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Original Article

Efficient mutagenesis targeting the *IFNAR1* gene in mice using a combination of Cas9 protein and dual gRNAs

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Abstract: We injected mouse zygotes with combinations of Cas9 protein, *Cas9* mRNA, and two gRNAs targeting a single exon of type I interferon receptor (*IFNAR1*) to determine the gene targeting efficiencies. Cas9 protein produced on-target mutations more efficiently than *Cas9* mRNA when each was used with a single gRNA, regardless of which gRNA was used. When *Cas9* mRNA and Cas9 protein were co-injected, the on-target efficiency could reach 97.0% when both gRNAs were used, which was higher than when either gRNA was used alone (61.3% and 75.5%, respectively; $P<0.05$). Co-injection of Cas9 protein with both gRNAs produced the highest on-target mutation rate of any combination (100.0%). Most on-target mutations were deletions of 2 to 113 nucleotides, and there were few off-target mutations in mutant animals. The expression intensity of IFNAR1 was reduced in heterozygous *IFNAR1*$^{+/-}$ mice (IF) and almost or completely absent in homozygous null *IFNAR*$^{-/-}$ mice compared with that in wild-type mice (IF and Western blot). When both gRNAs targeting *IFNAR1* were used simultaneously with two gRNAs targeting *FVII*, the on-target editing efficiency on each gene was 96.8% and 85.5%, respectively. Co-injection of dual gRNAs and Cas9 protein is an efficient approach for *IFNAR1* knockout and multi-gene editing in mice and may be applied in other animal models and breeding livestock.

Keywords: Mice, Cas9 protein, dual gRNAs, knockout, *IFNAR1*

Introduction

The clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas system is an adaptive immune response in archaea and bacteria that recognizes and cleaves foreign DNA from plasmids and phages [1, 2]. The *Streptococcus pyogenes* Cas9 nuclease (Cas9) cleaves a target sequence determined by a guide RNA (gRNA) that is complementary to a 20 nucleotide (nt) target region at the 5' end of the target. The only requirement of the DNA target is the existence of a protospacer-adjacent motif (PAM) sequence (-NGG for Cas9 from *S. pyogenes*) directly at downstream of the seed region of the target site [3, 4]. Repair of RNA-guided nuclease (RGN)-induced double-stranded breaks by nonhomologous end-joining or homology-directed repair introduces insertion or deletion mutations (indels) or other specific sequence alterations into the target DNA [5]. Multiple gRNAs can be used to target different genes [6-8], different exons [9-13], and even different loci in the same exon [14, 15], so as to generate deletions, insertions, or to knock out multiple genes. Dual gRNAs and *Cas9* mRNA can facilitate CRISPR/Cas9 targeting of individual genes both in mice and in embryonic stem cells (ESCs) [14, 16]. In addition, Cas9 can be introduced into target cells directly as a protein or as *Cas9* mRNA, which is subsequently translated [17-19]. Previous work showed that electroporation of Cas9 protein and a single gRNA directly into mouse zygotes can generate non-mosaic mutants [20]. Cas9 protein is believed to be degraded rapidly in cells, reducing the

chance of off-target effects [21]. Gene editing by CRISPR/Cas9 has been used for genetic modification and breeding of domestic animals [22-24].

IFNAR1 is a component of the receptor for type I interferons that is essential for responses to IFN-α and -β [25, 26]. IFNAR1 deficiency results in reduced myelopoiesis and impaired defense against most viral infections [27, 28]. Mice with *IFNAR1* knockout mutations are used to study antiproliferative response and susceptibility to viral infections [28]. Previously, chimeric *IFNAR1*-knockout mice were generated by homologous recombination in mouse ESCs [25, 29].

In this study, we utilized CRISPR/Cas9 gene editing to generate novel *IFNAR1* knockout mutations in mice. The targeting efficiency was compared by which the mouse zygotes were injected with single or dual gRNAs along with Cas9 protein, *Cas9* mRNA, or both Cas9 protein and *Cas9* mRNA. Co-injection of Cas9 protein with dual gRNAs might be the most efficient approach to generate founder animals with *IFNAR1* knockout.

Materials and methods

Chemicals and reagents

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

All animal protocols were approved by the Animal Care and Use Committees of Nanjing Normal University (IACUC-20201209). This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were maintained in an SPF animal facility at Nanjing Normal University and bred in individually ventilated cages (four mice per cage) with free access to food and water. The mice were kept in a 12:12 h light/dark cycle and maintained at 24±2°C and 50±20% relative humidity. All animal experiments were performed using proper anesthesia.

Construction of the targeting vector

Potential gRNA target sites in the second exon (Table S1) of *IFNAR1* were identified using the MIT CRISPR Design Tool (http://crispr.mit.edu). Off-target sites were identified in parallel, and the candidate gRNAs with the highest scores (among the scores above 60) were chosen. Oligomers encoding the selected gRNAs were synthesized (Table S2), annealed, and cloned into the PX459 V2.0 vector (62988, Addgene) at the *Bbs*I site. The recombinant plasmids were sequenced and used for cell transfection.

gRNA and Cas9 mRNA in vitro transcription

The gRNAs of *IFNAR1* (Table S3) and *FVII* (Table S4) were synthesized *in vitro*. The targeting sites of dual gRNAs were located in Exon 2, and not overlapped. A T7 promoter was inserted into upstream of the gRNAs by PCR using oligomers, and a 5'-GG transcription start region was arbitrarily created according to the kit recommendations (Thermo Fisher Scientific, Waltham, MA). The T7-gRNAs were gel-purified for *in vitro* transcription with the MEGAshortscript Kit (AM1354, Thermo Fisher Scientific). The pCAG-T3-hCAS-pA (48625, Addgene) plasmid was digested by SphI, and the DNA fragment encoding Cas9 was recovered by gel extraction for *in vitro* transcription with the mMESSAGE mMACHINE® T3 Transcription Kit (AM1348, Thermo Fisher Scientific). All RNAs were purified with phenol and chloroform and stored at -80°C until use (Details in Supplementary Data). Commercial Cas9 protein (M0646T, New England Biolabs, NEB) was diluted with TE Buffer (300 ng/μL) and stored at -80°C until use.

Transfection of NIH/3T3 cells

Mouse embryonic fibroblast cells (NIH/3T3; ATCC, CRL-1658) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone) at 37°C with 5% CO_2. Cells were transfected at 70-80% confluency using lipidosome (Lipofectamine®2000, Thermo Fisher Scientific). The ratio of DNA (g) to lipidosome (L) was 1 to 3. Then, the cells were transfected with 2 μg DNA and 6 μL Lip 2000 for 6 h. Cells that reached 80% confluency were recovered for genomic

DNA extraction in phenol, chloroform, and ethanol. The upper clear liquid was recycled after centrifugation, and the DNA was precipitated and dissolved in ultrapure water (details in Supplementary Data).

