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Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system

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Introducing a point mutation is a fundamental method used to demonstrate the roles of particular nucleotides or amino acids in the genetic elements or proteins, and is widely used in *in vitro* experiments based on cultured cells and exogenously provided DNA. However, the *in vivo* application of this approach by modifying genomic loci is uncommon, partly due to its technical and temporal demands. This leaves many *in vitro* findings un-validated under *in vivo* conditions. We herein applied the CRISPR/Cas9 system to generate mice with point mutations in their genomes, which led to single amino acid substitutions in proteins of interest. By microinjecting gRNA, hCas9 mRNA and single-stranded donor oligonucleotides (ssODN) into mouse zygotes, we introduced defined genomic modifications in their genome with a low cost and in a short time. Both single gRNA/WT hCas9 and double nicking set-ups were effective. We also found that the distance between the modification site and gRNA target site was a significant parameter affecting the efficiency of the substitution. We believe that this is a powerful technique that can be used to examine the relevance of *in vitro* findings, as well as the mutations found in patients with genetic disorders, in an *in vivo* system.

Introducing point mutations is a widely used experimental approach to evaluate the roles of specific nucleotides or amino acids in the function of the genetic elements or in proteins of interest. Using this technique, various nucleotides and amino acids have been proved to be indispensable for promoter or enhancer activity, as well as for the function or regulation of enzymes, transcription factors and signaling molecules. However, these findings were achieved mainly by *in vitro* experiments using exogenously provided DNA, such as plasmids or virus vectors, and the *in vivo* introduction of defined point mutations at endogenous genomic loci has been uncommon, largely because of the technical and temporal costs associated with such techniques. Thus, the *in vivo* context or relevance of *in vitro* findings has remained unexplored in many cases.

The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated 9) system is a recently developed genome-engineering tool based on the bacterial CRISPR immune system, in which guide RNA (gRNA) recruits the Cas9 nuclease to the target locus in the genome by sequence complementarity, and induces double strand breaks (DSBs)[1,2]. These DSBs cause small insertions or deletions (indels) following non-homologous end-joining (NHEJ) repair, or can be utilized to introduce defined sequence modifications through a homology-dependent repair (HDR) mechanism[1,2]. While the use of HDR-dependent genomic engineering has been reported in *in vitro* cell culture systems, whether this could be applied in *in vivo* systems to introduce defined point mutations and what parameters affect its efficiency were less explored.

In the present study, we applied the CRISPR/Cas9 system to create a single amino acid substituted mouse model. By microinjecting synthesized RNAs and single-strand oligodeoxynucleotide (ssODN) donor sequences into mouse zygotes, we were able to introduce defined point mutations in the mouse genome, which led to single amino acid substitutions in the proteins of interest, within as short as one to two months. Using this technique, we can now evaluate the *in vivo* relevance of particular nucleotides/amino acids with a low cost and within a short time, which facilitates the elucidation of the *in vivo* context of the findings in cultured cell systems, as well as the *in vivo* screening for the relevant mutation among those found in human patients with various diseases.

Results

Substitution by single gRNA and wild-type hCas9. To generate an amino acid substituted mouse model using the CRISPR system, we first took the simplest approach: microinjecting single gRNA, wild type (WT) hCas9 mRNA and a ssODN with a single base mismatch to the genomic sequence, which caused single amino acid conversion, into mouse zygotes. As a target, a c.274C>T mutation of the *Steroidogenic Factor 1* gene (*Sf-1*, also known as *Ad4BP* or *Nr5a1*)[3], which causes a p.R92W amino acid substitution in the SF-1 protein, was chosen, and gRNAs and ssODNs were designed as shown in Figure 1A.

To examine whether the sequence or location of the gRNAs could affect the efficiency of the substitution, we designed three independent gRNAs (Sf-1gRNA1, 2 and 3), which had their Protospacer-Adjacent Motif (PAM) sequence 52 or 7 bases upstream, or 12 bases downstream, of the target point mutation (Fig. 1A). Three independent ssODNs were also designed, placing the mismatched nucleotide or PAM sequence of gRNA1 and gRNA2 in their center, respectively (Fig. 1A).

Various combinations of gRNAs and ssODNs (Table 1) were mixed with hCas9 mRNA and microinjected into pronuclear stage embryos. The injected zygotes were transferred to pseudo-pregnant females at the two-cell stage, and the obtained pups were examined for the sequence around the *Sf-1* locus. As shown in Table 1, we found total 26 mice with the C>T substitution generated from three out of four experiments (experiments 2, 3 and 4), and 7 of them had biallelic substitutions (Fig. 1B and Table 1). These results show that microinjecting gRNA, WT hCas9 mRNA and the ssODN donor into mouse zygotes is sufficient to introduce a defined single base substitution in their genome.

