phenotype observed. Thus, further experiments would be required to investigate why overexpression of pH2B-GFP leads to Wallerian degeneration. However, protein misfolding, aberrant degradation pathways, abnormal axonal transport, and/or protein accumulation are seen in many neurodegenerative diseases [72] so it is likely that excess levels of pH2B-GFP are triggering apoptosis of the neurons by one of the above mentioned mechanisms.

In conclusion, we describe here a viable pH2B-eGFP pig model generated using CRISPR-Cas9 driven homologous recombination system. This large animal model represents a valuable tool in the field of translational research, with multiple potential applications in the fields of stem cell transplantation, cell tracking/lineage fate studies, and for studying chromosome/histone 2B dynamics in different somatic cell types and during early embryogenesis of reconstructed SCNT embryos.

Supporting Information
S1 Fig. 1RES pH2B-eGFP CRISPR-Cas9 mediated homology directed repair (HDR) into the pig β-actin locus. Fluorescence-activated cell sorting of porcine fetal fibroblast 4 days post co-transfection with CRISPR-Cas9 and targeting vector. A 2.4% GFP positive population was sorted for generation of single cell colonies. Non-transfected cells served as negative control, cells transfected with pMAX(GFP) served as positive control.

(PDF)

S1 Table. Summary of pregnancies and outcomes from Somatic cell nuclear transfer (SCNT) and in vitro fertilization (IVF) of both Model I and Model II lines.

(DOCX)
SI File. Nucleotide sequence of genomic insertion site for Model I lines. Sequence of flanking genomic region and the predicted genome mapping insertion site of the transgene for both Model I lines.

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References

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CHAPTER 2: GENERATION OF A SEVERE COMBINED IMMUNODEFICIENT (SCID) PIG MODEL

2.1 INTRODUCTION.

Severe combined immunodeficient (SCID) mouse models have allowed researchers to answer fundamental questions regarding the immune system and cell specific development. They also serve as a crucial bridging model to fulfill translational discoveries, especially in the field of stem cell transplantation, leading to new discoveries with potential pre-clinical application in human medicine (Shultz et al. 2012). Popular SCID mice models include nonobese diabetic/SCID mice (NOD.Cg-Prkdc<sup>SCID</sup>Il2RG<sup>tm1Wjl</sup> or NSG mice), NODShi.Cg-Prkdc<sup>SCID</sup>Il2RG<sup>tm1Sug</sup> (NOG mice) and C:129S4RAG-2<sup>tm1Flv</sup>IL2RG<sup>tm1Flv</sup> (BRG mice) (Shultz et al. 2012). These models harbor multiple genetic modifications abolishing adaptive immunity development, in addition to human transgenes such as cytokines and major histocompatibility complexes, to assist with engraftment and functionality of human lymphoid cells. (Shultz et al. 2007). The majority of these mouse SCID models harbor mutations in either the RAG-1 or RAG-2 genes and the common gamma chain (IL2RG) gene. These mutant mice are commonly known as double knockout (DKO) mice.

Functionally, RAG-1 and RAG-2 proteins catalyze V(D)J recombination of immunoglobins/B cell receptor genes (Ig) for B cells in the bone marrow and spleen, and also for the T cell receptor (TCR) genes for T cells in the thymus (McBlane et al. 1995; de Villartay et al. 2003). IL2RG, located on the X chromosome, encodes a subunit of the IL-2 receptor (Takeshita et al. 2016), also present in the IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Kondo et al. 1993; Noguchi et al. 1993; Kimura et al. 1995; Giri et al. 1994; Asao et al. 2001). The result of IL2RG...
inactivation is broadly impaired cytokine signaling, blocking T cell thymic development (prior to RAG-mediated recombination), impaired thymus and peripheral lymph node development, and lack of IL-15 Natural killer (NK) cell-mediated differentiation. (Cao et al. 1995; Pontzer et al. 1992). Combined, these IL2RG\textsuperscript{null} characteristics leads to XSCID syndrome, which in human accounts for 50 to 60% of all SCID cases (Basile 1997). The combination of both the IL2RG and RAG1 or RAG2 mutations leads a profound immunodeficient model lacking functional B, T, and NK cells allowing xenogenic engraftment of human stem cells of different sources.

SCID mice have been used for the last 3 decades to answer fundamental questions of ontogeny/biology of immune cells, for the development of new strategies for immunodeficiency therapy, and more importantly for xenotransplantation as a bridging model for humanized infectious diseases (HIV), human stem cell therapy and development of chimeric humanized organs (Shultz et al. 2012; Shultz et al. 2007). For instance, NOD/SCID/IL2RG\textsuperscript{null}, NOG, and RAG-2\textsuperscript{null}/IL2RG\textsuperscript{null} mice (DKO) permit the functional reconstitution of human hematopoietic and immune systems following the injection of purified human hematopoietic stem cells (Ishikawa et al. 2005; Traggiai et al. 2004).

Despite the fundamental role of SCID mice, certain limitations exist such as species-specific differences at the anatomical and physiological scale. An example of these species differences is that while the XSCID mice lacks T, B and NK cells, human XSCID lack T and NK cells but have B cells, albeit defective (Basile 1997; Cao et al. 1995).

In contrast, pigs closely resemble humans in terms of organ size, life span, anatomical, and physiological characteristics. SCID pigs can be critically important research tools to facilitate long-term follow-up studies of immune responses, xenotransplantation, stem cells-based therapies, and drug development for cancer. In addition, a SCID pig would allow the
development of procedures to assess the safety of allogeneic or xenogeneic stem cell therapies and gene therapy approaches, the development of humanized pig organs for xenotransplantation applications, and a large animal immunodeficient model for expansion of human stem cells. (Niemann & Petersen 2016)

In this chapter, I describe the development and characterization of a transgenic RAG-2^+/-. IL2RG^-y and RAG-2^-/ IL2RG^-y pig lines generated via CRISPR-Cas9 and somatic cell nuclear transfer (SCNT).

2.1.1 Current pig models of severe combined immunodeficiency (SCID).

A transgenic XSCID (IL2RG^-y) pig model has been previously described (Suzuki et al. 2012). This pig model exhibited a markedly reduction in T and NK cells in peripheral blood while having normal B cell numbers. It is important to note that NK and T cells were still detectable (but markedly reduced) in the spleen (~2.3%), where T cell numbers in thymus were undetected by flow cytometry or IHC. This model robustly recapitulated humans with XSCID syndrome (Basile 1997). This model was further capable of sustaining bone marrow-derived allogeneic hematopoietic stem cell engraftment via post-natal intravenous route utilizing stem cells from sibling donor (allogeneic, semi-matched). Post-natal human hematopoietic stem cell transplantation was also carried out, but did not lead to detectable engraftment, suggesting the presence of other immune barriers to xenotransplantation in a XSCID pig model.

Later a RAG-1^-/ and RAG-2^-/ transgenic pigs were described by Huang et al (2014a). These animals failed to perform V(D)J recombination of IgH locus (required for B cell development) and the TCR-β locus (required for T cell development). RAG-1/2 knockout pigs also showed significantly decreased levels of mature B and T lymphocytes compared with age-
matched WT pigs. It is important to note that CD3 + cells were markedly reduced in the spleen, but could be observed via immunohistochemistry (IHC). RAG-1⁻/⁻ and RAG-2⁻/⁻ pigs displayed hypoplastic thymus. The spleen showed germinal centers denuded of lymphocyte. These findings were consistent with RAG-1/2⁻/⁻ mice and humans (Mombaerts et al. 1992; Shinkai et al. 1992; Fischer et al. 1997).

A RAG2⁻/⁻ transgenic pig model has been described by lee et al. (2014). This model had a similar phenotype as described by Huang et al (2014a). While no allogeneic or xenogeneic engraftment with hematopoietic stem cells was carried out this model was, however, capable of developing mature teratomas representing all three germ layers when injected with xenogeneic human induced pluripotent stem cells (iPSCs). The pigs also tolerated grafts of allogeneic porcine trophoblast stem cells. These findings revealed the potential of immunodeficient pig model as a new large animal model to be explored in the field of allogeneic and xenogeneic transplantation using stem cells from various sources.

DKO pig model (RAG null IL2RG null, or BRG) have not yet been described. Equally important, substantial human hematopoietic stem cell engraftment has not been described in a SCID pig model.

2.2 MATERIALS AND METHODS.

2.2.1 Generation of RAG-2⁺/⁻ IL2RG⁻/⁻ and RAG2⁻/⁻ IL2RG⁻/⁻ porcine fetal fibroblast line.

A previous porcine IL2RG⁻/⁻ fetal fibroblast line had been previously developed in the Piedrahita lab using TALENs (Marks 2014). This cell line harbors a 5 base pair deletion of exon
1 of the IL2RG locus (IL2RG<sup>Δ79-83/Y</sup>). Lack of IL2RG was confirmed by western blot. This cell line was selected for further targeting of the RAG-2 locus via CRISPR-Cas9.

For generating RAG-2<sup>+/−</sup> IL2RG<sup>−/−</sup> and RAG2<sup>2−</sup> IL2RG<sup>−/−</sup> lines, a CRISPR-Cas9 was custom designed to target the 5’ region of exon 2 of the RAG-2 porcine locus of IL2RG<sup>Δ79-83</sup> SCNT porcine fetal fibroblast. A reported plasmid was also generated, which originally expresses red fluorescence protein (RFP) through a CMV promoter. A 2A-Hygromycin-GFP fusion sequenced is downstream of RFP and out of frame. A cloning site for nucleases is present between RFP and 2A. Single strand complementary oligos containing the RAG-2 CRISPR-Cas9 binding sequence with further modification (addition of an extra A base pare adjacent to PAM sequence to maintain downstream sequence of RFP out of frame) flanking a EcoRI and BamHI were annealed, digested, and cloned into the reporter plasmid. IL2RG<sup>−/−</sup> porcine fetal fibroblast line was thawed and transfected via Amaza system with RAG-2 CRISPR-Cas9 (1µg) and RAG-2 reporter plasmid (250 ng), followed by exposure to Hygromycin-B at 2mg/ml for 2 days post transfection for 2 days. A HpyCH4V restriction site is at the double strand break site of RAG-2 CRISPR-Cas9 binding site, and was used for restriction digest detection of indels. Genomic DNA from single cell colonies were isolated, primers were designed to amplify a 190 bp amplicon containing the restriction site, followed by HpyCH4V digestion and DNA sequencing. One colony (# 12) was selected for generation of RAG-2<sup>+/−</sup> IL2RG<sup>−/−</sup> pigs (5 base pair monoallelic mutation) via somatic cell nuclear (SCNT) transfer. Another colony (# 29), containing biallelic mutations containing one insertion and a 4 base pair deletion, was selected to generate RAG-2<sup>+/−</sup> IL2RG<sup>−/−</sup> transgenic pigs via the SCNT.
2.2.2 Generation of RAG-2+/IL2RG−/y and RAG-2−/IL2RG−/y pigs via Somatic Cell Nuclear Transfer (SCNT).

RAG-2+/IL2RG−/y and RAG-2−/IL2RG−/y pregnancies were generated using the same SCNT protocol described for generation of β-ACTIN-IRES-pH2B-eGFP pigs [30]. A total of four pregnancies were generated (Table 1). One pregnancy was used for RAG-2+/IL2RG−/y phenotype characterization (3 pigs), one for RAG-2−/IL2RG−/y phenotype characterization (3 pigs), one for allogeneic fetal liver pH2B-eGFP (3 pigs) and one for CD34 + xenogeneic (3 pigs), both via IUHCT.

2.2.3 Animal Welfare.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animals used in this study were obtained from a university-owned herd, and all animal procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University (Raleigh, NC). Animals were sacrificed by one of two methods, intravenous injection of sodium pentobarbital, or penetrating captive bolt euthanasia followed by jugular exsanguination. Both methods meet the recommended guidelines of the American Veterinary Medical Association for euthanasia in pigs. All surgeries were performed under isoflurane anesthesia, and a post-surgical regimen of bupivacaine, Banamine-S was administered to minimize pain. Xenotransplantation procedure and management of xenografted pigs was performed at an approved facility within the College of Veterinary Medicine under proper guidelines in concordance with IACUC protocols.
2.2.4 Genotyping of RAG-2<sup>+/−</sup> IL2RG<sup>+/−</sup> and RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> porcine fetal fibroblast and pigs.

For the IL2RG genotyping gDNA was isolated from porcine fetal fibroblast colonies and generated pigs and the primers 5’ CCACTGGAGTTTTTTCATTTTGATG 3’ and 5’ ATCCGAAAGCTCATTATTTGGTGT 3’ used to amplify the TALEN binding site and flanking region (~1Kb) under the following conditions 98°C for 1 min; 35 cycles (98°C for 10 s, 64.5°C for 10 s, 72°C for 15 s), 72°C for 1 min. Restriction digest with AvaII was performed to detect mono allelic indels of the IL2RG locus.

For RAG-2 genotyping gDNA was isolated from porcine fetal fibroblast colonies and generated pigs and the primers 5’ CCACTGGAGTTTTTTCATTTTGATG 3’ and 5’ ATCCGAAAGCTCATTATTTGGTGT 3’ used to amplify the CRISPR-Cas9 binding site and flanking region (190bp) under the following conditions 98°C for 1 min; 35 cycles (98°C for 10 s, 64.5°C for 10 s, 72°C for 10 s), 72°C for 1 min. Restriction digest with HypCH4V was performed to detect mono and biallelic indels of the RAG-2 locus.

2.2.5 Histological and immunofluorescence analyses.

Euthanized RAG-2<sup>+/−</sup> IL2RG<sup>+/−</sup> and aged matched wild type controls pigs were euthanized and lymphoid organs isolated. For hematoxylin and eosin (H&E) staining the thymus and spleen (and lymph nodes when present) were fixed in 10% neutral buffer formalin for 24h, followed by 70% ethanol for 24 hours. For IHC analysis of paraffin embedded tissue, samples were stained with mouse anti-human CD79α unconjugated (BioRad/AbDSerotec #MAC2538), anti-human CD3 (Dako #A0452), and rabbit anti-human CD335 (BIOSS #bs-10027R). For immunofluorescence analysis, fresh samples from spleen and thymus (and lymph nodes when present) were placed in 30% sucrose at 5°C overnight, followed by OCT embedding and
freezing. For pig cells samples were immunostained with mouse anti-human CD79α-Alexa Fluor-647, this antibody cross reacts with pig CD79α (BioRad/AbDSerotec #MCA2538A647) and mouse anti-porcine CD3-Alexa Fluor 405 (Novus # NBP1-28225AF405). Immunohistochemistry and immunofluorescence cell quantification was performed using NIH ImageJ software.

2.2.6 Flow cytometry and fluorescence activated cell sorting.

Peripheral blood (PB) collected into EDTA containing tubes and single cell suspension from thymus and spleen from RAG-2<sup>-/-</sup> IL2RG<sup>-/-</sup> were processed and prepared for antibody staining. For detection of pig cells in RAG-2<sup>-/-</sup> IL2RG<sup>-/-</sup> pigs and allogeneic engrafted animals the following antibodies were used. For T cells, mouse anti pig CD3 epsilon Alexa Fluor 405 labeled (Novus # NBP1-28225AF405), mouse anti pig CD8 PE labeled (BD # 559584) and mouse anti pig CD4 PE-Cy7 labeled (BD# 561473). For detection of pig B cells mouse anti human CD79α Alexa Fluor 647 Labeled (BioRad/AbDSerotec #MCA2538A647) using LEUCOPERM kit (BioRad/AbDSerotec #BUF09) for intracellular staining. For detection of pig NK cells, mouse anti pig CD335 (BioRad/AbDSerotec # MCA5972GA) conjugated with APC-Cy7 with the kit LNK#131PACCY7 (BioRad/AbDSerotec), and mouse anti porcine CD3 epsilon Alexa Fluor 405 labeled (Novus # NBP1-28225AF405). Samples were incubated for 1h at 5°C in staining buffer (BD#554657), followed by red blood lysis and fixation with lysing solution (BD#349202) and washing. All flow data was gated and displayed as percentage of gated mononuclear cells.
2.2.7 PCR assay for detection of pig V(D)J recombination.

