SUMMARY

Mice carrying mutations in multiple genes are traditionally generated by sequential recombination in embryonic stem cells and/or time-consuming interscrossing of mice with a single mutation. The CRISPR/Cas system has been adapted as an efficient gene-targeting technology with the potential for multiplexed genome editing. We demonstrate that CRISPR/Cas-mediated gene editing allows the simultaneous disruption of five genes (Tet1, 2, 3, Sry, Uty - 8 alleles) in mouse embryonic stem (ES) cells with high efficiency. Coinjection of Cas9 mRNA and single-guide RNAs (sgRNAs) targeting Tet1 and Tet2 into zygotes generated mice with biallelic mutations in both genes with an efficiency of 80%. Finally, we show that coinjection of Cas9 mRNA/sgRNAs with mutant oligos generated precise point mutations simultaneously in two target genes. Thus, the CRISPR/Cas system allows the one-step generation of animals carrying mutations in multiple genes, an approach that will greatly accelerate the in vivo study of functionally redundant genes and of epistatic gene interactions.

INTRODUCTION

Genetically modified mice represent a crucial tool for understanding gene function in development and disease. Mutant mice are conventionally generated by insertional mutagenesis (Copeland and Jenkins, 2010; Kool and Berns, 2009) or by gene-targeting methods (Capecchi, 2005). In conventional gene-targeting methods, mutations are introduced through homologous recombination in mouse embryonic stem (ES) cells. Targeted ES cells injected into wild-type (WT) blastocysts can contribute to the germline of chimeric animals, generating mice containing the targeted gene modification (Capecchi, 2005). It is costly and time consuming to produce single-gene knockout mice and even more so to make double-mutant mice. Moreover, in most other mammalian species, no established ES cell lines are available that contribute efficiently to chimeric animals, which greatly limits the genetic studies in many species.

Alternative methods have been developed to accelerate the process of genome modification by directly injecting DNA or mRNA of site-specific nucleases into the one-cell embryo to generate DNA double-strand break (DSB) at a specified locus in various species (Bogdanove and Voytas, 2011; Carroll et al., 2008; Urnov et al., 2010). DSBs induced by these site-specific nucleases can then be repaired by error-prone nonhomologous end joining (NHEJ) resulting in mutant mice and rats carrying deletions or insertions at the cut site (Carbery et al., 2010; Geurts et al., 2009; Sung et al., 2013; Tesson et al., 2011). If a donor plasmid with homology to the ends flanking the DSB is coinjected, high-fidelity homologous recombination can produce animals with targeted integrations (Cui et al., 2011; Meyer et al., 2010). Because these methods require the complex designs of zinc finger nucleases (ZNFs) or Transcription activator-like effector nucleases (TALENs) for each target gene and because the efficiency of targeting may vary substantially, no multiplexed gene targeting in animals has been reported to date. To dissect the functions of gene family members with redundant functions or to analyze epistatic relationships in genetic pathways, mice with two or more mutated genes are required, prompting the development of efficient technology for the generation of animals carrying multiple mutated genes.

Recently, the type II bacterial CRISPR/Cas system has been demonstrated as an efficient gene-targeting technology with the potential for multiplexed genome editing. Bacteria and archaea have evolved an RNA-based adaptive immune system that uses CRISPR (clustered regularly interspaced short palindromic repeat) and Cas (CRISPR-associated) proteins to detect and destroy invading viruses and plasmids (Horvath and
Please cite this article in press as: Wang et al., One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering, Cell (2013), http://dx.doi.org/10.1016/j.cell.2013.04.025
RESULTS

Simultaneous Targeting up to Five Genes in ES Cells

To test the possibility of targeting functionally redundant genes from the same gene family, we designed sgRNAs targeting the Ten-eleven translocation (Tet) family members, *Tet1*, *Tet2*, and *Tet3* (Figure 1A). Tet proteins (*Tet1/2/3*) convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in various embryonic and adult tissues and mutant mice for each of these three genes have been produced by homologous recombination in ES cells (Dawlaty et al., 2011; Gu et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011). To test whether the CRISPR/Cas system could produce targeted cleavage in the mouse genome, we transfected plasmids expressing both the mammalian-codon-optimized Cas9 and a sgRNA targeting each gene (Cong et al., 2013; Mali et al., 2013) into mouse ES cells. The number of total alleles mutated in each ES cell clone is listed from 0 to 2 for single-targeting experiment, and 0 to 6 for triple-targeting experiment. The number of clones containing each specific number of mutated alleles is shown in relation to the total number of clones screened in each experiment. See also Table S1.

