Supplemental information

Engraftment of human iPS and allogeneic porcine cells into pigs with inactivated *RAG2* and accompanying severe combined immunodeficiency (SCID)

Kiho Lee, Deug-Nam Kwon, Toshihiko Ezashi, Yun-Jung Choi, Chankyu Park, Aaron C. Ericsson, Alana N. Brown, Melissa Samuel, Kwang-wook Park, Eric Walters, Dae Young Kim, Jae-Hwan Kim, Craig L. Franklin, Clifton N Murphy, R. Michael Roberts, Randall S. Prather, and Jin-Hoi Kim

Supplemental Materials and Methods

Animal care

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Missouri approval number 7641.

Cell transfection and gene targeting

For gene targeting, 2-3 million cells were transfected with TALEN constructs along with a reporter vector; 2 µg of each construct per million cells. The cells were electroporated with the constructs at 490 V, 1 msec, 3 pulses by using a BTX Electro Cell Manipulator (Harvard Apparatus, Holliston, MA). The cells were plated in T75 flasks for 48 h and then sorted for GFP positive cells by using a Beckman Coulter MoFlo XDP. Among GFP positive cells, the top 10% of cells expressing the highest level of GFP were sorted and placed in 96-well plates; a single cell in a well. After ten days, half of the cells were used for genotyping. To investigate the presence of insertions/deletions (indels) after introducing TALENS, a fragment of genomic DNA flanking the TALEN cutting site was amplified by PCR **(Supplemental Table 2).** Genomic DNA for PCR amplification was isolated by using a cell lysis buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5, 0.5 % Nonidet P40, 0.5% Tween, and 400 µg/ml Protease K). Conditions for PCR were 2 min at 95°C followed by 32

cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, and 30 min at 72°C for extension. Expected size of the PCR products was 426 bp for *RAG2*. The PCR products were sequenced to identify the presence of indels.

Somatic cell nuclear transfer

To produce SCNT embryos, sow-derived oocytes were purchased from ART, Inc (Madison, WI). The oocytes were shipped overnight in maturation medium (TCM199 with 2.9 mM Hepes, 5 µg/ml insulin, 10 ng/ml EGF, 0.5 µg/ml p-FSH, 0.91 mM pyruvate, 0.5 mM cysteine, 10% porcine follicular fluid, 25 ng/ml gentamicin) and transferred into fresh medium after 24 h. After 40-42 h of maturation, cumulus cells were removed from the oocytes by vortexing in the presence of 0.1% hyaluronidase. During manipulation, oocytes were placed in the manipulation medium supplemented with 7.0 µg/ml cytochalasin B. The polar body along with a portion of the adjacent cytoplasm, presumably containing the metaphase II plate, was removed and a donor cell was placed in the perivitelline space using a thin glass capillary [1]. The reconstructed embryos were then fused in a fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM Hepes buffer, pH 7.2) by two DC pulses (1-sec interval) at 1.2 kV/cm for 30 µsec using a BTX Electro Cell Manipulator (Harvard Apparatus). After fusion, fused embryos were fully activated with 200 µM thimerosal for 10 min in the dark and 8 mM dithiothreitol for 30 min [2]. Embryos were then incubated in Porcine Zygote Media 3 (PZM3) [3] with 0.5 µM Scriptaid (Sigma-Aldrich, S7817), a histone deacetylase inhibitor, for 14-16 h. The next day, the SCNT embryos were transferred into surrogates. For transfer number 3, the embryos were washed from the Scriptaid and cultured for five additional days in PZM3 in the presence of 10 ng/ml CSF2. Blastocyst stage SCNT embryos were surgically transferred into the oviductal ampullary-isthmic junction of the surrogate[4].

Absence of exogenous DNA in RAG2 mutants

To detect the presence of the DNA construct, we amplified a fragment present in the TALEN

sets (*FokI*)[5] and a part of reporter (*RFP*, red fluorescent protein) as shown previously [6]. *HPRT1* (hypoxanthine phosphoribosyltransferase 1) served as an internal control to verify the quality of PCR reaction from each isolated genomic DNA. Primers for amplification are listed in **Supplemental Table 3.** Fifty ng of DNA was used for PCR analysis. Conditions for PCR amplification were initial denaturation for 2 min at 95°C followed by 34 cycles of 30 sec at 94°C for denaturation, 30 sec at 52°C for annealing, and 30 min at 72°C for extension. Expected sizes of the PCR products were 170 bp for *FokI* (engineered endonuclease), 180 bp for *RFP*, and 798 for *HPRT1*. The PCR products were loaded on a 2.0% agarose gel.

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from spleen of wild-type and *RAG2* biallelic mutant pigs by using a RNeasy mini kit (Qiagen, Valencia, CA, USA). Quantitative Real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was conducted by using an ABI ViiA^{TM} 7 system (Applied Biosystems, Foster City, CA, USA) and SYBR Green as the double-stranded DNA-specific fluorescent dye (Bio-Rad, Hercules, CA, USA) and appropriate primers (**Supplemental Table 5 & 6)**. The pig *ACTB* gene was used as an internal control to normalize the qRT-PCR efficiency and to quantify the relative expression of the genes in wild-type and *RAG2* biallelic mutant pig derived mRNA. After normalization with actin mRNA, the relative expressions of each mRNA in the *RAG2* biallelic mutant pig-derived genes was compared with those of the controls by performing RT-PCR on each sample independently and in triplicate. The quantitative real-time PCR results were compared by general linear model (PROC GLM) by using Statistical Analysis System (SAS Institute; Cary, NC). Differences with $p < 0.05$ were considered significantly different.

Off-target analysis

To identify putative off-target sequences from the TALENs, bioinformatics tools were used to identify sequences similar to each TALEN binding site from the most recent pig genome

assembly (Sscrofa10.2). Primers for the PCR were designed flanking the most likely off - target sites based on similarities to the *RAG2* TALEN binding sites. These regions were amplified in the founder animals and tested for off-targeting events by using the Surveyor nuclease assay as shown previously[6] **(Supplemental Table 4).** After PCR amplification, 300-500 ng of PCR products (10-15 µL were transferred to a fresh tube, denatured and reannealed according to the following thermocycler program: 95ºC for 2 min, 95ºC to 85ºC - 2ºC per sec, 85ºC to 25ºC -0.1ºC per sec. One µL of the Surveyor nuclease and 1 µL of the Surveyor enhancer were added and incubated at 42ºC for 30 min. Then the reactions were immediately placed on ice and 6X Surveyor nuclease stop buffer and 6X dye were added to the reactions. The samples were analyzed by electrophoresis on a 2.0% agarose gel.

Genotyping of RAG2 mutants

Genomic DNA was isolated from cells or tail of *RAG2* mutant pigs by DNeasy Blood and Tissue kit by employing the manufacturer's protocol (Qiagen, USA). Conditions for the PCR amplification was initial denaturation for 2 min at 95°C followed by 32 cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, and 30 min at 72°C for extension. Products from the PCR were analyzed on a 2.0 % agarose gel. Expected size of the PCR product was 426 bp. To identify potential indels on *RAG2* from TALENs, the PCR products were purified and sequenced. To identify mutations on each allele, PCR products flanking *RAG2* TALEN cutting site were cloned into TOPO sequencing vector (Invitrogen, USA). Then the TOPO plasmids containing *RAG2* PCR products were sequenced and compared to reference pig *RAG2* sequence.