Genomic DNA was isolated from peripheral blood from all time points used for flow cytometry. In addition, left over cell suspension from thymus and spleen at the time of pig euthanasia were used to isolate gDNA. The previous reported primers D1J1-F and D1J1-R (Huang et al. 2014b) were used to identify D-J rearrangement of the TCR-β locus in peripheral blood and thymus of RAG-2\(^{-/}\) IL2RG\(^{-/}\) pigs with Phire Hot Start II DNA Polymerase (Thermofisher # F122L) under the following conditions, 98°C for 1 min; 35 cycles (98°C for 10 s, 68°C for 10 s, 72°C for 30 s), 72°C for 1 min. IgH rearrangement was detected by PCR utilizing the previous reported primers FR1 and JH (rearranged IgH) and D4-F and J3-R (germinal control) (Huang et al. 2014b) with Phire Hot Start II DNA Polymerase under the following conditions 98°C for 1 min; 35 cycles (98°C for 10 s, 62.5°C for 10 s, 72°C for 30 s), 72°C for 1 min.

2.2.8 Statistical analysis

Flow cytometry data obtained for T, B and NK cells percentage from gated mononuclear cells from peripheral blood of age matched wild type controls and RAG-2\(^{-/}\) IL2RG\(^{-/}\) pigs for cells at 3 weeks were compared via two-tailed student's t-test with a significance value of \( p < 0.05 \). The same test was applied to cell quantification of immunohistochemistry/immunofluorescence obtained using NIH ImageJ software.
2.3 RESULTS.

2.3.1 Generation of $RAG-2^{+/IL2RG^{-/}}$ and $RAG-2^{+/-IL2RG^{-/}}$ transgenic pigs via CRISPR-Cas9 and Somatic Cell Nuclear Transfer.

$RAG-2$ gene editing was performed on the previously reported $IL2RG^{-/}$ porcine fetal fibroblast line (Marks 2014). Following hygromycin treatment cells were analyzed under fluorescence microscopy (hygromycin only and hygromycin plus CRISPR-Cas9). RFP (texas red channel) and GFP (FITC channel) positive cells were present only in the hygromycin plus CRISPR-Cas9 group (Fig. 12C), supporting $RAG-2$ CRISPR-Cas9 activity to the $RAG-2$ target sequence. Following selection, single cell colonies were generated by seeding at limiting dilutions, and screening performed by PCR followed by $HypCH4V$ restriction digestion. A high targeting efficiency, with indel frequency reaching 79% (51% monoallelic and 28% monoallelic) was obtained (Fig. 12E). Four homozygous and two heterozygous colonies were submitted for DNA sequencing, revealing deletion, point mutations and insertions (Fig. 12F). Sequencing analysis of one colony (#29) revealed biallelic mutations of 4 base pair deletion from one allele and a 1 base pair insertion in the other allele. In silico analysis of both allelic mutations predicted multiple premature stop codons immediately downstream of the CRISPR-Cas9 binding site, leading to lack of $RAG-2$ protein expression. This colony, therefore, was selected as SCNT donor to generating $RAG-2^{+/IL2RG^{-/}}$ transgenic pigs. An additional colony (#12) that contained a 5 base pair deletion in one allele, also predicted to generate multiple premature stop codons immediately downstream of the CRISPR-Cas9 binding site, was used to generate $RAG-2^{+/-IL2RG^{-/}}$ pigs.
Figure 12. Generation of RAG-2+/−IL2RGβγ and RAG-2−/−IL2RGβγ transgenic donor cell lines.

(A) Schematic representation of the RAG-2 pig locus, the red line indicates the CRISPR-Cas9 binding site, arrows indicate primers used for PCR, the amplicon contains 3 HpyCH4V restriction sites used for screening. (B) Reporter plasmid used for enrichment of RAG-2 modification, containing CMV promoter, RPF, cloned RAG-2 CRISPR-Cas9 gRNA target binding site followed by 2A peptide and hygromycin fused with GFP. (C) IL2RGβγ transfection analysis by fluorescence microscopy, cells transfected with reporter alone only express RFP (texas red channel) (transfection control), while few cells transfected with RAG-2 CRISPR-Cas9 and reporter co-expresses RFP and GFP (white arrow), indicating reporter activity. (D) Single cell colony PCR and HpyCH4V assay for detection of RAG-2+/−IL2RGβγ and RAG-2−/−IL2RGβγ cells lines. Wildtype (WT) PCR amplicons digested and undigested with HpyCH4V served as controls. The presence of three bands indicates heterozygous lines for RAG-2, while the presence of two bands in conjunction with the disappearance of the 190 bp band indicates homozygous lines. (E) Overall targeting efficiency of the pig RAG-2 locus, indicating total indels frequency (79%), heterozygous (51%) and homozygous (28%). (F) DNA sequencing of target region of selected RAG-2+/−IL2RGβγ and RAG-2−/−IL2RGβγ colonies. A WT sequence is indicated as a reference.
As shown in Table 1, a total of 555 reconstructed embryos were transferred and 4 pregnancies carried all the way to term for all experiments. These pregnancies include RAG-2+/− IL2RG+/− and RAG-2−/− IL2RG−/− (no transplant), RAG-2−/− IL2RG−/− for allogeneic transplants (discussed in chapter 3) and RAG-2−/− IL2RG−/− for xenogeneic transplant (discussed in chapter 3). We generated total of 12 viable pigs at birth (Table 1).

<table>
<thead>
<tr>
<th>Pregnancy</th>
<th>Embryos</th>
<th>Genotype</th>
<th>Type Transplant</th>
<th>Number of fetuses injected</th>
<th>Live pigs</th>
<th>Pigs life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>157</td>
<td>RAG-2+/− IL2RG+−</td>
<td>No Injection</td>
<td>-</td>
<td>3</td>
<td>3 weeks</td>
</tr>
<tr>
<td>2</td>
<td>134</td>
<td>RAG-2−/− IL2RG−/−</td>
<td>No Injection</td>
<td>-</td>
<td>3</td>
<td>3 weeks</td>
</tr>
<tr>
<td>3</td>
<td>135</td>
<td>RAG-2−/− IL2RG−/−</td>
<td>Allogeneic Fetal liver pH2B-eGFP cells</td>
<td>4</td>
<td>3</td>
<td>1 week</td>
</tr>
<tr>
<td>4</td>
<td>129</td>
<td>RAG-2−/− IL2RG−/−</td>
<td>Human CD34 + cells</td>
<td>4</td>
<td>3</td>
<td>1 day, 2 weeks and 3 weeks</td>
</tr>
</tbody>
</table>

2.3.2 Genotypic and RAG-2 dependent recombination analysis of thymus and spleen.

All animals were genotyped for the IL2RG and RAG-2 locus (Fig. 13B) to confirm correct homozygous or heterozygous inactivation of the RAG-2 locus and complete inactivation of the IL2RG locus. While the absence of the IL2RG protein was confirmed previously by Western blots during development of IL2RGnull pig (Marks 2014), a RAG-2 antibody that identified the pig RAG-2 protein could not be identified. As a result, I RAG-2 activity was examined using recombination assays reported by others (Huang et al. 2014). These assays are
used to detect IgH locus rearrangement in spleen, and TCR-β rearrangement in the thymus. Lack of recombination of both genes indicates lack of RAG-2 activity. Molecular analysis of genomic DNA from wild type and RAG-2<sup>+</sup>IL2RG<sup>−/−</sup> spleen showed the presence of IgH rearrangement (presence of ~ 500bp amplicon), indicating RAG-2 activity (Fig 13C). However, this band was absent in RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> spleen, confirming the lack of RAG-2 activity in RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs (Fig. 13C). PCR detection of D-J rearrangement of the TCR-β locus revealed the lack of this rearrangement for both RAG-2<sup>+</sup>IL2RG<sup>−/−</sup> and RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> lines, while wild type thymus shows rearranged bands (>750bp) (Fig 13D). Combined the data confirm the lack of RAG-2 activity in RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs in the IgH locus. TCR-β recombination was absent from both homozygous and heterozygous lines, likely due to lack of rearranged T-cells in the heterozygous RAG-2 pigs due effect of the IL2RG<sup>−/−</sup> on T cell development (but not B cell development).

![Figure 13](image_url)

**Figure 13.** Genotypic analysis, IgH and TCR-β locus rearrangement analysis of wild type, RAG-2<sup>+</sup>IL2RG<sup>−/−</sup> and RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs. (A) Two-week old RAG-2<sup>+</sup>IL2RG<sup>−/−</sup> and RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs. (B) Diagnostic restriction length fragment polymorphism (RFLP) of mutant pigs gDNA following PCR and digestion with HpyCH4V (RAG-2) or AvaI (IL2RG). Wild type digested and non-digested gDNA and SCNT donor cell gDNA serve as controls. For RAG-2 the
presence of three bands indicates heterozygous \textit{RAG-2}, while the presence of two bands, in conjunction with the disappearance of the 190 bp band, indicates homozygous \textit{RAG-2} null pigs. For the \textit{IL2RG} locus the presence of 2 bands indicates no mutation, while one band indicates an \textit{IL2RG} null genotype (X-linked, male cell line). (C) Detection of V(D)J IgH locus rearrangement in spleen gDNA. A germinal band is present in all samples (~1500bp), while the presence of a 500bp (indicated by the red box) indicates rearranged IgH locus. This band is present in the spleen of wild type and \textit{RAG-2^{+/−}} \textit{IL2RG^{−y}} pigs, while absent in the spleen of \textit{RAG-2^{−y}} \textit{IL2RG^{−y}} pigs. This indicates the lack of \textit{RAG-2} activity. (D) Detection of D-J TCR-β locus rearrangement in thymus gDNA. A germinal band is present in all samples (~1500bp), while the presence of bands smaller than 750bp (indicated by the red box) indicates rearranged TCR-β locus. This band is present in the thymus of wild type pigs and absent in the thymus of \textit{RAG-2^{+/−}} \textit{IL2RG^{−y}} and \textit{RAG-2^{−y}} \textit{IL2RG^{−y}} pigs.

\textbf{2.3.3 Histological analysis of thymus and spleen of wild type, \textit{RAG-2^{+/−}} \textit{IL2RG^{−y}} and \textit{RAG-2^{−y}} \textit{IL2RG^{−y}} pigs.}

Analysis of \textit{RAG-2^{+/−}} \textit{IL2RG^{−y}} and \textit{RAG-2^{−y}} \textit{IL2RG^{−y}} at necropsy identified a poorly developed thymus-like structure in both genotypes (Fig. 14). Hematoxylin and eosin (H&E) staining of this thymic remnant revealed a poorly developed, thymus-like lymphoid tissue, lacking proper cortex zonal development (Fig. 14). The thymic remnant from \textit{RAG-2^{+/−}} \textit{IL2RG^{−y}} and \textit{RAG-2^{−y}} \textit{IL2RG^{−y}} pigs had a very similar appearance.

H&E staining of the \textit{RAG-2^{+/−}} \textit{IL2RG^{−y}} spleen revealed hypocellular periarterial lymphatic sheath within germinal center when compared to wild type spleen (Fig. 14). More importantly, the spleen from \textit{RAG-2^{−y}} \textit{IL2RG^{−y}} had hypoplastic periarterial lymphatic sheath and contained clusters of parenchymal cells, and increased areas of red pulp. Germinal centers were completely devoid of lymphocytes (Fig. 14). Finally, no lymph nodes could be detected in \textit{RAG-2^{+/−}} \textit{IL2RG^{−y}} and \textit{RAG-2^{−y}} \textit{IL2RG^{−y}} pigs.
Figure 14. Histological analysis of thymus and spleen of wild type, RAG-2+/ IL2RG+/y and RAG-2+/ IL2RG+/? pigs. Thymus from a wild type pig could be easy visualized (black arrow) while in age matched RAG-2+/ IL2RG+/y and RAG-2+/ IL2RG+/? pigs it was nearly undetectable. H&E microscopic evaluation of the thymus revealed a poorly developed thymus lacking proper cortical zone development in RAG-2+/ IL2RG+/? and RAG-2+/ IL2RG+/? pigs. H&E microscopic evaluation of the RAG-2+/ IL2RG+/? spleen showed germinal center with hypocellular periarterial lymphatic sheath surrounding central arteries (black arrows) when compared to wild type spleen. The spleen of RAG-2+/ IL2RG+/? had hypoplastic periarterial lymphatic sheath (black arrows) and contained clusters of parenchymal cells, and increased areas of red pulp. Germinal centers were devoid of lymphocytes.
2.3.4 Peripheral blood lymphoid cell population analysis of RAG-2+/IL2RG−/y and RAG-2−/IL2RG−/y pigs.

Lymphoid cell analysis of peripheral blood from wild type, RAG-2+/IL2RG−/y and RAG-2−/IL2RG−/y pigs was performed via flow cytometry at 3 weeks of age with specific antibodies for pig CD3, CD4, CD8, CD79α and Nkp46 (CD335). Analysis was performed by gating flow cytometry data within mononuclear cells (MNC). RAG-2−/IL2RG−/y pigs had undetectable levels of CD79α. RAG-2+/IL2RG−/y animals had detectable, but reduced percentages of CD79α cells (Figure 15). These differences were statistically significant (p < 0.05). The data indicate the inactivation of the RAG-2 gene in RAG-2−/IL2RG−/y pigs, while also indicating a potential role of IL2RG in B cell development in RAG-2+/IL2RG−/y, thus resembling a XSCID phenotype.
Figure 15. B cell (CD79α positive) levels in peripheral blood of wild type, RAG-2^{+/--}IL2RG^{-/y} and RAG-2^{+/--}IL2RG^{-/y} pigs at 3 weeks of age. (A). Peripheral blood flow cytometry data showing CD79α versus side scatter for wild type, RAG-2^{+/--}IL2RG^{-/y} and RAG-2^{+/--}IL2RG^{-/y} pigs at 3 weeks of age. A red box indicates CD79α positive population. (B) Histogram of % CD79α + obtained from gated MNC, data represents mean, bars indicate standard deviation (N=3); * indicates significance at p < 0.05.

Analysis of T cells populations in peripheral blood via expression of CD3, CD4 and CD8 revealed that RAG-2^{+/--}IL2RG^{-/y} and RAG-2^{+/--}IL2RG^{-/y} pigs behaved similarly, having nearly undetectable levels of CD3 +, CD3 + CD4 + (CD4 SP), CD3 + CD8 + (CD8 SP) and CD3 + CD4 + CD8 + (DP) (not statistically different) when compared to wild type controls (Fig. 16). These data reinforce the inactivation of the IL2RG gene, which is acting as a second blocker of T cell development in the presence of an RAG-2 active allele (different scenario than the one
observed for the B cell lineage). The B cell and T cell flow cytometry data are in concordance with the PCR assay for detection of IgH and TCR-β DNA rearrangement.

Figure 16. T cell levels in peripheral blood of wild type, RAG-2+/IL2RG−/y and RAG-2−/−IL2RG−/y pigs at 3 weeks of age. (A). Representative peripheral blood flow cytometry data showing CD4 versus CD8 for wild type, RAG-2+/+IL2RG−/y and RAG-2−/−IL2RG−/y pigs at 3 weeks of age. Red boxes indicate CD4 SP, CD8 SP and DP population. (B) Histogram of %CD3+ obtained from gated MNC, data represents mean, bars indicate standard deviation (N=3). (C) Histogram of CD4 SP, CD8 SP and DP obtained from gated MNC, data represents mean, bars indicate standard deviation (N=3). * indicates significance at p < 0.05.