Plasmids encoding Cas9 and sgRNAs targeting *Tet1*, *Tet2*, and *Tet3* were transfected separately (single targeting) or in a pool (triple targeting) into ES cells. The number of total alleles mutated in each ES cell clone is listed from 0 to 2 for single-targeting experiment, and 0 to 6 for triple-targeting experiment. The number of clones containing each specific number of mutated alleles is shown in relation to the total number of clones screened in each experiment. See also Table S1.

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**Table 1. CRISPR/Cas-Mediated Gene Targeting in V6.5 ES Cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutant Alleles per Clone / Total Clones Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td><strong>Tet1</strong></td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Tet2</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Tet3</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Tet1+ Tet2 + Tet3</strong></td>
<td>20/96</td>
</tr>
</tbody>
</table>

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**Figure 1. Multiplexed Gene Targeting in mouse ES cells**

(A) Schematic of the Cas9/sgRNA-targeting sites in *Tet1*, 2, and 3. The sgRNA-targeting sequence is underlined, and the protospacer-adjacent motif (PAM) sequence is labeled in green. The restriction sites at the target regions are bold and capitalized. Restriction enzymes used for RFLP and Southern blot analysis are shown, and the Southern blot probes are shown as orange boxes.

(B) Surveyor assay for Cas9-mediated cleavage at *Tet1*, 2, and 3 loci in ES cells.

(C) Genotyping of triple-targeted ES cells, clones 51, 52, and 53 are shown. Upper: RFLP analysis. *Tet1* PCR products were digested with SacI, *Tet2* PCR products were digested with EcoRV, and *Tet3* PCR products were digested with XhoI. Lower: Southern blot analysis. For the *Tet1* locus, SacI digested genomic DNA was hybridized with a 5’ probe. Expected fragment size: WT = 5.8 kb, TM (targeted mutation) = 6.4 kb. For the *Tet2* locus, SacI, and EcoRV double-digested genomic DNA was hybridized with a 3’ probe. Expected fragment size: WT = 4.3 kb, TM = 5.6 kb. For the *Tet3* locus, BamHI and XhoI double-digested genomic DNA was hybridized with a 5’ probe. Expected fragment size: WT = 3.2 kb, TM = 8.1 kb.

(D) The sequence of six mutant alleles in triple-targeted ES cell clone 14 and 41. PAM sequence is labeled in red.

(E) Analysis of 5hmC levels in DNA isolated from triple-targeted ES cell clones by dot blot assay using anti-5hmC antibody. A previously characterized DKO clone derived using traditional method is used as a control. See also Figure S1.
most clones displaying two mutant alleles for each gene with small insertions or deletions (indels) at the target site (Figure 1D). To test whether these mutant alleles would abolish the function of Tet proteins, we compared the 5hmC level of targeted clones from loss of function alleles, we found a significant reduction of 5hmC levels in all clones carrying biallelic mutations in the three genes (Figure 1E).

To further test the potential of multiplexed gene targeting by CRISPR/Cas system, we designed sgRNAs targeting two Y-linked genes, Sry and Uty (Figure S1C). Short PCR products encoding sgRNAs targeting all five genes (Tet1, Tet2, Tet3, Sry, and Uty) were pooled and cotransfected with a Cas9 expressing plasmid and the PGK puroR cassette into ES cells. Of 96 clones that were screened using the RFLP assay, 10% carried mutations in all eight alleles of the five genes (Figure S1D and Table S1), demonstrating the capacity of the CRISP/Cas9 system for highly efficient multiplexed gene targeting.

One-Step Generation of Single-Gene Mutant Mice by Zygote Injection

We tested whether mutant mice could be generated in vivo by direct embryo manipulation. Capped polyadenylated Cas9 mRNA was produced by in vitro transcription and coinjected with sgRNAs. Initially, to determine the optimal concentration of Cas9 mRNA for targeting in vivo, we microinjected varying amounts of Cas9-encoding mRNA with Tet1 targeting sgRNA at constant concentration (20 ng/μl) into pronuclear (PN) stage one-cell mouse embryos and assessed the frequency of altered alleles at the blastocyst stage using the RFLP assay. As expected, higher concentration of Cas9 mRNA led to more efficient gene disruption (Figure S2A). Nevertheless, even embryos injected with the highest amount of Cas9 mRNA (200 ng/μl) showed normal blastocyst development, suggesting low toxicity.