Flow cytometry

Portions of the spleen from freshly euthanized mono-allelic and bi-allelic piglets were collected into RPMI-1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum, minced with a scalpel blade, aspirated multiple times through a 20 gauge

needle, and then forced through a 70 µm nylon mesh cell strainer (BD Biosciences, San Jose, CA). The splenocyte suspension was then incubated for 15 min with Pharm Lyse solution (BD Biosciences) to lyse erythrocytes, and was then pelleted at 200 × g for five min. After discarding the supernatant, the pellet was resuspended in cold staining buffer (BD Pharmingen) and cells counted on a hemacytometer. Cells were then resuspended and divided into aliquots of 5×10^6 cells in 200 µL staining buffer. FITC-conjugated mouse anti-pig CD21, mouse anti-pig CD3ε, and mouse anti-T-2 mycotoxin IgG1κ (isotype control) (SouthernBiotech, Birmingham, AL) were added to cells at 0.5 µg/1×10^6 cells and allowed to incubate in the dark at 4°C for 30 min. Cells were then washed twice and resuspended in fresh staining buffer. Cells were analyzed at the University of Missouri Cell and Immunobiology Core Facility using a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA). Data were analyzed by using Summit v4.3 software (Beckman Coulter).

TUNEL assay

Tissues were fixed in 4% (w/v) paraformaldehyde in 0.01 M PBS (pH 7.4), washed in PBS, dehydrated in ethanol (70%, 90%, and 100%) and embedded in paraffin wax. The sections (6 µm) were rehydrated (xylene 5 min; ethanol 100%, 95%, 70%, 2 min each) and washed in distilled water prior to TUNEL staining. The sections were incubated for 30 min with proteinase K (20 µg/ml in 10 mM Tris/HCl, pH 7.5) at room temperature. Sections were incubated for 10 min at room temperature in a moist chamber with the TUNEL mix (In situ Cell Death Detection kit, Roche, Swiss). After three PBS washes, slides were mounted in VECTASHIELD Mounting Media with DAPI (VECTOR, USA).

Cellular proliferation assay

Cellular proliferation of fibroblast cells derived from wild-type, *RAG2* monoalleic, and *RAG2* biallelic pigs was measured by counting number of cells in culture after 24 h and 48 h. The cells were seeded at 1×10^4 cells/well on 12-well plates coated with laminin. The number of

cells in each well was counted at 0, 24, and 48 h. Cells were stained with 0.4% trypan blue dye (Bio-Rad) to verify their viability. The number of cells at each time point were measured by using TC10 automated cell counter (Bio-Rad); three independent samples from each pig were used. Differences in the numbers of cells at 24 and 48 h were compared by using the Statistical Analysis System (SAS Institute, Cary, NC).

Derivation of human umbilical cord fibroblasts

Human umbilical cord tissues were collected freshly and aseptically in University Hospital (University of Missouri, Columbia, MO). The tissue collection (project #1201132) has been approved by the University of Missouri Health Sciences Institutional Review Board. The tissues were washed twice or more with phosphate buffered saline (PBS) to remove blood cells and minced into 1–2 mm^3 fragments with scissors in DMEM medium to deliver adherent cells by the explants method[7,8]. The fragmented tissues were placed into a 48-well plate (one piece per well) coated with 0.1% gelatin in DMEM medium (Thermo) containing 10% FBS, 1% Non Essential Amino Acids, 2 mM glutamine, 0.1 mM 2-mercaptoethanol and 4 ng/ml FGF2, followed by culturing in an incubator containing a humidified atmosphere of 4% O_2/5% CO_2/91% N_2 at 37°C. The cultures were kept undisturbed for the first 5-7 days and supplemented with Primocin (InvivoGen, San Diego, CA) to reduce risk of bacterial and fungal growth in the primary culture. The medium without Primocin or other antibiotics was refreshed every two days thereafter until the fibroblastic adherent cells from the tissue fragments developed outgrowths in the wells. The fibroblasts outgrowths started appearing at the periphery of the minced tissues after a week of culture. By 10-11 days, the fibroblasts were passaged from the 48-well plate into T25 flasks by using TrypLE™ (Invitrogen). The cells reached confluence in the flask by ~14 days and were expanded for reprogramming to iPSC.

Generation of iPSCs from umbilical cord fibroblasts with episomal vectors

A protocol developed by Okita et al[9] with episomal vectors carrying shRNA for p53 suppression and nontransforming L-MYC, in addition to the usual reprogramming genes *POU5F1, SOX2, KLF4* and *LIN-28,* was employed to reprogram the fibroblasts. Three micrograms of Y4[9] combination of the episomal plasmids was electroporated into 6×10^5 cells with a Nucleofector II device (Lonza, Basel, Switzerland) and Amaxa NHDF Nucleofector kit (Lonza) according to the manufacturer's instructions. An electroporation program 'U-020' in the device was used. The cells were allowed to recover for 2 to 4 days by culturing in the above conditions. Cells (2×10^5) were placed into 100 mm dishes previously coated with Matrigel (BD Bioscience, San Jose, CA). The following day the culture medium was switched to mTeSR1 (StemCell Technologies, Vancouver, Canada). Colonies resembling human ESC emerged around 14 days post-transduction **(Supplemental Fig. 12A)** and the colonies were mechanically isolated around day 20 and expanded into feeder-free condition on a Matrigel substratum **(Supplemental Fig. 12B).**

Immunohistochemical Examination of iPSC

Images of iPSC were captured with an Olympus CKX41 inverted microscope equipped with a digital camera Coolpix 5000 (Nikon, Melville, NY). For immunofluorescent analysis, cells were grown on coverslips coated with Matrigel. After fixation in 4% paraformaldehyde/ PBS for 10 min and permeabilization in 1.0% Triton X-100/PBS for 30 min, coverslips were placed in 5% goat serum/5% BSA in PBS for 1 h. Next, the cells were incubated overnight at 4°C with appropriately diluted primary antibodies, POU5F1 (1:200, sc-5279, Santa Cruz Biotechnology), NANOG (1:100, ab109250, Abcam), and SSEA4 (1:100, #4755p, Cell Signaling Technology) and followed by incubation with Alexa Fluor 568 or 488-labeled goat anti-mouse or rabbit antibody (1:500). Images were captured with an Olympus IX70 inverted microscope equipped with an ORCA-AG CCD camera (http://www.biotech.missouri.edu/mcc/Olympus.html).

