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Research Article

Efficient Production of Biallelic RAG1 Knockout Mouse Embryonic Stem Cell Using CRISPR/Cas9

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Background: Recombination Activating Genes (RAG) mutated embryonic stem cells are (ES) cells which are unable to perform V (D) J recombination. These cells can be used for generation of immunodeficient mouse. Creating biallelic mutations by CRISPR/Cas9 genome editing has emerged as a powerful technique to generate site-specific mutations in different sequences.
Objectives: The main purposes of this study were to achieve complete knock-out of RAG1 gene by investigating the nature of mutations in mutant mESC and to generate RAG1 knock-out mESCs containing homozygous indels with the aim of creating desired and specific RAG-1 -/- mutant mouse in a shorter period of time.
Materials and Methods: Here, we first utilized CRISPR/Cas9 system to target RAG1/RAG2 genes in NIH3T3 cells to test the activity and efficiency of our CRISPR system. Then we used the system for targeting RAG1 gene in mouse embryonic stem cell (mESCs) to generate knock-out embryonic stem cells. This method combined with highly active single guide RNA (sgRNA) is an efficient way to produce new RAG1-knockout mESCs in the selected regions of early coding DNA sequence, approximately between nucleotide c. 512-c. 513 and nucleotide c. 725-c. 726 of RAG1 coding sequence that had not been targeted previously.
Results: CRISPR gene editing resulted in a multitude of engineered homozygous and compound heterozygous mutations, including both in-frame and out-of-frame indels in 92% of mES cell clones. Most of the mutations generated by CRISPR/Cas9 system were out-of-frame, resulting in a complete gene knockout. In addition, 59% of the mutant ES cell clones carried out-of-frame homozygous indel mutations. The RAG1-knockout mESC clones retained normal morphology and pluripotent gene expression.
Conclusions: Our study demonstrated that CRISPR/Cas9 system can efficiently create biallelic indels containing both homozygous and compound heterozygous RAG1 mutations in about 92% of the mutant mESC clones. The 59% of mutant ES cell clones carried out-of-frame homozygous indel mutations.

Keywords: CRISPR-Associated Protein 9; Gene Editing; Homozygote; Mouse Embryonic Stem Cells

1. Background

Embryonic stem cells (ESCs) are derived from the inner cell mass of an embryo (blastocyst stage) (1, 2). Mouse embryonic stem cell (mESCs) have been widely used as vehicles to transfer site-specific genetic modifications to the mouse germline for producing mutant mouse models (3). Currently a variety of mouse models with natural immune system defects can be applied for the immunology and genetic studies. Among immunodeficiency mice models, recombination activating gene (*RAG*) deficient mice with targeted gene deletion can be applied for studies such as the xenografts, cancer, vaccines generation, autoimmune, infectious diseases, and graft-versus-host disease (GVHD) (4). *RAG1* and *RAG2* genes play an important role in the rearrangement and recombination of immunoglobulin and T-cell receptor genes during the process of V (D) J recombination.

Among the immunodeficiency knockout mouse models, *Rag1−/−* (5) and *Rag2−/−* (6) mice, which are characterized by complete absence of T and B cells.

RAG-1-homozygous-mutant mice containing mutation at nucleotide c. 2169 exhibit impaired lymphocyte development and decreased V(D)J rearrangements (7) and *RAG1*-mutant mouse with mutation around coding nucleotide 2916 had oligoclonal T cells, and demonstrated elevated levels of IgE (8). Mouse models of *RAG1* deficiency contain mutation around nucleotide c. 2514 and show complete absence of peripheral T and B cells (9).

In all the studies mentioned above, the regions related to catalytic core of RAG proteins were targeted. However, in order to produce early frame shifts and stop codons, target sites near the 5′ end of coding DNA sequence (CDS) are more efficient (10).

In present study, the early region of the second exon in CDS around nucleotides 512 and 725 of the *RAG1* locus was simultaneously targeted.

In recent years, programmable site-specific nucleases have been used to create immunodeficient models with recombination-activating genes (*RAG-1/RAG-2*) (4, 11-13).