Micro-injection of one-cell zygotes

Mouse (C57BL/6n, 6-8 weeks old) embryos were obtained from donor females mated with males following superovulation with the PMSG/hCG protocol [30]. The fertilized embryos were harvested from the oviducts in M2 medium and cultured in potassium simplex optimized medium (KSOM, MR-121-D) for 20 h post hCG injection. Microinjection was performed using an Olympus IX71 inverted microscope with the Narishige microinjection system. Solutions containing different combinations of gRNAs (50 ng/μL), Cas9 protein (30 ng/μL), and/or *Cas9* mRNA (30 ng/μL) were injected into the cytoplasm of one-celled zygotes in M2 medium. The injection was performed 21-23 h post hCG administration. The injected embryos were cultured in KSOM medium for 0.5 h and subsequently transferred into the oviducts of pseudopregnant ICR females. In order to collect all live pups on the due day, cesarean section was performed on all pregnant mice 18 days post embryo transfer. The tails of the newborns were collected for extraction of genomic DNA.

Analysis of on-target and off-target mutations

PCR was performed to amplify the target fragment of 513 base pairs (Table S5) using DNA extracted from transfected cells and mouse tails. In addition, predicted off-target fragments were amplified with designated primers (Table S5).

T7 endonuclease I (T7EI) cleavage assays were performed using the amplified on-target and off-target fragments. The hybridized PCR products were digested with T7EI (M0302L, NEB) for 60 min at 37°C. The reaction products were analyzed by 1% agarose gel electrophoresis (details in Supplementary Data).

T-cloning and sequencing to identify sequence modifications

PCR products of the target DNA from the newborn mice were cloned into a T-cloning vector (E1202S, NEB) and transformed into *Escherichia coli* Trans5α competent cells. Single colonies were selected, and DNA fragments containing the targeted loci were amplified by PCR and sequenced (details in Supplementary Data).

Immunofluorescence staining to detect IFNAR1 expression in cultured mouse cells

Ear tissues from wild-type (*IFNAR1*$^{+/+}$), heterozygous mutant (*IFNAR1*$^{+/-}$), and homozygous mutant (*IFNAR1*$^{-/-}$) mice were implanted on cell culture dishes. A thin cover slip was placed over the tissues to ensure close contact between the tissues and the bottom of the dishes. Primary cells usually grew out of the tissues 5-6 days post implantation. After 3-4 passages, the suspended fibroblast cells were transferred into empty zona pellucida using a micromanipulation procedure, which minimized the use of antibody reagents. The cells inside the zona pellucida were then fixed with 4% paraformaldehyde (16005) for 10 min, washed three times in DPBS, incubated for 20 min with 0.5% Triton-X100 (T8200, Solarbio, China), and treated with 0.25% Tween 20 (9005-64-5, Sangon Biotech, China) in DPBS for 30 min. Immunofluorescence staining was performed by incubation with primary antibody for IFNAR1 (1:200 dilution; ab62693, Abcam, UK) supplemented with 2% FBS overnight at 4°C, followed by incubation with goat anti-rabbit IgG (H+L) (1:200 dilution; A0516, Beyotime, China) for 2 h at 37°C. The cells were then washed in DPBST for 20 min and stained with 100 ng/mL DAPI (SN321-1-1, Shengxing Biological, China) for 10 min at room temperature. The stained cells were observed under a fluorescence microscope. The intensity of IFNAR1 staining was analyzed using Image J.

Western blot

Brain and lung tissues were collected from wild-type, heterozygous *IFNAR1*$^{+/-}$, and homozygous *IFNAR1*$^{-/-}$ mice. The tissues were lysed with radio-immunoprecipitation assay buffer (RIPA) and phenylmethanesulfonyl fluoride (PMSF; RIPA:PMSF=9:1). Total proteins were quantified with the BCA assay kit (GK5011, Shanghai Generay Biotech, China). About 10 μg protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane. Immunoblot was carried out with IFNAR1-specific antibody at 4°C overnight (1:2000 goat anti-mouse IFN-