Teratoma formation

Two Human iPSC lines (passage numbers between 4 and 9) from two individuals were injected (5 or 10 million cells per site) in 0.2 ml volume with 25% Matrigel solution subcutaneously into five pigs, 3 of them with a biallelic *RAG2* modification and 2 with monoallelic modification of *RAG2* as a control on day 1. The cultured iPSC were detached by dispase (StemCell Technologies) and scraping. After centrifugation (200 x g, 5 min), the cell pellet was resuspended with 0.1 ml of mTeSR1 medium and mixed with same volume of 50% Matrigel. The cells were then chilled on ice and loaded into a 1 ml syringe (BD, Franklin Lakes, NJ) and injected into two sites per pig, one ear and one lateral flank, through 22 gauge needles. The subsequent tumors were dissected out and fixed in 10% (v/v) neutral buffered formalin. Paraffin-embedded tissue was sectioned and then stained with hematoxylin and eosin (H&E). Porcine cells expressing trophoblast phenotypes generated from porcine iPSC (iTR)[10] were transplanted to one of the biallelic *RAG2* mutant pig. Ten million cells of the iTR subtype line (p29) were detached by TrypLE and scraping. The cell suspension was prepared as human iPSC and injected subcutaneously in the left ear. The cell transplant procedures were conducted in a blind format, with the individual performing the procedures unaware of the genetic status of the pigs.

Immunohistochemical analysis of Teratomas

For immunohistochemistry (IHC), tissues were fixed in 10% formalin in neutral buffer (Fisher, 99-909-14), embedded in paraffin, and sections (5 µm) prepared on glass slides. Endogenous peroxidase activity was first blocked by treating the tissues sections in 3% hydrogen peroxidase for an hour. Then the samples were pretreated with Borg Decloaker (Biocare Medical, CA) solution for antigen retrieval, and then blocked in Background Sniper (Biocare Medical, CA) solution. After washing, samples were incubated with primary antibodies **(Supplemental Table 7).** After incubation, samples were washed and incubated with horse radish peroxidase (HRP) conjugated secondary antibodies. The EnVision^{TM}+

system (Dako, Carpinteria, CA) was employed for detection. Either 3, 3-diaminobenzicine (DAB) or Romulin AEC Chromogen (Biocare Medical, Concord, CA) was used to visualize the signal. The samples were also stained with IP FLX hematoxylin to provide background. All photomicrographs were acquired by using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Olympus DP70 high-resolution digital microscope camera (Olympus, Center Valley, PA). The Borg, Sniper, Romulin Red and IP FLX hematoxylin were all purchased from Biocare (Concord, CA).

Source of the teratoma

To identify the source of teratomas, the human specific mitochondrial mitofusin 1 gene (*MFN1*) was amplified by using PCR. Genomic DNA was isolated from human iPSC, the teratomas, and blood from the tail of the *RAG2* mutant pig carrying the teratoma by using a DNeasy Blood and Tissue kit (Qiagen, USA). Conditions for the PCR amplification was initial denaturation of for 2 min at 96°C followed by 32 cycles of 30 sec at 95°C for denaturation, 30 sec at 52°C for annealing, and 30 min at 72°C for extension. Products from the PCR were loaded on 2.5% agarose gel. Expected size of the PCR products was 236 bp. Primers for the analysis were F: GCTGGCTAAGAAGGCGATTA and R: TCCCCTTCTGGAGGTTAGAAA.

Supplemental Tables

Supplemental Table 1: Efficiency of somatic cell nuclear transfer. A total of nine embryo transfers were performed for the study. All resulted in term development. Piglets born from 1-3 were used to study the SCID phenotype; 4-6 were used for teratoma formation; 7-9 have been retained for future breeding purposes.

Transfer	Cell types	Number of embryos generated (transferred)	Piglets born alive (still born)
1	RAG2 #14 (♂)	248 (243)	3 (1)
2	RAG2 #14 (♂)	180 (180)	4 (2)
3	RAG2 #12, 14 (♂)	95 (48)	6
4	RAG2 #14 (♂)	251 (243)	2 (1)
5	RAG2 #14 (♂)	204 (194)	2 (4)
6	RAG2 #14 (♂)	260 (244)	1 (1)
7	RAG2 #32 (♀)	259 (249)	1
8	RAG2 #32 (♀)	262 (252)	1
9	RAG2 #32 (♀)	260 (250)	2

Supplemental Table 2: Primers used to genotype *RAG2* mutations introduced by TALENs.

Gene	**Primers**	**Product**
RAG2	F: AAGGATTCCTGCTACCTTCCTCCT R: AGATAGCCCATCTTGAAGTTCTGG	426

Supplemental Table 3: Primers used to identify integration of exogenous DNA in *RAG2* mutants.

Gene	Primers (5'-3')	Product
FokI	CGGACGGAGCAATTTATACT CCACCATTCATTAGGGTTGA	170
RFP	TACTTGAAGCTGTCCTTCC CATGGTCTTCTTCTGCATTAC	180
HPRT1	GACTAGCATTCCTACTGCTTGCTG CCATGCTACTCAGGACAAGTTGAC	798

Supplemental Table 4: Primer sets used to identify off-site targeting of pig *RAG2*.

Gene	Abbreviation	Primer	Product
Mannosidase 2, alpha 1	*MAN2A1*	F:TGCCACATGATGCCTTTCTA R: TGCTGGTTCAAGATGCTGTC	205
Family with sequence similarity 82, member A2	*RMDN3*	F: CCTAGGCCTGAGTGTGGGTA R: GCCCTGACGCTTTTATTCTG	194
Metadherin	*MTDH*	F: TCCTTGCTTCCCTTGACTGT R: CGAGAGCATTTCTCGTAGCC	155
Tripartite motif-containing 31	*TRIM31*	F: TGTGCAGTTTTCAACCATCC R GTCTTTCAGTCCCCCTTTCC	163
Proprotein convertase subtilisin/kexin type 2	*PCSK2*	F: ACAAGTGGCCTTTCATGACC R: CTCTTCCTCCAGCTCCTCCT	218
Protein kinase, cAMP-dependent, catalytic, beta	*PRKACB*	F: CACTAAATAGTGGCCTTCTTGGA R: ACACACCCATCCTTTTCCAG	352
GC-rich sequence DNA-binding factor 2	*GCFC2*	F: GAAATGGGTTTGTTGAGTCCA R: ACGGTGGCAGAGCTGAATAG	417

Supplemental Table 5: Primer set used to detect expression of inflammatory-related genes

Genes	Primers	PCR product
IL6	ATGGCAGAAAAAGACGGATG GTGGTGGCTTTGTCTGGATT	215
TNF	CCACCAACGTTTTCCTCACT CCAAAATAGACCTGCCCAGA	247
IL1β	CCAAAGAGGGACATGGAGAA TTATATCTTGGCGGCCTTTG	160
IFNG	AAGCAGCTAAGCAGCCTCAC TGCCTTTTGTTTCTCCCCTA	180
CCL2	TCTCCAGTCACCTGCTGCTA TCCAGGTGGCTTATGGAGTC	185
ACTB	GAGGGCAGTAGCATCGCTTTAGTG GCACCTCAACCCGCTCCTAG	179

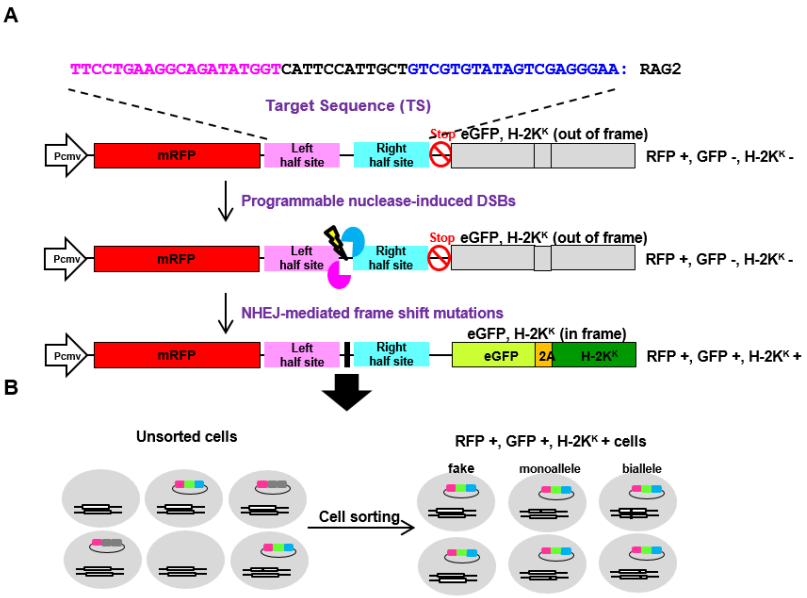
Supplemental Table 6: Primer lists for apoptosis-related genes.