Although, mutant mouse models can be directly generated by microinjection of programmable site-specific nuclease into zygotes, the production of mutant mice via mESCs may be suitable to derive gene-targeted mice since the nature of mutations after zygote injection cannot be determined prior to the mouse development. With ESCs, however, one could screen for the desired mutations before the creation of the genome-modified mice (14) and it may be easier to identify the genetic alterations in the ES cell clones than directly in the newly-modified mice. Besides, CRISPR/Cas9-mediated mutagenesis by zygote injection in mice creates somatic mosaicism in embryos (15, 16), resulting in allele complexity in the same animal. Thus, reaching the specific mutant or knock-out gene requires crossing animals which takes a longer period of time. One of the possible hypotheses for mosaic DNA mutation phenomenon is that the prolonged expression of Cas9 in embryos could cleave DNA continuously (16).

Thus in order to optimally and potentially use the CRISPR system for generating knockout mice it may be better to use ES cells instead of embryos.

2. Objectives

In this study, we generated various *RAG1* knock-out mouse ES cell lines containing in-frame and out-of-frame mutations by targeting a region at the beginning of exon1 using CRISPR/Cas9 genome editing system with two purposes: 1) investigating the nature of mutations in mutant mESC in order to achieve complete knock-out of RAG1 gene. 2) generation of *RAG1* knock-out mESCs containing homozygous indels with the aim of creating desired and specific *RAG*-1 $^{-/-}$ mutant mouse in a shorter period of time.

3. Materials and Methods

3.1. Design of Specific Single Guide RNAs (sgRNAs)

To design effective sgRNAs for targeting the region around the beginning of exons near the ATG start site of *RAG1/RAG*2 genes, a series of sgRNAs were analyzed through the online CRISPR RGEN tools program (https://*www.rgenome.net*).

sgRNAs were selected based on the rules for targeting efficiency and limited off-target sites. We targeted two regions of the *RAG1* and *RAG2* genes. For the *RAG1* gene two different gRNAs (gRNA F1 and F2) were selected to introduce a DSB between nucleotide c. 512 and c. 513 (gRNA-F2) and between nucleotide c. 725 and c. 726 (gRNA-F1). For the RAG2 gene, DSBs were introduced between nucleotide c. 405 and c. 406 (gRNA-F1) and between nucleotide c. 452 and c. 453 (gRNA-F2) (**Fig. 1**).

sgRNA positioning of the two proto-spacer regions (sgRNA target sites) in each *RAG1/RAG2* gene were assembled in separate expression vectors. To achieve this, pX330 vector (Addgene # 42230) and pLenti-Cas-Guide vector (Origene, GE100010), both consist of sgRNA scaffold and Cas9, were used. pX330 expression vector was digested with *Bbs*I (thermo scientific, ER1011) and pLenti-Cas-Guide vector was digested with *Bam*HI (thermo scientific, ER0051) and *Bsm*bI (thermo scientific, ER0451) restriction enzymes. Afterwards, both vectors were treated with Alkaline Phosphatase (thermo scientific, EL0011).

Complementary oligos; RAG1-F1, RAG1-F2, RAG2-F1 and RAG2-F2 (**Fig. 1**) for each target sequence were heated at 95 °C for 5 min, and annealed by decreasing 0.5 °C/second to 22 °C using a thermocycler (eppendorf, USA). Then, the short double strand DNA fragments (RAG1-F1R1, RAG2-F1R1 and RAG2-F2R2) were ligated into linearized vectors. In order to confirm ligation and correct direction of inserts, digestion with restriction enzymes and sequencing were done.

3.2. Validation of the CRISPR/Cas9 Genome Editing for Introduction Targeted DSBs

To test whether CRISPR system can achieve targeted cleavage of mouse chromosomes, NIH3T3 cell line was co-transfected using Lipofectamine2000 (Invitrogen, 11668), according to manufacturer's protocol, and GFP plasmid was used to monitor the transfection efficiency. Puromycin selection was carried out over 3-5 days and genomic DNA was isolated. Fragments of *RAG1* and *RAG2* genes were amplified using PCR, checked on a 1.5-2% agarose gel and subjected to Sanger sequencing using primers that had been designed for regions surrounding the target sites in *RAG1* and *RAG2* genes (**Table 1**).

