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Short communication

One-step generation of different immunodeficient mice with multiple gene modifications by CRISPR/Cas9 mediated genome engineering

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ABSTRACT

Taking advantage of the multiplexable genome engineering feature of the CRISPR/Cas9 system, we sought to generate different kinds of immunodeficient mouse strains by embryo co-microinjection of Cas9 mRNA and multiple sgRNAs targeting mouse *B2m*, *Il2rg*, *Prf1*, *Prkdc*, and *Rag1*. We successfully achieved multiple gene modifications, fragment deletion, double knockout of genes localizing on the same chromosome, and got different kinds of immunodeficient mouse models with different heritable genetic modifications at once, providing a one-step strategy for generating different immunodeficient mice which represents significant time-, labor-, and money-saving advantages over traditional approaches. Meanwhile, we improved the technology by optimizing the concentration of Cas9 and sgRNAs and designing two adjacent sgRNAs targeting one exon for each gene, which greatly increased the targeting efficiency and bi-allelic mutations.

1. Introduction

The fundamental understanding of many biological processes in humans has resulted from the development of 'humanized' mouse strains that are based on severely immunodeficient mice which carry multiple mutations in different immune response-associated genes, including *B2m*, *Foxn1*, *Il2rg*, *Prf1*, *Prkdc*, *Rag1*, *Rag2*, *etc.* (Shultz et al., 2007, 2012). Advances in the ability to generate humanized mice have depended on a systematic progression of genetic modifications to develop immunodeficient mice with multiple mutations (Dow and Lowe, 2012; Shultz et al., 2007, 2012). Apart from the formidable task to produce and characterize these mutants, the process of intercrossing individual allele or sequential targeting to generate different immunodeficient mouse models with multiallelic mutations is even more tedious, time-consuming, and expensive. Therefore, efficient strategies for triggering multiple genetic modifications are extremely desirable.

By NHEJ (non-homologous end joining), Cas9, a DNA endonuclease (now referred to as RNA-guided nucleases, RGENs) of the clustered, regularly interspaced, short palindromic repeats (CRISPR) system in bacterial and archaeal immunity (Jinek et al., 2012), provides the potential to engineer the eukaryote genome in a multiplexed way because its DNA-cleaving activity can be programmed with small RNA molecules to recognize specific DNA sequences, thus sparing the need to engineer a new protein for each new DNA target sequence. This potential was first achieved in cells (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013; Qi et al., 2013). Recently, Wang et al. made the potential become reality in mice (Wang et al., 2013). Such strategy would find broad application in the development of multigenic mouse models.

Taking advantage of the multiplexable genome engineering feature of the RGEN system, we sought to generate different kinds of immunodeficient mouse strains by co-microinjection of Cas9 mRNA and multiple sgRNAs targeting mouse *B2m*, *Il2rg*, *Prf1*, *Prkdc*, and *Rag1* into embryos. We successfully achieved multiple gene modifications, fragment deletion, double knockout of genes localizing on the same chromosome, and got different kinds of immunodeficient mouse models with different heritable genetic

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modifications at once, providing a one-step strategy for generating different immunodeficient mice with multiple gene modifications by CRISPR/Cas9 mediated genome engineering. Meanwhile, we improved the efficacy of the CRISPR/Cas9 technique in mouse genome engineering beyond the current standard by optimizing the concentration of Cas9 and sgRNAs and designing two adjacent sgRNAs targeting one exon for each gene.

2. Materials and methods

2.1. Animals

Mice were housed in standard cages in an Assessment and Accreditation Of Laboratory Animal Care credited SPF animal facility on a 12-h light/dark cycle. All animal protocols are approved by the Animal Care and Use Committee of the Model Animal Research Center, the host for the National Resource Center for Mutant Mice in China, Nanjing University.

2.2. DNA constructs

pST1374-Cas9-N-NLS-flag-linker (AddgeneID 44758) was described as before (Shen et al., 2013). sgRNA oligos were annealed into the pUC57-sgRNA expression vector with a T7 promoter. The sequences of sgRNA oligos and pUC57-sgRNA expression vector are listed in Supplementary Table 3 and Supplementary Sequence.

2.3. In vitro transcription

The pST1374-Cas9-N-NLS-flag-linker vector was linearized with Age1 and *in vitro* transcribed using T7 Ultra Kit (Ambion, AM1345). Cas9-N-NLS-flag-linker mRNA was purified using the RNeasy Mini Kit (Qiagen, 74104). sgRNA expression vectors were linearized with Dra I and transcribed *in vitro* using the MEGAshortscript Kit (Ambion, AM1354). sgRNAs were purified using the MEGAclear Kit (Ambion, AM1908) and concentrated by alcohol precipitation. For 5 (or 10) sgRNAs co-injection experiments, 2 μg each of the 5 (or 10) sgRNA expression vectors were mixed together and digested with Dra I. Digestion products were cleaned up with PCR Purification Kit (Qiagen, 28004), followed by transcription *in vitro*.