To investigate whether postnatal mice carrying targeted mutations could be generated, we coinjected sgRNAs targeting Tet1 or Tet2 with different concentrations of Cas9 mRNA. Blastocysts derived from the injected embryos were transplanted into foster mothers and newborn pups were obtained. As summarized in Table 2, about 10% of the transferred blastocysts developed to birth independent of the RNA concentrations used for injection suggesting low fetal toxicity of the Cas9 mRNA and sgRNA. RFLP, Southern blot, and sequencing analysis demonstrated that between 50 and 90% of the postnatal mice carried biallelic mutations in either target gene (Figures 2A, 2B, and 2C and Table 2).

Surprisingly, specific Δ9 Tet1 and specific Δ8 and Δ15 Tet2 mutant alleles were repeatedly recovered in independently derived mice. Preferential generation of these alleles is likely caused by a short sequence repeat flanking the DSB (see Figure S2B) consistent with previous reports demonstrating that perfect microhomology sequences flanking the cleavage sites can generate microhomology-mediated precise deletions by end repair mechanism (MMEJ) (McVey and Lee, 2008; Symington and Gautier, 2011) (Figure S2B). A similar observation was also made when TALEN mRNA was injected into one-cell rat embryos (Tesson et al., 2011).

We also derived blastocysts from zygotes injected with Cas9 mRNA and Tet3 sgRNA. Genotyping of the blastocysts demonstrated that of eight embryos three were homozygous and three were heterozygous Tet3 mutants (two failed to amplify) (Figure S2C). Some blastocysts were implanted into foster mothers and, upon C section, we readily identified multiple mice of smaller size (Figure S2D), many of which died soon after delivery. Genotyping shown in Figure S2E indicated that all pups with mutations in both Tet3 alleles died neonatally. Only 2 out of 15 mice survived that were either Tet3 heterozygous mutants or WT (Figure S2F). These results are consistent with the lethal neonatal phenotype of Tet3 knockout mice generated using traditional methods (Gu et al., 2011), although we have not yet established which of the Tet3 mutations produced loss of function rather than hypomorphic alleles.

One-Step Generation of Double-Gene Mutant Mice by Zygote Injection

To test whether Tet1/Tet2 double-mutant mice could be produced from single embryos, we coinjected Tet1 and Tet2 sgRNAs with 20 or 100 ng/μl Cas9 mRNA into zygotes. A total of 28 pups were born from 144 embryos transferred into foster mothers (21% live-birth rate) that had been injected at
the zygote stage with high concentrations of RNA (Cas9 mRNA at 100 ng/µl, sgRNAs at 50 ng/µl), consistent with low or no toxicity of the Cas9 mRNA and sgRNAs (Table 3). RFLP, Southern blot analysis, and sequencing identified 22 mice carrying targeted mutations at all four alleles of the Tet1 and Tet2 genes (Figures 2D and 2E) with the remaining mice carrying mutations in a subset of alleles (Table 3). Injection of zygotes with low concentrations of RNA (Cas9 mRNA at 20 ng/µl, sgRNAs at 20 ng/µl) yielded 19 pups from 75 transferred embryos (25% live-birth rate), which is a higher survival rate than from embryos injected with 100 ng/µl of Cas9 RNA. Nevertheless, more than 50% of the pups were biallelic Tet1/Tet2 double mutants (Table 3). These results demonstrate that postnatal mice carrying biallelic mutations in two different genes can be generated within one month with high efficiency (Figure 2F).

Although the high live-birth rate and normal development of mutant mice suggest low toxicity of CRISPR/Cas9 system, we sought to determine the off-target effects in vivo. Previous work in vitro, in bacteria, and in cultured human cells suggested that the protospacer-adjacent motif (PAM) sequence NGG and the 8 to 12 base "seed sequence" at the 3' end of the sgRNA are most important for determining the DNA cleavage specificity (Cong et al., 2013; Jiang et al., 2013; Jinek et al., 2012). Based on this rule, only three and four potential off targets exist in mouse genome for Tet1 and Tet2 sgRNA, respectively (Table S2 and Experimental Procedures), with each of them perfectly matching the 12 bp seed sequence at the 3' end and the NGG PAM sequence of the sgRNA (there is no potential off-target site for Tet3 sgRNA using this prediction rule). From seven double-mutant mice produced from injection with high RNA concentration we PCR amplified 400 to 500 bp fragments from all seven potential off-target loci and found no cleavage in the Surveyor assay (Figure S3), suggesting a high specificity of CRISPR/Cas system.