Of note, while the frequencies of DSB caused by hCas9/gRNAs, which are indicated by the frequencies of mutated alleles, are comparable among 4 experiments (68–89%, $P = 0.75$) (Table 1), the frequencies of designated substitutions vary significantly (0–29.6%, $P < 0.01$) (Table 1). This lack of a correlation between the frequency of the mutation and the substitution to occur suggests that there may be an optimal rate of DSB occurance to introduce HDR-dependent sequence incorporation (such like too frequent DSB might be unfavorable for HDR), or that the distance and/or the mutual position between the substitution target site and gRNA PAM sequences (surrogate DSB point) could affect the substitution efficiency. Our subsequent experiments support the latter possibility (see below). To explore the effect of mutual position between substitution target site and DSB point on substitution efficiency, we designed two additional ssODNs which have similar sequence to ssODN1, but carry point mutations in 36 base upstream or 12 base downstream to the PAM sequence of Sf-1 gRNA3 (Supplementary Fig. S1). ssODNs were microinjected into mouse zygotes with hCas9 mRNA and Sf-1 gRNA3, and the frequency of the designated substitution and the mutated allele in those mice were compared to experiment 4 (Supplementary Table S1). As a result, while 80–95% of mice had mutated alleles in all three experiments, the frequency of the substituted alleles differs substantially: 29.6% with ssODN3, 20.0% with ssODN4, and 0% with ssODN5 (Supplementary Table S1). These results indicate that the probability of the mutation in donor DNA to be incorporated into the genome declines with the distance between the DSB point and the mutation. In addition, the mutation which is at the downstream (3′ side) of PAM sequence showed slightly lower frequency of substitution than the mutation with the same distance from the PAM sequence but is at the upstream (5′ side). Together, these results show that the mutual location of gRNA target site and substitution target site is an important parameter for the efficient introduction of the designated point mutation.

Although there was an efficient generation of targeted point mutations, we observed that many mice with the C>T substitution also received additional random mutations in the flanking regions (Fig. 1C and Table 1). Moreover, it has previously been reported that single gRNA/WT hCas9 may introduce undesired mutations at the genomic loci with similar sequences to the target site (off-target effect)[4–6], which prompted us to test the double nicking strategy[5] to induce more accurate genome modifications in mouse zygotes.

Substitution using two gRNAs and hCas9 D10A (double nicking). Combining a pair of gRNAs with mutated hCas9 (hCas9 D10A), double nicking cause DSB only at the genomic loci where both of the gRNAs bind at an appropriate distance, thus making it highly specific to the target site[5]. To examine whether this set-up could be used for the HDR-dependent genome editing *in vivo*, we aimed to induce the c.1187A>G mutation of the *Sox9* gene, which results in a p.K396R amino acid substitution in the SOX9 protein. Lysine 396 is an evolutionary-conserved post-translational modification site of the SOX9 protein, but its *in vivo* relevance has not been fully examined[7,8]. We designed two gRNAs with a five-base offset where their PAM sequences are positioned about 40 bases upstream and three bases downstream of the A>G substitution site (Fig. 2A). Two ssODNs were designed with several silent mutations within the recognition sequence of gRNA2 to avoid repetitive digestions. An additional silent mutation to eliminate the *Pvu*II site was also designed to facilitate the genotyping (Fig. 2A). Sox9-ssODN1 causes both silent mutations and c.1187A>G substitution (K-to-R substitution), while Sox9-ssODN2 causes only silent mutations (K-to-K substitution) (Fig. 2A, lower panel). The two gRNAs, hCas9 D10A mRNA and 2 ssODNs were mixed and microinjected into zygotes to generate amino acid substituted mice (by Sox9-ssODN1) and negative control mice (by Sox9-ssODN2) simultaneously.

The obtained pups were genotyped as before, and we found that five pups out of 24 (20.8%) with the designated substitution, two of which had a K-to-R substitution (Fig. 2B) and three of which had a K-to-K substitution (Table 2). A frame shift mutation caused by an indel was observed in only one mouse. Thus, double nicking, together with donor ssODN, can also introduce defined point mutations in mouse zygotes. The frequency of the substitution was lower than that of the single gRNA/WT hCas9 condition shown in previous section, but was sufficient to generate mice with two genotypes in a single microinjection experiment by mixing different donor DNAs. Of note, a *Pvu*II site substitution was never observed in the mice with upstream substitutions (Fig. 2B and data not shown). This, together with the results of the experiment 4–6 with *Sf-1*, suggest that only a part of the sequence of the donor DNA near the DSB site will be incorporated through the CRISPR-mediated HDR pathway. Therefore, it is important to place the gRNA target site close enough to the substitution site to achieve efficient substitution.

SOX9-K396R gRNA2 and WT hCas9 mRNA with ssODNs were also microinjected into zygotes to compare the efficiency with double nicking (Table 2). As shown in Table 2, single gRNA with WT hCas9 resulted in a higher mutation frequency (76.9%) as well as substitution frequency (34.6%), and as in the case of *Sf-1*, biallelic substitution and additional random mutations were also observed (Supplementary Fig. S2 and Table 2).

