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RAG1/2 Knockout Pigs with Severe Combined Immunodeficiency

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Pigs share many physiological, biochemical, and anatomical similarities with humans and have emerged as valuable large animal models for biomedical research. Considering the advantages in immune system resemblance, suitable size, and longevity for clinical practical and monitoring purpose, SCID pigs bearing dysfunctional RAG could serve as important experimental tools for regenerative medicine, allograft and xenograft transplantation, and reconstitution experiments related to the immune system. In this study, we report the generation and phenotypic characterization of RAG1 and RAG2 knockout pigs using transcription activator-like effector nucleases and then used to provide donor nuclei for somatic cell nuclear transfer. We obtained 27 live cloned piglets; among these piglets, 9 were targeted with biallelic mutations in RAG1, 3 were targeted with biallelic mutations in RAG2, and 10 were targeted with a monoallelic mutation in RAG2. Piglets with biallelic mutations in either RAG1 or RAG2 exhibited hypoplasia of immune organs, failed to perform V(D)J rearrangement, and lost mature B and T cells. These immunodeficient RAG1/2 knockout pigs are promising tools for biomedical and translational research. The Journal of Immunology, 2014, 193: 000–000.

RAG1/2 genes encode the enzymes that catalyze the V(D)J recombination of Ig and TCR genes in B and T lymphocyte precursors to generate the diversity of B and T cell primary immune repertoires (1, 2). RAG1 and RAG2 are two adjacent genes in autosomes, and their encoded enzymes function synergistically as a protein complex (3). If either of the two genes is disrupted, V(D)J recombination is compromised and the B and T cell development arrested in an immature status.

RAG1/2 function is conserved among different mammalian species. In humans, mutations in RAG1 or RAG2 result in a complete absence of both B and T cells (T-B-SCID) or Omenn syndrome, in which abnormal T cells without sufficient recombination can be autoreactive and the patients show symptoms similar to those of graft-versus-host disease (4–6). RAG1/2 knockout mice are both immunodeficient and characterized by the absence of mature B and T cells (7, 8). Such mice are commonly used for immunological, inflammation, oncology, and stem cell transplantation studies (9–12). Immunodeficient mice have also been used for the functional reconstitution of the human hematopoietic and immune systems by transplanting human hematopoietic stem cells (13). RAG1 knockout rats (14) and RAG1/2 knockout rabbits have been reported as well (15, 16). However, rodent models and other midsize animals as rabbits are not always suitable to sufficiently imitate the human genetic and physiological states. In fact, there are significant differences in every aspect of the immune system function and regulation between mice and humans (17). Likewise, the genomic responses to inflammation are quite different in mice compared with humans (18). In addition, their small size and short longevity make it hard to carry out surgical and clinical procedures or enable long-term tracking and evaluation posttissue or postcell transplantation. Therefore, immunological assessments and procedures developed in SCID mice may not translate directly into the same outcomes in humans.

Pigs resemble humans in terms of organ size, life span, anatomical, and physiological characteristics. Thus, they are considered as important laboratory animal models for biomedical research, especially for tissue engineering and human organ transplantation involving xenograft procedures (19). SCID pigs can be potentially important research tools to facilitate long-term follow-up studies of immune responses, xenotransplantation, stem cells, and cancer over clinically relevant time frames. Furthermore, SCID pigs can help develop procedures to assess the safety of stem cell therapies, or the effects of surgery and radiation therapy in the transplanted tumors, thus constituting a remarkable preclinical model.

To date, no natural SCID pig strains have been identified. This study aimed to produce SCID pig strains by gene modification to

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; IF, immunofluorescence; PB, peripheral blood; PFF, porcine fetal fibroblast; SCNT, somatic cell nuclear transfer; TALEN, transcription activator-like effector nuclease; WT, wild type.

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satisfy the demands of biomedical research. Transcription activator-like effector nucleases (TALENs) have been reported as a powerful genome-editing tool to introduce targeted gene disruption in many species (20). We thus applied TALEN technology to target RAG1/2 in porcine fetal fibroblasts (PFFs), and then the targeted PFFs were used as nuclear donor to produce pigs by somatic cell nuclear transfer (SCNT). As a result, SCID pig models without mature B and T lymphocytes were established.