Multiplexed Precise HDR-Mediated Genome Editing In Vivo

The NHEJ-mediated gene mutations described above produced mutant alleles with different and unpredictable insertions and deletions of variable size. We explored the possibility of precise

Figure 2. Single- and Double-Gene Targeting In Vivo by Injection into Fertilized Eggs

(A) Genotyping of Tet1 single-targeted mice.
(B) Upper: genotyping of Tet2 single-targeted mice. RFLP analysis; lower: Southern blot analysis.
(C) The sequence of both alleles of targeted gene in Tet1 biallelic mutant mouse 2 and Tet2 biallelic mutant mouse 4.
(D) Genotyping of Tet1/Tet2 double-mutant mice. Analysis of mice 1 to 12 is shown. Upper: RFLP analysis; lower: southern blot analysis. The Tet1 locus is displayed on the left and the Tet2 locus on the right.
(E) The sequence of four mutant alleles from double-mutant mouse 9 and 10. PAM sequences are labeled in red.
(F) Three-week-old double-mutant mice. All RFLP and Southern digestions and probes are the same as those used in Figure 1. See also Figures S2 and S3.
The genetic manipulation of mice is a crucial approach for the study of development and disease. However, the generation of mice with specific mutations is labor intensive and involves gene targeting by homologous recombination in ES cells, the production of chimeric mice, and, after germline transmission of the targeted ES cells, the interbreeding of heterozygous mice to produce the homozygous experimental animals, a process that may take 6 to 12 months or longer (Capecchi, 2005). To produce mice carrying mutations in several genes requires time-consuming intercrossing of single-mutant mice. Similarly, the generation of ES cells carrying homozygous mutations in several genes is usually achieved by sequential targeting, a process that is labor intensive, necessitating multiple consecutive cloning steps to target the genes and to delete the selectable markers.

As summarized in Figure 4, we have established three different approaches for the generation of mice carrying multiple genetic alterations. We demonstrate that CRISPR/Cas-mediated genome editing in ES cells can generate the simultaneous mutations of several genes with high efficiency, a single-step approach allowing the production of cells with mutations in five different genes (Figure 4A). We chose the three Tet genes as targets because the respective mutant phenotypes have been well defined previously (Dawlaty et al., 2011, 2013; Gu et al., 2011). Cells mutant for Tet1, 2 and 3 were depleted of 5hmC as would be expected for loss of function mutations of the genes (Dawlaty et al., 2013). However, we have not as yet established, which of the Cas9-mediated gene mutations produced loss of function rather than hypomorphic alleles.

We also show that mouse embryos can be directly modified by injection of Cas9 mRNA and sgRNA into the fertilized egg resulting in the efficient production of mice carrying biallelic mutations in a given gene. More significantly, coinjection of Cas9 with Tet1 and Tet2 sgRNAs into zygotes produced mice that carried mutations in both genes (Figure 4B, upper). We found that up to 95% of newborn mice were biallelic mutant in the targeted gene when single sgRNA was injected and when coinjected with two different sgRNAs, up to 80% carried biallelic mutations in both targeted genes. Thus, mice carrying multiple mutations can be generated within 4 weeks, which is a much shorter time frame than can be achieved by conventional consecutive targeting of genes in ES cells and avoids time-consuming intercrossing of single-mutant mice.

The introduction of DSBs by CRISPR/Cas generates mutant alleles with varying deletions or insertions in contrast to designed precise mutations created by homologous recombination. The introduction of point mutations into human ES cells, cancer cell lines, and mouse by ZNF or TALEN along with DNA oligo has

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Table 3. CRISPR/Cas-Mediated Double-Gene Targeting in BDF2 Mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cas9/sgRNA (ng/μl)</th>
<th>Blastocyst/Injected Zygotes</th>
<th>Transferred Embryos (Recipients)</th>
<th>Newborns (Dead)</th>
<th>Mutant Alleles per Mouse/Total Mice Testeda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet1 + Tet2</td>
<td>100 / 50</td>
<td>194/229</td>
<td>144(7)</td>
<td>31(8)</td>
<td>22/28</td>
</tr>
<tr>
<td></td>
<td>20 / 20</td>
<td>92/109</td>
<td>75(5)</td>
<td>19(3)</td>
<td>11/19</td>
</tr>
</tbody>
</table>