Genes	Primers	PCR product
BID	TTCAGGAACCAGAGCCTGTC	229
	AGTTGCGCAAGTAGGTGAGG	
BAX	TGGTCGCGCTTTTCTACTTT	250
	CAGCCCATCTTCTTCCAGAT	
BCL2L1	GAAACCCCTAGTGCCATCAA	196
	GGGACGTCAGGTCACTGAAT	
PUMA	GGTCCTCAGCCCTCACTCTC	210
	CTGCTGCTCCTCTTGTCTCC	
ACTB	GAGGGCAGTAGCATCGCTTTAGTG	179
	GCACCTCAACCCGCTCCTAG	

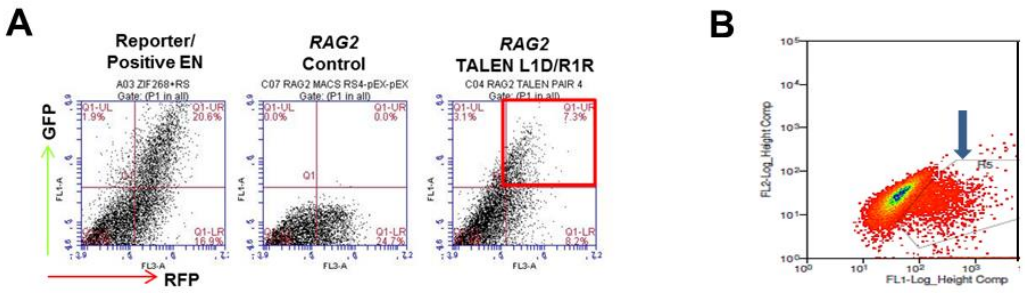
Supplemental Table 7: Antibodies used for IHC.

Antigen Targeted	Source	Dilution
CD79A	Diagnostic Biosystems- # Mob118	1:100
CD3	DAKO- # A0452	1:400
CD335	Bioss- #bs-2417R	1:100
CD34	Leica - #PA0212	Prediluted
CD45	Leica - #PA0042	Prediluted
GFAP	Leica - #PA0026	Prediluted
ENO2	Leica - #PA0435	Prediluted
CTNNB1	Leica - #PA0083	Prediluted
VWF	DAKO - #A0082	1:100
ACTG2	Leica - #PA0943	Prediluted
DES	Leica - #PA0032	Prediluted
CD 204	Transgenic Inc - #KT022	1:100

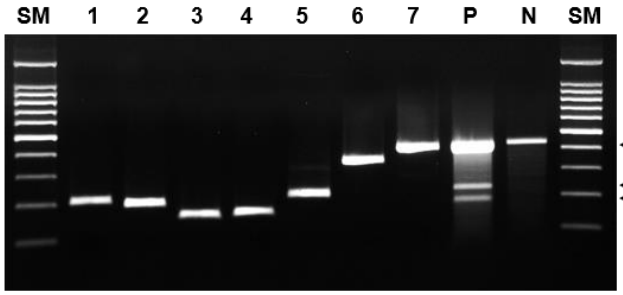
Supplemental Figures



Supplemental Figure 1: Construction of surrogate reporter vector for enrichment of cells targeted by TALENs. **(A)** The reporter vector consisted of the monomer *RFP* gene, the programmable nuclease's target sequence (left and right half-sites), the enhancer *GFP* and the *H-2KK* gene, a truncated mouse MHC class I molecule, (upper panel of a)[11]. If e*GFP* and *H-2KK* sequence are out of frame because of the absence of programmable nuclease activity, only the *RFP* gene is expressed. When a double-strand break is introduced into the target sequence by programmable nucleases, the break is repaired by non-homologous end-joining (NHEJ), which often causes frame shift mutations[12]. Such mutations can render e*GFP* into in frame with *RFP* within the reporter plasmid, inducing the expression of the mRFP-eGFP-H-2KK fusion protein (low panel of a). **(B)** Schematic illustrates enrichment of nuclease-induced mutations in *mRFP+eGFP+H-2KK+* cells sorted by two systems: magnetic separation by H-2KK antibody and flow cytometry by RFP and GFP expression. Within cells, reporter plasmids and chromosomal target loci are illustrated. Mutations are shown as black spots. When we cultured a single cell in a single 96-well, the average survivability and colony formation from the cells was around 35%; which was efficient enough to obtain colonies for screening.



Supplemental Figure 2: Targeting of *RAG2* by using a reporter guided TALEN system. **(A)** The activity of the designed TALENs was pre-validated by introducing the TALENs with a reporter into HEK 293T cells. Enrichment of cells expressing RFP/GFP, compared to controls of RFP/GFP positive cells, was detected when TALEN sets were introduced with a reporter. After validation, the constructs coding for TALENs and a reporter for *RAG2* were introduced into pig fibroblast cells by electroporation. **(B)** After 48 h post-transfection, the cells were sorted for GFP^+ cells by fluorescence-activated cell sorting (FACS) and individually plated into 96-well plates. The frequency of GFP positive cells from each transfection ranged from 23.0% to 38.0%. The box below the arrow indicates the gate used to sort GFP^+ cells.



Gene	Sequence
RAG2	TTCCTGAAGGCAGATATGGTcattccattgatGTCGTGTATAGTCGAGGGAA
MAN2A1	TTCCTGGAGGCAGATATGGTtgtccctggggaGTCCCCCCAGAAACAATTTA
RMDN3	ATCGTTTTCACTGATTAATAatattcaataccAAAGTATATAGTCTAAGATT
MTDH	CGGCGTCTGTGCGCCCCAGCgtcatcccgagaGTCCCCCTGACGGGAGGGAA
TRIM31	GTGCTACAATAAATAGTCTTgtgtggagtttGTCGTGTATTTGAAACTTTC
PCSK2	GTGCTGAAGGAGTGGACGCTgatgctgcacggCACGCAGAGCGCCCCTTACA
PRKACB	ATAGTAAAGGCAGAACCTGGacattatgtggcACTCCAGAATATTTGGCTCC
GCFC2	CCAAGAAAGACAGTTCTGGTcaggtctaaaggTAAGCAGAAAACTGCCAAAA

Supplemental Figure 3: Off-target analysis of *RAG2* TALENs from genomic DNA of *RAG2* mutated pigs. Upper) Surveyor nuclease digest of heteroduplex DNA revealed no additional off-target mutations at the 7 loci with highest homology to RAG2 gene; SM: size marker, lane 1, *MAN2A1*; 2, *RMDN3*; 3, *MTDH*; 4, *TRIM31*; 5, *PCSK2*; 6, *PRKACB*; 7, *GCFC2*; P, positive control – biallelic *RAG2* knock-out pig derived genomic DNA; N, negative control - wild-type pig derived genomic DNA. Lower) Genes and sequence homologies of *RAG2* related sequences to exclude off-target mutations. Upper case: TALEN binding sites; lower case; TALEN cut site; homolog base pairs in red.