Finally, we compared the off-target effects under the two conditions. Five putative off-target sites of SOX9-K396R gRNA2 were computationally predicted[6], and the sequences of those sites in gRNA/hCas9-injected mice were analyzed (Fig. 3A). As a result, we did not detect any mutations in the total of 250 loci (five off-target sites in 50 mice) examined (Fig. 3B and Table 3), indicating that both single gRNA with WT hCas9 and double nicking introduced designated genetic modification in highly specific manner. To examine if this high specificity could also be observed with other gRNAs, off-target effect of Sf-1 gRNAs were also analyzed. We focused on the mice which received designated substitution in first 4 experiments (total 26 mice shown in Table 1) and examined total of 121 loci, and did not find any off-target mutation (Supplementary Fig. S3 and

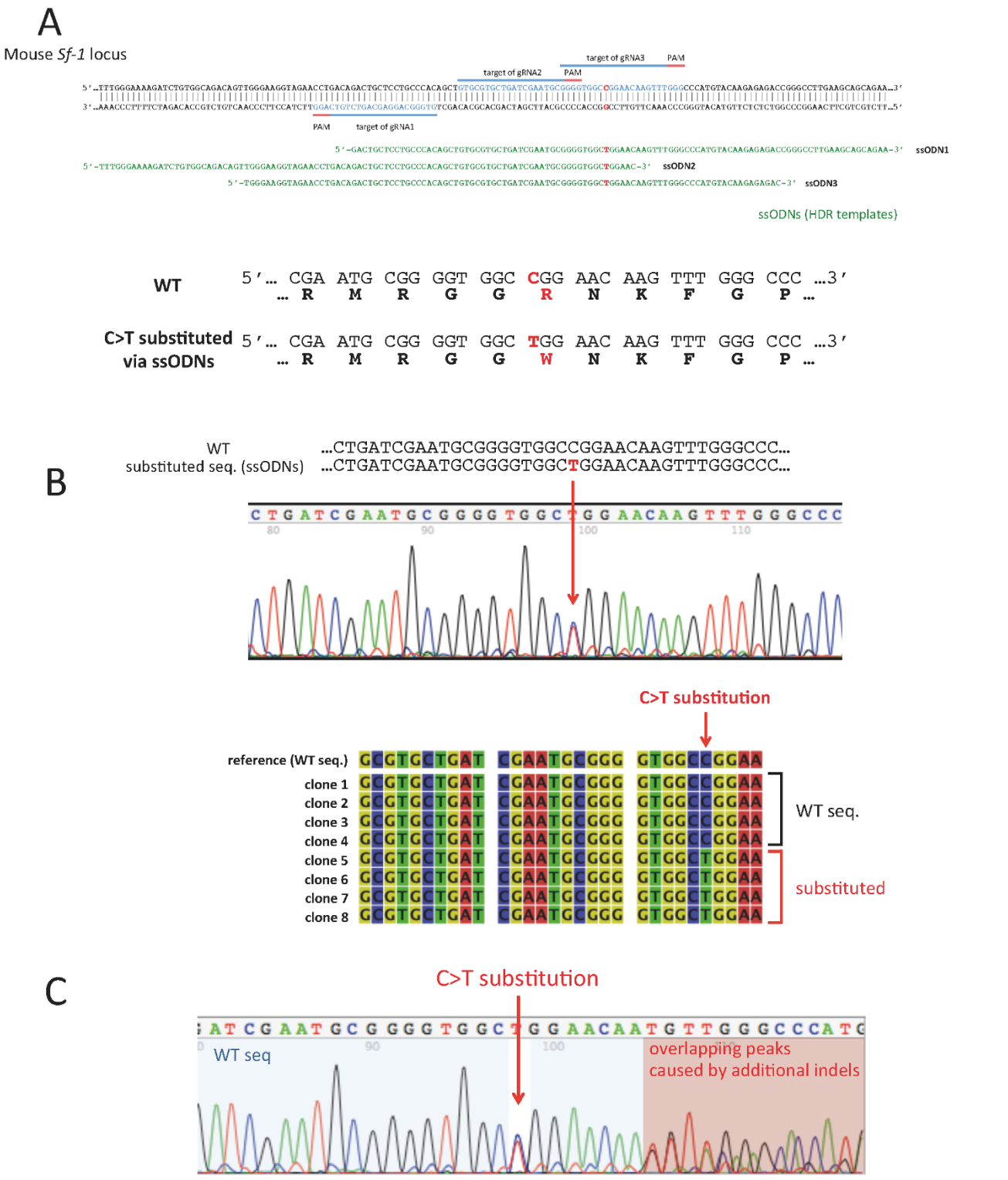


Figure 1 | Introduction of the C>T substitution in *Sf-1* locus. (A) A schematic illustration showing the locations of the gRNAs and ssODNs, along with the mouse *Sf-1* locus. Blue bars and letters indicate the position of the gRNA targets with red bars highlight the PAM sequences. ssODNs are shown in green letters at the corresponding position to the *Sf-1* locus. Red letters indicate the substitution target site in the *Sf-1* locus and corresponding mismatched nucleotides in the ssODNs. The deduced amino acid sequences from wild type (WT) and substituted sequences are shown at the bottom, with red letters indicating the target and the results of the substitution. (B) The results of the sequence analysis of the *Sf-1* locus of a representative mouse, with a monoallelic C>T substitution. The *Sf-1* locus around the gRNA target site was PCR amplified and directly sequenced (Upper panel), or cloned into the plasmid and sequenced independently (Lower panel). The upper panel shows the electropherogram of the direct sequencing results, in which one allele received a C>T substitution. WT and donor DNA sequences are shown on the top, with red letters indicating the mutated nucleotide. The red arrow indicates the overlapping peak caused by the monoallelic substitution. The lower panel shows the sequence alignment of eight independent clones. The eight sequences are separated into two types: the WT sequence and substituted sequences. (C) The results of the direct sequencing of the *Sf-1* locus around the gRNA target site of another representative mouse obtained in experiment 4 shown in Table 1, in which one alleles received C>T substitutions, followed by additional mutation(s) that caused continuous overlapping peaks.