Cas9 mRNA and sgRNAs targeting Tet1 and Tet2 were coinjected into fertilized eggs. The blastocysts derived from the injected embryos were transplanted into foster mothers and newborn pups were obtained and genotyped. The number of total alleles mutated in each mouse is listed from 0 to 4 for Tet1 and Tet2. The number of mice containing each specific number of mutated alleles is shown in relation to the number of total mice screened in each experiment.

aSome of the pups were cannibalized.
been demonstrated previously (Chen et al., 2011; Soldner et al., 2011; Wefers et al., 2013). We demonstrate that CRISPR/Cas-mediated targeting is useful to generate mutant alleles with predetermined alterations, and coinjection of single-stranded oligos can introduce designed point mutations into two target genes in one step, allowing for multiplexed gene editing in a strictly controlled manner (Figure 4B, lower). It will be of great interest to assess whether this targeting system allows for the production of conditional alleles, or precise insertion of larger DNA fragments such as GFP markers so as to generate conditional knockout and reporter mice for specific genes.

There are several potential limitations of the CRISPR/Cas technology. First, the requirement for a NGG PAM sequence of S. pyogenes Cas9 limits the target space in the mouse genome. It has been shown that the Streptococcus thermophilus LMD-9 Cas9 using different PAM sequence can also induce targeted DNA cleavage in mammalian cells (Cong et al., 2013). Therefore, exploiting different Cas9 proteins may enable to target most of the mouse genome. Second, although the sgRNAs used here showed high targeting efficiency, much work is needed to elucidate the rules for designing sgRNAs with consistent high targeting efficiency, which is essential for multiplexed genome engineering. Third, although our off-target analysis for the seven most likely off targets of Tet1 and Tet2 sgRNAs failed to detect mutations in these loci, it is possible that other mutations were induced following as yet unidentified rules. A more thorough sequencing analysis for a large number of sgRNAs will provide more information about the potential off-target sites. Last, oligo-mediated repair allows for precise genome editing, but the other allele is often mutated through NHEJ (Figures 3B, 3C, and S4C). We have shown that using lower Cas9 mRNA concentration generates more mice with heterozygous mutations. Therefore, it may be possible to optimize the system for more efficient generation of mice with only one oligo-modified allele. In addition, employment of Cas9 nickase will likely avoid this complication because it mainly induces...
DNA single-strand break, which is typically repaired through HDR (Cong et al., 2013; Mali et al., 2013).

It is likely that a much larger number of genomic loci than targeted in the present work can be modified simultaneously when pooled sgRNAs are introduced. The methods presented here open up the possibility of systematic genome engineering in mice, facilitating the investigation of entire signaling pathways, of synthetic lethal phenotypes or of genes that have redundant functions. A particularly interesting application is the possibility to produce mice carrying multiple alterations in candidate loci that have been identified in GWAS studies to play a role in the genesis of multigenic diseases. In summary, CRISPR/Cas-mediated genome editing makes possible the generation of ES cells and mice carrying multiple genetic alterations and will facilitate the genetic dissection of development and complex diseases.

EXPERIMENTAL PROCEDURES

Procedures for Generating sgRNAs Expressing Vector

Bicistronic expression vector expressing Cas9 and sgRNA (Cong et al., 2013) were digested with BbsI and treated with Antarctic Phosphatase, and the linearized vector was gel purified. A pair of oligos for each targeting site (Table S3) was annealed, phosphorylated, and ligated to linearized vector.

Cell Culture and Transfection

V6.5 ES cells (on a 129/Sv x C57BL/6 F1 hybrid background) were cultured on gelatin-coated plates with standard ES cell culture conditions. Cells were transfected with a plasmid expressing mammalian-codon-optimized Cas9 and sgRNA (single targeting), three plasmids expressing Cas9 and sgRNAs targeting Tet1, Tet2, and Tet3 (triple targeting), or five PCR products each coding for sgRNA targeting Tet1, Tet2, Tet3, Sry, and Uty, along with a plasmid expressing PGK-puroR using FuGENE HD reagent (Promega) according to the manufacturer’s instructions. Twelve hours after transfection, ES cells were replated at a low density on DR4 MEF feeder layers. Puromycin (2 \( \mu \)g/ml) was added 1 day after replating and taken off after 48 hr. After recovering for 4 to 6 days, individual colonies were picked and genotyped by RFLP and Southern blot analysis, and the leftover ES cells on plate were collected for Suveryor assay.