A

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WT      TTCCTGAAGGCAGATATGGT CATTCCATTGAT GTCGTGTATAGTCGAGGGAA
#12(♂) TTCCTGAAGGCAGATATGGT CATT-CATTGAT GTCGTGTATAGTCGAGGGAA
#14-1 (♂) TTCCTGAAGGCAGATATG-- ------------ GTCGTGTATAGTCGAGGGAA
#2-2 (♀) TTCCTGAAGGCAGATATGGT CATT--------- GTCGTGTATAGTCGAGGGAA
#2-6 (♀) TTCCTGAAGGCAGATATGGT ----------GAT GTCGTGTATAGTCGAGGGAA
#2-32 (♀) ---------------------- ---------- -TCGTGTATAGTCGAGGGAA
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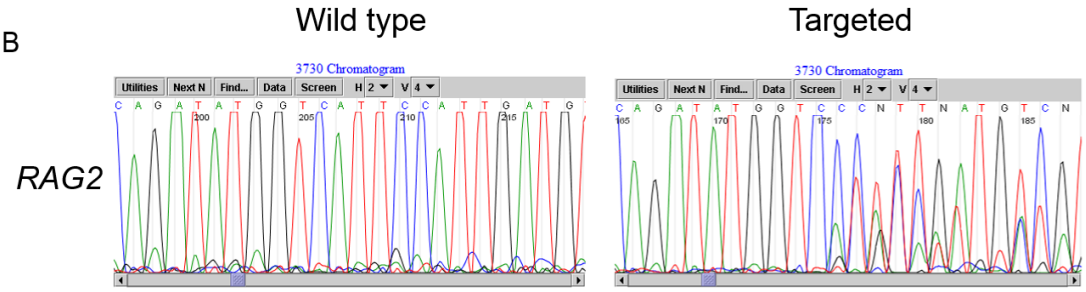
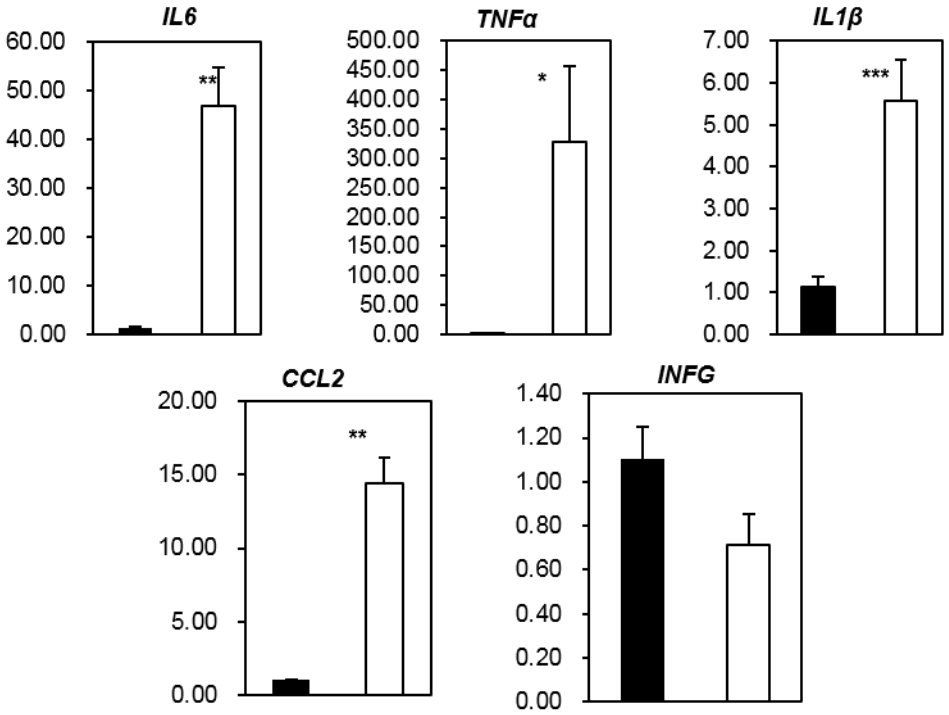
B

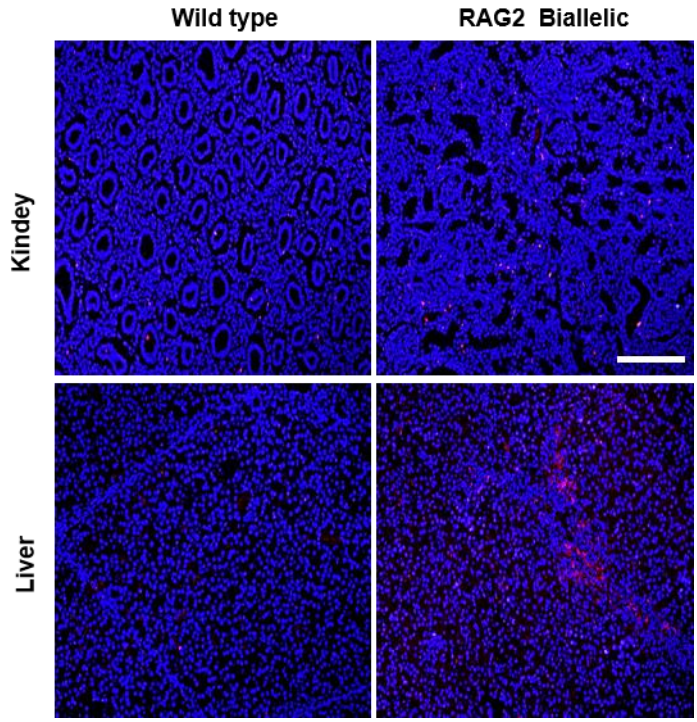


Supplemental Figure 4: Sequencing of PCR amplicons to identify candidate cell colonies for SCNT after introducing TALEN-induced polymorphisms **(A)** Different mutations identified in *RAG2*. The mutant allele #2-32, in which a 43 bp deletion resulted in formation of a premature stop codon, was used to produce monoallelic female *RAG2* knockout pigs. **(B)** Sequence read of *RAG2* (Targeted) from colony #14. When PCR was used to amplify a fragment of *RAG2* flanking the TALEN binding sites in genomic DNA isolated from colony #14, multiple nucleotide reads, not present in the Wild type DNA, were observed adjacent to the projected TALEN binding/cutting site. Cells from colony (#14) provided one of the sources employed for somatic cell nuclear transfer to produce *RAG2* mutant piglets (Supplemental Table 1).