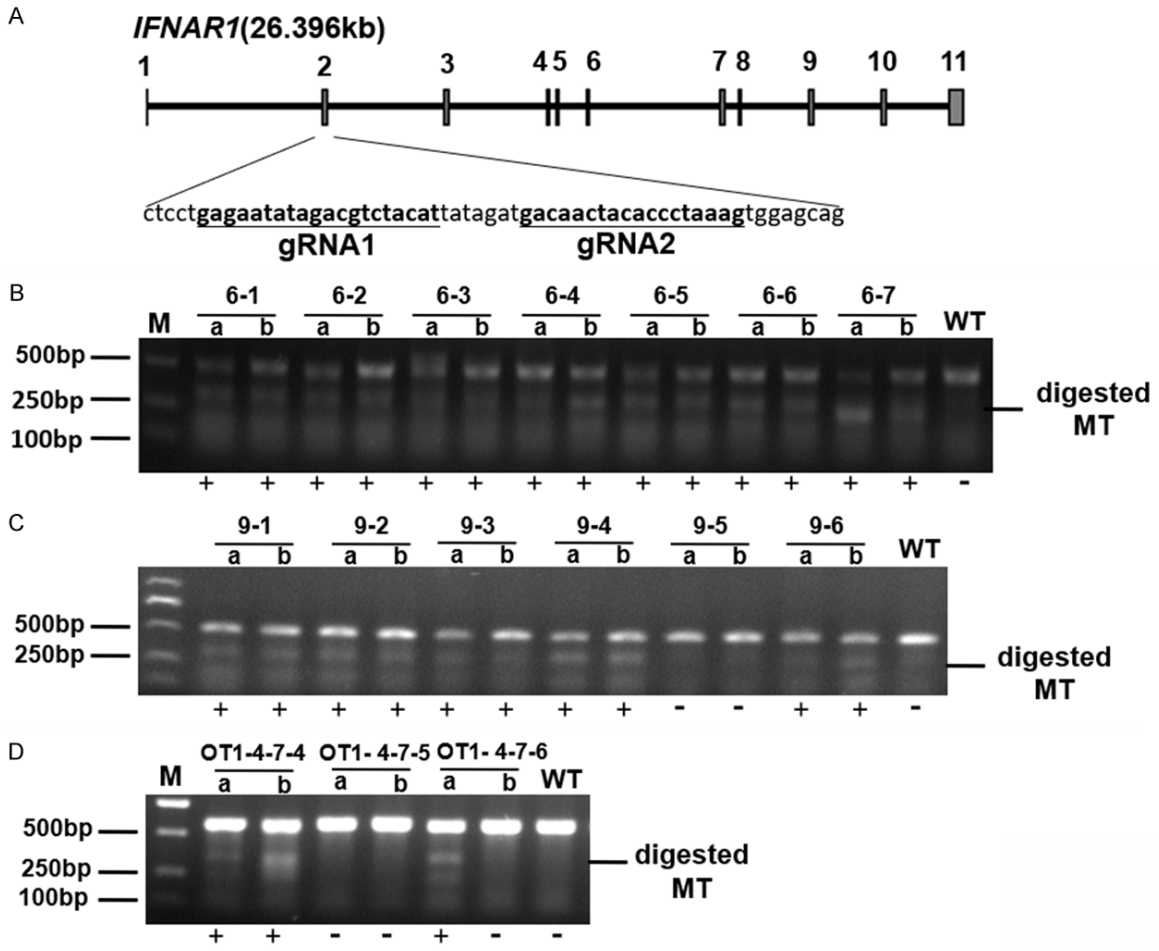

Figure 1. Generation and analysis of IFNAR1 gene targeting with CRISPR/Cas9 in mice. (A) Structure of the *IFNAR1* gene and a schematic diagram of target sites in Exon2. The sequences targeted by gRNA1 and gRNA2 are underlined. (B) PCR amplicons of target sites in the mice of Group 6 (dual gRNAs+Cas9 protein) were subjected to T7EI assay. Amplicons from targeted mice mixed 1:1 with wild-type (WT) amplicons (a) and amplicons from targeted mice (b) were digested by T7EI enzyme to identify mutations. Amplicons from WT mice served as negative controls. Mouse lines 6-1, 6-2, 6-3, 6-4, 6-5, 6-6, and 6-7 carried on-target mutations. (C) PCR amplicons of target sites in the mice of Group 9 (dual gRNAs+Cas9 protein+*Cas9* mRNA) were subjected to T7EI assay as described in (B). Lines 9-1, 9-2, 9-3, 9-4, and 9-6 carried on-target mutations. (D) PCR amplicons of off-target sites (OT1-4) in the mice of Group 7 (gRNA1+Cas9 protein+*Cas9* mRNA) were subjected to T7EI assay as described in (B). Lines OT1-4-7-4 and OT1-4-7-6 carried off-target mutations.

α/β R1 antibody, BAF3039, R&D, Biotechne). Primary antibodies were visualized with rabbit anti-goat IgG-HRP secondary antibody (BS30-503, Bioworld Technology, Inc.) using an Enhanced ECL Chemiluminescence Detection Kit (E411-04, Vayme). The membrane was photographed for analysis.

Statistical analyses

The on-target and off-target mutation frequencies were analyzed using SPSS software (SPSS 18.0, IBM). The percentage data in each replicate were arcsine transformed and subjected to one-way ANOVA. Means were compared by Fisher's least significant difference test (PLAS). The threshold for statistical significance was $P<0.05$. Significant difference was indicated by a, b, and c among the groups ($P<0.05$).

Results

Gene editing in cultured cells to verify the activity of the gRNAs

We designed two gRNAs targeting Exon 2 of *IFNAR1*, which naturally contains a PAM-NGG motif (**Figure 1A**). One gRNA (gRNA1) contained

20 nucleotides, whereas the other (gRNA2) was truncated to 18 nucleotides. Plasmids containing the gRNAs were transfected into NIH/3T3 cells, and on-target mutations of *IFNAR1* were determined by T7EI assay. The resulting *IFNAR1* editing frequencies were 33.2% (gRNA1) and 38.3% (gRNA2), respectively. In sequenced ten mutant NIH/3T3 TA cloning colonies, five samples (50%) exhibited 2-16 nt deletions, one sample had a 2 nt insertion, and two showed nt transversion.

Comparison of the on-target efficiencies of different RGN systems

We performed a 3×3 factorial experiment in which we injected nine separate groups of mouse zygotes with different combinations of gRNA1, gRNA2, *Cas9* mRNA, and Cas9 protein (**Table 1**). We performed T7EI analysis to examine the resulting IFNAR1 mutations (**Figure 1B** and **1C**). Cas9 protein produced higher on-target mutation rates than *Cas9* mRNA when each was used with either gRNA1 or gRNA2. When *Cas9* mRNA was used with both gRNAs, the on-target mutation rate (36.9%) was higher than the rates produced by *Cas9* mRNA with each single gRNA (14.5% and 23.3%, respectively, $P<0.05$); however, it was still lower than the rates produced by Cas9 protein with each single gRNA (57.5% and 77.6%, respectively, $P<0.05$). When *Cas9* mRNA and Cas9 protein were co-injected, the on-target efficiency was higher when both gRNAs were used (97.0%) than when either gRNA was used alone (61.3% and 75.5%, respectively; $P<0.05$). When Cas9 protein was used with both gRNAs, the on-target mutation rate was 100%, which was higher than the rate produced by any other combination. The truncated gRNA2 produced a higher on-target mutation rate (77.6%) than the standard-length gRNA1 (57.5%) when each was used with Cas9 protein alone ($P<0.05$), whereas there was no difference in the on-target mutation rates produced by the gRNAs when each was used with *Cas9* mRNA alone. Mice with mutations in both *IFNAR1* alleles were obtained in several of the experimental groups (**Table 1**).

In Group 6 (gRNA1+gRNA2+Cas9 protein), all 17 targeted F0 mice were analyzed by DNA sequencing. Sixteen out of the 17 mice (94.1%) exhibited deletions ranging in size from 3 nt to 85 nt, and 11/17 mice (64.7%) carried mutations within or after the binding site of gRNA2 (**Table 2**). In Group 9 (gRNA1+gRNA2+Cas9 protein+*Cas9* mRNA), 15 targeted F0 mice were analyzed by DNA sequencing. All mutations were located in Exon 2 of *IFNAR1* gene. Thirteen out of the 15 mice in Group 9 (86.7%) exhibited deletions ranging in size from 2 nt to 113 nt, whereas one mouse had a 2 nt insertion and one had a C to T transversion (**Table 3**). Nine of the 15 mutations in Group 9 (60.0%) were located within or after the binding site of gRNA2. Sixteen of the 17 mutations in Group 6 (94.1%) resulted in premature termination of protein translation (Supplementary Data, Table S6), whereas 10 of the 15 mutations in Group 9 (66.7%) resulted in premature termination (Supplementary Data, Table S7).