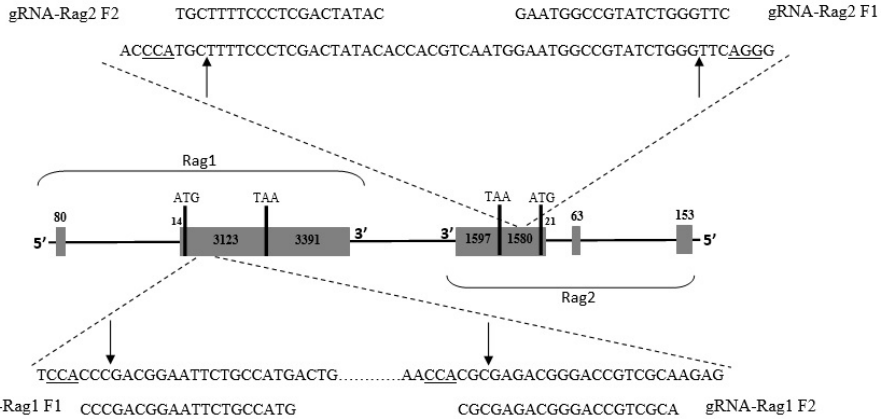

Figure 1. RAG1 structure and gRNA design. Two gRNAs (gRNA F1 and gRNA F2) for each RAG1/RAG2 genes were designed to target the selected regions. PAM sequence (NGG) is underlined. The regions at the beginning of the exons near the ATG start site were targeted.

Table 1. Primers used to amplify fragments of RAG-1 and RAG-2 genes

Primer names	Sequence 5′ to 3′
RAG1-primer F	GAA GAA GCA CAG AAG GAG AAG
RAG1-primer R	ATC GGC AAG AGG GAC AAT AGC
RAG2-primer F	ATTCCTCCTGGCAAGACT
RAG2-primer R	GCATAGACTCTGACAAGCA

3.3. ES Cell Culture and Transfection

Male ES cells, strain 129 mouse embryonic stem cells, were derived from the inner cell mass of strain 129 mouse blastocyst (at 3.5 days post coitus) in our laboratory. Cells were grown under feeder-free-culture condition in mESC proliferative medium supplemented with R2i: Dulbecco's Modified EagleMedia-F12 (DMEM-F12) (invitrogen, 21331) supplemented with 15% Knockout Serum (KOSR) ,1% non-essential amino acids, 2 mM L-glutamine, 0.1 mM 2-β-mercaptoethanol,100 $U.mL^{-1}$ penicillin, and 100 $mg.mL^{-1}$ streptomycin, 1000 $U.mL^{-1}$ mouse leukemia inhibitory factor (LIF), R2i (R2i including 1 μM PD0325901 and 10 μM SB431542), and 2% ES-FBS (ES Cell Qualified FBS). Mouse ESCs were grown on 0.1%-gelatin-coated support in the absence of feeder cells.

Transfection in mES cells was done as described for NIH3T3 cells using *Lipofectamine*2000 *according to manufacturer's instructions*. Cells were co-transfected with sgRNA and Cas9 expressing vector and a puromycin resistant plasmid, for subsequent selection, by lipofectamine 2000 as described in the previous section. The genomes of transfected ES cells were then examined.

3.4. RAG1 Knockout ES Cell Production by CRISPR/Cas9 System

To isolate mES single colonies several gelatin-coated 96-well plates were prepared and serial dilutions were performed across the entire plates. Clones were obtained by serial dilution of ES population in 96-well plates. After 4 to 5 days, full wells containing single cells were identified by microscopy and the clones were transferred into a new gelatin-coated 96-well plate. DNA was extracted from ES cell clones and the required regions of *RAG1* gene were extracted and amplified by PCR. The PCR products were sequenced by Sanger sequencing with the desired *RAG1* primers and mutations were confirmed by sequencing (Macrogen, Korea).

3.5. Real-Time-Reverse-Transcription PCR

OCT4, SOX2, and NANOG gene expression levels were analyzed by real-time quantitative PCR based on SYBR Green detection with the QIAGEN's real-time PCR machine. Total RNA was extracted from mESCs and mouse embryonic fibroblast (MEF) cells, as the control, using TRIzol reagent. First-strand cDNA synthesis was completed using a cDNA Synthesis Kit (Takara; Kyoto, Japan).

The PCR primers used to amplify OCT4, SOX2, and NANOG genes are listed in **Table 2**. Real-time PCR was performed with SYBR Green in a final reaction volume of 20 μL. Finally, relative gene expression levels were quantified by normalizing to the respective GAPDH mRNA level. Experiments were conducted in duplicates.