Flow cytometric analysis of the NK cell marker Nkp46 was also carried out. The data revealed that both RAG-2+/IL2RG−/y and RAG-2−/−IL2RG−/y pigs lack detectable levels of Nkp46 +
cells in peripheral blood (Fig. 17). This finding was expected, since NK levels are influenced by \( IL2RG \) inactivation, but not by \( RAG-2 \) inactivation.

![Diagram of Nkp46 cell levels in peripheral blood of wild type, \( RAG-2^{+/IL2RG^{-/y}} \) and \( RAG-2^{-/-IL2RG^{-/y}} \) pigs at 3 weeks of age.](image)

Figure 17. Nkp46 cell levels in peripheral blood of wild type, \( RAG-2^{+/IL2RG^{-/y}} \) and \( RAG-2^{-/-IL2RG^{-/y}} \) pigs at 3 weeks of age. (A). Representative peripheral blood flow cytometry data showing Nkp46 versus side scatter for wild type, \( RAG-2^{+/IL2RG^{-/y}} \) and \( RAG-2^{-/-IL2RG^{-/y}} \) pigs at 3 weeks of age. A red box indicates Nkp46 positive population. (B) Histogram of %Nkp46 + obtained from gated MNC, data represents mean, bars indicate standard deviation (N=3). * indicates significance at \( p < 0.05 \).

The \( RAG-2^{+/IL2RG^{-/y}} \) pig model presents similar characteristics to the human and pig XSCID syndrome with presence of peripheral blood B cells, but lack of T and NK cells (Suzuki et al. 2012; Fischer et al. 1997). The \( RAG-2^{+/IL2RG^{-/y}} \) model, analogous to the mouse DKO model (Shultz et al. 2007) goes one step further, leading to the lack of peripheral blood T, B and NK
cells, and impaired lymphoid tissue development (no lymph nodes and poorly developed thymus).

2.3.5 Spleen and thymus lymphoid cell population analysis of RAG-2<sup>+/−</sup>IL2RG<sup>−/−</sup> and RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs.

To better understand the model, immunohistochemistry and flow cytometry analysis of the lymphoid organs, thymus (primary site for T cell development) and spleen (common site final maturation of B cell) was performed. Analysis via immunohistochemistry (Fig. 18) and flow cytometry (Fig. 19) from the spleen of three weeks old RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs showed no detectable levels of B cells unlike wild type age matched controls (p < 0.05). RAG-2<sup>+/−</sup>IL2RG<sup>−/−</sup> animals had detectable, but reduced, B cell levels (Fig. 18B) and reduced percentages (Fig. 19) of CD79α cells when compared to wild type (p < 0.05). This further confirms the RAG-2 deficiency, while also reflecting a role of IL2RG in B cell generation, where a marked reduction in B cells percentages was found in RAG-2<sup>+/−</sup>IL2RG<sup>−/−</sup> pigs.
Figure 18. Immunohistochemistry analysis of B cells from the spleen of wild type, RAG-2<sup>+-</sup>IL2RG<sup>-y</sup> and RAG-2<sup>-/-</sup>IL2RG<sup>-y</sup> pigs. (A) Representative immunohistochemistry data for CD79α from spleen of wild type, RAG-2<sup>+-</sup>IL2RG<sup>-y</sup> and RAG-2<sup>-/-</sup>IL2RG<sup>-y</sup> pigs at 3 weeks of age. (B) Histogram of number of CD79α + cells per high power field microscopy obtained with ImageJ analysis from spleen from each group, data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at p < 0.05.
Figure 19. Flow cytometry analysis of B cells from the spleen of wild type, RAG-2⁺/⁻IL2RG⁻/⁻ and RAG-2⁺/⁻IL2RG⁻/⁻ pigs. (A) Representative flow cytometry data showing CD79α versus side scatter for spleen of wild type, RAG-2⁺/⁻IL2RG⁻/⁻ and RAG-2⁻/⁻IL2RG⁻/⁻ pigs at 3 weeks of age. (B) Histogram of % CD79α + cells obtained from gated MNC from spleen from each group, data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at p < 0.05.

Immunohistochemistry for CD3 in the spleen showed a marked reduction of CD3 (p < 0.05) cell numbers in RAG-2⁺/⁻IL2RG⁻/⁻ and RAG-2⁻/⁻IL2RG⁻/⁻ compared to age-matched wild type; however they could still be identified in the mutant pigs (Figure 20). Flow cytometry analysis using pig specific antibodies for CD4 and CD8 was carried out to reveal the phenotype of this CD3 + cells. The flow cytometry data showed these CD3 + cells to be exclusively CD4 SP, while CD8 SP and double positive (DP) cells were lacking in both RAG-2⁺/⁻IL2RG⁻/⁻ and RAG-2⁻/⁻IL2RG⁻/⁻ pigs (Fig. 21). Spleen RAG-2⁺/⁻IL2RG⁻/⁻ and RAG-2⁻/⁻IL2RG⁻/⁻ T cell profiles
were similar, with \( RAG-2^{+/}IL2RG^{+/} \) showing an increased in cell numbers (Fig. 20) and % CD3 + cells (Fig. 21) when compared to \( RAG-2^{-/-}IL2RG^{-/-} \) \((p < 0.05)\).
Figure 20. Immunohistochemistry for CD3 in wild type, RAG-2⁺/⁻ IL2RG⁻/⁻ and RAG-2⁻/⁻ IL2RG⁻/⁻ spleen. (A) Representative immunohistochemistry data showing CD3 staining for spleen of wild type, RAG-2⁺/⁻ IL2RG⁻/⁻ and RAG-2⁻/⁻ IL2RG⁻/⁻ pigs at 3 weeks of age. A marked reduction in CD3⁺ cells is observed in RAG-2⁺/⁻ IL2RG⁻/⁻ and RAG-2⁻/⁻ IL2RG⁻/⁻ pigs, (red arrow). (B) Histogram of number of CD3⁺ cells per high power field microscopy obtained with ImageJ analysis from spleen from each group, data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at p < 0.05. Spleen from RAG-2⁻/⁻ IL2RG⁻/⁻ pigs had a distinct architecture when compared to wild type and RAG-2⁺/⁻ IL2RG⁻/⁻ due to poor developed germinal centers.
Next, analysis for the thymus T cell profile was carried out. Similarly to the spleen, immunohistochemistry for CD3 cells revealed a mark reduction of CD3 + cells in RAG-2<sup>+/−</sup> IL2RG<sup>−/−</sup> and RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> when compared to aged-matched wild type pigs (Fig. 22). Further flow cytometry analysis of thymus CD3 + cells revealed a similar picture to that observed in the spleen. RAG-2<sup>+/−</sup> IL2RG<sup>−/−</sup> and RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> thymus had significant reduction of CD3 + when
compared to aged matched wild type pigs (Fig. 23) \((p < 0.05)\). \textit{RAG-2}^{+/}/IL2RG^{-/-} \text{ and } \textit{RAG-2}^{-/-}/IL2RG^{-/-}\) thymus showed a similar profile lacking CD8 SP and DP cells compared to aged matched wild type pigs (Fig. 23).

\textbf{Figure 22. Immunohistochemistry for CD3 in thymus of wild type, RAG-2}^{+/}/IL2RG^{-/-} \text{ and } \textit{RAG-2}^{-/-}/IL2RG^{-/-} \text{ pigs.} \text{ (A)} \text{ Representative immunohistochemistry data showing CD3 staining for thymus of wild type, RAG-2}^{+/}/IL2RG^{-/-} \text{ and } \textit{RAG-2}^{-/-}/IL2RG^{-/-}\) pigs at 3 weeks of age. A marked reduction in CD3 + cells is observed for \textit{RAG-2}^{+/}/IL2RG^{-/-} \text{ and } \textit{RAG-2}^{-/-}/IL2RG^{-/-}\), but could still be detected in both lines. \text{ (B)} \text{ Histogram of number of CD3 + cells per high power}
field microscopy obtained with ImageJ analysis from spleen from each group, data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at p < 0.05.

The spleen and thymus data combined reveal that:

1) The CD3 population was affected in both RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) and RAG-2\(^{+/-}\) IL2RG\(^{-/-}\) pigs. The spleen and thymus of the RAG-2\(^{+/-}\) IL2RG\(^{-/-}\) pigs had highly reduced levels of CD3 + cells compared to wild type (p < 0.05); Fig 20 and 22). In addition the lack of RAG-2 also affected the levels of CD3 in both spleen and thymus (p < 0.05); Fig 20 and 22).

2) Levels of both CD8 SP and DP cells were significantly affected by the IL2RG inactivation but not by the RAG-2 inactivation (p < 0.05; Fig 21 C-D and Fig 23 C-D) supporting the overriding effect of the IL2RG mutation on T cell differentiation.

3) The reduced CD3 + cells observed in the spleen and thymus of RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) and RAG-2\(^{+/-}\) IL2RG\(^{-/-}\) pigs had an exclusive CD4 SP profile, while not differing between each other (p < 0.05); Fig 21E and 23E).

These data suggest that in the presence an active RAG-2 allele and the absence of an active IL2RG gene, there is presence of CD3 + cells (Fig. 20 and 22), but the inactive IL2RG gene still prevents CD3 + cells to further develop into CD8 SP and DP cells, both pathways of T cell development dependent on IL2RG subunit stimulation (IL-7 and IL15) (Ellmeier et al. 2013).
Figure 23. T cell levels (CD3, CD4, CD8) in the thymus of wild type, RAG-2<sup>−/+</sup>IL2RG<sup>y</sup>, and RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs. (A) Representative flow cytometry data showing CD4 versus CD8 from gated CD3<sup>+</sup> cells for thymus of wild type, RAG-2<sup>−/+</sup>IL2RG<sup>y</sup> and RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs at 3 weeks of age. (B-E) Histogram of each cell population (%) obtained from gated MNC from thymus from each group. Histogram data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at \( p < 0.05 \).

Next, the expression profile of Nkp46<sup>+</sup> cells within the spleen of RAG-2<sup>−/+</sup>IL2RG<sup>y</sup>, RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs and age matched controls was examined. As shown in Fig. 24, Nkp46<sup>+</sup> cells were detected in both mutant lines but in reduced levels when compared to wild type controls \( (p < 0.05) \). In contrast, flow cytometry data showed an increased percentage of Nkp46<sup>+</sup> cells in the mutants when compared to wild type control (Fig. 25). The IHC data (Fig. 24) is in concordance with the peripheral blood data where Nkp46<sup>+</sup> cells were markedly reduced in both
mutant lines (Fig 17). The IHC also revealed a different distribution pattern of Nkp46 + cells in the spleen of RAG-2<sup>−/−</sup>IL2RG<sup>+/−</sup>. In contrast, the distribution pattern of RAG-2<sup>+/−</sup>IL2RG<sup>+/−</sup> resembles wild type spleen. This is most likely due to the abnormal appearance of the spleen in RAG-2<sup>−/−</sup>IL2RG<sup>+/−</sup> with poor developed germinal centers (Fig. 5). Despite the different distribution, RAG-2<sup>+/−</sup>IL2RG<sup>+/−</sup> and RAG-2<sup>−/−</sup>IL2RG<sup>+/−</sup> had similar spleen Nkp46 + cell numbers as demonstrated by IHC (Fig. 24).

When expressed as percent of mononuclear cells, the flow cytometry data revealed the spleen of RAG-2<sup>+/−</sup>IL2RG<sup>+/−</sup> and RAG-2<sup>−/−</sup>IL2RG<sup>+/−</sup> to have increased levels of Nkp46 + cells when compared to wild type controls. However, this effect could be due to the lack of T cells, and reduced levels of B cells in both mutant lines, thus reducing the overall numbers of mononuclear cells. This would result in increased % of Nkp46 + cells within gated MNC. However, the IHC data confirmed reduced levels of spleen Nkp46 + cells.
Figure 24. Immunohistochemistry analysis of Nkp46 + cells from the spleen of wild type, RAG-2\(^{+/−}\)IL2RG\(^{−/−}\) and RAG-2\(^{+/−}\)IL2RG\(^{−/−}\) pigs. (A) Representative immunohistochemistry data showing Nkp46 staining for spleen of wild type, RAG-2\(^{+/−}\)IL2RG\(^{−/−}\) and RAG-2\(^{+/−}\)IL2RG\(^{−/−}\) pigs at 3 weeks of age. (B) Histogram of number of Nkp46 + cells per high power field microscopy obtained with ImageJ analysis from spleen from each group, data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at p < 0.05.
Figure 25. Flow cytometry analysis of Nkp46 + cells from the spleen of wild type, RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) and RAG-2\(^{-/-}\)IL2RG\(^{-/-}\) pigs. (A) Representative flow cytometry data showing Nkp46 versus side scatter for spleen of wild type, RAG-2\(^{-/-}\)IL2RG\(^{-/-}\) and RAG-2\(^{-/-}\)IL2RG\(^{-/-}\) pigs at 3 weeks of age. (B) Histogram of % Nkp46 cells obtained from gated MNC from spleen for each group, data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at \(p < 0.05\).

2.4 DISCUSSION.

The pig represents an attractive large animal model for the development of a SCID model since it more closely resembles humans regarding anatomy, hematology, physiology, size, and longevity. Thus, they are considered as important laboratory animal models for biomedical research, especially for tissue engineering and human organ transplantation involving xenografts.
The similar anatomical size also provides a unique opportunity for the development and investigation of innovative approaches for immunodeficiency therapy via stem autologous/allogeneic stem cell transplantation or gene therapy.

While $IL2RG^{+/y}$ and $RAG1/2^{-/-}$ transgenic pigs have been described previously (Suzuki et al. 2012; Huang et al. 2014; Choi et al. 2014), a $RAG-2^{-/-} IL2RG^{+/y}$ pig model has not been previously described. This model would represent a new platform for the engraftment of human stem cells of various sources, while also allowing the study of autologous/allogeneic immune reconstitution into a such complex immunodeficient phenotype.

The $RAG-2^{-/-} IL2RG^{+/y}$ pig model described here exhibit characteristics of a NOD/SCID/$IL2RG^{null}$, NOG, and $RAG-2^{null}/IL2RG^{null}$ mice (Ishikawa et al. 2005; Traggiai et al. 2004), by having; impaired lymph node development, thymus lacking cortical zone development (Fig. 14), and no evidences of IgH and TCR-β rearrangement (Fig. 13). These animals also lacked peripheral blood B, T and NK cells (Fig. 15-17). A $RAG-2^{+/-} IL2RG^{+/y}$ model that served as a control for the $RAG-2$ mutation was also described. When one $RAG-2$ allele was active, B cell development was evident in the $IL2RG^{+/y}$ background (and IgH rearrangement) (Fig. 13, Fig. 15, Fig.18, Fig. 19), although B cell levels were reduced in peripheral blood and spleen of these animals when compared to aged matched wild type. This was also confirmed via IHC for CD79α cells within the spleen (Fig. 18). These data indicate a role of $IL2RG$ in B cell development in the pig, although it’s absence does not completely block this cell lineage maturation. This is consistent with previous results (Choi et al. 2014), suggesting a role of $IL2RG$ in pig B cell development.

Despite B cell development in $RAG-2^{+/-} IL2RG^{+/y}$, it was determined that $RAG-2^{+/-} IL2RG^{-/-}$ and $RAG-2^{-/-} IL2RG^{+/y}$ behave similarly regarding T cell phenotype in the blood (Fig. 8), spleen
CD8 SP and DP cells were absent from RAG-2+/− IL2RG/y and RAG-2−/− IL2RG/y pigs. These data support the role of IL2RG in T-cell development, since CD8 SP cell development depends on IL-7 and IL-15 stimulation (Ellmeier et al. 2013).