Suveryor Assay and RFLP Analysis for Genome Modification

Suveryor assay was performed as described by (Guschin et al., 2010). Genomic DNA from treated and control ES cells or targeted and control mice was extracted. Mouse genomic DNA samples were prepared from tail biopsies. PCR was performed using Tet1-, Tet2-, and 3-specific primers (Table S3) under the following conditions: 95°C for 5 min; 35 x (95°C for 30 s, 60°C for 30 s, 68°C for 40 s); 68°C for 2 min; hold at 4°C. PCR products were then denatured, annealed, and treated with Suveryor nuclease (Transgene). DNA concentration of each band was measured on an ethidium bromide-stained agarose gel (2%). For sequencing, PCR products were cloned using the Original TA Cloning Kit (Invitrogen), and sequenced on an ethidium bromide-stained 10% acrylamide Criterion TBE gel (BioRad) and quantified using ImageJ software. The same PCR products for Suveryor assay were used for RFLP analysis. Ten microliters of Tet1, Tet2, or Tet3 PCR product was digested with ScaI, EcoRV, or XhoI, respectively. Digested DNA was separated on an ethidium bromide-stained agarose gel (2%). For sequencing, PCR products were cloned using the Original TA Cloning Kit (Invitrogen), and mutations were identified by Sanger sequencing.

Dot Blot

DNA was extracted from ES cells following standard procedures. DNA was transferred to nylon membrane using BioRad slot blot vacuum manifold apparatus. Anti-5hmC (Active Motif 1:10,000) was used to detect 5hmC following manufacturer’s protocol.

Production of Cas9 mRNA and sgRNA

T7 promoter was added to Cas9 coding region by PCR amplification using primer Cas9 F and R (Table S3). T7-Cas9 PCR product was gel purified and used as the template for in vitro transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). T7 promoter was added to sgRNAs template by PCR amplification using primer Tet1 F and R, Tet2 F and R, and Tet3 F and R (Table S3). The T7-sgRNA PCR product was gel purified and used as the template for IVT using MEGashortscript T7 kit (Life Technologies). Both the Cas9 mRNA and the sgRNAs were purified using MEGAClear kit (Life Technologies) and eluted in RNase-free water.

One-Cell Embryo Injection

All animal procedures were performed according to NIH guidelines and approved by the Committee on Animal Care at MIT. B6D2F1 (C57BL/6 X DBA2) female mice and ICR mouse strains were used as embryo donors and foster mothers, respectively. Superovulated female B6D2F1 mice (7–8 weeks old) were mated to B6D2F1 stud males, and fertilized embryos were collected from oviducts. Cas9 mRNAs (from 20 ng/\( \mu \)l to 200 ng/\( \mu \)l) and sgRNA (from 20 ng/\( \mu \)l to 50 ng/\( \mu \)l) was injected into the cytoplasm of fertilized eggs with well recognized pronuclei in M2 medium (Sigma). For oligos injection, Cas mRNA (100 ng/\( \mu \)l), sgRNA (50 ng/\( \mu \)l), and donor oligos (100 ng/\( \mu \)l) were mixed and injected into zygotes at the pronuclei stage. The injected zygotes were cultured in KSOM with amino acids at 37°C under 5% CO2 in air until blastocyst stage by 3.5 days. Thereafter, 15–25 blastocysts were transferred into uterus of pseudopregnant ICR females at 2.5 dpc.

Southern Blotting

Genomic DNA was separated on a 0.8% agarose gel after restriction digests with the appropriate enzymes, transferred to a nylon membrane (Amersham) and hybridized with \(^{32}P\) random primer (Stratagene)-labeled probes.