Analysis of off-target effects

We analyzed the five off-target sites in the mouse genome with the highest homology to each gRNA by T7EI assay (**Figure 1D**). Analysis of the 10 potential off-target sites across all relevant experimental groups revealed a total of two off-target mutations, both of which occurred in Group 7 (**Table 4**).

Phenotypic analysis of the IFNAR1-knockout mice

When IFNAR1-null adult mice (*IFNAR1*$^{-/-}$) generated using our RGN systems were transferred from a specific pathogen-free (SPF) environment into a non-SPF environment, their back skin became susceptible to infection leading to skin ulceration and eye blindness (**Figure 2A**). In addition, the F0 *IFNAR1*$^{-/-}$ mouse in Group 9 (**Table 1**) showed ulceration of adjacent toes after toe clipping and became blind after 1 year. Immunofluorescence staining of cultured skin cells from heterozygous *IFNAR1*$^{+/-}$ mice (F1 of Line 9-1, fluorescence intensity =12.8) and homozygous *IFNAR1*$^{-/-}$ mice (F2 of Line 9-1, fluorescence intensity =0.08) revealed that the IFNAR1 protein levels were decreased to 42.8% and 0.26%, respectively, compared with that in cultured skin cells from wild-type *IFNAR1*$^{+/+}$ mice (fluorescence intensity =29.9, defined as 100%; $P<0.0001$; **Figure 2B**). Western blots indicated that a band representing IFNAR1 at 47.5 KD was absent in lung tissue (normally characterized by moderate IFNAR1 expression) and brain tissue (normally characterized by

Table 1. Generation of IFNAR1-knockout mice using different combinations of gRNAs and Cas9 mRNA and protein

Group	CRISPR/Cas9 system	Injected embryos	Transferred embryos	Replicates	Recipients	Newborns generated	Newborns containing on-target mutation	Newborns with two mutant alleles	Percentage of newborn mice with on-target mutation (Mean ± SEM)
1	gRNA1+*Cas9* mRNA	72	64	3	3	14	2	0	14.5±2.1[a]
2	gRNA2+*Cas9* mRNA	76	70	3	3	17	4	1	23.3±2.9[a]
3	gRNA1+gRNA2+*Cas9* mRNA	70	67	3	3	16	6	0	36.9±3.4[b]
4	gRNA1+Cas9 Protein	67	60	3	3	10	6	1	57.5±6.6[c]
5	gRNA2+Cas9 Protein	91	85	3	4	27	21	2	77.6±2.5[d]
6	gRNA1+gRNA2+Cas9 Protein	68	62	3	3	17	17	2	100.0±0[e]
7	gRNA1+*Cas9* mRNA+Cas9 Protein	70	62	3	3	20	12	0	61.3±4.9[c]
8	gRNA2+*Cas9* mRNA+Cas9 Protein	71	70	3	3	16	12	0	75.5±4.3[d]
9	gRNA1+gRNA2+*Cas9* mRNA+Cas9 Protein	88	86	3	4	26	25	1	97.0±3.0[e]

Note: [a,b,c,d,e]different superscript letters within same column indicate significant differences ($P<0.05$).

Table 2. DNA sequences of IFNAR1 mutations induced by dual gRNAs+Cas9 protein in mice

Founder	Target sequences (5'-3')	Indels
Dual gRNAs+*Cas9* Protein		
WT	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCCTAAAGTGGAGCAGCCACGGAG	
6-1	CCTGAGAATATAGACGTCTACATTATAGATGACAACTA--------AGTGGAGCAGCCACGGAG	Δ8 nt
6-2	CCTGAGAATATAGACGTCTACATTATAGATGACA----------AAGTGGAGCAGCCACGGAG	Δ11 nt
6-3	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACC-----GTGGAGCAGCCACGGAG	Δ5 nt
6-4	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACC---AAGTGGAGCAGCCACGGAG	Δ3 nt
6-5	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCCTAAAGT----------GGAG	Δ11 nt
6-6	CCTGAGAATATAGACG----------AAAGTGGAGCAGCCACGGAG	Δ28 nt
6-7	CCTGAGAATATA----------GTGGAGCAGCCACGGAG	Δ35 nt
6-8	GGAGA----------///----------GAGA	Δ74 nt
6-9	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCC----GTGGAGCAGCCACGGAG	Δ4 nt
6-10	CCTGAGAATATAGACGTCTACATTATAGATGACAACTC-------AAGTGGAGCAGCCACGGAG	Δ7 nt
6-11	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCCTAAAGTGGAGCAGCCACGGAG ↑A	+1 nt
6-12	CCTGAGAATATAGACGTCTACATTATAGATGACAACTAC--------GTGGAGCAGCCACGGAG	Δ8 nt
6-13	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACA-----AAGTGGAGCAGCCACGGAG	Δ5 nt
6-14	CCTGAGAATAT----------TGGAGCAGCCACGGAG	Δ37 nt
6-15	AATCTG----------///----------TGTG	Δ85 nt
6-16	CCTGAGAA----------GCAGCCACGGAG	Δ44 nt
6-17	CCTGAGAATATAGACGTCTACATTATAGATGACAACTA--------AGTGGAGCAGCCACGGAG	Δ8 nt

Note: The sequences targeted by the two gRNAs are underlined in the wild type. Deletions are marked as "-". Insertion is labeled in grey highlight.

Table 3. DNA sequences of IFNAR1 mutations induced by dual gRNAs+Cas9 protein+Cas9 mRNA in mice

Founder	Target sequences (5'-3')	Indels
Dual gRNAs+Cas9 Protein+*Cas9* mRNA		
WT	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCCTAAAGTGGAGCAGCCACGGAG	
9-1	CCTGAGAATATAGACGTCTACATTATAGATGACAACTA-------AAGTGGAGCAGCCACGGAG	Δ7 nt
9-2	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCCTA--GTGGAGCAGCCACGGAG	Δ2 nt
9-3	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCTTAAAGTGGAGCAGCCACGGAG	C→T
9-4	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCC----GTGGAGCAGCCACGGAG ↑AT	+2 nt Δ4 nt
9-6	CCTGAGAATATA----------AGTGGAGCAGCCACGGAG	Δ33 nt
9-7	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCCTAAAGT----------GGAG	Δ11 nt
9-8	CCTGAGAA----------GTGGAGCAGCCACGGAG	Δ39 nt
9-9	GTGG----------///----------TTCAGCA	Δ102 nt
9-10	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACACCCT---GTGGAGCAGCCACGGAG	Δ3 nt
9-11	CCTGAGAATATAGACGTCTACATTATAGATGACAACTC----CTAAAGTGGAGCAGCCACGGAG	Δ4 nt
9-12	CCTGAGAATATAGACGTCTACATTATAGATGACA----------GTGGAGCAGCCACGGAG	Δ13 nt
9-13	G----------///----------AATATCG	Δ113 nt
9-14	CCTGAGAATATAGACGTCTACATTATAGATGACAACTACA-----AAGTGGAGCAGCCACGGAG	Δ5 nt
9-15	CCTGAGAATATAG----------AAAGTGGAGCAGCCACGGAG	Δ31 nt
9-16	CCTGAGAATATAGACGTCTACA----------GTGGAGCAGCCACGGAG	Δ25 nt

Note: The sequences targeted by the two gRNAs are underlined in the wild type (WT). Deletions are marked as "-". Insertion and transversion (C→T) are labeled in grey highlight.