Table 2. Primers used to amplify fragments of OCT4, SOX2 and NANOG genes

Primer names	Sequence 5′ to 3′
Oct4-primer F	CGTGTGAGGTGGAGTCTGGA
Oct4-primer R	GCTGATTGGCGATGTGAGTG
SOX2-primer F	GGACTGCGAACTGGAGAAGG
SOX2-primer R	CGTTAATTTGGATGGGATTGGT
Nanog-primer F	CTGAGGAGGAGGAGAACAAGGTC
Nanog-primer R	CATCTGCTGGAGGCTGAGGTA

3.6. Statistical Analysis

All experiments were repeated at least three times. The results were expressed as the means ± S.D, using SPSS version 22.0 statistical software for data analysis of

variance (ANOVA; Tukey test). $P < 0.05$ was considered statistically significant.

4. Results

4.1. sgRNA-Cas9 Guided Genome Editing in RAG Genes

To test whether the designed sgRNAs can achieve targeted cleavage we used CRISPR/Cas9 system for targeting the *RAG1* and *RAG2* locus in mouse embryonic fibroblast cells (NIH3T3).

We targeted two regions in each of the *RAG1* and *RAG2* genes. Two sgRNAs were used to introduce DSBs at the beginning of the exon near the ATG start site of each gene (**Fig. 2A**). Because of the greater ability of *RAG1* sgRNAs vs *RAG2* gRNAs to induce Cas9-mediated introduction of DSBs at the *RAG* locus and the easy detection of *RAG1* sgRNAs-induced indels by PCR (data not shown), we selected *RAG1* sgRNAs for transfection into the mES cells to target the same two sites simultaneously. As previous experiments have revealed, disruption of *RAG1* or *RAG2* blocks the initiation of V(D)J recombination in mice (5, 6).To analyze genome editing by sgRNA-Cas9, genomic DNA was isolated from ES cells harvested 3-5 days after transient transfection. The extracted DNA was analyzed for the presence of site-specific gene modification by the PCR amplification of regions surrounding the target sites (**Fig. 2B**).