Prediction of Potential Off Targets

Potential targets of CRISPR sgRNAs were found using the rules outline in (Mali et al., 2013). For a 20 nt sgRNA targeting sequence of nnnn nnmNM MMMM MMMM MMMM, where M are the seed bases preceding the PAM sequence NGG, four search sequences (MMM MMMM MMMM GG; MMM MMMM MMMM GG; MMM MMMM MMMM GG; MMM MMMM MMMM GG) were generated. Exact matches to these search sequences in the mouse genome (mm9) were found using bowtie and reported as potential targets of the CRISPR sgRNA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.04.025.

ACKNOWLEDGMENTS

We thank Ruth Flannery and Kibibi Ganz for support with animal care and experiments. We thank Jaenisch lab members for helpful discussions on the manuscript. We are also grateful to G. Grant Wielstead and Daniel B. Dadon for the help of editing the manuscript. M.M.D is a Damon Runyon Postdoctoral Fellow. A.W.C. is supported by a Croucher scholarship. R.J. is an adviser to Stemgent and a cofounder of Fate Therapeutics. This work was supported by NIH grants R37-HD045022 and R01-CA084198 to RJ.

Received: March 29, 2013
Revised: April 13, 2013
Accepted: April 14, 2013
Published: May 2, 2013

REFERENCES


Figure S1. Single-, Triple-, and Quintuple-Gene Targeting in mES Cells, Related to Figure 1

(A) RFLP analysis of clones from each single-targeting experiment (1 to 17 are shown).

(B) RFLP analysis of triple-gene-targeted clones (37 to 53 are shown). Tet1 PCR products were digested with SacI, Tet2 PCR products were digested with EcoRV, and Tet3 PCR products were digested with XhoI. WT control is shown in the last lane. Genotyping of clone 51, 52, and 53 are also shown in Figure 1C.

(C) Schematic of the Cas9/sgRNA-targeting sites in Sry and Uty. The sgRNA-targeting sequence is underlined, and the protospacer-adjacent motif (PAM) sequence is labeled in green. The restriction sites at the target regions are bold and capitalized. Restriction enzymes used for RFLP analysis are shown.

(D) RFLP analysis of quintuple-gene-targeted clones (1 to 10 are shown). Sry PCR products were digested with BsaJI, Uty PCR products were digested with AvrII. WT control is shown in the last lane. RFLP analysis of Tet1, 2, 3 loci are not shown.
Figure S2. One-Step Generation of Single-Gene Mutant Mice by Zygote Injection, Related to Figure 2

(A) RFLP analysis of blastocysts injected with different concentration of Cas9 mRNA and Tet1 sgRNA at 20 ng/μl. Tet1 PCR products were digested with SacI.
(B) Commonly recovered Tet1 and Tet2 alleles resulted from MMEJ. PAM sequence of each targeting sequence is labeled in green. Microhomology flanking the DSB is bold and underlined in WT sequence.
(C) RFLP analysis of eight Tet3-targeted blastocysts demonstrated high targeting efficiency (embryo 3 and 5 failed to amplify). Tet3 PCR products were digested with XhoI.
(D) Some Tet3-targeted mice show smaller size and all homozygous mutants died within 1 day after birth.
(E) RFLP analysis of Tet3 single-targeted newborn mice. Mouse 8 and 14 survived after birth. Sample 2 and 6 failed to amplify.
(F) Sequences of both Tet3 alleles of surviving Tet3-targeted mouse 14. PAM sequences are labeled in red.
Figure S3. Off-Target Analysis of Double-Mutant Mice, Related to Figure 2

(A) Three potential off targets of Tet1 sgRNA and four potential off targets of Tet2 sgRNA are shown. The 12 bp perfect matching seed sequence is labeled in blue, and NGG PAM sequence is labeled in red.

(B) Surveyor assay of all seven potential off-target loci in seven double-mutant mice derived with high concentration of Cas9 mRNA (100 ng/μl) injection. WT control is included as the eighth sample. The weak cleavage activity at Ubr1 locus is not due to off-target effect because sequences of these PCR products show no mutations.
Figure S4. Multiplexed Precise HDR-Mediated Genome Editing In Vivo, Related to Figure 3
(A) RFLP analysis of single oligo injection embryos with HDR-mediated targeting at Tet1 and Tet2 locus.
(B) RFLP analysis of double oligo injection embryos with multiplexed HDR-mediated targeting at both Tet1 and Tet2 loci.
(C) Sequences of both alleles of Tet1 and Tet2 in embryo 2 show simultaneously HDR-mediated targeting at one allele of both genes, and NHEJ-mediated gene disruption at the other allele of each gene.