Perhaps the most surprising finding was that nearly all CD3+ cells observed in RAG-2+/− IL2RG/y and RAG-2−/− IL2RG/y spleen and thymus were CD4 SP cells, (Fig. 21, Fig. 23). This distribution pattern was similar to Nkp46 positive cells seen by flow cytometry and IHC. Nkp46+ cells were present in the spleen of RAG-2+/− IL2RG/y and RAG-2−/− IL2RG/y pigs (Fig. 24, Fig. 25), while absent in peripheral blood (Fig. 17). These novel observations were not described previously in the IL2RG/y pig with reported absence of NK cells in all tissues examined (Suzuki et al. 2012a). However, Suzuki et al. (2012a) examined NK cells using expression of CD16. The CD16 is a Fc receptor present in NK cells (Romee et al. 2013). CD16 is also present in pig dendritic cells (Piriou-guzylack & Salmon 2008), making it not an exclusive NK marker. Nkp46, in contrast, is highly specific for NK cells, as it is part of the natural cytotoxicity receptors (NCRs) family (Bo et al. 2011).

Co-expression of Nkp46+ cells and CD3 CD4 are phenotypic features of Natural Killer T cells (NKT). NKT cells are a specialized subset of T cells that share surface markers with the NK cells. Type-II NKT cells comprise CD3 + CD4+, and appears to share receptors of the cluster of NK cell markers, including receptors of the C-lectin Ly49 and NKR-families (Godfrey et al. 2010; Lantz et al. 1997). Although NKT cells are present in the thymus, the developmental origin of these cells is controversial. The intrathyhmic development of NKT cells depends on CD1d expression by DP thymocytes rather than thymic epithelial cells (Godfrey et al. 2010; Coles & Raulet 2000). Some investigators have argued that NKT cells are present in thymus-
deficient nude mice or in bone marrow–repopulated, thymectomized adult mice (Pellicci et al. 2002). Furthermore, Lantz et al. (1997) demonstrated that NKT cells appeared to partly develop in IL2RG deficient mouse thymus. A fraction of these NKT cells have also been demonstrated to express NKp46 in mouse and human (Bo et al. 2011). NKT cells are often known for their immunosuppressive properties (Coles & Raulet 2000). In pigs it has been shown that inactivation of the CD1d gene results in complete absence of NKT cell in all organs tested (Yang et al. 2015). Based on these observations I hypothesized that detectable CD4 SP cells and Nkp46 + cells in both mutant lines could represent a subset of NKT cells. This question will be addressed in more detail in Chapter 3 utilizing the cell tracking model described in Chapter 1.

In summary, in this Chapter I described a RAG-2+/− IL2RG+/− and RAG-2−/− IL2RG−/− pig model, with RAG-2−/− IL2RG−/− pigs exhibiting characteristics of NOD/SCID/IL2RG-null mice (lack of peripheral blood lymphocytes and poor lymphoid tissue development). Next, in Chapter 3 I investigate the ability of this model to sustain allogeneic and xenogeneic stem cells engraftment via in utero hematopoietic stem cell transplantation.

2.5 REFERENCES


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CHAPTER 3: ALLOGENEIC AND XENOGENEIC IN UTERO HEMATOTOPOIETIC STEM CELL TRANSPLATATION (IUHCT) OF RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> PIGS.

3.1 INTRODUCTION.

In humanized SCID or DKO mice hematopoietic stem cell transplantation is normally done postnatally after some form of treatment that enhances engraftment. Normally this is done by radiation conditioning where the host bone marrow cells are partially ablated enhancing the engraftment of the incoming cells. (Holtick et al. 2015; Lang et al. 2013). In previous experiments in Dr. Piedrahita’s lab it had been determined that postnatal engraftment, even after radiation conditioning, was a difficult and inefficient method of engrafting either allogeneic and xenogeneic cells in pigs. As a result, IUHCT was examined as an alternative method. The potential advantages of IUHCT over postnatal transplantation are largely based on the immunologic immaturity of the early gestational fetus, providing the opportunity for induction of donor-specific tolerance to allogeneic and even xenogeneic hematopoietic stem cells. This method had been used previously in both sheep and pigs with promising results even when using xenogeneic cells as will be discussed in detail below. In pigs, the injections are done at gestation
day 42. As animals are born approximately 70 days later this provides a significant amount of
time for cells to engraft and proliferate before the animals are exposed to infectious agents.

Human IUHCT is a nonmyeloablative alternative to hematopoietic stem cell
transplantation for congenital hematologic disorders that can be diagnosed early in gestation
(Merianos et al. 2008; Vrecenak & Flake 2013). This is because in humans, levels of
engraftment after postnatal allogeneic stem cell transplantation have been below what might be
expected to be therapeutic for most hematologic diseases, and clinical success has been limited
to X-linked severe combined immunodeficiency (XSCID) (Merianos et al. 2008). This has been
largely attributed (as demonstrated in certain immunodeficient mice strains) to niche competition
between host and donor niche stem cells, with the first having an advantage over transplanted
donor stem cells (Waldschmidt et al. 2002; Blazar et al. 1995).

Currently, the most popular large animal model of IUHCT is the sheep, where early
gestational transplantation of allogeneic hematopoietic stem cells into wild type sheep fetuses
results in sustained multilineage hematopoietic chimerism (Flake et al. 2017). The fetal sheep
model is also permissive for xenogeneic engraftment, as multilineage hematopoietic chimerism
has been documented after transplantation of human hematopoietic stem cells. However, the
reported degree of engraftment is very low, usually below 1%, making this an impractical model
(Zanjani et al. 1992; Zanjani et al. 1994). IUHCT has also been successfully performed in
immunocompetent pig fetuses (Fujiki et al., 2003; Ogle, Knudsen, Nishitai, Ogata, & Platt, 2009)
with similar results to those obtained in sheep.

To date, however, IUHCT of hematopoietic stem cell has never been explored in an
immunodeficient pig model. Moreover, combining the RAG-2\(^{-}\) /IL2RG\(^{+}\) pig and IUHCT of
allogeneic pH2B-eGFP hematopoietic stem cell provides a unique opportunity for precise
analysis of the ability of allogeneic hematopoietic stem cells to engraft the different lymphoid compartments. This would allow detailed assessment of chimerism via different experimental techniques (Flow cytometry, immunofluorescence), without the need for analysis of different SLA surface molecules to differentiate donor and host cells.

In addition to allogeneic IUHCT, the \textit{RAG-2\textsuperscript{-/-}IL2RG\textsuperscript{+/y}} model described here would serve as an attractive candidate model for IUHCT with human CD34 + cells (xenogeneic engraftment). Human hematopoietic stem cells require a specific niche containing defined surface-surface molecule interaction and cytokine stimulation. Currently, assessment of hematological stem cell potential (functional analysis) is measured via in vitro assays such as colony formation assay and long-term culture (Eaves et al. 1989). However these assays have limitation as they do not examine engraftment or long term proliferation and differentiation ability. IUHCT of human CD34 + cells into \textit{RAG-2\textsuperscript{-/-}IL2RG\textsuperscript{+/y}} would potentially result in a model that can used not only for studying the hematological ability of human hematopoietic stem cells in a pig model, but also as a potential bio reactor for expansion of human CD34 + cells. Furthermore, human T cell lineage differentiation can lead to human T cell selection in a pig thymus environment, which would lead to the development human T regulatory (Treg) cells capable of tolerating pig antigens. These cells would have tremendous clinical value since they could lead to pig organ xenograft tolerance when transplanted into humans.

In this chapter, I describe allogeneic and xenogeneic IUHCT of \textit{RAG-2\textsuperscript{-/-}IL2RG\textsuperscript{+/y}} pigs. IUHCT of allogeneic pH2B-eGFP hematopoietic stem cells was carried out to demonstrate the ability of this model to sustain engraftment of SLA mismatched lymphoid tissue engraftment for B and T cells (missing niches), while also assisting with identification/correlation of CD3 + CD4 + and Nkp46 + cells. IUHCT of human CD34 + cells were also performed to explore the ability
of this model to sustain xenogeneic engraftment. Finally, I contrasted allogeneic and xenogeneic engraftment of lymphoid tissue (thymus, spleen) of \textit{RAG-2}^{-/-} \textit{IL2RG}^{-/-} pigs to identify additional pig innate barriers to long-term xenogeneic engraftment.

3.2 MATERIALS AND METHODS

3.2.1 SLA typing of donor cells and host \textit{RAG-2}^{-/-} \textit{IL2RG}^{-/-} line.

In human Histocompatibility testing between unrelated donors and recipients prior to hematopoietic stem cell transplantation includes five human leukocyte antigen (HLA) loci: HLA-A, -B, -C, -DRB1, and -DQB1. Optimal transplantations are defined as 10/10, perfect match between the critical 5 HLA locus cited above are shared between donor and recipient. The 8/8 refers to high-resolution matching at the four loci HLA-A, -B, -C, and -DRB1. Minimum requirements for adult bone marrow and peripheral blood mobilized cells transplantation are 6 of 8 loci match for HLA-A, -B, -C and DRB1 (Spellman et al., 2012). In addition, the presence of donor antigens or alleles not shared by the recipient determines host versus graft allore cognition, while the presence of recipient alleles or antigens not shared by the donor determines graft versus host allore cognition, both individual scenarios are classified as unidirectional mismatch and may lead to failure of engraftment (Petersdorf, 2016). Both scenarios may occur simultaneously between a single donor and recipient (each one having a allele that the other one does not have), leading to a bidirectional mismatch and failure of engraftment (Petersdorf, 2016).

There are seven classical class I genes and three non-classical class I genes mapped to the SLA complex. The constitutively expressed classical SLA class I genes are SLA-1, SLA-2 and SLA-3, while the rest are pseudogenes. SLA class II loci in the pig include DRB1, DQA, DQB1, DOB1, DMB, DMA and DOA (Gao et al., 2014). To ensure that the allogeneic transplants were
done across different swine leukocyte antigen complex (SLA) haplotypes, both the pH2B-eGFP hematopoietic stem donor cells and the recipient \( RAG-2^{-/-} IL2RG^{-/-} \) line were typed via a previously described PCR assay using site specific primers (PCR-ssp) (Ho et al. 2010) (Gao et al. 2014; Martens et al. 2003). The assay included primers specific for identification of pig MHC-I pig loci SLA-1, SLA-2 and SLA-3, and MHC-II pig loci DRB1, DQB1 and DQA (Fig. 26), therefore testing the pig SLA analogues to classical HLA regulating the success of hematopoietic stem cell transplantation (Petersdorf, 2016).

3.2.2 Allogeneic IUHCT of pH2B-eGFP fetal liver hematopoietic stem cells into \( RAG-2^{-/-} IL2RG^{-/-} \) fetuses.

Forty-two days pH2B-eGFP fetal liver hematopoietic were isolated using a previous reported protocol for isolation of fetal liver hematopoietic cells (Bility et al. 2012). Briefly, 42 days pH2B-eGFP fetal male liver (3 separate donors) were minced and digested in liver digest medium (Gibco # 17703034), and washed with IMDM (Gibco # 12440053). Fetal liver parenchymal and hematopoietic stem cells were separated by density centrifugation and hematopoietic stem cells cryopreserved in in Hanks' Balanced Salt Solution (HBSS) containing 5% DMSO, 0.3M Sucrose and no calcium/magnesium. At day 42 of gestation a wildtype sow carrying a transgenic \( RAG-2^{-/-} IL2RG^{-/-} \) pregnancy was prepared for surgery using the surgical protocol for somatic cell nuclear transfer. pH2B-eGFP fetal liver cells were thawed and ran through a live dead magnetic isolation system (Miltenyibiotec # 130-090-101) using LS Columns (Miltenyibiotec # 130-042-401) placed in a QuadroMACS Separator (Miltenyibiotec #130-090-976). Approximately 3.5 \( \times \) 10^6 viable pH2B-eGFP fetal liver hematopoietic cells resuspended in 30 µL of PBS containing 5mg/ml Penicillin-G (Sigma # P7794-10MU) were sterile injected into
the liver of 42 days developing \( RAG-2^{-/-} IL2RG^{+/y} \) transgenic fetuses (N=4) (Table 1). In utero transplantation was performed sterile via ultra sound guided (Aloka) procedure, using a PAN chiba (25 gauge X 6 cm) cytological aspirating needle (Gallini Medical Devices ref# PA25-6).

3.2.3 Xenogeneic IUHCT of human CD34 + cells into \( RAG-2^{-/-} IL2RG^{+/y} \) fetuses.

The same procedure described above was utilized to deliver human CD34 + cells into the liver of 42 days \( RAG-2^{-/-} IL2RG^{+/y} \) developing fetuses. Peripheral blood mobilized stem cells (PBMC) from a male adult donor were utilized as the source of stem cells. Frozen PBMC were thawed and washed in autoMACS Rinsing Solution (Miltenyibiotec #130-091-222) containing MACS BSA Stock Solution (# 130-091-376) at 1:20 dilution. Mononuclear cells were isolated via centrifugation in Ficoll-Paque PLUS (GE Healthcare #71-7167-00 AG). Mononuclear cells were properly incubated with CD34 MicroBead Kit -UltraPure (Miltenyibiotec #130-100-453), and CD34 + human cells isolated with magnetic system described above for allogeneic fetal liver hematopoietic isolation. Xenotransplantation procedure and management of xenografted pigs was performed at an approved facility within the College of Veterinary Medicine under proper guidelines in concordance with IACUC protocols. Approximately \( 7 \times 10^6 \) viable CD34 + human cells were injected into the liver of 42 days developing \( RAG-2^{-/-} IL2RG^{+/y} \) transgenic fetuses, using the same approach described above (N=4). Column enrichment was confirmed via flow cytometry by staining pre and post column separation cells with anti-human CD34-PE, (Miltenyibiotec # 130-081-002). Genomic DNA from human cells was isolated and sexing confirmed by PCR to amplify the human ALT1 gene from X chromosome (5’-

CCCTGATGAAGAACCTTGATCTC - 3’ and 5’- GAAATTACACACATAGGTG GCCACT-
3'), and the human SRY from the Y chromosome (5'-CATGAACGCATTCATCGTGTGGTC-3’ and 5’-CTGCAGGAAGCAATGCATCTT-3’). PCR conditions were 98°C for 1 min; 35 cycles (98°C for 10 s, 58°C for 10 s, 72°C for 8 s), 72°C for 1 min.

3.2.4 PCR assay for detection of human TREC recombination.

To demonstrate human TCR rearrangement in the thymus of xenotransplanted RAG-2−/−IL2RG−/− pigs, previous reported primers (Al-harthi et al. 2000) were used to amplify the δRec-ΨJα segment of the human coding joint T cell receptor excision circle (TREC). This extrachromosomal DNA circle indicates proper δRec-ΨJα recombination. Amplification of the IL2RG locus was used as control for DNA loading. Ten ng of gDNA from thymus and blood were used as templates, thymus of a RAG-2−/−IL2RG−/− noninjected pig served as negative control, while gDNA from donor cells used for xenoengraftment was used as positive control. PCR conditions for δRec-ΨJα were 98°C for 1 min; 35 cycles (98°C for 10 s, 69°C for 10 s, 72°C for 15 s), 72°C for 1 min.