Table 4. Analysis of potential off-target sites in each group of experimental mice

Target sites	Recognition sites	Mutant mice/total mice tested (%)								
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
gRNA1 (on-target)	ATGTAGACGTCTATATTCTC AGG									
OT1-1	ATGTAG CTC TC A ATATTCTC TA G	0	-	0	0	-	0	0	-	0
OT1-2	ATGT CT AC T TCTAT T TTCTC CA G	0	-	0	0	-	0	0	-	0
OT1-3	ATGTAG C CGTCT G TAT A CT GG GG	0	-	0	0	-	0	0	-	0
OT1-4	ATGTAGACGTCT TC ATTCT G CAT	0	-	0	0	-	0	33.3	-	0
OT1-5	ATGTA T ACGTC A AT A TC T TC AGG	0	-	0	0	-	0	0	-	0
gRNA2 (on-target)	GACAACTACACCCTAAAG TGG									
OT2-1	G G CAACTACA G CCTAAA A TGG	-	0	0	-	0	0	-	0	0
OT2-2	GA AG ACT C C T CCCTAAAG A GG	-	0	0	-	0	0	-	0	0
OT2-3	GA T A TA TACACCCTAA T G AA G	-	0	0	-	0	0	-	0	0
OT2-4	GA A AA AA ACACC G TAAAG T A G	-	0	0	-	0	0	-	0	0
OT2-5	G C CAAC AC CACC A TAAAG CA G	-	0	0	-	0	0	-	0	0

Note: The frequencies of mutation at predicated off-target (OT) sites were compared separately in newborn mice between each group. Group1 to Group9 correspond to the groups in **Table 1**. At each off-target site, the nucleotides that mismatch with the on-target sequence are labeled in grey highlight.

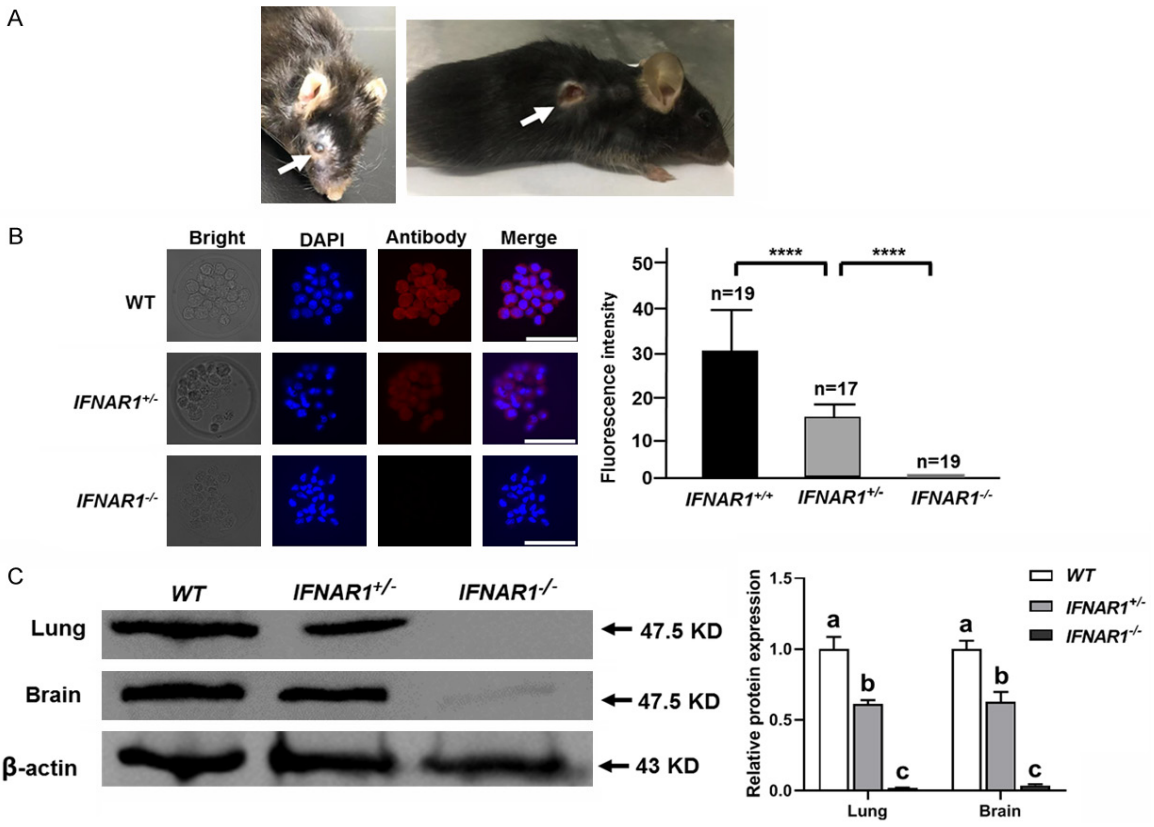

Figure 2. Phenotypes of IFNAR1-mutant mice. A. *IFNAR1*$^{-/-}$ null mice displayed blindness (arrow) and skin ulceration (arrow). B. Left panel: The IFNAR1 protein in cultured skin cells of wild-type (WT), heterozygous *IFNAR1*$^{+/-}$ (F1 of Line 9-1), and homozygous *IFNAR1*$^{-/-}$ (F2 of Line 9-1) mice were examined by immunofluorescence staining. Before immunostaining, cultured cells were transferred into empty zona pellucida by micromanipulation. Bar =50 μm. Right panel: Compared with that in WT cells, the intensity of IFNAR1 expression was 42.8% in *IFNAR1*$^{+/-}$ cells and 0.26% in *IFNAR1*$^{+/-}$ cells. ****, indicates a highly significant difference (P<0.0001). C. Left panel: IFNAR1 protein (47.5 KD) was present in both lung tissue (normally characterized by moderate IFNAR1 expression) and brain tissue (normally characterized by abundant IFNAR1 expression) in wild-type and *IFNAR1*$^{+/-}$ mice but was not detectable in *IFNAR1*$^{-/-}$ null mice. Right panel: the gray scale analysis of IFNAR1 expression. There was a significantly reduced IFNAR1 protein expression in *IFNAR1*$^{+/-}$ animals compared to wide-type controls.

abundant IFNAR1 expression) [31] of *IFNAR1*$^{-/-}$ null mice, but present in those of wild-type mice and heterozygous *IFNAR1*$^{+/-}$ mice (**Figure 2C**). However, there was a significantly reduced IFNAR protein expression in *IFNAR1*$^{+/-}$ animals compared to Controls (**Figure 2C**).

