Supplementary Information, Data S1

All operations and experiments involving rabbits were approved by the Animal Research Ethics Committee of the Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences.

Materials and methods

Construction of TALENS

The TALENs for targeting rabbit RAG1 exon and RAG2 exon were individually designed and assembled according to the Golden Gate TALEN assembly method[1] and contained ELD/KKR derived from Fok I nuclease domains[2]. We acquired the plasmids for TALEN assembly from Addgene (MA, USA). All repeat variable di-residues were assembled and transferred into pCS2+KKR and pCS2+ELD.

Collection of rabbit zygotes

New Zealand white rabbits were treated with pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG) as previously described[3]. Sexually mature donor rabbits were superovulated by intramuscular injection with 100 U of PMSG (Ningbo Renjian Pharmaceutical Co., Ltd., Ningbo, China). On the fourth day, donor rabbits were mated and intravenously injected with 100 U of hCG (Ningbo Renjian Pharmaceutical Co., Ltd., Ningbo, China). The fertilized oocytes were flushed from oviducts with pre-warmed M199 18 h to 20 h after mating. Medium 199 includes Hanks' salts and 25 mM4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (12350; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS); SH0070.03; Hyclone, Logan, UT, USA) and transferred into Earle's balanced salt solution (EBSS) complete medium (SH30029.09; Hyclone) supplemented with non-essential amino acids, essential amino acids (BME), 1 mM L-glutamine,

0.4 mM sodium pyruvate, and 10% FBS[4].

Microinjection with TALEN-coding mRNA and in vitro cultivation of injected embryos

The TALEN-coding mRNA was synthesized and purified using an AmbionmMessagemMachine kit. The RNA concentration was determined by spectrophotometry, and the quality was analyzed by electrophoresis on 1% denaturing agarose gel.

All manipulations ofembryos were performed in M199 covered with mineral oil on a heated microscope stage set at 37.5 °C. The mRNA diluted in RNase-Free tris-ethylenediaminetetraacetic acid buffer, approximately 5-10 pL (50 ng/μL), was injected into the cytoplasm of pronuclear-stage embryos as described in Flisikowska et al.[5]. The injected embryos were cultured in EBSS complete medium at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 5 d to test whether or not the developmental competence of embryos was affected by injection of TALEN-coding mRNA.

Embryo transfer

Embryo transfers were conducted according to our published procedures[3]. The surrogate rabbits and donor rabbits were injected with 100 U hCG on the same day. Six to fourteen injected embryos were transferred into the oviducts through the fimbriae.

Mutation detection of RAG locus in injected embryos and newborns

Morula or blastocyst stage embryos injected with mRNA were individually collected and lysed with NP40 solution (1% NP40 plus 50 ng/μLProteinase K in 1 X Taq Buffer). PCR amplification of the RAG target site was conducted according to the published protocol[6]. The genome DNA of newborn rabbit ear tissue was extracted and used as template for PCR. DreamTaq DNA Polymerase (Thermo Scientific) was used to amplify the target site with the following

conditions:95 °C for 5 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and then 72 °C for 5 min. The PCR products were recovered and purified using a DNA purification kit (Tiangen, Beijing, China). The recovered PCR products were sequenced (BGI, Shenzhen, China) to preliminarily determine the events of mutation. The PCR products showing a different curve compared with those of wild type animals were cloned into the pMD-18T simple plasmid vector (Takara, Dalian, China). At least 10 colonies selected from each transformation were used for sequencing to obtain detailed information of the mutation.