3.2.5 Histological and immunofluorescence analyses.

Immunofluorescence analysis of human cells, fresh OCT embedded thymus and spleen were immunostained with mouse anti-human CD45 PE-CF594 labeled (BD #562279), mouse anti-human CD19-FITC (BD #564456) and mouse anti-human CD335-FITC (BD#564536). Detection of pig macrophage/neutrophil infiltrates was performed by staining thymus and spleen of allografted and xenografted animals with mouse anti-pig monocyte/granulocyte antibody (SouthernBiotech #4525-01) conjugated with Readilink 350/440 antibody labeling kit (Biorad #135-1001). Membrane attack complex (MAC) was detected using mouse anti-C5b-C9
(Invitrogen # DIA 011-01-02) conjugated with Readilink 350/440 antibody labeling kit (Biorad #135-1001).

3.2.6 Flow cytometry and fluorescence activated cell sorting.

For the detection of human lymphocytes from peripheral blood, thymus, spleen and bone marrow of xenotransplanted pigs mouse anti-human CD45-PE-CF594 (BD #562279), for T cell detection, mouse anti-human CD3 PE labeled (BD # 555333), mouse anti-human CD8 APC labeled (BD # 340584) and mouse anti-human CD4 FITC labeled (BD# 561842) were used. For detection of human B cells, mouse anti-human CD19 FITC labeled (BD #564456), for human NK cells mouse anti-human CD335 FITC labeled (BD#564536), and mouse anti-human CD14 PE labeled (BD#562691) were used. All flow cytometry samples were incubated for 1h at 5°C in stain buffer (BD#554657), followed by red blood lysis and fixation with lysing solution (BD#349202) and washing with PBS. All flow data was gated and displayed as percentage from mononuclear cells (MNC).

3.3 RESULTS

3.3.1 Allogeneic IUHCT of pH2B-eGFP labeled hematopoietic stem cells into RAG-2⁻/⁻IL2RG⁻/⁻ fetuses.

3.3.1.1 SLA typing of donor cells and host RAG-2⁻/⁻IL2RG⁻/⁻ line.

SLA typing of donor and recipients demonstrated transplants to be mostly bidirectional mismatches (0/8) for donor 1 and 2, with only one allele being shared (SLA-3 03xx) and mostly unidirectional mismatch (0/8) for donor 3 with 7 alleles being common shared (SLA-1 04xx, SLA-2 04xx, SLA-3 03xx, 04xx, DRB1-07xx, DQB1 02xx, 09xx). Only one perfect haplotype
match was observed for DQA between donor 3 and recipients. Therefore all donors were classified as poor hematopoietic stem cells donors using the classifications described above (Fig. 26). Three viable RAG-2/ IL2RG-Δ pigs were euthanized within 1 week of life for analysis of lymphoid organs.

Figure 26. SLA typing for porcine MHC-I and MHC-II haplotypes of pH2B-eGFP pig donors and RAG-2/ IL2RG-Δ host line. (A) Results for RAG-2/ IL2RG-Δ line, and all three pH2B-eGFP (Donors 1, 2 and 3). Red boxes indicate the MHC-I (SLA-1, SLA-2, SLA-3) and MHC-II (DRB1, DQB-1, and DQA) PCR reactions with different primers. An internal control (porcine α-ACTIN, 516 bp) is present in all reactions. Each well also contains a pair of primers specific for the multicopy alleles for each sub-haplotype group. The presence of a smaller band than α-ACTIN indicates a positive signal for the corresponding MHC allele. (B) Haplotype summary for donors and recipients. The only perfect match was between donor 2 and recipient for the DQA haplotype, while other haplotypes differed between recipients and donor.
3.3.1.2 Analysis of B cell population in spleen of allografted *RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs.

B cell engraftment was assessed via immunohistochemistry, flow cytometry and PCR detection of a rearranged IgH locus. The B cell receptor (BCR) complex is composed of membrane immunoglobulin (Ig) heavy and light chain with heterodimers of CD79α (Igα) and CD79β (Igβ), and is expressed in the surface of B cells (Cho et al. 2007). CD79α is routinely used for identification of B cells. The pH2B-eGFP positive donor cells could be easily identified under GFP fluorescence microscopy analysis and were absent from aged matched wild type spleen and noninjected *RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs (Fig. 27). The presence of CD79α was also confirmed by conventional immunohistochemistry, and allografted pigs displayed increased CD79 α when compared to a noninjected animal (Fig. 27). As expected IgH rearrangement was detected in the spleen of allografted pigs (Fig 27).
Figure 27. Spleen immunostaining of B cells (CD79α) and IgH locus rearrangement analysis of wild type and allografted RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs at 1 week of age. (A) Wild type spleen contains CD79α positive cells, but not pH2B-eGFP positive cells. Spleen of noninjected RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pig has neither CD79α nor pH2B-eGFP positive cells. The spleen of an allografted RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pig with CD79α positive cells, which all overlap with pH2B-eGFP cells indicating donor origin. Note that the number of pH2B-eGFP positive cells appears higher than the CD79α positive cells, indicated non-B cell allografted cells are also present. (B) Conventional immunohistochemistry for CD79α from three allografted pigs. (C) PCR assays for
the identification of the rearranged IgH locus from spleen gDNA. Noninjected RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pig’s spleen served as negative controls, and a wild type as positive control. All three allografted pigs displayed the IgH locus rearrangement. CD79α (red), and pH2B-eGFP (green). (D) Histogram of number of CD79α + cells per high power field microscopy obtained with ImageJ analysis from thymus of a noninjected RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pig and the three RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs that receive IUHCT.

Flow cytometry analysis for CD79α was also carried out for spleen of allografted RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs to examine the presence of B cells and the level of donor/host chimerism using pH2B-eGFP to identify the donor cells. A CD79α positive population was identified in the spleen of RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) allografted pigs (Fig. 28). Gating on CD79α positive cells, followed by pH2B-eGFP analysis revealed that over 90% of the CD79 positive cells were of donor origin (Fig. 28). Together these results not only supported the lack of proper B cell lineage differentiation in RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs, but also demonstrated that IUHCT can be used to obtain SLA mismatched B cell engraftment.

Figure 28. Flow cytometry analysis of B cells from spleen of allografted RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs. (A) Representative flow cytometry data from spleen of allografted RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pig. A red box indicates the CD79α positive cells within the spleen. A gate was created on CD79α positive cells (indicated by red arrow) followed by analysis of GFP expression. (B) Histogram showing percentage of CD79α positive cells from the spleen of all 3 RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs, while a wild type serves as positive control. Green histogram represents the levels of GFP.
chimerism, indicating the percentage of GFP positive (donor origin) from the CD79α positive population.

3.3.1.3 Analysis of thymus T cell engraftment in RAG-2$^{-/-}$ IL2RG$^{+/y}$ pigs.

To examine the reconstitution of the developmental niche responsible for development of T cells, thymus T cell engraftment was assessed via immunohistochemistry, flow cytometry and PCR detection of rearranged TCR-β locus. As for B cells, allogeneic T cell engraftment was confirmed by detection of pH2B-eGFP and staining the thymus of a RAG-2$^{-/-}$ IL2RG$^{+/y}$ pig for CD3. The pH2B-eGFP positive cells were easily identified (Fig. 29), and supported robust engraftment. In contrasts, pH2B-eGFP positive cells were absent from the thymus of aged-matched noninjected RAG-2$^{-/-}$ IL2RG$^{+/y}$ pigs (Fig. 29). Immunohistochemistry for CD3 showed increase numbers of CD3 + cells in all allografted pigs when compared to a not injected pig (Fig. 29). In addition, TCR-β rearrangement was detected in the thymus of allografted pigs (Fig. 29).
Figure 29. Immunostaining of thymus T cells (CD3) and TCR-β locus rearrangement analysis of wild type and allografted RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs. (A) Wild type thymus contains CD3 positive cells, but no pH2B-eGFP positive cells. Thymus of noninjected RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs contains neither CD3 nor pH2B-eGFP positive cells. The thymus of allografted RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs contain CD3 positive cells, all overlapping with pH2B-eGFP cells indicating that they are of donor origin. (B) Immunohistochemistry staining for CD3 from the thymus of allografted RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs. CD3 positive cells are present in all allografted pigs, while markedly reduced in a noninjected pig as had been previously discussed in Fig. 22 where CD3
cells where seen in null pigs. (C) Identification of rearranged TCR-β locus in thymus gDNA. Noninjected RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pig thymus served as negative control, and wild type pig as positive control. All allografted pigs displayed TCR-β locus rearrangement. Immunofluorescence staining for CD3 (blue), and pH2B-eGFP (green). (D) Histogram of number of CD3 + cells per high power field microscopy obtained with ImageJ analysis from thymus of a noninjected RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pig and the three RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs that receive IUHCT.

Flow cytometry analysis of co-expression of CD3 and pH2B-eGFP revealed greater than 90% of CD3 + cells to co-express pH2B-eGFP. This was also the case for CD8 SP and DP cells (Fig. 30), However, I observed that allografted pigs contained variable mixed chimerism (host-donor) for CD4 SP cells. This confirmed our previous observation that the thymus of RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) contained a CD3 population of CD4 SP cells. Allografted animals, therefore contained a mixture of host and donor CD3 and CD4 SP cells (Fig. 20).

Figure 30. Flow cytometry analysis of T cell subtypes from thymus of allografted RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs. (A) flow cytometry data from the thymus of allografted RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) pigs,
showing the presence of CD4 SP, CD8 SP, DN and DP population. (B) Representative flow cytometry data gated for CD3 + and pH2B-eGFP, showing greater than 90% of the CD3 cells are of donor origin. (C-D) Representative flow cytometry data for CD8 SP/pH2B-eGFP, and DP/pH2B-eGFP showing all detectable cells were of donor origin. (E) Representative flow cytometry data for CD4 SP/pH2B-eGFP, showing mixed/variable chimerism with both host and donor cell detected in all allografted animals. (F-I) Histogram showing percentages and chimerism of CD3 + cells (F), CD8 SP cells (G), DP cells (H) and CD4 SP cells (I) from thymus of RAG-2⁻/⁻ IL2RG⁻/⁻ allografted pigs, while a wild type serves as positive control. Histogram represents the proportion host/donor cells from the respective population.

To investigate if the CD4 SP population consisted of NKT cells, CD4 SP cells from the thymus and spleen of allografted pigs were stained with anti-Nkp46 antibody. As shown in Fig 31 these cells were indeed NKT cells and were both of host and donor origin supporting that the RAG-2⁻/⁻ IL2RG⁻/⁻ pigs can generate NKT cell in the absence of both RAG-2 and IL2RG. Additionally, I was able to demonstrate that all T helper cells (CD4 SP, Nkp46 –) were of donor origin (Fig. 31). This pattern was identified in the allografted thymus and spleen of RAG-2⁻/⁻ IL2RG⁻/⁻ pigs (Fig. 31). This observation clarifies that all CD4 SP cells observed in RAG-2⁻/⁻ IL2RG⁻/⁻ pigs represent NKT cells. While NKT cells have been demonstrated in IL2RGnull mice (Lantz, Sharara, Tilloy, Andersson, & DiSanto, 1997) this is the first time this cellular phenotype has been demonstrated in a pig severe combined immunodeficient model.
Figure 31. Flow cytometry analysis of CD4 SP cells for co-expression of Nkp46 in allografted RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pig's thymus and spleen. Flow cytometry data of 3 allografted thymus (A) and spleen (B) showing gated CD4 SP analysis for co expression of Nkp46. Host CD4 SP (GFP -) cells express Nkp46, confirming their NKT phenotype (CD4 SP Nkp46 + GFP -). Note also the presence of donor NKT (CD4 SP Nkp46 + GFP +). Importantly, CD4 SP Nkp46 - (T-Helper cells) are all GFP + (donor origin), where T helper cells from the host are absent.
3.3.1.4 Analysis of Nkp46 cell engraftment in spleen of RAG-2<sup>-/-</sup> IL2RG<sup>+/y</sup> pigs.

Analysis of Nkp46 expression from the spleen of RAG-2<sup>-/-</sup> IL2RG<sup>+/y</sup> pigs was also carried out via immunohistochemistry and flow cytometry. RAG-2<sup>-/-</sup> IL2RG<sup>+/y</sup> allografted pigs contained increased overall numbers of Nkp46 + cells when compared to a noninjected RAG-2<sup>-/-</sup> IL2RG<sup>+/y</sup> pig (Fig. 32). While Nkp46 + cells were identified via immunohistochemistry, the levels of donor-derived Nkp46 + cells were below 20% for all allografted pigs (Fig. 32). This low donor-derived Nkp46 cells was expected, since RAG-2<sup>-/-</sup> IL2RG<sup>+/y</sup> spleen contained Nkp46 + cells (Fig. 24). This limited engraftment was also observed for the CD4 SP population, since RAG-2<sup>-/-</sup> IL2RG<sup>+/y</sup> pigs also contained these cells within the spleen (Fig. 23). As for the thymus, I demonstrated the presence of host NKT cells, donor NKT cells, and donor T helper cells, while host T helper cells were absent (Fig 31).

![Figure 32. Spleen immunohistochemistry and flow cytometry analysis of Nkp46 cells from allografted RAG-2<sup>-/-</sup> IL2RG<sup>+/y</sup> pigs.](image)
spleen of 3 allografted RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs, Nkp46 positive cells are present in all 3 allografted spleens, while also present in the noninjected pig. (B) Histogram of number of Nkp46 + cells per high power field microscopy obtained with ImageJ analysis from spleen of a noninjected RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pig and the three RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pig that receive IUHCT. (C) Representative flow cytometry data of Nkp46 and CD4 SP, CD8 SP and DP (gated CD3 + cells) from spleen of allografted RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs. A red box indicates the Nkp46 positive cells within the spleen, T cell subtypes are also indicated. A gate was created on Nkp46 positive cells (indicated by red arrow) and CD4 SP, followed by analysis of GFP expression analysis. (D-E) Histogram showing percentage of Nkp46 (D) and CD4 SP (E) positive cells from the spleen of all 3 RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs, while a wild type served as positive control. Green histogram represents the levels of GFP chimerism, indicating the percentage of GFP positive (donor origin) from the gated positive population.

3.3.1.5 Summary of allogeneic IUHCT.

Our results demonstrate that IUHCT of RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs sustain allogeneic engraftment of SLA mismatched hematopoietic stem cells. All 3 donors were classified as poor donors, since a perfect match was not observed for SLA-1, 2, 3 and DRB1 and DQB1. This approach allows for future in vivo experiments investigating the immune cell interactions between cells of different of SLA groups. The lack of B cell, CD8 SP and DP cells in RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs allowed substantial engraftment of these lineages. Variable mix of host and donor cells was observed within Nkp46 + and CD4 SP cell population. Further analysis revealed host CD4 SP to be NKT cells. Taken together, these data reflect the immunodeficient phenotype of the RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pig model, its ability to engraft with SLA-mismatched allogeneic cells while also highlighting the utility of the pH2B-eGFP model.
3.3.2 Xenogeneic IUHCT engraftment of RAG-2^{−/−}IL2RG^{−/−} fetuses using human CD34 + cells isolated from mobilized peripheral blood.

3.3.2.1 IUHCT of RAG-2^{−/−}IL2RG^{−/−} fetuses using human CD34 + cells.

As shown above, the RAG-2^{−/−}IL2RG^{−/−} pig allows robust engraftment with allogeneic hematopoietic stem cells via IUHCT. Therefore, to explore their ability to sustain xenogeneic engraftment, I performed IUHCT with human CD34 + cells isolated from mobilized peripheral blood stem cells (Fig. 33). Mobilized peripheral blood stem cells were enriched for CD34 + cells using immunomagnetic purification as described in materials and methods. Purification to 99% was confirmed via flow cytometry (Fig. 33). In addition, donor cells were identified as male by detection of the human X and Y chromosome (Fig. 33). A total of 7 x 10^6 viable human CD34 + cells were transplanted via IUHCT into 42 days RAG-2^{−/−}IL2RG^{−/−} developing fetuses. Resulting pigs were analyzed at birth and at 1 and 2 weeks.