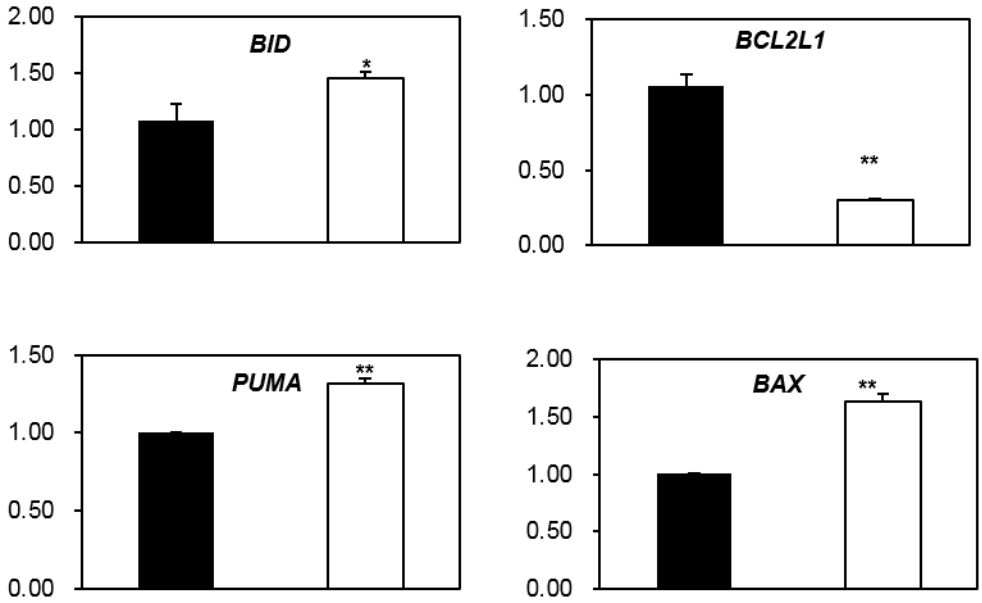
Supplemental Figure 5: The second mutation introduced within the conserved polypeptide domain of *RAG2*. In the *RAG2*$^{\Delta 140,S141H/\Delta 140\text{-}527}$ piglets, the second allele had become modified by a deletion of three nucleotides in adjacent codons, resulting in a single amino acid deletion and one amino acid replacement (S to H). The mutation occurred at a site within a 160 aa domain that is highly conserved across species ranging from mammals to birds, strongly implying that the mutation occurred at a site likely essential for the proper functioning of RAG2. The site of the mutation is highlighted with the red box.



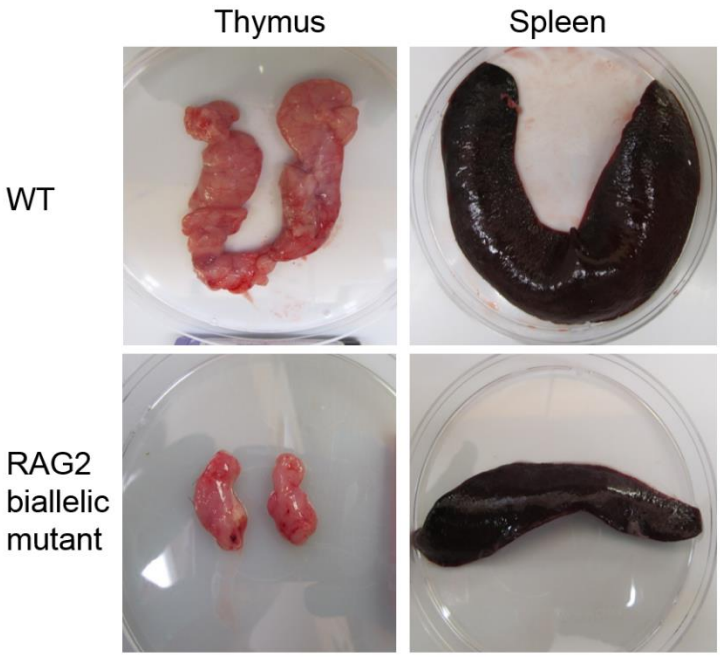
Supplemental Figure 6: Expression of genes indicative of inflammation in spleens of wild-type (control) and biallelic mutant pigs. Open bars are values for the *RAG2*$^{\Delta 140,S141H/\Delta 140\text{-}527}$ pigs; and solid bars are values for controls. The Y axis in each case indicates relative expression of mRNA relative to *ACTB*. * indicates statistical difference (*p<0.05, **p<0.01, and ***p<0.005).



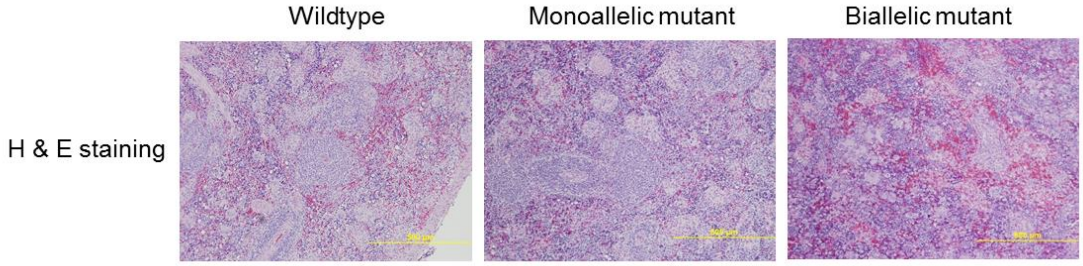
Supplemental Figure 7: Apoptosis, indicated by the pink nuclei, in wild-type and *RAG2*$^{\Delta 140,S141H/\Delta 140-527}$ pigs at day 29 as revealed by TUNEL staining of histological sections. Bar = 500 µm.