Simultaneous editing of IFNAR1 and FVII with Cas9 protein and dual gRNAs for each gene

In order to examine whether dual gRNAs and Cas9 protein can be applied to edit multiple genes simultaneously, we designed an experiment in which Cas9 protein and both gRNAs targeting *IFNAR1* were co-injected into mouse zygotes along with one of two gRNAs targeting *FVII* (F7-2, standard 20 nt; tF7-1, truncated 18 nt) or with both gRNAs targeting *FVII* (Table S4) previously used in our laboratory [35]. As shown in **Table 5**, the editing efficiency for *IFNAR1* was high (90.5-96.8%) regardless of which gRNA was used for *FVII*; however, the *FVII* editing efficiency was only 38.4% when only the F7-2 gRNA was applied, whereas it increased to 48.6% when only the tF7-1 gRNA was applied ($P<0.05$). The efficiency for *FVII* editing reached as high as 85.5% when both gRNAs targeting *FVII* were used, it was significantly different than the efficiency achieved using only the F7-2 or tF7-1 gRNA (**Table 5**).

Discussion

We have demonstrated that co-injection of dual gRNAs and Cas9 protein in CRISPR/Cas9 can effectively improve the efficiency of gene targeting in mice. In this study, gene editing in mice using Cas9 protein was more efficient than that using *Cas9* mRNA, regardless of whether one gRNA or dual gRNAs were applied. *Cas9* mRNA has been routinely used as a CRISPR/Cas9 reagent with a satisfactory result [32-35]. Theoretically, *Cas9* mRNA can be replaced by Cas9 protein, as Cas9 protein is expected to be degraded rapidly and has a shorter half-life [17]. Intracytoplasmic injection of Cas9 protein and a single gRNA produced on-target mutations of the *Foxn1* gene in up to 71% of experimental mice [18]. In cultured cells, it took about 6 h for genomic DNA to be cut after transfection with a plasmid containing *cas9* DNA, whereas it took 1 h for genomic DNA to be cut after Cas9 protein was introduced directly into cells [21]. It was also reported that Cas9 protein started cutting genomic DNA 4 h after injection into pronuclear zygotes, whereas *Cas9* mRNA took 6 h to induce cutting of genomic DNA [18]. In another study, it has been shown that electroporation of Cas9 protein/sgRNA into early pronuclear zygotes generated non-mosaic mutant mice [20]. The time between translation and function may delay gene editing in the fast pace of the cell cycle in zygotes, leading to a decrease in editing efficiency. Because of the short interval between first cell divisions in zygotes, delayed gene editing resulted in mosaics of sister embryonic cells in which some blastomeres carried desired mutations while others did not [36]. Injection of RNA at the two-cell stage of an embryo greatly increased the chance of insertion of large foreign DNA fragments at Cas9 cutting sites, because embryos at the two-cell stage usually hold a relatively longer G2 phase (about 12 h) in which the open chromatin structure increases the likelihood of homologous recombination [37]. We observed that female mice began to ovulate 10 h after HCG injection and caging with male mice (data not shown). Therefore, according to the scheme for cell cycle progression in mouse zygotes [37], the zygotes in our study were at S-phase when the CRISPR/Cas9 reagents were injected into the cytoplasm 21-23 h post HCG injection. By the time the injected Cas9 protein began to work, the zygotes should have been in early or middle G2 phase, whereas they were probably in late G2 phase or metaphase by the time genomic DNA was cut following injection of *Cas9* mRNA. In future studies, we will determine if the editing efficiency can be increased by injecting the CRISPR/Cas9 reagents into the cytoplasm of zygotes at the G1 phase instead of the G2 phase.

The truncated gRNA2 (18 nt) resulted in a higher editing efficiency than the standard-length gRNA1 (20 nt) when Cas9 protein was used with or without additional *Cas9* mRNA. We previously demonstrated and confirmed in this study that truncated gRNAs (18 nt) generated *FVII*-knockout mice more efficiently than standard length gRNAs (20 nt) in a site-dependent manner [38].

Previous studies showed that simultaneous use of dual or multiple gRNAs with *Cas9* mRNA to target one or multiple exons in a single gene increased the editing efficiency in mouse zy-

Table 5. Efficiency of simultaneous targeting of two genes (*IFNAR1* and *FVII*) using combinations of dual gRNAs per gene and Cas9 protein

Treatment (dual gene targeting)			No. Injected zygotes	Replicates	No. Tested fetuses	Number of mice with on-target *IFNAR1* mutations			Number of mice with on-target *FVII* mutations		
Cas9	*IFNAR1*	*FVII*				One allele	Two alleles	Total Efficiency% (Mean±SEM)	One allele	Two alleles	Total Efficiency% (Mean±SEM)
Cas9 Protein	gRNA1+gRNA2	F7-2 (20 nt)	88	3	15	13	0	90.5±16.5[a]	5	0	38.4±24.8[a]
		tF7-1 (18 nt)	96	3	21	19	1	96.8±6.2[a]	11	0	48.6±14.5[a]
		F7-2+tF7-1	105	3	27	24	1	93.3±6.4[a]	22	1	85.5±2.1[b]

Note: [a,b]different superscript letters within same indicate significant differences (P<0.05).

gotes compared with the use of a single gRNA with *Cas9* mRNA [14, 15]. Similar results were obtained in mouse ESCs [16]. Zou et al. used multiple gRNAs to target multiple genes both in mice and in monkeys and obtained on-target mutation rates in monkey embryos up to 100% for the *Arntl* gene and 91% for the *Prrt2* gene [15]. We found that the use of dual gRNAs to target one exon of *IFNAR1* resulted in significantly higher efficiency compared with the use of single gRNAs. This is possibly due to the fact that multiple gRNAs targeting different loci in a single exon increase the target sites available to the CRISPR/Cas9 machinery. We have further demonstrated that both *IFNAR1* and *FVII* gene, which was available in our laboratory, were simultaneously targeted with higher efficiencies when Cas9 protein and dual gRNAs for each gene were included in co-injection. Therefore, it is effective to target not only single gene, but also multiple genes in the simultaneous CRISPR/Cas9 system.