Table 1 | Summary of experiments targeting the *Sf-1* locus

	Microinjection		Survival of zygotes			Result of genotyping				Ratio of genotyping	
Experiment	gRNA	ssODN	2 cell embryo/ injected zygotes	Transferred	Genotyped	Monoallelic substitution (+indel)	Biallelic substitution (+indel)	Indel	WT	Mouse with substituted alleles (%)	Mouse with mutated alleles (%)
1	1	1	128/256	128	39	0 (0)	0 (0)	31	8	0 (0)	31 (79.5)
2	1	2	101/203	101	37	1 (1)	0 (0)	32	4	1 (2.7)	33 (89.2)
3	2	3	124/169	124	51	8 (7)	1 (1)	26	16	9 (17.6)	35 (68.6)
4	3	1	157/217	144	54	10 (7)	6 (5)	28	10	16 (29.6)	44 (81.5)

Supplementary Table S2). Together, these results suggest the ratio of on/off-target mutation is substantially high in this experimental set up.

Discussion

Introducing point mutations is widely used technique in cultured cell based experiments, but uncommon in *in vivo* system partly because of its temporal and technical demands. The standard approach to examine the function of particular nucleotides or amino acids in *in vivo* background has been to create a knock-in or transgenic mouse that carries cDNA or other genetic element with defined point mutation(s). However these techniques require long and laborious procedures such as constructing targeting vectors, cloning of ES cell and/or establishing multiple transgenic mouse lines. Our method overcome those difficulties and possible problems and is ideal to observe the effect of subtle genetic modification under physiological condition.

The CRISPR/Cas9 system has been used to introduce defined genetic modification in cultured cell system[5,9], but whether this could be applied to *in vivo* system and what parameter(s) is influential for its efficiency was less explored. We demonstrated that the CRISPR/Cas9 system, both single gRNA/WT hCas9 and double nicking set-ups, was effective in mouse zygotes with the former having slightly higher efficiency. In our best condition, microinjection of single gRNA with WT hCas9 introduced the point mutation in more than 30% of mice examined, in which 10% of them received biallelic modification, allowing researcher to analyze the mouse model in the founder generation (Note that the possible mosaicism should be considered in analyzing the phenotype of founder generation and we believe that the confirmation in descendant generation is indispensable to conclude genotype-phenotype relationship). We observed higher efficiency with single gRNA with WT hCas9 than double nicking set-up, which is expected because the latter set-up requires two gRNA-hCas9 complexes to be recruited simultaneously to the target site to introduce DSB while one complex is enough for the former. In addition, to inject the same amount of RNAs in total, the concentration of each gRNA and hCas9 mRNA was lower in our double nicking set-up, which might also affect the efficiency (see Methods for the details).

The mutual position of substitution target sites and gRNA target sites (DSB points) seems to affect the substitution efficiency. Using same gRNA and ssODNs with various point mutations, we found that the mutations which are closer to the gRNA target site is incorporated more often than that are farther from DSB point (Supplementary Fig. S1 and Supplementary Table S1). This is in line with the previous report in which the efficiency of gene conversion was examined using plasmid donor DNA and restriction enzyme in cell culture system, and found that the frequency of the substitution declines with the distance from the digestion site[10]. Whether the frequency of substitution varies by using sense (i.e. the same strand of gRNA target) or anti-sense strand sequence for ssODN could be another point to be examined in the future studies.

Surprisingly, we did not observe any off-target effect in our experiments. The total of 371 loci were examined but no indels were detected. This was an unexpected result, since frequent off-target mutagenesis has been reported in human cells[4,5], but is consistent with a rare off-target effect observed in mouse zygotes microinjected with hCas9 and gRNA plasmids[11]. These results do not exclude off-target effects in general, but imply that the cultured cells and mouse zygotes have distinct risks for off-target effects, possibly due to the different stability of the CRISPR/Cas9 components and/or the nuclear/chromatin dynamics between stable cell lines and zygotes under early developmental process. Further accumulation of data is necessary to understand the kinetics of CRISPR mediated genome modification to optimize the experimental parameters for obtaining highest efficiency with minimum off-target effect in various back ground.

In summary, we have established an experimental system to introduce defined point mutations into the mouse genome using the CRISPR/Cas9 system, which enabled us to generate an amino acid substituted mouse model at a low cost and in a short time. The WT hCas9 and double nicking strategy were both effective and the distance between the DSB site and substitution site seems to be an important parameter for determining the substitution efficiency. We believe this technique will be useful to reveal the *in vivo* relevance of particular nucleotides/amino acids identified in *in vitro* systems, as well as to reproduce the genetic variations found in patients of genetic disorders in mouse models.