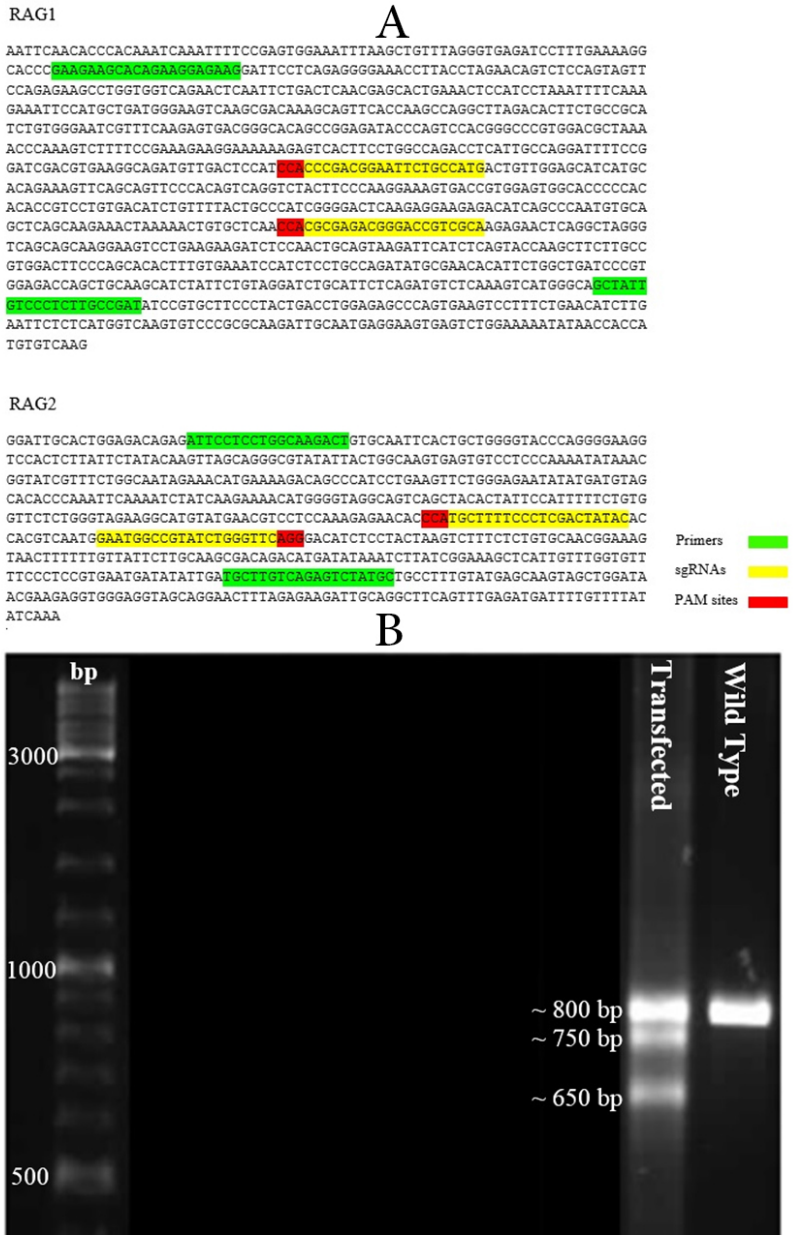

Figure 2. RAG1 locus and CRISPR-mediated cleavage activity in mESCs. (A) Target selection and sequences corresponding to the RAG1 and RAG2 fragments. (B) Gel-electrogram Image of RAG1 fragments after CRISPR-mediated cleavage activity in mESCs. PCR products of RAG1 were amplified and directly analyzed by 2 % agarose gel. The presence of ~750 and ~650 bp fragments showed that deletions have been occurred in ~800 bp RAG1 fragment.

4.2. RAG1 Knockout mES Clones

To isolate single knock out clones, after 3 weeks of growing mES cells colonies in 96-well plates, a total of 13 clones were isolated from the single-cell culturing of four 96-well plates. Upon performing PCR with primers around the cleavage sites in *RAG1* gene, indels were detected in the 12 of 13 clones. Based on the resulting DNA fragment bands on the agarose gel, 9 of them showed deletions and 3 of them showed insertions (**Fig. 3**).

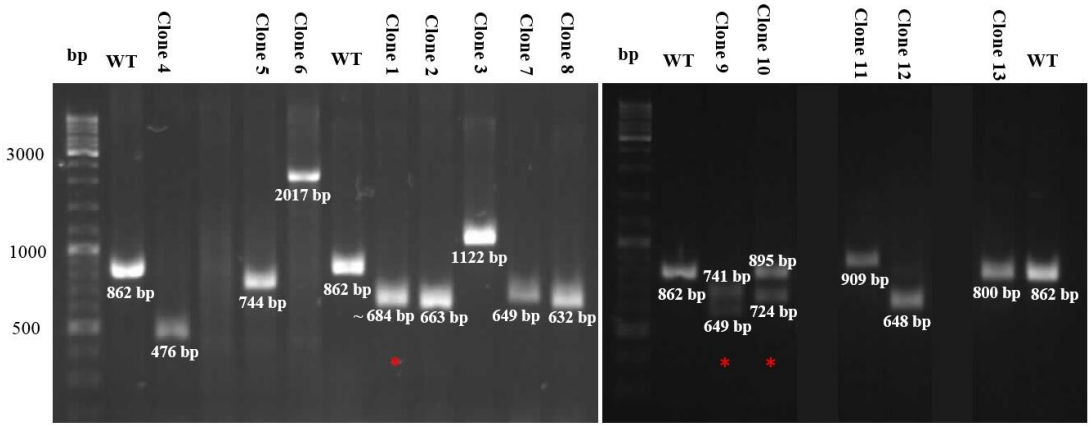

Figure 3. PCR Products of RAG1 from 13 clones were amplified and directly analyzed on 2 % agarose gel. The presence of RAG1 PCR amplicon in variouse sizes showed that deletions in different sizes have been occurred in 862bp RAG1 fragment. Compound heterozygote clones are indicated by red asterisks.

To confirm the results of cleavage by CRISPR system in mES clones, Sanger sequencing showed that different indels were detected at target sites with various mutation sizes (**Fig. 4A-B**).

12 of 13 ES cell clones showed biallelic targeting of two regions in the *RAG1* locus. 7 of 12 mutated ES cell clones showed deletions of varying sizes, one showed only one insertion, 4 showed a combination of deletions and insertions. 10 out of 12 mutant-mES-cell clones (all the mutant cell clones except clone 3 and 7) had biallelic out-of-frame insertions/deletions in this region, leading to frameshift and early termination and hence gene disruption. Also, we identified novel out-of-frame homozygous insertions/deletions in about 59% of the mutant mESCs (knock-out clones 2, 4, 5, 6, 8, 11, 12).