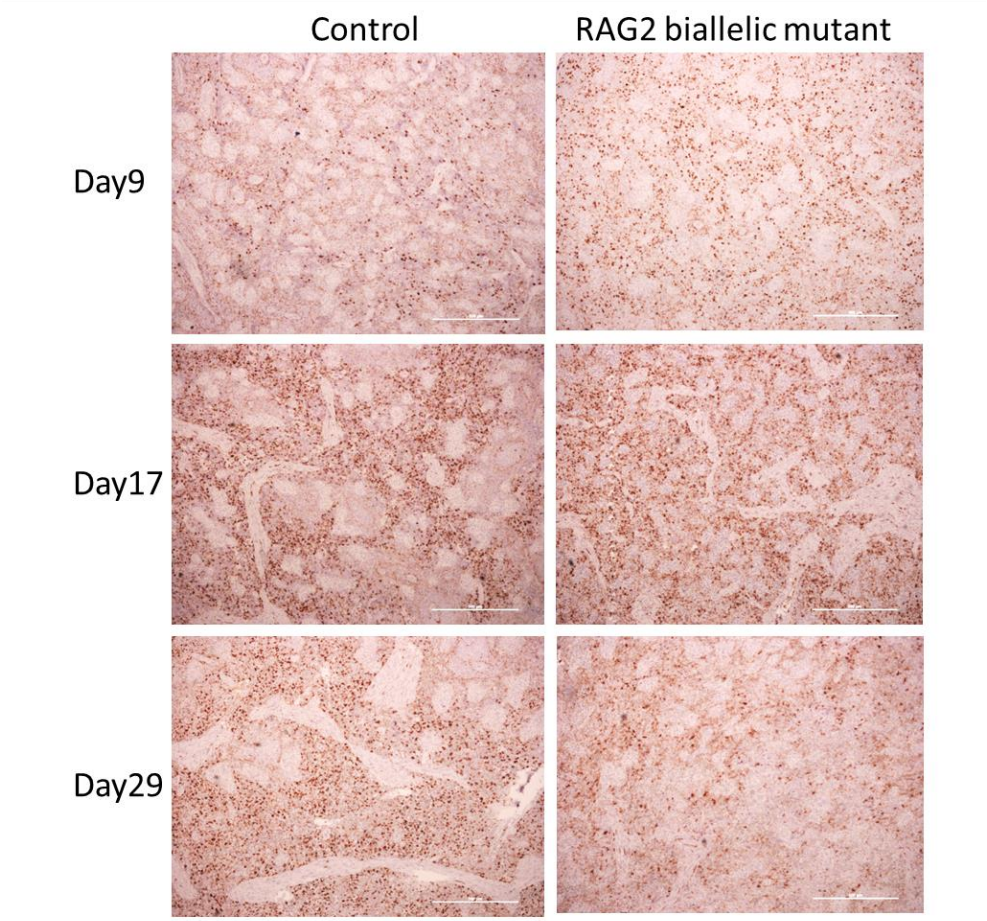
Supplemental Figure 8: Expression of genes related to apoptosis in spleens of control and *RAG2*$^{\Delta 140,S141H/\Delta 140\text{-}527}$ pigs. The expression of anti-apoptotic regulatory factor BCL-2 like protein 1 (*BCL2L1)* was lower in spleen from *RAG2* biallelic mutants than in wild-type pigs (Fig. 6B, $p<0.01$), while that of the pro-apoptotic factors *BAX*, *BID*, and *PUMA* was increased ($p<0.05$). Open bars are the biallelic mutants and solid bars the controls. Y axis indicates relative expression of mRNA relative to *ACTB*. * indicates statistical difference (*$p<0.05$ and **$p<0.01$).



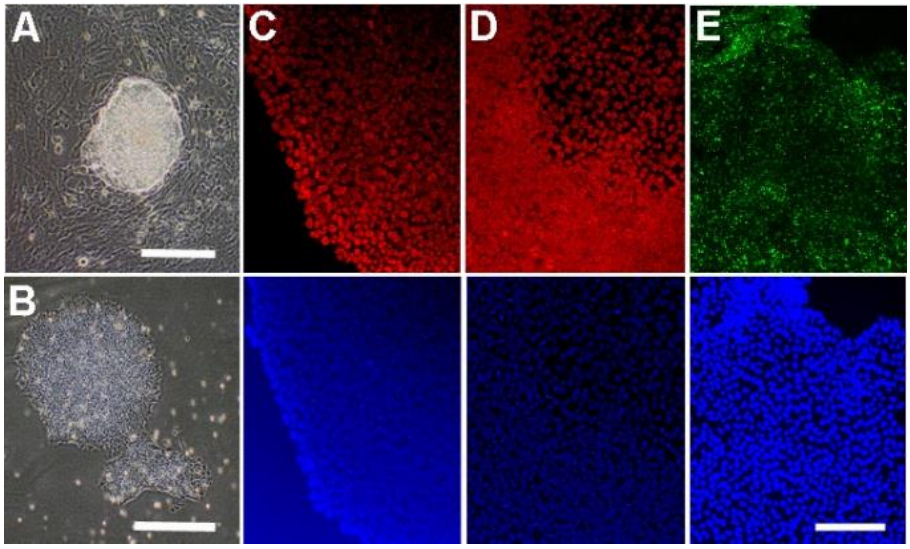
Supplemental Figure 9: Morphological comparisons of thymus glands and spleens of wild-type WT) and *RAG2*$^{\Delta 140,S141H/\Delta 140-527}$ pigs at day 29 of age. Hypoplastic development of thymus in this particular mutant pig and a smaller spleen distinguished the animal from the control. The size difference of the two spleens was reflected in their respective weights (*RAG2* biallelic mutant, 5.56 g versus control 21.99 g). Weights of these pigs were 2.22 kg (*RAG2* biallelic mutant) and 4.10 kg (wild-type). Analogous differences were noted in *RAG2*$^{\Delta 140,S141H/\Delta 140-527}$ pigs euthanized at d 17, except there was no sign of a thymus in the two mutant animals.



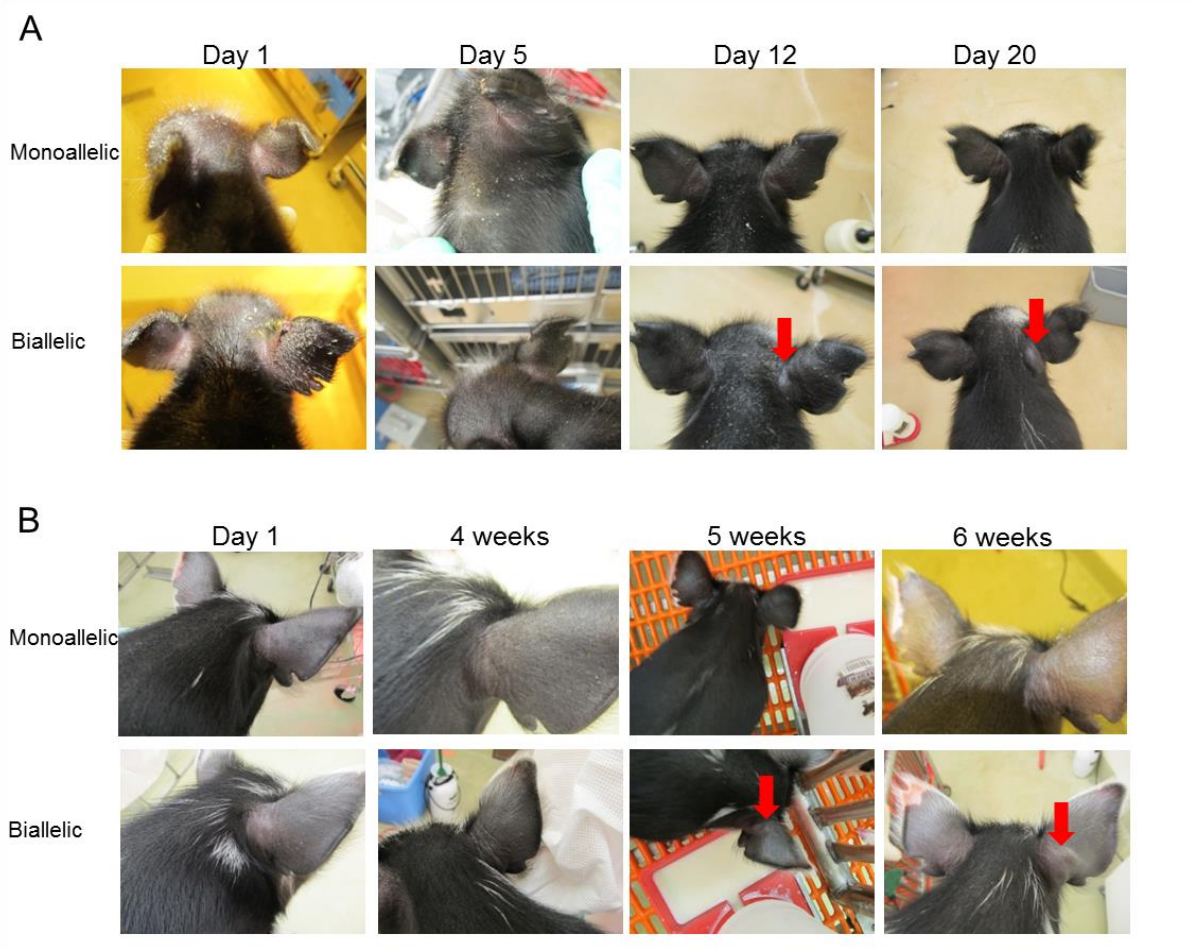
Supplemental Figure 10: Developmental defects in spleens of *RAG2*$^{\Delta 140,S141H/\Delta 140-527}$ pigs. H & E-stained sections from biallelic mutants revealed marked white pulp hypoplasia with lack of a germinal center and periarteriolar lymphoid sheath formation. The scale bar indicates 500 µm.