**Figure 33.** Purification and sexing of human CD34 + cells used for IUHCT.
(A) Flow cytometry analysis of CD34 + cells utilized for IUHCT procedure. Peripheral blood mobilized stem cells were stained with anti-human CD34 antibodies prior to magnetic isolation (pre CD34 column) and after purification in magnetic columns (post CD34 column), revealing a 100 fold enrichment (0.9 to 99%) of CD34 + cells. (C) Detection of X and Y chromosome from donor human cells. PCR primers were used to amplify the human X and Y chromosome. Donor cells were found positive for X and Y chromosome, revealing donor cells to be of male sex. Male (M) and female (F) control gDNA was used as gender controls.
### 3.3.2.2 Analysis of peripheral blood of xenografted RAG-2⁻/⁻IL2RG⁺/- pigs.

Peripheral blood was collected from cord blood of xenografted RAG-2⁻/⁻IL2RG⁺/- pigs at birth prior to colostrum ingestion. Xenografted pigs (N=3) contained between 794 to 1040 cells/100µL human CD45 (hCD45) in peripheral blood, accounting for 4 to 5% of total gated MNC (Fig. 34). However, compared to adult control blood levels, RAG-2⁻/⁻IL2RG⁺/- xenografted levels were approximately 6.5-fold lower. Similar numbers were obtained for human T cell lineages. Xenografted pigs contained between 751 to 967 cells/100µL human CD3 (hCD3), between 54 to 309 cells/100µL human CD4 (hCD4) and between 250 to 387 cells/100µL human CD8 (hCD8). From human CD3 + cells the majority were human CD8 (61.3 to 71.06%), where human CD4 represented a smaller proportion (3.68 to 22.2%). Human DP cells were also identified (0.84 to 3.9%). A previous report of IUHCT of human CD34 + cells into wild type pigs resulted in no detection of peripheral blood human CD45 cells (Fujiki et al. 2003). Ogle and colleagues have also attempted IUHCT of human T cell-depleted of bone marrow and umbilical cord blood wild type pig fetuses, leading to mean TCRVα (T cells) of 170 cells/100µL of peripheral blood, and a marked decline in chimerism was observed after 1 week from birth (Ogle et al., 2009). Thus, the RAG-2⁻/⁻IL2RG⁺/- pigs had approximately 2.4-fold greater levels of human T cells than wild type pigs.

In addition to T cells, I examined for the presence of human CD19 (B cells), human Nkp46 (NK cells) and human monocytes (CD14). As shown in Fig. 35, All these cell lineages were absent indicating that peripheral blood engraftment was limited to the T cell lineage.
Figure 34. Flow cytometry analysis of peripheral blood from xenograft RAG-2⁻/⁻ IL2RG⁻/⁻ pigs at birth. (A) Representative flow cytometry analysis for human CD45 and human CD3. Blood from a xenografted pig shows a distinct human CD45⁺ and human CD3⁺ population, revealing human T cell lineage engraftment. (B) Flow cytometry analysis of human CD3⁺ cell for the co-expression of human CD4 and human CD8 in three xenografted pigs. Xenografted pigs contained human CD3 cells co-expressing human CD4 and human CD8 markers. (C-F) Histogram showing number of human cells/100µL of pig blood for human CD45, human CD3, human CD4 and human CD8. Human blood served as positive control while blood of a noninjected pig served as negative control.
Figure 35. Flow cytometry analysis for human B, NK cells and monocytes from peripheral blood from xenografted RAG-2⁻/⁻ IL2RG⁻/⁻ pigs. Flow cytometry analysis from xenografted pigs at birth for human CD19 + cells (A), human Nkp46 + cells (B) and human monocytes (CD14). Xenogeneic pigs did not contain human B, NK cells and monocytes. Human blood served as positive control while blood of a noninjected pig served as negative control, showing no cross-reactivity of human antibodies.

To determine the persistence of the human CD45 cells in the circulation, blood was sampled once a week for up to 2-3 weeks after birth. Peripheral blood was collected every 7 days until the time of euthanasia for flow cytometry analysis for the presence of human CD45 cells (2 and 3 weeks). As seen in Fig 36, human cell clearance was observed from peripheral blood by week 1 suggesting the presence of an immune barrier against human cells in RAG-2⁻/⁻ IL2RG⁻/⁻ xenografted pigs after birth.
Figure 36. Flow cytometry analysis from peripheral blood of RAG-2<sup>-/-</sup> IL2RG<sup>−/−</sup> of two xenografted pigs between birth and week 2. Flow cytometry analysis from RAG-2<sup>-/-</sup> IL2RG<sup>−/−</sup> xenografted pigs, showing the presence of human CD45 cells in the peripheral blood at day 1. Subsequent blood collections performed at week intervals after birth shows the absence of human CD45 cells, indicating human cell clearance from peripheral blood after birth.

3.3.2.3 Analysis of spleen and bone marrow of xenografted RAG-2<sup>-/-</sup>IL2RG<sup>−/−</sup> pig at birth.

As in peripheral blood, flow cytometry analysis of human CD45<sup>+</sup> in spleen and bone marrow revealed the presence of human cells most of which were CD3<sup>+</sup> cells (Fig.37). The detected human CD3<sup>+</sup> cells were CD4 SP, CD8 SP and DP. In contrasts to peripheral blood where CD8 SP was the dominant population, in spleen the CD4 SP was dominant (Fig. 37). Similarly, bone marrow analysis of xenografted pig at birth revealed the presence of low levels of CD45<sup>+</sup> CD3<sup>+</sup> cells. From spleen CD3<sup>+</sup> cells, 51% were CD4 SP, 29.5% were CD8 SP and 7.2% DP cells. From bone marrow CD3<sup>+</sup> cells, 45% were CD4 SP, 37% were CD8 SP and
4.7% DP cells. As for peripheral blood, analysis of spleen and bone marrow for human B, NK and monocytes revealed nearly undetectable levels and lack of defined population, therefore revealing their absence (Fig. 37).

Figure 37. Human xenogeneic engraftment in the spleen and bone marrow of RAG-2\(^{-/-}\) IL2RG \(^{-/-}\) pig at birth. (A) Immunofluorescence staining for human CD45 (green signal) and H&E in the spleen of a RAG-2\(^{-/-}\) IL2RG \(^{-/-}\) xenografted pig at the time of birth. (B) Flow cytometry analysis confirming the immunofluorescence findings for human CD45, showing analysis and presence of human CD3, human CD4 SP, CD8 SP and DP cells, while also showing nearly undetectable levels of human CD19, human Nkp46 and human CD14 (C). Flow cytometry analysis from bone marrow showing the presence of human CD45, human CD3, human CD4 SP, CD8 SP and DP cells, while showing nearly undetectable levels of human CD19, human Nkp46 and human CD14.
3.3.2.4 Analysis of RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> thymus after human hematopoietic stem cell engraftment at birth.

The thymus of a xenografted pig was examined for the presence of human T cells at birth via immunohistochemistry and flow cytometry. Staining the thymus of a noninjected and the xenografted pig with human CD45 (FITC) revealed robust engraftment of human cells (Fig. 38). Flow cytometry analysis for human CD3 showed xenografted thymus at day 1 revealed the thymus to be 58% human T cells. Further analysis of the human CD3 cells showed the presence of human CD4 SP (40%), human CD8 SP (49%) and human DP (5.5%) cells (Fig. 38). Previous reports of IUHCT in wild type pig fetus resulted in human CD3 cell levels between 0.1 to 0.9% (Fujiki et al., 2003; Ogle et al., 2009), RAG-2<sup>−/−</sup>IL2RG<sup>−/−</sup> pigs’ thymus microenvironment revealed a greater advantage for human T cell engraftment, capable of supporting engraftment of the main T cell subtypes. These data is in concordance experiments performed by Boris Nikolic and colleagues, where fetal thymus, when co-transplanted with human fetal thymus and fetal liver into SCID mice led to the development of human CD4 SP, CD8 SP and DP cells at the time of analysis (13 weeks post transplantation) (Nikolic et al., 1999).
Figure 38. Human T cell engraftment in the thymus of xenografted RAG-2\(^{-/-}\)IL2RG\(^{-/-}\) pig at birth. (A) Flow cytometry analysis with human CD3 antibody showing human T cell engraftment of the thymus of xenografted pigs at birth. (B) Flow cytometry analysis of gated human CD3 + cells for expression of human CD4 and human CD8. Xenografted thymus contained the three main T cell subtypes, CD4 SP, CD8 SP, and DP cells. (C) Histological structure of thymus from noninjected pig compared to the thymus of a xenografted pigs at birth. Immunofluorescence staining for human CD45 (green signal) is also shown. Noninjected thymus served as negative control, showing no antibody cross-reactivity with pig cells. Xenografted pig contained robust human CD45 engraftment.

3.3.2.5 Analysis of spleen and bone marrow of xenografted RAG-2\(^{-/-}\)IL2RG\(^{-/-}\) pig after birth.

To determine if human cells were being cleared from solid organs as was observed in peripheral blood (Fig 36) spleen and bone marrow from xenografted pigs were examined at 2
and 3 weeks of age. Although detected in spleen and bone marrow at birth, human CD45 and CD3 cells could not be identified in the spleen and bone marrow of xenografted pigs at 2 and 3 weeks (Fig. 39 and 40), suggesting a similar pattern of human cell clearance observed in peripheral blood after birth. Like peripheral blood at birth, human CD19, human Nkp46 and human CD14 were not detected (Fig. 39 and 40).

Taken together these data suggest that *RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup>* pigs barriers to xenogeneic engraftment also limits long-term spleen and bone marrow engraftment after IUHCT of human CD34<sup>+</sup> cells. The barrier appeared to be specific to the post-natal period, as human cells could persist within the developing *RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup>* fetuses for approximately 73 days (days between fetal injection and parturition).

Figure 39. Analysis of human xenogeneic engraftment in the spleen of *RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup>* pigs after birth. (A, B). H&E and immunofluorescence staining for human CD45 in the spleen.
of RAG-2−/ IL2RG −/y xenografted pigs at 2 weeks (A) and 3 weeks (B). Human CD45 cell were absent. (C, D) Flow cytometry analysis of spleen also revealed nearly undetectable levels of human CD45, CD3, CD19, Nkp46 and CD14 at week 2 (C) and at week 3 (D).

Figure 40. Analysis of human xenogeneic engraftment in the bone marrow of RAG-2−/ IL2RG −/y pigs after birth. (A, B) Flow cytometry analysis of human CD45, CD3, CD19, Nkp46 and CD14 at week 2 (C) and at week 3 (D). Human CD45, CD3, CD19, Nkp46 and CD14 were nearly undetectable in the bone marrow at week 2 and 3.
3.3.2.6 Analysis of RAG-2\textsuperscript{-/-}IL2RG\textsuperscript{+/y} thymus after human hematopoietic stem cell engraftment after birth.

Immunofluorescence staining for human CD45 and flow cytometry analysis with human CD3, CD4 and CD8 revealed xenografted RAG-2\textsuperscript{-/-} IL2RG\textsuperscript{+/y} pigs have high levels of human T cells after birth (week 2 and 3) (Fig. 41). Like the data obtained at day 1 I identified via flow cytometry the presence of human CD3, human CD4 SP, human CD8 SP and human DP cells within the thymus of RAG-2\textsuperscript{-/-} IL2RG\textsuperscript{+/y} pigs. These data indicate the thymus of xenografted pigs RAG-2\textsuperscript{-/-} IL2RG\textsuperscript{+/y} can sustain all three major T cell phenotypes (CD4 SP, CD8 SP, DP) after birth. More importantly, I did not observe human cell clearance from the thymus of xenografted pigs, suggesting the thymus of RAG-2\textsuperscript{-/-} IL2RG\textsuperscript{+/y} may provide a protective microenvironment for human cells when compared to peripheral blood, spleen and bone marrow. Previous report of IUHCT of human CD34 + cells into wild type pigs resulted in 0.1% (Ogle et al., 2009) and 0.9% human CD3 engraftment of thymus (Fujiki et al. 2003), compared to up to 80% in the RAG-2\textsuperscript{-/-} IL2RG\textsuperscript{+/y} background. This was confirmed by IHC showing that the thymus in the xenografted pigs was mostly comprised of human cells (Fig 38 and 41).
Figure 41. Human T cell engraftment in the thymus of xenografted RAG-2⁻/⁻ IL2RG⁻/⁻ pigs after birth. (A) Flow cytometry analysis with human CD3 antibody showing human T cell engraftment of the thymus of xenografted pigs at 2 and 3 weeks. (B) Flow cytometry analysis of gated human CD3 + cells for expression of human CD4 and human CD8. Xenografted thymus contained the three main T cell subtypes, CD4 SP, CD8 SP, and DP cells. (C) Histological structure of thymus from noninjected pig compared to the thymus of 2 xenografted pigs at 2 weeks and 3 weeks. Immunofluorescence staining for human CD45 (green signal). Noninjected thymus served as negative control, showing no antibody cross-reactivity with pig cells. All xenografted pigs contain robust human CD45 engraftment after birth.

3.3.2.7 Detection of human T cell receptor excision circle (TREC).

To demonstrate T cell thymopoiesis, T cell receptor excision circles (TREC), were detected as described previously (Al-harthi et al. 2000) (Ogle et al., 2009). TRECs are generated during V(D)J gene recombination, a process responsible for the diversity of T cell antigen receptor (TCR) repertoire. This complex end-to-end fusion of gene segments is mediated by recombination-activating genes RAG-1/2 that recognize ‘heptamer–spacer–nonamer’ recombination signal sequences (RSSs) flanking each V, D and J gene segment. Generation of a coding TCR chain results in the excision of extrachromosomal DNA circles (TREC). Therefore,
its identification correlates with T cell differentiation (Mensen et al. 2013; Al-Harthi et al. 2000). I selected previously reported human specific primers capable of detecting TCRδ to TCRα recombination (δRec-ΨJα), since δRec-ΨJα represents approximately 67% of recombination events (Zhang et al. 1999) (Ogle et al., 2009).

Human TREC's were not detected from peripheral blood (at the time of birth) in any xenografted RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs, even though these animals contained significant numbers of blood human T cell when examined. However, human TREC's were detected in the thymus of xenografted RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs at all stages examined. As duplication of TREC circles within replicating mature T cell does not occur (Al-Harthi et al. 2000), the presence of human TREC signal is highly indicative of de novo human T cell thymopoiesis.

![Figure 42](image.png)

**Figure 42. Detection of human TREC signal from thymus and blood of xenografted RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs.** (A) PCR assay with primers specific for human TREC (~400 bp) and an internal, loading control primer (~1Kb). Human peripheral blood mobilized stem cells gDNA served as positive control, while gDNA from the thymus of a noninjected RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pig served as negative control. Human TREC signal is present in the thymus of all xenografted pigs but absent from peripheral blood at day 1, even though human T cells were present at that stage.
3.3.2.7 Summary of xenogeneic IUHCT.

Our results demonstrate that \( RAG-2^{-/-} IL2RG^{+/y} \) pigs can sustain robust, never reported before human T cell engraftment levels post IUHCT of human CD34 + cells. Human B, NK and monocytes were nearly undetectable via flow cytometry (<0.1%) and a defined population was not observed as for human CD45 and human CD3 data. The thymus was the only lymphoid organ capable of sustaining human engraftment after birth, and pig immunological barriers were evident, limiting the detection of human cell in peripheral blood, spleen and bone marrow after birth. Taken together these data reflects the immunodeficient phenotype of \( RAG-2^{-/-} IL2RG^{+/y} \), while also surprisingly revealing the presence of other immunological barriers to human engraftment.