Methods

Plasmids. hCas9, hCas9 D10A and the gRNA cloning vector[1] were purchased from Addgene (Plasmid ID #41815, #41816 and #41824, respectively). Since the original gRNA cloning vector lacks the partial sequence of the U6 promoter and gRNA scaffold, we first modified it by adding the following sequence: 5′-GTGGAA-AGGACGAAACACCGGCTAGCAGGCCTATCGATGTTTTAGAGCTAGAA-ATAGC-3′ into the *Afl*II site to fill the missing sequence and to facilitate further cloning. gRNA expression vectors were constructed by inverse PCR using this modified vector and the primer pairs shown in the Table below. The PCR products were *Dpn*I digested and used for the transformation of *E. coli* (DH5a). The sequences of the obtained constructs were validated by sequencing.

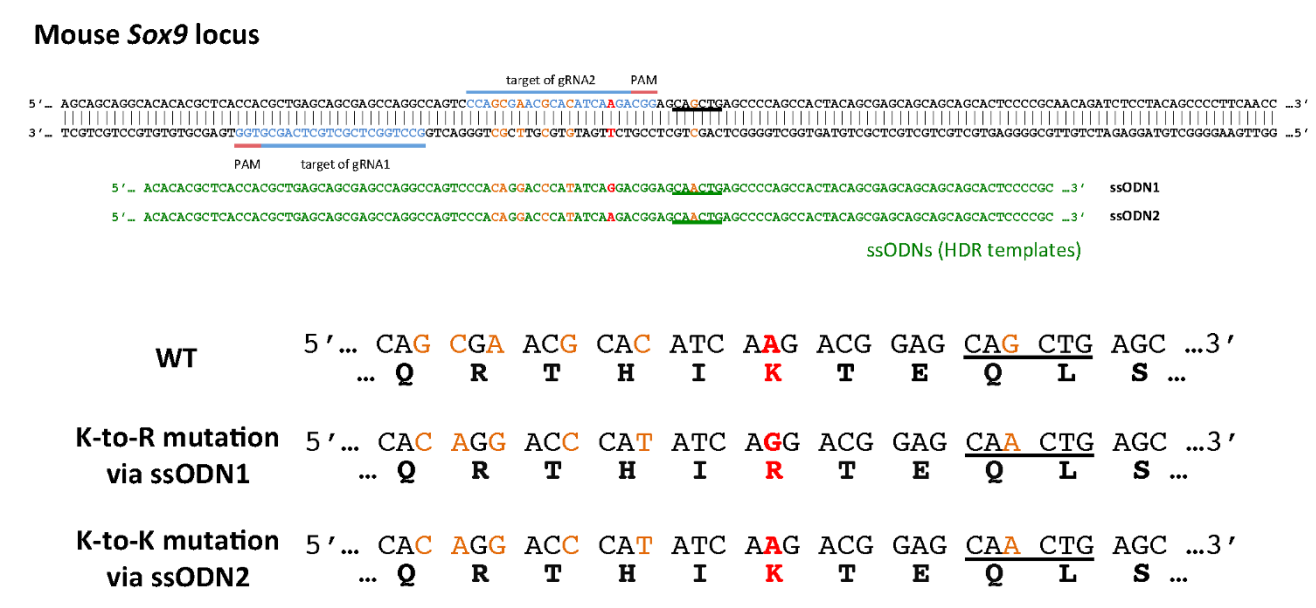
gRNA design. The gRNAs were designed by searching for "GG" or "CC" sequences near the point mutation target sites, and were defined as N(21)GG or the reverse complement sequence of CCN(21). The gRNA pairs used for double-nicking were designed by searching for CCN(40–48)GG sequences, and the pair closest to the substitution site was chosen.

The gRNAs used in this study were: Sf-1 gRNA1 5′-GTGGGCAGGAGC-AGTCTGTCAGG-3′, Sf-1 gRNA2 5′-GTGCGTGCTGATCGAATGCGGGG-3′, Sf-1 gRNA3 5′-GGGGTGGCCGGAACAAGTTTGGG-3′, SOX9-K396R gRNA1 5′-GCCTGGCTCGCTGCTCAGCGTGG-3′, SOX9-K396R gRNA2 5′-CCAGCGAAC-GCACATCAAGACGG-3′.

RNA synthesis. Template DNAs for *in vitro* transcription were generated by PCR amplification of the ORFs of hCas9 and hCas9 D10A, as well as gRNAs (i.e. guide sequences and scaffold sequences) from each plasmid using PrimeSTAR (TaKaRa) and the primer sequences shown below (the T7 RNA polymerase recognition sequence was attached to the 5′ end of the fw primers). The PCR products were purified and used for the *in vitro* RNA synthesis using mMessage mMachine T7 kit (lifetechnologies). The reaction scale was doubled, and the reaction time was extended to over-night to obtain a sufficient amount of gRNA. The synthesized RNAs were purified using an RNeasy mini kit (Qiagen) with an additional ethanol precipitation.

ssODN. The synthesized single stranded oligonucleotides (110 bases, PAGE purified) were purchased from fasmac.