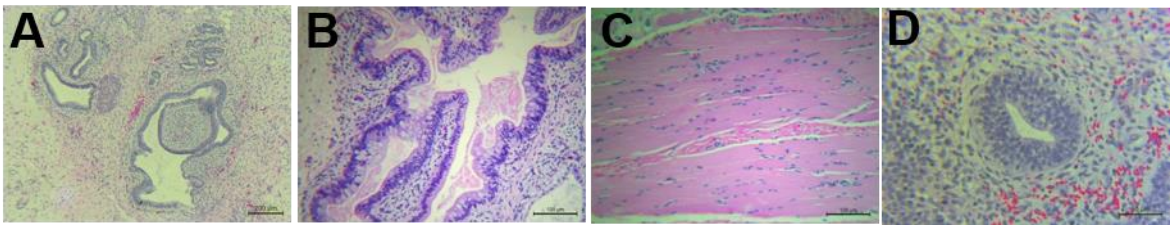
Supplemental Figure 11: Presence of macrophages in the spleen of wild-type (control) and *RAG2*$^{\Delta 140,S141H/\Delta 140\text{-}527}$ biallelic mutants. Presence of macrophage is shown by IHC with CD204 as a macrophage-specific antigen. There was no difference in abundance of macrophages between the controls and the RAG2 mutant animals. Scale bar: 500 µm.



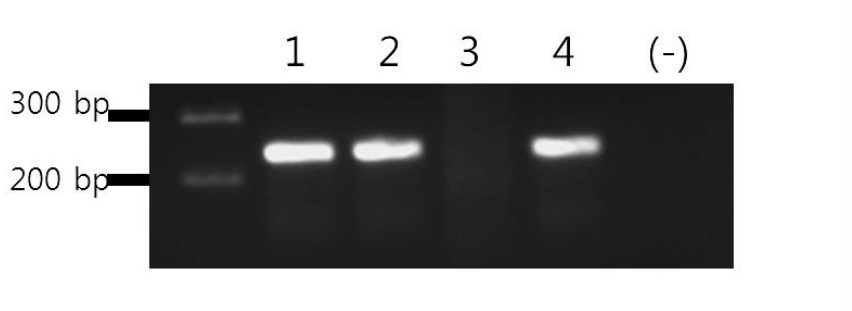
Supplemental Figure 12: Generating human iPSC with episomal plasmids. **(A)** Phase contrast image of an iPSC colony at 14 days after the plasmid transfections to umbilical cord outgrowth. **(B)** Image of the isolated iPSC colonies on feeder-free culture conditions. **(C-E)** Pluripotent nuclear protein POU5F1 **(C)**, NANOG **(D)** and cell surface molecule SSEA-4 **(E)** were expressed in the cells. Lower panels show nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). Bars = 200 µm in **A** & **B,** and 100 µm in **E**.



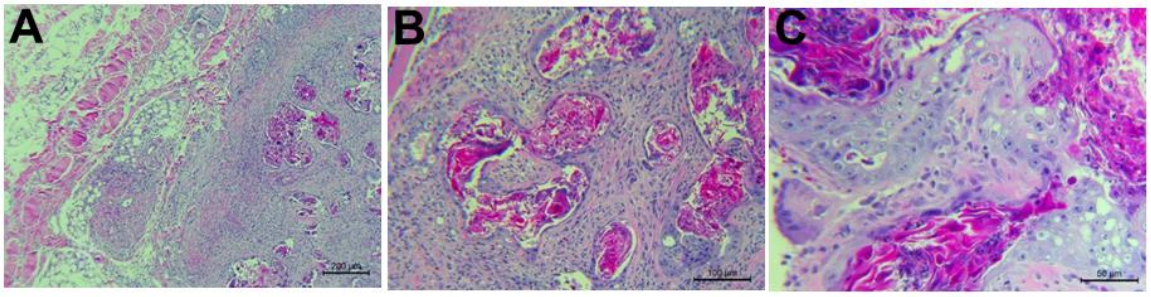
Supplemental Figure 13: Progression of the growth of teratomas in *RAG2* mutant pigs. **(A)** Pigs received 10 million cells. A growth was detected on the injection site around 12-14 days post-injection. **(B)** Pigs received 5 million cells. A growth was detected after 5 weeks post-injection. The arrows indicate sites of potential teratoma growth. No growth was observed from monoallelic *RAG2* mutants.



Supplemental Figure 14: Histological analysis of the teratoma that formed on the lateral flank area of the second *RAG2*$^{\Delta 140,S141H/\Delta 140\text{-}527}$ pig, where tumor progression was relatively slow. The tumor section contained a disorganized mixture of tissues with proportionately less muscle tissue and more cystic structures than observed in the teratomas of the first mutant pig (Fig. 4C) **(A)** Low magnification view of a section of the teratoma; **(B-D)** Representative sections illustrating the presence of the three germ layers (B endoderm; C mesoderm; D ectoderm) Bar = 100 µm.



Supplemental Figure 15: Human origin of the teratoma. A fragment of human specific mitochondrial mitofusin 1 (*MFN1*) was amplified by using PCR. Expected size of the human amplicon was 236 bp. 1, genomic DNA from human iPSC #1 line; 2, genomic DNA from human iPSC #2 line; 3, genomic DNA from *RAG2*$^{\Delta 140,S141H/\Delta 140\text{-}527}$ pig used as the recipient; 4, genomic DNA from the teratomas; (-), - negative control (no DNA).



Supplemental Figure 16: Tumor formation from transplanted porcine induced trophoblast cells injected into a *RAG2*$^{\Delta 140,S141H/\Delta 140\text{-}527}$ pig. Histological sections were stained with H&E. (**A**) the central region of the tumor (right) consisted epithelial layers of cells; the marginal region (left) contained striated muscle tissue. Bar = 200 µm. (**B**) and (**C**) are images of higher magnification at the central regions. Bars = 100 µm and 50 µm, respectively.

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