3.3.3 Comparison of immune responses/barriers in allografted and xenografted \( RAG-2^{-/-} IL2RG^{+/y} \) pigs.

Some of the major limitations to engraftment of human cells in immunodeficient mice strains are host humoral and innate immune responses. In CB17-SCID (\( Prkd^{scid} \)) mice, major impediments to human cell engraftment are due to innate host system, including robust macrophage and neutrophil cell function, and high levels of hemolytic complement activity (Shultz et al. 2003; Greiner et al. 1998). The development of NOD-SCID mice overcame some of these limitation, as these mice harbor additional defects in myeloid and dendritic cell development and lack hemolytic complement due to a 2–base pair deletion in the coding region of the hemolytic complement (Hc) gene that encodes the C5 complement component (Shultz et al. 1995). This mutation prevents the formation of the C5b-9 membrane attack complex (MAC). Complement activation leads to the cleavage of C5 into C5a and C5b. C5b may be found free in
the plasma, or as part of the MAC complex and its identification indicates complement activation (Ricklin & Lambris 2013; Smith 2015).

When transplanting pig cells/tissues into a human/primate (xenotransplantation models), an immediate rejection process takes place, mainly driven by human natural antibodies and human complement system against porcine cells (hyper acute rejection) (Li et al. 2009). Therefore, it was scientifically sound to question the role of pig complement system against human xenotransplanted cells. To our knowledge this has never been investigated previously in a human to pig xenograft pig model.

Macrophage function against xenogeneic engraftment is mediated through the SIRPα-CD47 receptor-ligand axis. SIRPα (Signal Regulatory Protein-α), encoded by the SIRPα gene, is expressed by macrophages (and neutrophils in pig). Its interaction with ubiquitously expressed CD47 provides a “do not eat me” signal (phagocytosis) (Brehm et al. 2010). The NOD mice strain has a polymorphism in SIRPα that is very similar to human SIRPα but the SIRPα in BALB/c and C57BL6 have very low homology to human SIRPα (Brehm et al. 2010). As a result, the BALB/c and C57BL6 DKO strains can phagocytize engrafted human cell. When BALB/c or C57BL6 RAG-2<sup>null</sup> IL2RG<sup>null</sup> mice are modified to express human SIRPα they show increased levels of human hematopoietic stem cell engraftment and immune development (Takenaka et al. 2007).

The role of pig SIRPα and its interaction with human CD47 has been recently investigated in vitro. It has been reported that recombinant human SIRPα proteins bind to porcine CD47 (Subramanian et al. 2006). However, the protein-binding assay does not necessarily reveal whether porcine CD47 can deliver inhibitory signaling via SIRPα to human macrophages. In fact, the demonstrated inability of porcine red blood cells to stimulate SIRPα
tyrosine phosphorylation in human macrophages indicated a lack of functional interaction between porcine CD47 and human SIRPα (Ide et al. 2007). In agreement with this possibility, porcine cells transfected with human CD47 showed markedly reduced sensitivity to in vitro phagocytosis by human macrophages (Ide et al. 2007). Therefore, it is possible that pig macrophages expressing pig SIRPα could limit human engraftment, by phagocytosis of human cells expressing incompatible human CD47. Thus, I investigated, via immunohistochemistry the pattern of complement C5b and pig SIRPα staining in allografted and xenografted pig frozen tissue sections to further understand the involvement of this pig humoral and innate system components in xenogeneic engraftment rejection.

3.3.3.1 Analysis of allografted and xenografted tissues for pig complement.

Allografted and xenografted pig thymus and spleen were immunostained with anti-C5b antibody. Results revealed xenografted thymus contained increased C5b positive cells when compared to allografted thymus (Fig. 43). C5b signal in allografted thymus was mainly restricted to blood vessels, and co-localization with allografted cells (pH2B-eGFP) was not detected. In contrast, xenogeneic thymus contained human CD45 (CD45- FITC antibody) co-localized with C5b staining. While complement activation has been suggested to play a role in allograft rejection (Ricklin & Lambris, 2013), this phenomenon is not fully understood. Regardless, our goal was to investigate if xenografts and allografts would differ in the ability to activate complement system. To demonstrate that xenograft and allograft differ in the degree of complement activation, the number of C5b + cells was normalized to the number of GFP + cells (nC5b/nGFP) from allografts and xenografts. This normalization allowed more accurate
interpretation, as C5b deposition may be influenced by number of engrafted cells. Xenografted thymus contained 10-fold increase in the numbers of C5b + cell when compared to allografted thymus (mean of 0.4 ± 0.2 versus 0.02 ± 0.01) (p < 0.05) (Fig. 43). These data highly suggest human cells to activate complement system in a greater degree when compare to pig cells, leading to C5 cleavage and C5b deposition in the surface of human cells.
Figure 43. Immunofluorescence staining for C5b in xenografted and allografted thymus of RAG-2–/– IL2RG–/– pigs. (A) Allografted thymus immunofluorescence staining and overlay images for DAPI (blue) and C5b (red). Allografted cells are green (pH2B-GFP). Note in all pigs that C5b (red) is mainly localized within blood vessels, not co-localized with allografted cells (green). (B) Xenografted thymus immunofluorescence staining and overlay images for human CD45 (green), DAPI (blue) and C5b (red). Note in all xenografted pigs, co-localization of C5b (red) with human CD45 cells (green). (C) Histogram showing normalization of number of C5b + cells/number of GFP + cells from xenografted and allografted thymus, data are presented as mean value, bars indicate standard deviation (N=3). * indicates significance at p < 0.05.
Similarly, immunofluorescence staining of C5b was also carried out for the spleen of xenografted and allografted \textit{RAG-2\textsuperscript{−/−} IL2RG\textsuperscript{−/−}} pigs. Only one xenografted (N=1) pig displayed human cells, and was compared to allografted samples (N=3). Despite the insufficient samples size for statistical analysis, C5b staining from xenografted spleen was remarkable distinct from all allografted spleens, with multifocal C5b + regions (plasma free C5b) and co-localized with human CD45 cells.
Figure 44. Immunofluorescence staining for C5b in xenografted and allografted spleen of RAG-2<sup>-/-</sup> IL2RG<sup>-/-</sup> pigs. (A) Allografted spleen immunofluorescence staining and overlay images for pH2B-eGFP (green), DAPI (blue) and C5b (red) (N=3). (B) Xenografted spleen immunofluorescence staining and overlay images for human CD45 (green), DAPI (blue) and C5b (red). Note in the xenografted spleen (N=1) multifocal staining of C5b. Co-localization of C5b (red) with human CD45 cells (green) is also present. (C) Histogram showing normalization of n C5b + cells/n GFP + cells for xenografted (N=1) and allografted (N=3), data are presented as mean value, bars indicate standard deviation (except for xenogeneic).

Combined the spleen and thymus C5b immunofluorescence data point a predisposition of xenografted tissues to trigger complement activation when compared to allografted tissues, leading to deposition of C5b. Xenografted thymus contained C5b co-localized within human cells, suggesting the presence of MAC complex in the surface of human cells. Xenografted spleen contained both co-localized C5b with human cells, while also containing C5b positive areas away from human cells.
3.3.3.2 Analysis of allografted and xenografted tissues for pig SIRPα.

Next I investigated the infiltration of pig SIRPα cells within allografted and xenografted thymus and spleen via immunohistochemistry. Immunofluorescence staining was performed against pig SWC3a (SIRPα, also known as CD172a). The antibody was tested on peripheral blood to confirm the expression in macrophages and neutrophils. As expected flow cytometry analysis of a wild type blood sample revealed expression of SIRPα to be within the monocytes/macrophages and granulocytes population (neutrophils) (Fig. 45).

![Flow cytometry analysis for the expression of pig SIRPα in peripheral blood.](image)

**Figure 45. Flow cytometry analysis for the expression of pig SIRPα in peripheral blood.** Peripheral blood from a wild type pig was collected and stained for pig SIRPα. Data shows flow cytometry peripheral blood forward versus side scatter (left), and forward scatter versus SIRPα signal. Lymphocytes, monocytes, and granulocytes gates are indicated by red structures. A sample without antibody shows no positive signal for SIRPα on the x axis (middle). The addition of antibody results in the shift of gated monocytes and granulocytes population, confirming them positive for SIRPα (right). The gated lymphocyte population does not shift, as lymphocytes do not express SIRPα.

Immunofluorescence analysis of xenografted (N=3) and allografted (N=3) thymus revealed xenografted thymus to contained increased infiltration of SIRPα+ cells (pig macrophages and neutrophils) when compared to allografted thymus (Fig. 46). These cells were not human macrophages, as I have demonstrated these animals do not contain human CD14 (date...
not shown). Normalization was carried out as previously described for C5b assay (number of SIRPα + cells/GFP + cells), showing xenografted thymus to contain approximately 2.4-fold greater of SIRPα + positive cells when compared to allografted thymus (mean of 0.43 ± 0.19 versus 0.09 ± 0.03 (p < 0.05). The presence of SIRPα + cells in the allografted thymus is expected as the thymus contain resident macrophages (Gordon et al. 2014).
Figure 46. Immunofluorescence staining for SIRPα in xenografted and allografted thymus of RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs. (A) Allografted thymus immunofluorescence staining and overlay images for DAPI (blue) and SIRPα (red), allografted cells are naturally green (pH2B-eGFP). (B) Xenografted thymus immunofluorescence staining and overlay images for human CD45 (FITC, green), DAPI (blue) and SIRPα (red). (C) Histogram showing normalization of number SIRPα + cells/number GFP + cells from xenografted and allografted thymus, data are presented as mean value, bars indicate standard deviation (N=3). SIRPα + cells are present in both, but in increased proportion for xenografted thymus. * indicates significance at p < 0.05.
Similarly, immunofluorescence staining of SIRPα was also carried out for the spleen of xenografted and allografted RAG-2-/- IL2RG-/-y pigs. The one xenografted (N=1) pig spleen displayed increased SIRPα staining when compared to the allografted spleens (Fig. 47), suggesting a similar pattern as observed in the thymus.
Figure 47. Immunofluorescence staining for SIRPα in xenografted and allografted spleen of RAG-2⁻/⁻ IL2RG⁻/⁻ pigs. (A) Allografted spleen immunofluorescence staining and overlay images for pH2B-eGFP (green), DAPI (blue) and SIRPα (red) (N=3). (B) Xenografted spleen immunofluorescence staining and overlay images for human CD45 (green), DAPI (blue) and SIRPα (red). (C) Histogram showing normalization of n C5b + cells/n GFP + cells for xenografted (N=1) and allografted (N=3), data are presented as mean value, bars indicate standard deviation (except for xenogeneic).

These data indicate that the presence of human engrafted cells results in an increased infiltration of pig SIRPα + cells (macrophages and neutrophils). The presence of SIRPα + pig cells, combined with the possible lack of proper interaction between the pig SIRPα receptor and the human CD47 could lead to phagocytosis of human cells, resulting in contribution to the clearance of human cells over time.
3.4 DISCUSSION.

3.4.1 Allogeneic IUHCT of RAG-2\(^{-/-}\) IL2RG\(^{-/-}\) of pigs.

Large animal models for studying allogeneic IUHCT of hematopoietic stem cells are currently limited. To date, the most popular animal model for allogeneic transplantation studies is the sheep. Early gestational transplantation of allogeneic hematopoietic stem cells into normal sheep fetuses results in sustained multilineage hematopoietic chimerism (Flake et al., 1986). Other large animal models such as primates (Harrison et al. 1989; Shields et al. 1995), goats (Pearce et al. 1989) and dogs (Blakemore et al. 2004) have also been used, albeit less successfully, for allogeneic engraftment via IUHCT. While the sheep is to date the most commonly used large animal for IUHCT in part to the large body of research on fetal surgery in this species (Flake et al., 1986; E. O. Zanjani et al., 1992), it suffers from multiple drawbacks including, seasonal breeder, small litter (1-2) size, and high sensitivity to SCNT (Hosseini et al., 2008) making it more difficult to develop complex transgenic lines.

The pig, in contrast, has litters with multiple offsprings, it’s not seasonal, and responds well to SCNT, facilitating the development of complex transgenic lines as we have demonstrated here. In addition, it resembles humans at the genetic, anatomical and physiological levels, making it an attractive model for investigation of IUHCT. Others (Lee et al., 2005) demonstrated that immunocompetent pig fetuses are capable of sustaining SLA mismatched engraftment after hematopoietic stem cell IUHCT, with levels of chimerism reaching up to 8% in the spleen and thymus of allografted pigs after birth. IUHCT was also useful for inducing tissue allograft (kidney) tolerance across a full major histocompatibility complex barrier (Lee et al., 2005), supporting the ability of this approach to also induce immune tolerance.
Following allogeneic transplantation analysis in pigs, host versus donor cell differentiation is commonly assessed through the expression of different SLA surface molecules or detections of the Y-chromosome in male to female transfers. The SLA detection approach has limitations such as the lack of proper antibodies available for the detection of multiple SLA surface molecules (Lee, et al. 2005). The Y-chromosome detection method requires fluorescent in situ hybridization and it is difficult to quantify by a flow cytometry (Khan et al., 2004). To overcome these limitations I developed the $\beta$-ACTIN-IRES-pH2B-eGFP gene edited pig. This line, when combined with the $RAG-2^{-/-}$ $IL2RG^{-/-}$ line, allowed us to carefully examine allogeneic engraftment after IUHCT and determine that the immunodeficient models resulted in:

1) greater than 90% donor engraftment of B cell lineage in the spleen (Fig. 27-28); 2) greater than 90% donor engraftment of T cell lineage within the thymus (CD3, CD8 SP and DP) (Fig. 29-30); 3) address the mix chimerism (donor/host) of CD4 SP and Nkp46+ cells in the thymus and spleen, revealing the NKT cell developmental ability of $RAG-2^{-/-}$ $IL2RG^{-/-}$ pigs (Fig. 31-32).

This was accomplish using poor pH2B-eGFP pig donors due to the mismatch of SLA haplotypes regulating engraftment of hematopoietic stem cells (SLA-1, 2, 3, DRB1, DQB1) (Fig. 26) (Petersdorf, 2016). The robust allogeneic T and B cell lineage engraftment observed was possible since the niche competition was absent due to the lack of host $RAG-2^{-/-}$ $IL2RG^{-/-}$ B and T cells, confirming the proper inactivation of $RAG-2$ and $IL2RG$ genes. However, that was not the case for NKT cells, as host NKT cell development likely had a niche competition advantage over donor NKT cells.

NKT cells are mostly CD1d-depedent, lipid antigen–reactive, immunoregulatory T lymphocytes that can promote cell-mediated immunity to tumors and infectious organisms, including bacteria and viruses, yet paradoxically they can also suppress the cell-mediated immunity associated with
autoimmune disease and even allograft rejection/tolerance (Godfrey et al. 2010). Several studies support a thymus-dependent origin for these cells, as they are significantly reduced in neonatally thymectomized mice (Hammond et al. 1998). While conventional T cells are selected through affinity for MHC molecules, NKT cells follow a different pathway, being selected on the bases of the interaction of CD1, a surface molecule responsible for presenting glycolipid antigens, with the prototypic antigen being the glycosphingolipid α-galactosylceramide (α-GalCer) (Godfrey et al. 2010). NKT cells are comprised of two main subsets; Type I and Type II. Most Type I NKT cells express a semi-invariant T cell receptor (TCR) and are referred to as invariant NKT (iNKT) cells. Type II NKT cells recognize different antigens using an oligoclonal TCR repertoire (MacDonald, 2002). A third group of NKT cells (NKT-like cells) have also been described and are CD1d independent (Hammond et al., 1999), they are by far the most heterogeneous and the least characterized. In mice type I and type II NKT are often CD4 SP or DN (double negative) (Godfrey et al. 2010). The mutant RAG-2−/− IL2RG−/− and RAG-2−/− IL2RG−/− pigs exhibit a similar NKT profile (CD4 SP). NKT cells have been identified in IL2RGnull mouse models, revealing their developmental capability independent of IL2RG cytokine stimulation (Lantz et al. 1997). Surprisingly, in our model these cells were also capable of developing under a RAG-2 deficient phenotype in the absence of TCR-β recombination (Fig. 13). In mice, RAG-1 and RAG-2 deficiency result in complete absence of NKT cells (Junqing Cui, et al., 1997) and RAG-2 hypomorphs in humans results in reduced levels of NKT cells (Matangkasombut et al., 2008), and a hypomorphic R229Q RAG-2 mouse mutant recapitulates human Omenn syndrome (Marrella et al., 2007). While I only investigated the rearrangement of TCR-β, further analysis of the TCR repertoire through DNA sequencing of purified NKT cells from RAG-2−/− IL2RG−/−...
would be beneficial to further reveal the TCR repertoire of these cells. To our knowledge this is the first report of NKT cells in RAG-2 deficient pig model.