Materials and Methods

Animals

A local strain of Bama miniature pigs from Southern China was used as subjects of gene targeting. Pigs were maintained under conventional housing conditions. All experiments were performed in accordance with the guidelines for animal experiments of the Department of Science and Technology of Guangdong Province (China) and approved by the Animal Research Committee of the Guangzhou Institutes of Biomedicine and Health.

Selection of RAG1/2 knockout fibroblasts

PFFs were isolated and prepared, as previously described (21). They were cultured in 10-cm culture dishes until they reached 90% confluency. Afterward, 10^5 PFFs in 300 μl PBS containing 50 μg linearized pcDNA3.1 and 50 μg RAG1/2 TALEN paired plasmids were electroporated at 230 V/cm and 500 μg by using a Gene Pulse Xcell electroporator (Bio-Rad, Hercules, CA). The transfected PFFs were diluted in 100 ml DMEM (HyClone, Logan, UT) supplemented with 15% FBS (HyClone, Logan, UT). PFFs were then placed in 10-cm dishes. After 12 h of recovery, the attached cells were selected with 500 μg/ml G418 (Merck, Darmstadt, Germany) for ~10 d. Cell colonies with compact appearance were obtained, and then we cultured them in 48-well plates. After the cells reached confluency, one-sixth of the cells in each well were removed and subjected to genotyping. The remaining cells were transferred to 24-well plates and cultured until confluency was reached. Gene-targeted cell colonies were frozen for future use.

Genotyping of cell colonies and cloned piglets

Genomic DNA samples were extracted from the cell colonies by incubating with lysis buffer (0.45% Nonoidet P-40 v/v; 60 μg/ml protease K) at 56°C for 1.5 h and then 95°C for 15 min. The genomic DNA of the cloned pigs was extracted from ear biopsies. The following primers were used to amplify and analyze the sequences of the DNA fragment: for the targeted region of RAG1 TALENs, 5'-CTGACCTGGAGAGTCCAGTG-3' and 5'-GCA-CTAGCGAGGAAAGCTGC-3'; and for the targeted region of RAG2 TALENs, 5'-CAACGACATTCAAGGAATG-3' and 5'-TAACATGG-CCTAATTCAGC-3'.

Somatic cell nuclear transfer and embryo transfer

SCNT was performed as described in previous studies (21, 22). Pig ovaries were transported from a local slaughter house to the laboratory in 0.9% saline at 35–39°C. Oocytes were aspirated from ovarian antral follicles and cultured in maturation medium at 39°C for 42–44 h. The polar body and the adjacent cytoplasm of oocytes were gently aspirated using glass pipettes in micromanipulation medium supplemented with 0.3% BSA (A 8022, Sigma-Aldrich) and 7.5 mg/ml cytochalasin B (C6762, Sigma-Aldrich). The mutant cells were prepared as a cell suspension. A single cell was aspirated and injected into the perivitelline space of an oocyte.

Anticoagulated peripheral blood (PB) was obtained, and cell suspensions were prepared from the thymus, the spleen, and the bone marrow (BM) of RAG1/2 knockout pigs and age-matched control pigs. RBCs were eliminated with lysis buffer (555899; BD Pharmaning, San Diego, CA). We selected the mononuclear cells as a gate for the analysis. To identify CD3+ cells, CD4+ and CD8+ T cells and IgM+ B cells, we used mouse anti-pig CD3e Ab (561476; BD Pharmaning), CD4a (559585; BD Pharmaning), CD8a (559584; BD Pharmaning), and goat anti-pig IgM (AAI39F; AbD Serotec). A total of 5 × 105 cells was incubated with the indicated Abs for 45 min at 4°C and washed twice with PBS. Samples were analyzed using an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI). At least 10,000 cells were analyzed per run.

PCR analysis to identify V(D)J recombination

DNA samples were extracted from the thymus, the spleen, whole blood, and the BM of the mutant and control pigs. The primers are shown in Supplemental Table I. TCR-β recombination was analyzed by PCR using KOD-plus-neo DNA polymerase (Toyobo, Osaka, Japan) under the following conditions: 98°C for 5 min; 30 cycles of 98°C for 10 s, 68°C for 30 s, and 68°C for 1 min; and a final step at 68°C for 7 min. A fragment of IgH gene was amplified by PCR using KOD-plus-neo with the same thermal cycle condition as TCR-β. Premixed Ex Taq (Takara, Dalian, China) was used to identify the rearranged IgH locus by PCR using FR1 and JH primers. The thermal cycler parameters were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s; and a final step at 72°C for 7 min.