4.3. Real-Time PCR Results for Pluripotency Genes

The mESCs clones had normal morphology and were carefully cultivated in medium supplemented with R2i in a pluripotent state throughout the gene targeting experiments.However, for confirming the undifferentiated state of the cells the expression of stemness markers were investigated. Total RNA was extracted from the ESCs cultured in feeder-free conditions. Expression of Nanog, Oct4, and Sox2 in the ES cells versus the MEF cells. Quantitative analysis of pluripotency markers, Nanog, Oct4, and Sox2 in ESCs cultured in the presence of LIF was performed. GAPDH was used as the loading control.

The gels have been run under the same experimental condition. Real-time PCR and agarose gel electrophoresis results for the three key markers for pluripotent stem cell including Oct4, Sox2, and Nanog revealed that the *RAG1* knock-out mES cell line was positive for the all three stemness markers (**Fig. 5**).

5. Discussion

Embryonic stem cells with inability to perform V(D)J recombination are mutated embryonic stem cells of the *RAG* (recombination activating gene) genes. *RAG* genes include *RAG1* and *RAG2*, which play an important role in the rearrangement and recombination of immunoglobulin and T-lymphocyte-receptor genes during the VDJ recombination process. The most important applications of the *RAG*-mutant mouse embryonic stem cells, other than investigating the function of *RAG* genes, are the production of specified *RAG*-knockout mice, and the studying the mechanism of cell and molecular differentiation of the lymphatic system (17). Here, we generated specified*RAG1*-knockout mES clones within a matter of weeks using CRISPR system with following approach: 1) guide sequence design and sgRNA construction, 2) testing the CRISPR sgRNAs 3) clone isolation, 4) clone screening and validation, and 5) confirming the undifferentiated state of the mutant ES cells.