A



WT	5′…	CAG	CGA	ACG	CAC	ATC	AAG	ACG	GAG	CAG	CTG	AGC	…3′
	…	Q	R	T	H	I	K	T	E	Q	L	S	…
K-to-R mutation via ssODN1	5′…	CAC	AGG	ACC	CAT	ATC	AGG	ACG	GAG	CAA	CTG	AGC	…3′
	…	Q	R	T	H	I	R	T	E	Q	L	S	…
K-to-K mutation via ssODN2	5′…	CAC	AGG	ACC	CAT	ATC	AAG	ACG	GAG	CAA	CTG	AGC	…3′
	…	Q	R	T	H	I	K	T	E	Q	L	S	…

B

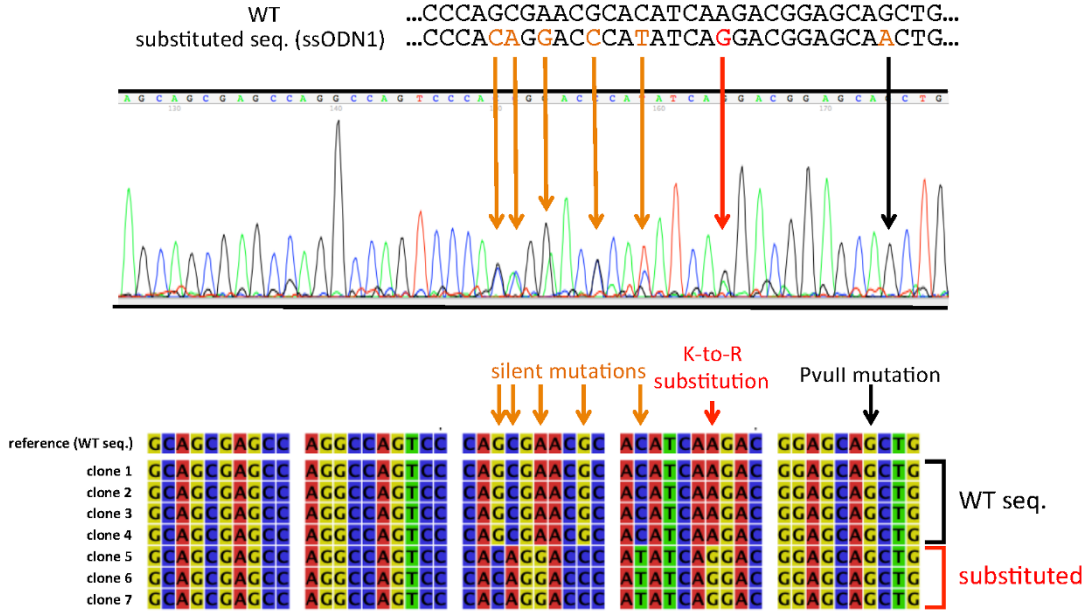


Figure 2 | Introduction of the K396R substitution in *Sox9* locus. (A) A schematic illustration demonstrating the locations of the gRNAs and ssODNs along with the mouse *Sox9* locus. The blue bars and letters indicate the positions of gRNA targets, while red bars highlight the PAM sequences. The sequences of ssODNs are shown in green letters at the corresponding positions to the *Sox9* locus. Red letters indicate the substitution target site in the *Sox9* locus and the corresponding mismatched nucleotides in the ssODNs. The deduced amino acid sequences from wild type (WT) and substituted sequences are shown at the bottom, with red letters indicating the target and the results of the substitution. Orange letters in the sequences are the targets of silent mutations, which do not cause amino acid conversion. The *Pvu*II site is underlined in the *Sox9* loci which had mutated in ssODNs. (B) The results of the sequence analysis of the *Sox9* locus of a representative mouse, which had a monoallelic K-to-R substitution. The *Sox9* locus around the gRNA target site was PCR amplified and directly sequenced (Upper panel), or was cloned into the plasmid and sequenced independently (Lower panel). The upper panel shows the electropherogram of the direct sequencing result of a mouse obtained in experiment 1 shown in Table 2, in which one allele received a K-to-R substitution. The WT and donor DNA sequences are shown on the top, with orange and red letters indicating mutated nucleotides. The orange and red arrows indicate the overlapping peaks caused by monoallelic substitutions, and a black arrow indicates the non-overlapping peak despite the fact that the ssODN (donor DNA) had a mutated nucleotide. The lower panel shows the sequence alignment of seven independent clones. The seven sequences were separated into two types: the WT and substituted sequences.

Table 2 | Summary of experiments targeting the *Sox9* locus

	Microinjection			Survival of zygotes			Result of genotyping				Ratio of genotype	
Experiment	gRNA	hCas9	ssODN	2 cell embryo/ injected zygotes	Transferred	Genotyped	Monoallelic substitution (+indel)	Biallelic substitution (+indel)	Indel	WT	Mouse with substituted alleles (%)	Mouse with mutated alleles (%)
1	1 & 2	D10A	1 & 2	144/188	144	24	5 (0)	0 (0)	1	18	5 (20.8)	6 (25.0)
2	2	WT	1 & 2	116/139	90	26	6 (1)	3 (1)	11	6	9 (34.6)	20 (76.9)