Results

Generation of RAG1/2 knockout pigs

Using the Golden Gate assembly method (23), we designed and assembled two pairs of TALENs targeting the exon regions of RAG1 or RAG2 (Fig. 1A). The TALEN plasmids were cotransfected with pcDNA3.1, which can provide a neomycin resistance, into PFFs derived from 35-d-old fetuses by electroporation. After selection with G418, 181 cell colonies from RAG1 TALEN-transfected cells and 454 from RAG2 TALENs were obtained from several rounds of electroporation and were cultured further. Sequencing of PCR-amplified fragments was employed to analyze the integrity of the target site in these expanded colonies. Monoaallelic and biallelic RAG mutant colonies were identified. For RAG1, 55 (30.4%) colonies were monoaallelic (RAG1+/−) and 14 (7.7%) were biallelic (RAG1−/−). For RAG2, 98 (21.6%) colonies were monoaallelic (RAG2+/−) and 6 (1.3%) colonies were biallelic (RAG2−/−). Deletions (1–56 bp) and insertions (2–7 bp) were detected in the RAG1 locus. Likewise, deletions (2–108 bp) and insertions (1–4 bp) were found in the RAG2 locus.

The following colonies were selected as donor cells for SCNT: one with RAG1+/− mutation, six with RAG1+/− mutation, four with RAG2+/− mutation, and five with RAG2+/− mutation (Table I). The reconstructed embryos were then introduced into 24 surrogate mothers. Among these mothers, 13 developed to full term and gave birth to 42 cloned piglets (Table II). Among these piglets, 15 were stillborn and 27 were alive. Among the 27 live-born piglets, 9 (5 males and 4 females) were RAG1−/−, 3 (2 males and 1 female) were RAG2−/−, 10 (5 males and 5 females) were RAG2+/−, and 5 were wild type (WT; Table II). One of the 5 male RAG1−/− pigs harbored two different mutant forms in the homologous chromosomes. The 11 other homozygous RAG1/2 knockout piglets were monoaallelic and exhibited different mutant forms in their targeted sites (Fig. 1B). All of these mutations in the exons caused frameshift, therefore producing a nonfunctional RAG1/2 enzyme.

Flow cytometry analysis

Anticoagulated peripheral blood (PB) was obtained, and cell suspensions were prepared from the thymus, the spleen, and the bone marrow (BM) of RAG1/2 knockout pigs and age-matched control pigs. RBCs were eliminated with lysis buffer (555899; BD Pharmaning, San Diego, CA). We selected the mononuclear cells as a gate for the analysis. To identify CD3+, CD4+, and CD8+ T cells and IgM+ B cells, we used mouse anti-pig CD3e Ab (561476; BD Pharmaning), CD4a (559585; BD Pharmaning), CD8a (559584; BD Pharmaning), and goat anti-pig IgM (AAI39F; AbD Serotec). A total of 5 × 105 cells was incubated with the indicated Abs for 45 min at 4°C and washed twice with PBS. Samples were analyzed using an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI). At least 10,000 cells were analyzed per run.

Histological and immunofluorescence analyses

Dead RAG1/2 knockout pigs and euthanized age-matched control pigs were dissected. The thymus and the spleen were removed and then soaked in 4% paraformaldehyde for 3 d. The fixed tissues were embedded in paraffin and sectioned at 3 μm for H&E staining and immunofluorescence (IF) analyses. In IF staining, Ag was retrieved in 1 M citrate buffer (pH = 6.0) bath for 20 min. Tissues were immunostained with FITC-labeled goat anti-pig IgM (AAI39F; AbD Serotec, Kidlington, U.K.) and rabbit anti-CD3e Abs (17617-1-AP; Proteintech, Chicago, IL) and visualized using an Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary Ab (A-11004; Invitrogen, Eugene, OR) with a fluorescence microscope.
The specificity of the TALENs was tested by electronic PCR (http://www.ncbi.nlm.nih.gov/sutils/e-pcr), which predicts the potential off-target sites in the pig genome. Using reported criteria (24), we identified three potential off-target sites for RAG1 TALENs and one for RAG2 TALENs (Supplemental Table II).