3.4.2 Xenogeneic IUHCT of RAG-2 \(^{-/-}\) IL2RG \(^{-/-}\) of pigs.

The sheep is the most popular large animal model of IUHCT of human hematopoietic stem cells. This model has been demonstrated to sustain xenogeneic engraftment levels of \(~3.3\%\) post IUHCT of human hematopoietic stem cells (Zanjani et al. 1994; Zanjani et al. 1992). However, as mentioned previously, limitations related to the ovine specie (cloning efficiency, litter size) reveal the need to explore other large animal species for IUHCT of human hematopoietic stem cells. Here I demonstrated how transgenic immunodeficient pigs may be generated with efficiency and predictability, allowing robust engraftment of hematopoietic stem cells after IUHCT. Xenogeneic engraftment of human hematopoietic stem cells in an immunodeficient porcine model via IUHCT has never been reported. Yataka Fujiki and colleague (2003) performed human CD34 + IUHCT into immunocompetent pig fetuses and identified human cells in the thymus (1.6\% hCD45) and bone marrow (1.1\% hCD45) of a fetus collected at approximately 90 days of gestation, peripheral blood levels of human CD45 at birth were 0.58\%. This work demonstrates the limitation of IUHCT to establish human xenogeneic engraftment into an immunocompetent pig model, even when delivering the cells in the immunologically premature fetus and supports the development of an immunodeficient pig. In our model IUHCT of human CD34 + cells into the liver of day 42 RAG-2 \(^{-/-}\) IL2RG \(^{-/-}\) fetuses led to significant engraftment of human CD45 cells at birth ranging from 5\% in peripheral blood to over 80\% in thymus. This is the first report of significant (>1\%) human xenogeneic engraftment at such levels in a post-natal pig model. However, while significant, there were still differences
between xenogeneic and allogeneic engraftment supporting the need for additional genetic modifications. While allogeneic transplants resulted in the engraftment of donor T, B and NKT cells, xenogeneic engraftment mainly of T cell lineage, with the identification of human CD3, CD4 SP, CD8 SP and DP cells in peripheral blood (Fig. 34), thymus (Fig. 38), spleen and bone marrow (Fig. 37). The thymus of RAG-2⁻/⁻ IL2RG⁻/⁻ pigs sustained robust human T cell engraftment (Fig. 38 and 41). Ogle and colleagues have demonstrated the thymus of wild type pigs to sustain human T cell thymopoiesis post IUHCT via detection of human CD3 and human TREC signal (Ogle et al., 2009). Similarly, I detected human CD3 and CD4 SP, CD8 SP, DP and TREC signal in the thymus of all xenografted RAG-2⁻/⁻ IL2RG⁻/⁻ pigs. This indicates RAG-2⁻/⁻ IL2RG⁻/⁻ pigs thymus to mainly sustain and maintain T cell engraftment post IUHCT, being in concordance with previous works reported by others (Fujiki et al., 2003; Ogle et al., 2009). These findings are also in concordance with experiments performed by Hannes Kalscheuer and colleagues, where fetal thymus from immunocompetent pigs, when transplanted into NOD/SCID mice, was found to sustain human T cell lineage engraftment post human CD34 + cell transplantation (Kalscheuer et al. 2014).

I did not perform assays for the identification of human T regulatory cells (Treg, CD25 + Foxp3 +) (Sakaguchi, et al., 2008), but I anticipate the thymus of RAG-2⁻/⁻ IL2RG⁻/⁻ pigs to sustain the development of these cells, as human Treg cells developed in the fetal pig thymus transplanted into NOD/SCID mice, revealing the ability of the pig fetus thymus to support Treg differentiation (Kalscheuer et al., 2014). Therefore IUHCT of RAG-2⁻/⁻ IL2RG⁻/⁻ may further be explore in the future for the generation of human Treg cells capable of tolerating pig antigens, these cells would have clinical value in the field on xenotransplantation, as they may promote tolerance to pig antigens when transplanted into humans receiving pig organs xenografts.
Human B (hCD19), NK (hNkp46) and monocytes (hCD14) could not be detected via flow cytometry in peripheral blood, spleen and bone marrow at birth (Fig. 35, 37) or after birth (Fig. 39, 40). This in concordance with previous reports of IUHCT of immunocompetent pigs where the greater degree of engraftment was found to be towards human T cells (Fujiki et al., 2003; Ogle et al., 2009). While Fujiki and colleagues also reported low levels of human B (0.6%) and NK (0.2%), this was only evident in 90 days immunocompetent pig fetus. Ogle and colleagues also reported low levels of human B and NK cells via RT-PCR detection of human CD19 and CD16 mRNA. In contrary the sheep model was reported to contain human monocytes and B cells (E. D. Zanjani, et al., 1994). These findings suggest the pig to poorly sustain the engraftment of human monocytes, B and NK cells, and could be related to incompatibility of pig cytokines responsible for the development of these human cell lineages. Transgenic expression of human cytokines has been previously described in immunodeficient mouse models, where triple transgenic NSG mice expressing interleukin 3 (IL-3), granulocyte/macrophage-colony-stimulating factor (GM-CSF) and stem cell factor (SCF) engrafted with human hematopoietic stem cells display increased Treg cells and some increases in myeloid cells, specifically dendritic cells, in the circulation. The poor engraftment of human monocytes, B and NK cells could be overcome via transgenic approaches, where transgenic expression of human cytokines responsible for differentiation of: 1) human macrophages (macrophage-colony stimulating factor) (Geissmann, et al., 2010), 2) human B cells (B cell-activating factor) (Zhang et al., 2005) and 3) human NK cells (interleukin 15) (Huntington et al., 2009) may lead to increase levels of these human cell lineages in transgenic immunodeficient pigs. Although I could not detect these cells via flow cytometry, the use of more sensitive assays in the future such as RT-PCR may allow the identification of these cells at very low levels. Additional reasons for the lack of
Engraftment are: 1) quality of donor cells used; 2) the short-term engraftment of human cells in lymphoid organs other than the thymus, preventing post-natal maturation of these cell lineages. Further experiments using additional CD34+ donors are required in the future to further confirm the results obtained from this study. Regardless, here I demonstrated how RAG-2^{-/} IL2RG^{-/} fetuses sustain human lymphoid lineage engraftment post IUHCT of human CD34+ cells, opening a new window of possibilities for xenotransplantation investigation in a large animal model.

3.4.3 Comparison of pig immune barriers in allografted and xenografted RAG-2^{-/} IL2RG^{-/} pigs.

Despite the significant levels of human cells in the peripheral blood at birth, human cell clearance was observed 1 week after the initial analysis, revealing the presence of an immunological barrier in RAG-2^{-/} IL2RG^{-/} pigs (Fig. 36). Like in the peripheral blood human T cells were not detected in the spleen and bone marrow at 2 and 3 weeks (Fig. 39 and 40), suggesting the immunological barrier to also limit human engraftment within those organs after birth.

The immunological barrier observed towards human cells could not be due to host B and T cells, as RAG-2^{-/} IL2RG^{-/} pigs lacked these lymphoid cells (Fig. 15 to 23). The role of RAG-2^{-/} IL2RG^{-/} pig NKT cells towards human cell rejection is unclear, and it should be questioned, as NKT cells were the only lymphoid cells present in RAG-2^{-/} IL2RG^{-/} pigs. While NKT cell development has been previously prevented in transgenic pigs by disrupting the CD1d pig locus, CD4 SP NKT cells have been demonstrated to assist with human cell engraftment in a immunodeficient mice model of xenogeneic islet human transplantation, revealing their potential
role towards immunosuppression and protection or xenografted cells (Ikehara et al. 2000). Therefore I investigated the pig humoral (complement) and myeloid (SIRPα) system as potential immunological barriers to human cells.

IUHCT of pig pH2B-eGFP allogenic and human xenogeneic hematopoietic stem cells created a unique opportunity for us to compared immunological responses and contrast frozen section of allografts and xenografts (thymus and spleen) for pig complement system (C5b) (humoral) and pig SIRPα (myeloid) via immunofluorescence staining. Overall I observed increased C5b signaling in xenografted organs compared to allografted organs. Allografted C5b signaling was approximately 10-fold lower (Fig. 43 and 44). These data suggest that human cells attract binding and activation of C5, leading to C5b deposition xenografted organs. This could lead to formation of MAC complex and direct lysis of human cells, assisting with human clearance. In addition, C5a (result from cleavage of C5) is a chemotactic agent responsible for recruiting other innate cells such as neutrophils and macrophages (Smith, 2015), leading to a more potent inflammatory response and rejection. Solutions to address complement activation inhibition have been explored in pig models of xenotransplantation through the expression of human CD59. CD59 is a glycoprotein known as MAC-inhibitory protein and is responsible for inhibiting the formation of MAC complex (Cooper et al. 2016). Expression of human CD59 in pig xenografts followed by transplantation in primate models resulted in inhibition of MAC complex, delaying the xenograft rejection (Klymiuk et al. 2010). However, for this approach to work in our model, permanent expression of pig CD59 in transplanted human cells would be necessary since human cells are exposed to pig complement proteins. This is a difficult and impractical task to accomplish. A perhaps more approachable strategy is gene inactivation of complement proteins such as C3 and C5, however gene inactivation of complement proteins may
have detrimental phenotypic consequences (i.e., dysregulation of adaptive immune response, delayed fracture healing) (Ehrnthaller et al., 2013; Pekkarinen et al., 2015). Perhaps the fundamental biological question to address is what human surface proteins/antigens are being recognized by the pig complement proteins, to our knowledge such question has never been investigated in a human to pig xenogeneic model. In the future, further experiments assessing the ability of pig complement to induce lysis of human cell, as well as to which complement pathway could be directly involved with complement activation (classical, alternative, lectin) (Smith 2015) are required. The model reported provides opportunities for the investigation of pig complement recognition towards human cell surface antigens, which may lead to new discoveries and strategies to enhance human cell engraftment in RAG-2^{-/} IL2RG^{-/} pigs.

Analysis of SIRPα expression to identify pig macrophages and neutrophils supported increased infiltration by these two cell types in xenografted than allografted thymus and spleen (Fig. 46 and 47). Pig SIRPα cells were also detected in allografted tissue, as expected since neutrophils and macrophages are important myeloid cells of the immune defense found within the thymus and spleen (Gordon et al. 2014) Similar to the complement activation results, these data support that the xenogeneic human cells induce a greater immune rejection that allogeneic cells. One possibility of increased human cell clearance is the lack of interaction between pig SIRPα and human CD47 leading to human cell clearance by phagocytosis. In NOD mice SIRPα polymorphism makes it very similar to human SIRPα, allowing proper interaction with human CD47 and reduced phagocytic activity, where C57BL/6 and BALB/c mice have much less homology to human SIRPα, leading to increased phagocytic activity (Takenaka et al., 2007). SCID mice models with poor SIRPα homology expressing human SIRPα have showed increased
levels of engraftment and reduced macrophage phagocytic activity towards human cells (Takenaka et al. 2007). Experiments investigating the interaction of pig SIRPα and human CD47 are lacking, where the opposite scenario has been recently investigated (human SIRPα and pig CD47). As demonstrated by Kentaro Ide and colleagues, pig CD47 does not properly interact with human SIRPα (Ide et al., 2007) and inhibition of phagocytosis of pig cells by human macrophages may not occur. However our model would benefit from the information of the proper interaction between pig SIRPα and human CD47, as pig macrophages are the cells to encounter human cells expressing CD47 after IUHCT of human CD34 + cells in RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs. As the data obtained from C5b analysis, this finding highly suggests xenografted tissue to attract more macrophages and neutrophils than allografted tissues. The increased infiltration of pig SIRPα cells combined with the lack of proper interaction of pig SIRPα and human CD47 would lead to increased human cell phagocytosis, assisting with human cell clearance. In addition the increased infiltration of macrophages and neutrophils in xenografted tissues could be related with complement up regulation, as C5a works as chemotactic agent for macrophages and neutrophils (Ricklin & Lambris, 2013). Additional experiments to assess the interaction of pig SIRPα cells and human CD47 would be necessary to reveal if pig macrophages are indeed capable of performing phagocytosis of human cells. To our knowledge this has not been done previously. Transgenic expression of human SIRPα within pig’s macrophages is an attractive strategy to pursue and may lead to increased and sustained levels of human chimerism in the blood and other lymphoid tissues of RAG-2<sup>−/−</sup> IL2RG<sup>−/−</sup> pigs after IUHCT of human CD34 + cells.

Remarkable, despite the up regulation of the pig C5b and SIRPα towards xenografted tissues, complete human cell clearance was not observed in the thymus of all 3 xenografted pigs.
The thymus has long been proposed as an immunological privileged lymphoid tissue, with a distinct blood-thymus barrier formed by (1) the capillary endothelial cells and their basement membranes; (2) perivascular connective tissue; (3) epithelial reticular cells and their basement membranes; and (4) macrophages which eliminate any antigen that escapes from the capillary (Ribatti 2015). Since the thymus does not contain afferent lymphatic drainage (only efferent), this proposed privileged protection is mainly towards macromolecules of blood origin. Among macromolecules present in the blood are immunoglobins/antibodies. While humans naturally possess in their serum anti-pig immunoglobins capable of targeting sugar molecule called α-1,3-galactose (Klymiuk et al. 2010), experiments investigating naturally (or induced) anti-human pig antibodies are lacking. It is scientifically sound to proposed that anti-human antibodies (naturally or induced by IUHCT) could be transferred to pigs through the colostrum, leading to human cell clearance after colostrum ingestion through opsonization. Immunoglobin opsonization of human cells would potentially increase complement activity (complement binding to immunoglobins bound to antigens) and macrophage phagocytosis through macrophage complement receptors (Ricklin & Lambris 2013; Campagne et al. 2007). During IUHCT in mice, maternal anti-donor antibodies have been shown to play an important role in long-term allogeneic chimerism maintenance during the post neonatal period. Merianos et al. (2009) demonstrated that maternal sensitization during IUHCT and subsequent transfer of alloantibodies via colostrum induces an adaptive immune response in mouse pups, leading to allograft rejection during the postnatal period. This rejection was not observed if the pups were fostered by dams not subjected to the IUHCT procedure and thus did not produce alloantibodies (Merianos et al. 2009). To our knowledge, the role of induced xenoantibodies against human
CD34 + cells after IUHCT have not been investigated in mice or other large animal models. These are experiments to be considered in the future.

In summary, and despite the identified engraftment deficiencies, this model represents a new hallmark towards the development and application of a severe combined immunodeficient large animal model for multiple applications in the field of transplantation immunology and regenerative medicine. This model may not only be used for the IUHCT of human CD34 + cell engraftment, but also for the transplantation of human stem cells from different tissues/sources, allowing the development of severe combined immunodeficient pigs with chimeric human organs for xenotransplantation applications.

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