2.4. T7EN1 cleavage assay and sequencing

T7EN1 cleavage assay was performed as described (Shen et al., 2013). For the T7EN1 cleavage assay of *Il2rg*, which is localized on chromosome X, 50 ng wild-type PCR fragment and 150 ng PCR fragment from all male founders were mixed, then applied T7EN1 cleavage assay. Primers for amplifying B2m, Il2rg, Prf1, Prkdc and Rag1 sgRNAs targeted fragments are listed in Supplementary Table 4. PCR products with mutations detected by T7EN1 cleavage assay were sub-cloned into T vector (Takara, D103A). For each sample, at least 24 white colonies were picked up randomly and sequenced by M13-47 primer. Colonies with double peaks were taken out during alignment. Indel and mutation information were listed in Supplementary Table 2. For possible long fragment deletion, new primers were designed to amplify larger amplicon flanking the targeted site. The PCR products were cloned and sequenced by M13-47 and M13-48 primers for each colony.

2.5. Cas9/sgRNA injection of one-cell embryos

Cas9/sgRNA co-injection of one-cell embryos was performed as described (Shen et al., 2013). Briefly, mouse zygotes obtained by mating of CBA males with superovulated C57BL/6 J females, were injected with a mixture of Cas9 mRNA and sgRNAs as indicated in Table 1. Microinjections were performed in the cytoplasm and larger (male) pronucleus of fertilized oocytes. Injected zygotes were transferred to pseudopregnant CD1 female mice.

2.6. Flowcytometry analysis of leukocytes in peripheral blood

Three drops of whole blood drawn from 6-week-old founder's tail vein was collected in 30 μL of 50 mM EDTA, and red blood cells were lysed with ACK buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM EDTA, pH 7.2). The remaining cells were washed with cold PBS and stained with fluorescent antibodies against B220 (eBioscience, 11-0452-85), H2-kb (eBioscience, 12-5958-10), and CD90.2 (eBioscience, 25-0902-82) for subsequent analysis by flowcytometry (BD LSR II analyzer). Total 10,000 cell events were collected and analyzed in flowjo software. Even-aged mice in the same genetic background were used as control.

3. Results and discussion

3.1. sgRNA:Cas9-mediated modifications of 5 immuno-genes by single sgRNA targeting

Different immunodeficient mouse models are derived from multiallelic mutations of different immune-related genes, including *B2m*, *Foxn1*, *Il2rg*, *Prf1*, *Prkdc*, *Rag1*, *Rag2*, *etc.* (Shultz et al., 2007, 2012). For example, the most versatile immunodeficient strain, NSG, carries *Prkdc* and *Il2rg* double mutation. A recent paper described the multiplexable genome engineering feature of the RGEN system in mouse (Wang et al., 2013), suggesting the possibility to get different immune-related gene mutation mouse models by embryo co-microinjection of different kinds of sgRNAs. To test the possibility, 5 different sgRNAs each targeting one exon of mouse *B2m*, *Il2rg*, *Prf1*, *Prkdc*, and *Rag1* (Supplementary Figure 1 and Supplementary Table 1), were selected using previously described criteria (Cho et al., 2013; Cong et al., 2013; Hwang et al., 2013; Mali et al., 2013). The sgRNAs were *in vitro* transcribed as description (Shen et al., 2013).

The first test was performed by one-cell-stage mouse embryo co-microinjection with a mixture of 20 ng/μl mRNA of NLS-flag-linker-Cas9, a modified Cas9 easy for nuclear localization (Shen et al., 2013), along with 5 ng/μl each of 5 sgRNAs targeting *B2m*, *Il2rg*, *Prf1*, *Prkdc*, and *Rag1* (Table 1). A total of 27 pups (Founders #33–59) from 4 litters were born from 81 transferred embryos (Table 1). Modifications of the different loci from randomly selected 7 founders were first assayed by PCR (Fig. 1A), T7EN1 cleavage assay (Fig. 1B), and sequencing (Tables 2 and 3, and Supplementary Table

Table 1
Summary of embryo microinjection of Cas9 mRNA and sgRNAs.

	Inject mixture	Embryos injected	Embryos transferred	Recipient	Pups amount	Pups No.
1st	20 ng/μl Cas9 mRNA. 5 sgRNAs, 5 ng/μl each.	162	81	4	27	#33–#59
2nd	20 ng/μl Cas9 mRNA. 10 sgRNAs, 2.5 ng/μl each.	167	93	4	9	#60–#68
3rd	20 ng/μl Cas9 mRNA. 10 sgRNAs, 5 ng/μl each.	149	89	3	4	#90–#93