Sf-1 ssODN1 5′-GACTGCTCCTGCCCACAGCTGTGCGTGCTGATCGAATGCGGGGTGGCTGGAACAAGTTTGGGCCCATGTACAAGAGAGACCGGGCCTTGAAGCAGCAGAA-3′, Sf-1 ssODN2 5′-TTTGGGAAAAGATCTGTGGCAGACAGTTGGGAAGGTAGAACCTGACAGACTGCTCCTGCCCACAGCTGTGCGTGCTGATCGAATGCGGGGTGGCTGGAACAAGTTTGGGCCCATGTACAAGAGAGAC-3′, Sf-1 ssODN3 5′-TGGGAAGGTAGAACCTGACAGACTGCTCCTGCCCACAGCTGTGCGTGCTGATCGAATGCGGGGTGGCTGGAACAAGTTTGGGCCCATGTACAAGAGAGAC-3′, Sf-1 ssODN4 5′-GACTGCTCCTGCCCACAGCTGTGCGTGCTGATCGAATGCGGGGTGGCCGGAACAAGTTTGGGCCCATGTATAAGAGAGACCGGGCCTTGAAGCAGCAGAA-3′, Sf-1 ssODN5 5′-GACTGCTCCTGCCCACAGCTGTGTGTGCTGATCGAATGCGGGGTGGCCGGAACAAGTTTGGGCCCATGTACAAGAGAGACCGGGCCTTGAAGCAGCAGAA-3′, Sox9-K396R ssODN1 5′-ACACACGCTCACCACGCTGAGCAGCGAGCCAGGCCAGTCCCACAGGACCCATATCAGGACGGAGCAACTGAGCCCCAGCCACTACAGCGAGCAGCAGCAGCACTCCCCGC-3′, Sox9-K396R ssODN2 5′-ACACACGCTCACCACGCTGAGCAGCGAGCCAGGCCAGTCCCACAGGACCCATATCAAGACGG-

A

	chromosome	position	strand	sequence	# mismatches
Sox9 locus	chr11	112,785,156-112,785,178	+	CCAGCGAACGCACATCAAGACGG	0
OT-1	chrX	78,583,896-78,583,918	+	CCAGAGAACCCACATCAAGAGGG	2
OT-2	chr8	123,777,951-123,777,973	+	CCAGCGAGGGCACACCAAGAAGG	3
OT-3	chr6	15,067,966-15,067,988	+	CCAGCTAAAGCACATCTAGAGAG	3
OT-4	chr15	97,443,330-97,443,352	-	CCAGCGAGCTCACTTCAAGACAG	3
OT-5	chr13	72,438,524-72,438,546	+	CCAGTGAAAGCACATGAAGATGG	3

B

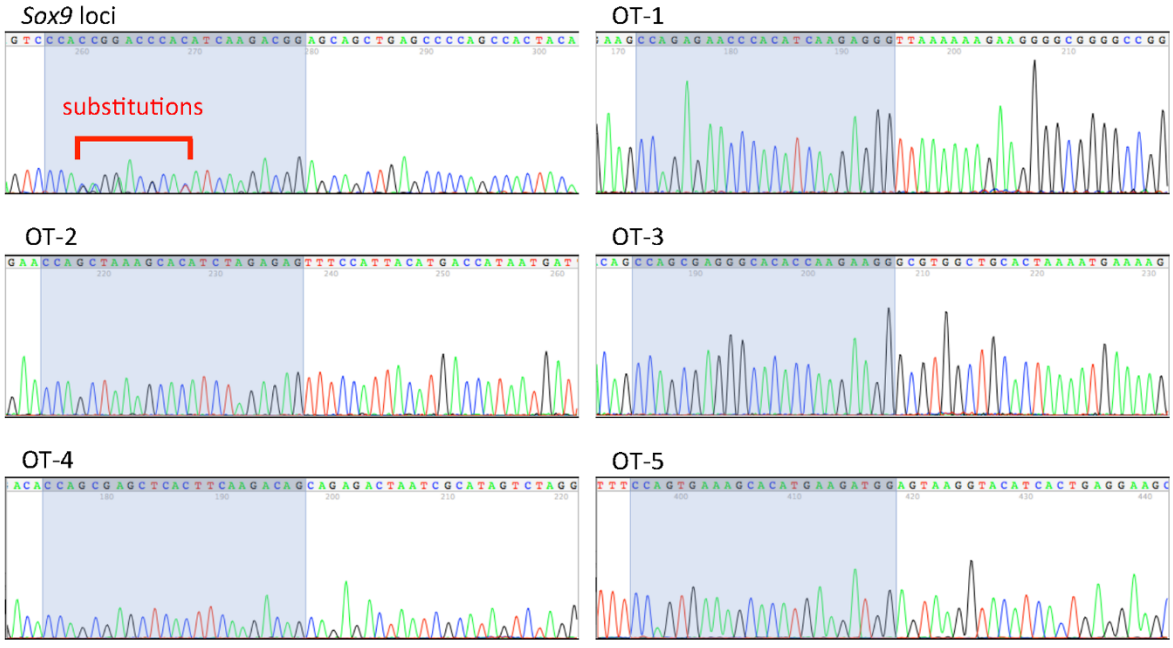


Figure 3 | Off-target analysis of SOX9-K396R gRNAs. (A) The sequences and positions of possible off-target sites for SOX9-K396R gRNA2. OT: Off-target site. (B) The results of the sequence analysis of the target site and off-target sites. Direct sequencing of the PCR-amplified *Sox9* locus and five putative off-target sites from single representative mouse are shown as representative results. Only the sequence of the *Sox9* locus showed overlapping peaks caused by substitution. The gRNA target and possible off-target sequences were shown at the fourth to twenty-sixth bases, as indicated by the darker background color.