Genomic DNA obtained from all of the RAG1/2 and RAG2/2 pigs was used as template in a PCR amplifying the potential off-target regions. Primers used for this purpose are shown in Supplemental Table I. DNA-sequencing results revealed that no mutations occurred in any of the potential off-target sites.

Macroscopic and histological analyses of RAG1/2 knockout pigs

All piglets were raised under conventional housing conditions. Consequently, one male RAG1/2 (1–12) and two male RAG2/2 (2–7, 2–8) pigs that were born weak survived <3 d. Similarly, the other homozygous knockout piglets, which looked normal and strong when born, died between 11 and 29 d after birth (Fig. 1B, 1C). In contrast, heterozygous RAG2 knockout and WT piglets grew normally and survived until they reached sexual maturity. Dead knockout pigs and sacrificed age-matched WT pigs were autopsied immediately. The results showed that homozygous knockout pigs had undersized thymuses compared with age-matched WT pigs (Fig. 1D). Moreover, in one male RAG2/2 pig, one lobe of the thymus was absent. Although there was no obvious difference in size, the spleens of homozygous RAG1/2 knockout pigs were much thinner and more loosely packed (data not shown) than those of age-matched WT pigs.
The thymuses and the spleens were fixed and embedded for H&E staining to further analyze the inner structure and cell composition. In the thymuses of homozygous knockout piglets, very few hypoplasic corupes were found, and the thymus lobules were atrophied (Fig. 1E, upper panel). The number of thymocytes in these animals was also remarkably decreased compared with age-matched WT pigs (Fig. 1E, upper panel). In the spleens of RAG1/2 knockouts, the periarterial lymphatic sheath around the central artery became hypoplastic, and the white pulp almost disappeared. The number of lymphocytes was also remarkably reduced in the RAG1/2 knockout pigs compared with the WT controls (Fig. 1E, lower panel).

**Homozygous RAG1/2 knockout pigs lacked mature B and T lymphocytes**

To detect the status of B and T lymphocytes in the homozygous RAG1/2 knockout pigs, we collected cells from thymus, spleen, PB, and BM. We then performed FACS assay. After staining the thymocytes with CD4, CD8, and CD3 Abs, we found that homozygous knockout pigs harbored almost no CD4/CD8 single- and double-positive (+) cells (<1% of the total number of cells) in their thymuses compared with WT controls (<45% CD4+ cells, ~55% CD8+ cells, and ~40% CD4+CD8+ cells of the total number of cells, respectively; Fig. 2A). The ratio of CD3+CD4+ thyic cells and CD3+CD8+ thymic cells also decreased drastically in mutants (<1%), whereas ratios of ~40 and 20% (respectively) were observed in the WT counterparts (Fig. 2B, 2C). In the spleen and PB of homozygous knockout pigs, CD3+CD4+ cells were absent as well, but a small number of CD8+ cells (0.5–7% in the spleen and 10–20% in the blood) remained (Supplemental Fig. 1). These CD8+ cells were neither CD3+ nor CD4+, indicating that they were not T cells.

To determine the status of B cells, we detected IgM expression in cells derived from BM, spleen, and PB. Homozygous knockout pigs lacked IgM+ B cells in the BM (<0.5%) compared with WT pigs (18.6%) (Fig. 3A). Furthermore, IgM+ cells were almost undetectable in the PB and spleen of homozygous knockout pigs (Fig. 3A). The status of B and T lymphocytes was further analyzed by IF. The spleen of homozygous knockout pigs lacked all of the IgM+ cells and harbored <1% of CD3+ cells; by comparison, the spleen of age-matched WT pigs contained 40–50% CD3+ cells and 3–5% IgM+ cells (Fig. 3B).

**V(D)J recombination analysis of homozygous RAG1/2 knockout pigs**

We analyzed the V(D)J rearrangements of TCR and BCR to verify whether the developmental arrest of lymphocytes is caused by the inhibited RAG1/2 function. We designed a pair of primers, namely, D11J1-F (upstream D1) and D11J1-R (between J1.3 and J1.4), for PCR analysis (Supplemental Table I) to identify D-J rearrangements in the TCR-β locus. DNA samples were prepared from thymus, spleen, and PB of RAG1−/−, RAG2−/−, and WT pigs. DNA from PFFs was used as a negative control. PCR products of knockout pigs and PFF only showed the germinal band (as an inner control) on the gel, whereas WT pigs showed additional shortened rearranged bands (Fig. 4A, Supplemental Fig. 2A, 2B). This finding indicates the lack of D-J rearrangement in the TCR-β locus of homozygous knockout pigs.