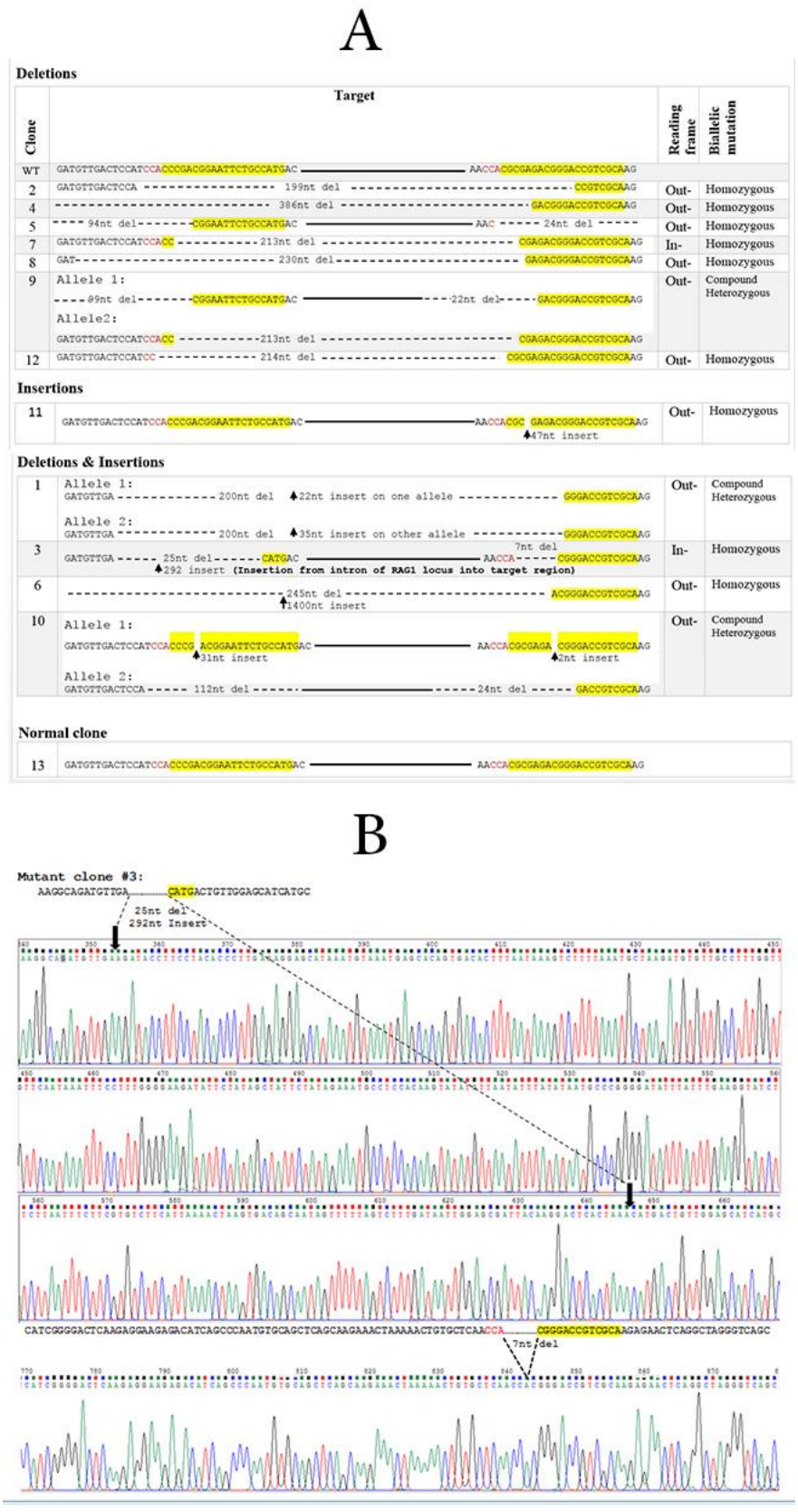

Figure 4. CRISPR/Cas9 mediated indels. The knock-out alleles of ES cell clones were amplified via PCR and sequenced. (A) Target sites marked in yellow, PAM sites shown in red, the vertical arrows represent insertions. Dashed and solid lines represent deleted and wild sequences, respectively. (B) sanger sequencing of PCR product of RAG1-target sites in clone #3 with both insertion and deletions. Target site indicated in yellow, PAM sequence in red

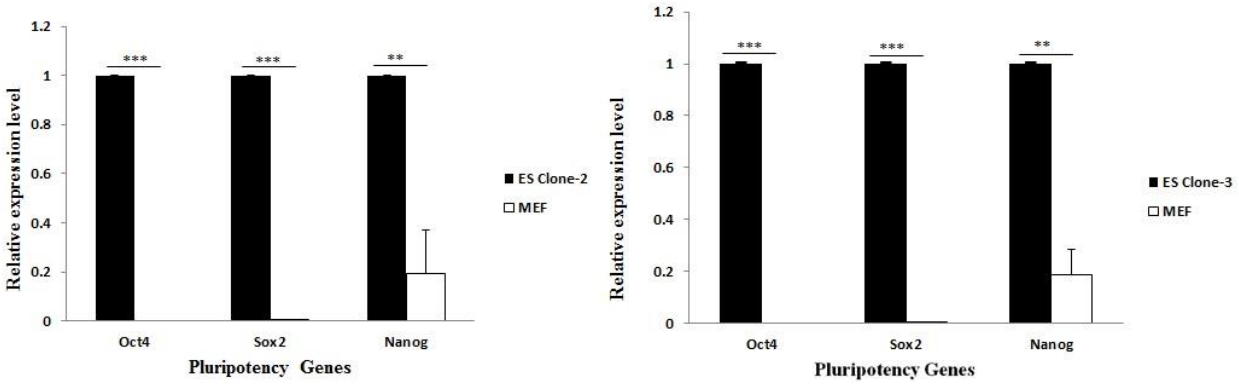

Figure 5. Stable pluripotent state in RAG1-knock-out mESCs versus MEFs. Expression of pluripotency marker genes in mESCs clone 2, clone 3 and MEF cells was analyzed by real-time PCR.

CRISPR/Cas9 is an efficient tool for editing the genome of mammalian cells and for generating animal models. However, using this technology in many fields still poses major problems, including mosaicism especially when using CRISPR in embryos and off-target mutations. The rate of off-target editing can be reduced with properly designed sgRNAs. *According to observation* in some studies based on the whole-genome analysis of gene-modified human stem cells, off-target effects were very rare (18) but mosaicism still remains a major concern. Recently, several strategies have been employed to limit mosaicism (16, 19-21).

However, a clear and assured strategy has yet to be proposed to eliminate the mosaic mutations resulting from CRISPR gene editing. May be it would be required to combine various solutions and use a combined strategy to avoid mosaic mutations in the future studies. If these strategies do not produce safe and precise results in clinical applications of CRISPR system, it may be needed to produce gene edited embryos using CRISPR system via mutant ES cells. This classic method may not be abandoned and offers some advantages over microinjection into zygotes, including removing mosaicism and screening for the desired mutations before the creation of genome modified animals, since the nature of mutations after zygote injection cannot be determined prior to animal development (14-16).