We identified two *IFNAR1* off-target events in mice that were co-injected with gRNA1, *Cas9* mRNA, and Cas9 protein. We did not detect any other off-target events, which suggest that dual gRNAs do not necessarily increase the risk of off-target mutations. These results are supported by previous findings that off-target mutations were rare in experiments that used dual gRNAs for gene editing [14, 15]. The low rates of off-target effects might be attributed to the selection of gRNAs with minimal homology to off-target sites. It was also reported that the application of Cas9 protein instead of *Cas9* mRNA reduced the risk of off-target effects without decreasing on-target efficiency [17-19, 21]. It is also possible that dominant lethal off-target mutations cause the termination of fetal development, resulting in a low incidence of off-target mutations in term-developed animals. A full-genome screen by DNA sequencing would be required to reveal all off-target mutations and thus prevent any potential mutations from adversely affecting offspring breeding.

In summary, we compared the gene editing efficiencies of different combinations of gRNAs, *Cas9* RNA, and Cas9 protein in mice. Co-injection of dual gRNAs and Cas9 protein into mouse zygotes was a highly efficient approach to generate *IFNAR1*-knockout mice as well as to simultaneously target multiple genes. The high efficiency of gene editing with dual gRNAs and Cas9 protein allows a small number of zygotes to be used, which is important if CRISPR/Cas9 is to be a feasible application for gene editing of large farm animals for the purpose of genetic improvement [22-24].

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Disclosure of conflict of interest

None.

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Supplementary Data

Materials and methods

gRNA and Cas9 mRNA in vitro transcription

Briefly, 1 μg linearized DNA template, T7 enzyme, and reaction buffer were added, mixed, and reacted at 37°C for 2 h. The reaction was terminated with DNA hydrolase, and RNA was purified with phenolic chloroform. The pCAG-T3-hCAS-pA (Addgene, 48625) plasmid was digested by SphI, and the DNA fragment encoding Cas9 was recovered by gel extraction for *in vitro* transcription with the mMESSAGE mMACHINE® T3 Transcription Kit (Ambion, AM1348). All RNAs were purified with phenol and chloroform and stored at -80°C until use. Specifically, equal volumes of phenol and chloroform were mixed and added into the RNA transcription reaction solution. The supernatant was then recovered after mixing and centrifugation. The RNA was precipitated with glacial ethanol and then diluted in DEPC-treated H_2O.

Transfection of NIH/3T3 cells

The ratio of DNA (g) to lipidosome (L) was 1 to 3. Briefly, cells were passaged and cultured for 24 h prior to transfection. The suspended cells were spread onto six-well culture dishes and cultured in 10% FBS DMEM for 12 h. Then, the cells were transfected with 2 μg DNA and 6 μl Lip 2000 for 6 h. The transfected cells were then cultured in medium supplemented with 2 μg/ml puromycin after replating at 24 h and addition of fresh medium after 48 h post transfection. Cells that reached 80% confluency were recovered for genomic DNA extraction in phenol, chloroform, and ethanol. Briefly, the cell suspension was mixed with proteinase K at 55°C for 3 h. Then, 600 μl phenol chloroform was added and mixed for 1 min. The upper clear liquid was recycled after centrifugation, and the DNA was precipitated and dissolved in ultrapure water.

Analysis of on-target and off-target mutations

T7 endonuclease I (T7EI) cleavage assays were performed using the amplified on-target and off-target fragments. Briefly, nearly 800 ng purified PCR product was denatured and annealed by incubation at 95°C for 5 min followed by cooling to 25°C at 5°C/min. For the genomic DNA obtained from the tails of newborn mice, 400 ng PCR product was mixed with 400 ng PCR product of wild-type genomic DNA and then denatured and annealed. The hybridized PCR products were digested with T7EI (NEB, M0302L) for 60 min at 37°C. The reaction products were analyzed by 1% agarose gel electrophoresis.

T-cloning and sequencing to identify sequence modifications

Briefly, 100 ng purified PCR product was added to 50 μl Trans5α cells. The cells were then cooled on ice for 30 min, heat-shocked at 42°C for 90 s, immediately kept on ice for 2 min, plated on Luria-Bertani (LB) agar containing ampicillin, and incubated at 37°C overnight. Single colonies were selected, and DNA fragments containing the targeted loci were amplified by PCR and sequenced.

Table S1. Targeting Exon 2 of *IFNAR1* gene

Targeted gene loci	Genomic DNA sequences of target region (5'-3')*
IFNAR1 Exon 2	gtggagaaaatctgaaacctcctgagaatatagacgtctacattatagatgacaactacaccctaaagtggagcagccacg-gagagtcaatgggcagtgtgacctttcagcagaatatcgaac

*, Two gRNAs that recognize the DNA sequences are labeled with under-lines.

Table S2. DNA sequences for constructing recombinant IFNAR1 gRNA expression vectors

gRNA name	Target site (5'-3')	Length (bp)	Synthesized DNA sequence	
			Sense (5'-3')	Antisense (5'-3')
gRNAs				
gRNA1	ATGTAGACGTCTATATTCTC	20	caccgATGTAGACGTCTATATTCTC	aaacGAGAATATAGACGTCTACATc
gRNA2	GACAACTACACCCTAAAG	18	caccGACAACTACACCCTAAAG	aaacCTTTAGGGTGTAGTTGTC

A Bbs I enzyme restriction site is artificially added by adding the sequence of "caccg" (gRNA1) or "cacc" (gRNA2) in synthesized DNAs of sense gRNAs (Sense, 5'-3'), and adding the "aaac" at antisense gRNAs (Antisense, 5'-3'). These sequences are used to clone it into PX459 V 2.0 expression vector.

Table S3. Oligomers used as templates for *in vitro* transcription of *IFNAR1* gRNAs

gRNA	Synthesized oligomer
Forward sequences for gRNA1 and gRNA2 (5'-3')	
IFN-gRNA1	GA TAATACGACTCACTATAGGG ATGTAGACGTCTATATTCTCGTTTTAGAGCTAGAAATA
IFN-gRNA2	GA TAATACGACTCACTATAGGG GACAACTACACCCTAAAGGTTTTAGAGCTAGAAATA
Reverse sequence for all gRNAs (5'-3')	
CTGCAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	

T7 promotor sequence is grey highlighted, and two protective nucleotides (GA) are added in front of the promoter. The sequences of gRNAs are underlined. DNA templets of gRNAs *in vitro* transcription are amplified by the way of PCR using forward and reverse oligomers.