For BCR, the IgH locus was selected to detect V-D-J recombination occurring in BM, spleen, and PB. Primers reported by others, namely, FR1 (upstream V1) and JH (in J5), were used to amplify the germinal internal control. The V-D-J rearrangement of IgH was performed by PCR in BM, spleen, and PB of the homozygous RAG1/2 knockout pigs. In contrast to rearranged bands in the WT control sample (Fig. 4B, Supplemental Fig. 2C, 2D). These results indicated that recombination did not occur in the IgH locus of RAG1−/− and RAG2−/− pigs.

**Discussion**

In mice, RAG genes can be manipulated by subjecting embryonic stem cells to traditional homologous recombination and chimera technology (7, 8). In pigs, germline-competent embryonic stem
cells are not available, but SCNT can be used to generate gene-targeted pigs (26). In rabbits, RAG genes have been efficiently knocked out by injecting mRNA-encoded TALENs to embryos (15, 16). However, the generated founder animals with this embryo injection approach are usually chimeric and contain cells with no mutation and/or with different mutations. To obtain genetically modified animals with a single mutation, one or two additional rounds of breeding should be conducted to select viable offspring. In contrast, TALEN injection is more applicable in small animals than in pigs, because the latter takes 6 mo to sexual maturity, exhibits longer gestation periods (~4 mo), and implies higher recipient costs than the former. In this study, porcine somatic cells were subjected to gene targeting by TALEN technology and then SCNT to produce RAG1/2 knockout pigs. Viable biallelic and monoallelic mutants were obtained through a single round of nuclear transfer.

Gene-targeting efficiency of PFFs was quite high in our study. Efficiencies of 48 and 23.6% were obtained for RAG1 and RAG2, respectively. Traditional homologous recombination methods cannot normally reach a sufficiently high efficiency to achieve Table II. SCNT results for the generation of RAG1/2 knockout cloned pigs

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Colonies</th>
<th>Transferred Embryos</th>
<th>No. Recipients</th>
<th>No. (%): Pregnancies</th>
<th>No. Born</th>
<th>No. Survived</th>
<th>No. (%): Heterozygotes</th>
<th>No. (%): Homozygotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAG1 D1 and D17</td>
<td>226</td>
<td>1</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C52 and C85</td>
<td>355</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>1 (m)</td>
<td>3 (f)</td>
</tr>
<tr>
<td>C85</td>
<td>233</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1 (m)</td>
<td>3 (f)</td>
</tr>
<tr>
<td>E1</td>
<td>263</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>1 (f)</td>
<td>3 (f)</td>
</tr>
<tr>
<td>E5</td>
<td>160</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1 (f)</td>
<td>3 (f)</td>
</tr>
<tr>
<td>E5 and E6</td>
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<td>1</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>1285</td>
<td>9</td>
<td>6 (66.7)*</td>
<td>24</td>
<td>12</td>
<td>0</td>
<td>9 (5 m, 4 f)</td>
<td>(75.0)*</td>
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<tr>
<td>RAG2 A206</td>
<td>1010</td>
<td>4</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A74 and A107</td>
<td>266</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3 (m)</td>
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<td>2 (m)</td>
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<tr>
<td>A63 and A107</td>
<td>229</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2 (m)</td>
<td>0</td>
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<tr>
<td>A151</td>
<td>818</td>
<td>3</td>
<td>1</td>
<td>2</td>
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<tr>
<td>A188</td>
<td>487</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>B2 and C1</td>
<td>399</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>2 (f)</td>
<td>1 (f)</td>
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<tr>
<td>B2 and C3</td>
<td>424</td>
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<td>2</td>
<td>4</td>
<td>3</td>
<td>3 (f)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3633</td>
<td>15</td>
<td>7 (46.7)*</td>
<td>18</td>
<td>15</td>
<td>10 (5 m, 5 f)</td>
<td>3 (2 m, 1 f)</td>
<td>(66.7)*</td>
</tr>
</tbody>
</table>

*aShown as percentage of recipients.

*bShown as percentage of survived piglets.

f, female; m, male.