Although, mutant mouse models can be directly generated by microinjection of programmable site-specific nuclease into zygotes, the production of mutant mice via mESCs may be suitable to derive gene-targeted mice with the desired alteration from a specific knockout mESC line (14). Furthermore, as mentioned above, injecting CRISPR/Cas9 machinery into even a single-celled embryo creates a mixture of edited cells (mosaicism) and this phenomenon in the mouse embryos prevents obtaining mouse models with the specific mutations in a short period of time (15, 16). Consequently, the *RAG*-mutant mESCs would be a good model for producing specified knockout mouse by isolating clonal populations of the mutant mESCs. Using this approach, it would be possible to avoid mosaicism, a frequent problem when microinjecting of CRISPR system into single-cell embryo (15, 16); the chimaeras derived from the homozygote-mutant-ES-cell clones can be directly used in the rapid process of generating mouse models as well.

Here we showed that CRISPR/Cas9 constructs with highly active sgRNAs can be easily used to generate several novel *RAG1*-knockout-mouse-ES cells for producing knockout mouse.

We tested pairs of sgRNA for *RAG1* and *RAG2* genes in NIH3T3 cells and selected the *RAG1* sgRNA for targeting *RAG1* gene in mES cells based on the DNA cleavage efficiency and the easy detection of induced indels by PCR. Mutation detection in *RAG1* gene based on the first result in all the ES cells lead to obvious deletions in a large number of cells. We also showed that the deletion of a gene fragment with high efficiency can occur in *RAG* genes by simultaneous cleavage of two targeted sites in one gene. Such results have been reported in other cases (22-26). In this report, significant insertion/deletion have been achieved in about 92% of the selected mES-cell clones, as seen previously for other genes using the same conditions (27) and through the homology-directed-repair (HDR) pathway to generate knock-in mES cells (28), but NHEJ is preferred over HDR in the case of CRISPR/Cas9 DSBs (29). In our study, using the CRISPR/Cas 9 system, different in-frame and out-of-frame insertions/deletions were detected in the target region of *RAG1* gene, resulting in different knock-out-ES-cell clones. 92% of the ES-cell clones showed biallelic targeting of the *RAG1* locus, among which 83% of them carried out-of-frame insertions/deletions and about 59% had out-of-frame homozygous insertions/deletions. All of the sites targeted by CRISPR system were in the both regions of the *RAG1* locus, except for clone 11 where only the sgRNA-F1 was targeted by CRISPR system. According to the nature of mutations generated by the CRISPR/Cas9 system in Exon 1 of the *RAG1* gene in mouse ES cells, not all alterations generated by this system are in-frame, which results in frameshift and gene disruption. Also,

considering the rate of homozygous mutant ESCs produced by this system, specific knockout mice with the desired mutations can be generated in less time. After validating the ES-cell clones, the undifferentiated state of the mutant-ES cells were determined. Although the cultivation of ES cells in medium supplemented with R2i efficiently maintains pluripotency state of the ES cells (30, 31), in order to ensure the undifferentiated state of our knockout-ES cells after genetic manipulation process and several passages, the expression of stemness markers were investigated, which successfully confirmed the ground state pluripotency of mutant ES cells. Efficient cleavage and wide-range of indels, such as biallelic mutations, in all mutant ES cell clones can be generated using CRISPR/Cas9 system with highly active and correctly-designed sgRNAs. Not all mutations generated by CRISPR/Cas9 system were in-frame, resulting in complete gene knockout. Most of the mutant-ES-*cell* clones carried out-of-frame homozygous indel *mutations,* which inactivated the protein-coding *RAG1* gene, thus generating specific gene knockouts.

6. Conclusions

The study demonstrates that the high-efficiency editing by CRISPR-Cas system can be achieved in mouse-cell-line genomes at targeted locations with efficient and well-targeted sgRNAs. Genome editing results indicated that CRISPR/Cas9 system with correctly-designed sgRNAs generates mutations in the desired genes and significant deletions can be achieved in the large number of cells using two exonic gRNAs targeting one gene. Using CRISPR/Cas9 system, we efficiently created out-of-frame indels, containing both homozygous and compound heterozygous *RAG1* mutations in about 84% of the mutant-mESC clones, resulting in the complete knock-out of protein activity. 59% of *RAG1* knock-out mES clones had homozygous indels, hence this method can be readily used for faster generation of *RAG1* knockout mice by creating chimera.

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