T7 endonuclease I (T7E1) assay

The genomic DNA was extracted from rabbit embryos lysed with NP40 solution as described above or survival founder rabbits peripheral blood using the Genomic DNA Extraction Kit (Tiangen, Beijing, China). PCR to amplify fragment encompassing the TALEN target site was performed with the following protocol: 95 °C for 7 min; 35 cycles of 98 °C for 10 s, 68 °C - 0.6 °C/cycle for 30 s; 68 °C for 3 min, and then 72 °C for 5 min in a 20 μL reaction using ExTaq (Takara, Dalian, China). Primers were listed in supplementary information, Table S1 (rRAG1-F1 and rRAG1-R1 for RAG1 target site, rRAG2-F2 and rRAG2-R2 for RAG2 target site). As previously described7, 2 μL of PCR product was melt and reanneals in NEBuffer 2 (New England Biolabs) with the following protocol: 95 °C for 10 min; 95 – 98 °C at – 2°C/s; 85 – 25 °C at – 0.1°C/s; hold at 4 °C. The annealed samples were treated with 10 units of T7 endonuclease I (New England Biolabs) for 15 min at 37°C in a reaction volume of 20μL. Reactions were stopped by the addition of 2 μL 0.5 M EDTA, and then analyzed by 10% polyacrylamide gel electrophoresis.

Histological analysis of thymus and spleen

The thymus and spleen collected from WT or KO rabbits were fixed in 10% neutral buffered

formalin for 48 h, followed by dehydration and treatment with xylene for 10 min. Each paraffin-embedded tissue was cut into 5 μm thick sections and then stained with H&E. Histological evaluation was performed with light microscopy.

Fluorescence-activated cellsorting (FACS) analysis ofT and B lymphocytes development

Approximately 1mL heparinized blood samples were collected from the rabbit auricular veins and lysed using hypotonic ammonium chloride. Thymocytes, splenocytes, and bone marrowcells were collected into RPMI1640 and single-cell suspensions were prepared by standard methods as previously described[8]. Anti-rabbit IgM (NRBM, IgG1), FITC-conjugated anti-rabbit CD4(KEN4, IgG2a) and anti-rabbit CD8 (12.C7, IgG1)were purchased from AbDSerotec (Düsseldorf, Germany). Anti-mouse IgG-phycoerythrin (PE) was obtained from Santa Cruz (CA, USA). Approximately 0.5×10^6 to 1×10^6 cells per sample were incubated with primary antibody in 50 μL of phosphate buffered saline (PBS) for 30 min on ice in the dark, washed twice by centrifugation at 400 *g* for 5 min with ice-cold PBS, and then incubated with a labeled second antibody under the same conditions. After centrifugation, the cells were resuspended with 200 μL of ice-cold PBS shortly before they were used for FACS analysis. An AccuriC6 flow cytometer (Becton Dickinson) and Flowjosoftware(Tree Star, Ashland, OR, USA)were used to collect and analyze the data. A population of more than 10,000 live cells was analyzed per sample.

Detection of V(D)J gene rearrangement

To confirm whether or not the recombination of V(D)J is deficient in RAG-KO rabbits, blood DNA was isolated from live RAG-KO rabbits and analyzed by PCR[9]. The PCR mixture (50 μL) included 1× long PCR buffer with 1.5 mM$MgCl_2$, 15 mMdNTP, 1 μM each primer, 1 μg of

template DNA, and 2.5 U of long PCR Enzyme Mix (Thermo Scientific). The reaction was performed under the following conditions: 7 min at 94 °C, 35 cycles of 98 °C for 10 s, 68 °C to 0.6 °C/cycle for 30 s, 68 °C for 4 min + 5 s/cycle, and 68 °C for 10 min.

Off-target analysis

Potential off-target cleavage sites of TALENs were predicted by e-PCR in the rabbit genome. The criteria for identifying off-target sites were referred from Miller[10]. No more than six mismatches and 2 bp gaps in the two effector binding elements (EBEs) and fewer than 1000 bp between the two putative off-target sites were observed. Up to 26RAG1-TALENs and 14 RAG2-TALENs potential off-target sites were identified. Among these, the spacer region from two RAG1 and six RAG2 potential sites was <100 bp. The potential off-target sites were amplified and sequenced as previously described[10].

References

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