FIGURE 2. FACS analysis of T lymphocytes in the thymus of homozygous RAG1/2 knockout pigs. (A) CD4/CD8 single- and double-positive cell defects in the thymus of homozygous knockout pigs. (B) CD3+CD4+ cell defects in the thymus of homozygous knockout pigs. (C) CD3+CD8+ cell defects in the thymus of homozygous knockout pigs. Samples from an age-matched WT pig were used as control.
adequate gene-targeted somatic cells that can be used for the subsequent nuclear transfer. Among the modified biallelic colonies, a high proportion was homozygous in the same indel. Other groups have reported similar findings using TALENs to target porcine somatic cells (27).

Among the 42 cloned piglets, 15 were stillborn. The reason for this high incidence remains unclear, but off-targeting or aberrant reprogramming may have contributed to this abnormality. Different mutant types, including RAG1<sup>−/−</sup>, RAG2<sup>−/−</sup>, and RAG2<sup>+/−</sup> on their targeted sites, were found among the 27 live-born piglets. RAG1/2 biallelic knockout pigs showed slower weight gain than the WT and heterozygous littermates and could not survive for >1 mo under conventional housing conditions. The early death of these homozygous pigs is probably because of deficient immune function. In contrast, the WT and RAG2<sup>+/−</sup> piglets were healthy and grew until they reach sexual maturity under the same conditions. We are now breeding them to establish stable heterozygous lines. Homozygous SCID pigs could then be produced conveniently by breeding heterozygotes whenever they are needed to perform experiments with a specific purpose.

V(D)J recombination in our knockout pigs is inhibited because of the lack of RAG1/2 enzymes. We also observed hypoplastic thymus and spleen in RAG1/2 knockout pigs, as in RAG1/2 knockout mice (7, 8) and Il2rg knockout pigs (26). RAG1/2 knockout pigs also showed significantly decreased proportions of mature B and T lymphocytes compared with age-matched WT pigs. The same finding has been found in mice (7, 8), rats (14), rabbits (15), and in some cases of human SCID (28). Phenotypic differences, such as a decrease in the number of B cells, which are observed in Il2rg null mice but not in humans, have not been observed in RAG1/2 knockout pigs.

**FIGURE 3.** FACS analysis of B lymphocytes and IF staining of B and T lymphocytes in homozygous RAG1/2 knockout pigs. (A) IgM<sup>+</sup> B cell defects in the BM, spleen, and PB of homozygous knockout pigs. (B) IgM and CD3 IF analysis showed that the spleen of homozygous knockout pigs has lost IgM<sup>+</sup> and CD3<sup>+</sup> cells. Representative IF image for FITC-labeled IgM<sup>+</sup> cells and Alexa Fluor 594-labeled CD3<sup>+</sup> cells. Samples from age-matched WT pigs were used as control. Scale bar, 200 μm.
Considering that B lymphocytes accounted for a very low percentage of cells in tested tissues (such as 3–5% IgM+ B cells in the WT spleen), we concluded that FACS data were insufficient to evaluate the depletion of lymphocytes in homozygous RAG1/2 knockout pigs. Therefore, we performed IF to confirm the FACS recombination analyses of TCR-β gene in the thymus and IgH gene in the BM of homozygous RAG1/2 knockout pigs. (A) TCR-β gene rearrangement in the thymus was detected using DJ1-F and DJ11-R primers; PCR products of homozygous knockout pigs showed only the germland bands (arrowhead) on the gel; WT pigs showed additional shortened bands (arrows). (B) IgH gene recombination in the BM was detected using FR1 and JH primers for the rearranged gene and D4-F and J3-R primers for the germland ones as an inner control. The rearranged bands (arrow) were evident in the WT pigs but not in any of the homozygous knockout pigs. G, PFFs; W, water.

FIGURE 4. V(D)J recombination analyses of TCR-β gene in the thymus and IgH gene in the BM of homozygous RAG1/2 knockout pigs. (A) TCR-β gene rearrangement in the thymus was detected using DJ1-F and DJ11-R primers; PCR products of homozygous knockout pigs showed only the germland band (arrowhead) on the gel; WT pigs showed additional shortened bands (arrows). (B) IgH gene recombination in the BM was detected using FR1 and JH primers for the rearranged gene and D4-F and J3-R primers for the germland ones as an inner control. The rearranged bands (arrow) were evident in the WT pigs but not in any of the homozygous knockout pigs. G, PFFs; W, water.

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Disclosures

The authors have no financial conflicts of interest.

References