Table S4. Oligomers used as templates for *in vitro* transcription of *FVII* gRNAs

gRNA	Synthesized oligomer
Forward sequences for std-gRNAs (5'-3')	
F7-2	GA TAATACGACTCACTATAG GCGTGCCAACTCACTCCTGGGTTTTAGAGCTAGAAATA
Forward sequences for tru-gRNAs (5'-3')	
tF7-1	GA TAATACGACTCACTATAGG CGTGCCAACTCACTCCGTTTTAGAGCTAGAAATA
Reverse sequence for all gRNAs (5'-3')	
CTGCAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	

T7 promotor is grey highlighted, and two protective nucleotides (GA) are added in front of the promoter. The sequences of gRNAs are underlined. Templets of gRNA transcription are amplified by PCR using forward and reverse oligomers.

Table S5. Primers of PCR detection of on-target and off-target mutations of *IFNAR1*

Primers	Sequences (5'-3')	gRNAs recognition site*
Primers for detecting on-target mutations in *IFNAR1* gene by PCR amplification		
IFN1-513-F	TGTGGTTTGTAGGCATCA	513 bp, Chr16: -91489960
IFN1-513-R	ACTTGCAGGAGTTGGTTC	
Primers for detecting off-target mutations induced by gRNA1		
OT1-1-f	CTGAGTGAGTGCAGTCGGTA	577 bp, Chr5: -150421144
OT1-1-r	GCACGTCAAGCCATAGGTGT	
OT1-2-f	GTGTGGCCATTCTAGCACCT	576 bp, Chr19: +42119326
OT1-2-r	ACTGGGCTGGTATAGGCTGA	
OT1-3-f	AGCTCACTGCTATGCAGCTC	593 bp, Chr10: +82975118
OT1-3-r	CAGTGAACAGGCCATGAGGA	
OT1-4-f	AAAACAGGCAGCCAAACACC	523 bp, Chr1: -158368067
OT1-4-r	ATGGCTTCCCCTTGCCTTTT	
OT1-5-f	AGATCAAGGTGATTCGGGGTG	635 bp, Chr2: -139415365
OT1-5-r	ATCCAGGGTCTCTCCTAACACA	
Primers for detecting off-target mutations induced by gRNA2		
OT2-1-f	CCCCCAGCCTTCTCACTTTT	621 bp, Chr3: +74794888
OT2-1-r	AAGCCACCTGGAGAAGAACG	
OT2-2-f	TTATACCCAGAAGGCGCAGC	504 bp, Chr8: +90339695
OT2-2-r	GAAGGCCTCCTGTCATCACC	
OT2-3-f	ACCTGAAACCCTGGGTCCTA	623 bp, Chr8: +12608082
OT2-3-r	AGAACAAAGCCAGCCAGGTT	
OT2-4-f	AGTGGGGCATGGAAGAAAGG	579 bp, Chr6: +114177350
OT2-4-r	AGCACACTGTCCAGGTTCAC	
OT2-5-f	CAGATGGGGCCTTGCTGTAT	586 bp, Chr2: +168307775
OT2-5-r	ATGGCTCTGCTTTCAGGTCC	

Forward primers (f) are designed at about 300 bp up-stream of gRNA targeting site. Reverse primers (r) are designed at about 300 bp down-stream of gRNA targeting site. Five sites with the highest potentials of off-target (OT) for each of gRNAs are designed. *, All sequences of recognition sites are searched from GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Table S6. Deduced amino acid sequence of mutant IFNAR1 proteins induced by 2g+Cas9 protein in mice

Mice		Amino acid (AA) sequences	Mutation type	No. AA
WT	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTLKWSSHGESMGSVTFSAEYRTKDEAKWLKVPECQH	Normal AA sequence	590
6-1	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDN	Early termination	43
6-2	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDKVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	72
6-3	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	74
6-4	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYT-KWSSHGESMGSVTFSAEYRTKDEAKWLKVPECQH	One AA deleted	589
6-5	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTLKWRVNGQCDLFSRISNKRRGEVVKSA	Early termination	72
6-6	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDESGAATESQWAV	Early termination	48
6-7	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	64
6-8	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGERVNGQCDLFSRISNKRRGEVVKSA	Early termination	51
6-9	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTRGAATESQWAV	Early termination	56
6-10	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDN	Early termination	43
6-11	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDEQLHPKVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	76
6-12	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	73
6-13	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYKVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	74
6-14	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIGAATESQWAV	Early termination	45
6-15	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENL	Early termination	29
6-16	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPEKQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	61
6-17	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDN	Early termination	43

The sequence of eighty amino acids (AA) of wild type (WT) IFNAR1 is presented and used to compare with those of mutant alleles. The deduced AA sequences, which do not match with that WT, is grey highlighted.

Table S7. Deduced amino acid sequence of mutant IFNAR1 proteins induced by 2g+Cas9 protein+Cas9 mRNA in mice

Mice		Amino acid sequences	Mutation type	No. AA
WT	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTLKWSSHGESMGSVTFSAEYRTKDEAKWLKVPECQH	Normal AA sequence	590
9-1	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDN	Early termination	43
9-2	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTLVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	75
9-3	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTLKWSSHGESMGSVTFSAEYRTKDEAKWLKVPECQH	Codon polymorphism	590
9-4	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNTTP	Early termination	46
9-6	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIEWSSHGESMGSVTFSAEYRTKDEAKWLKVPECQHTTTTKCEFSLL	Frame shift	579
9-7	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTLKWRVNGQCDLFSRISNKRRGEVVKSA	Early termination	72
9-8	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPEKWSSHGESMGSVTFSAEYRTKDEAKWLKVPECQHTTTTKCEFSLLDT	Frame shift	577
9-9	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGSAEYRTKDEAKWLKVPECQHTTTTKCEFSLLDTNVYIKTQFRVRAEEGNSTSSW	Frame shift	556
9-10	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYTL-WSSHGESMGSVTFSAEYRTKDEAKWLKVPECQH	One AA deleted	589
9-11	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNY	Early termination	44
9-12	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDSGAATESQWAV	Early termination	53
9-13	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGISNKRRGEVVKSA	Early termination	38
9-14	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYIIDDNYKVEQPRRVNGQCDLFSRISNKRRGEVVKSA	Early termination	74
9-15	aa-seq	MLAVVGAAALVLVAGAMLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIESGAATESQWAV	Early termination	47
9-16	aa-seq	MLAVVGAAALVLVAGAPWVLPSAAGGENLKPPENIDVYSGAATESQWAV	Early termination	49

The sequence of eighty amino acids (AA) of wild type (WT) IFNAR1 is presented and used to compare with those of mutant alleles. The deduced AA sequences, which do not match with that WT, is grey highlighted.