Table 3 | Summary of off-target effects by SOX9-K396R gRNAs

Target sites	Mutation rate with WT Cas9 (%)	Mutation rate with double nicking (%)
Sox9 locus	20/26 (76.9)	6/24 (25.0)
OT-1	0/26 (0)	0/24 (0)
OT-2	0/26 (0)	0/24 (0)
OT-3	0/26 (0)	0/24 (0)
OT-4	0/26 (0)	0/24 (0)
OT-5	0/26 (0)	0/24 (0)

OT: Off-target site.
Mutation rate: No. of mice with a mutated allele (including indels and substitutions)/No. of mice genotyped.

AGCAACTGAGCCCCAGCCACTACAGCGAGCAGCAGCAGCACTCCC-CGC-3′.

Microinjection. The microinjection of mouse zygotes was performed as described before[12,13]. Essentially, mouse zygotes were obtained by mating superovulated BDF1 females and WT BDF1 males (Sankyo lab service). RNAs and ssODNs were mixed just before microinjection into the cytoplasm or pro-nuclei of zygotes, and the injected embryos were incubated at 37°C until they were transferred into pseudo-pregnant females at the two-cell stage. The concentration of injected RNAs was always kept at 500 ng/μl in total. For the single gRNA/WT Cas9 condition, gRNA and hCas9 mRNA were mixed at a 1 : 1 ratio, and thus the final concentration was 250 ng/μl each, and for the double nicking condition, the gRNAs and hCas9 mRNA were mixed at 1 : 1 : 1 ratio, and thus a final concentration of 167 ng/μl each. The concentration of injected ssODNs was final 100 ng/μl. The protocols for animal experiments were approved by the Animal Care and Use Committee of the National Research Institute for Child Health and Development (Permit Numbers: A2004-003-C09, A2009-002-C04).

Genotyping. Genomic DNA was extracted from the tail tips of pups, and the genomic sequences around the gRNA target sites were PCR amplified using the primers shown below. The obtained PCR products were treated by ExoSAP-IT (USB) and sequenced directly, or were cloned into the plasmid and sequenced.

The criteria for the "mutation" and "substitution". The genomic sequences around the gRNA target sites were PCR amplified and served for direct sequencing (see above). The electropherograms of each sequence were classified into three categories: wild type (no overlapping peaks), mutated (continuous overlapped peaks, which started from the gRNA target site) and substituted (overlapped peak(s) at the designated position(s)) (Supplementary Fig. S2). The sequences were also aligned with the wild type sequence to check whether there was a homozygous mouse with identical indels on both alleles (which also produce a no overlapping peak pattern). PCR products were cloned into the plasmid, and multiple clones were sequenced to determine the sequences of each allele when necessary.

Off-target analysis. Possible off-target sites for SOX9-K396R gRNA2 and Sf-1 gRNA 1, 2 and 3 were predicted by an online-based tool (http://www.genome-engineering.org/)[6]. Four or five putative off-target sites were found allowing a maximum of three to four bases of mismatches (Fig. 3A and Supplementary Fig. S3). The off-target sites were PCR amplified from the genomic DNA of gRNA-injected mice and analyzed by direct sequencing.

Primers. The primer sequences used in this study are shown below:

For vector construction: gRNA vector adopter fw 5′-GCTAGCAGGCCTATCGATGTTTTAGAGCTAGAAATAGCAAGTTAAAATA-AGGCTAGTCCGTTATC-3′, gRNA vector adopter rev 5′-ATCGATAGGCCTGCTAGCCGGTGTTTCGTCCTTTCCACAAGATATATAA-AGCCAAGAAATCGAAATAC-3′.

For gRNA cloning: SOX9-K396R gRNA1 fw 5′-CCTGGCTCGCTGCTCAGC-GGTTTTAGAGCTAGAAATAGCAAG-3′, SOX9-K396R gRNA1 rev 5′-AACCGCTGAGCAGCGAGCCAGGCGGTGTTTCGTCCTTTCCAC-3′, SOX9-K396R gRNA2 fw 5′-CAGCGAACGCACATCAAGAGTTTTAGAGCTAG-AAATAGCAAG-3′, SOX9-K396R gRNA2 rev 5′-AACTCTTGATGTGCGTTC-GCTGCGGTGTTTCGTCCTTTCCAC-3′, Sf-1-gRNA1 fw 5′-TGGGCAGGA-GCAGTCTGTCGTTTTAGAGCTAGAAATAGCAAG-3′, Sf-1-gRNA1 rev 5′-AACGACAGACTGCTCCTGCCCACGGTGTTTCGTCCTTTCCAC-3′, Sf-1-gRNA2 fw 5′-TGCGTGCTGATCGAATGCGGTTTTAGAGCTAGAAA-TAGCAAG-3′, Sf-1-gRNA2 rev 5′-AACCGCATTCGATCAGCACGCACGG-TGTTTCGTCCTTTCCAC-3′, Sf-1-gRNA3 fw 5′-GGGTGGCCGGAACAA-GTTTGTTTTAGAGCTAGAAATAGCAAG-3′, Sf-1-gRNA3 rev 5′-AACAAAC-TTGTTCCGGCCACCCCGGTGTTTCGTCCTTTCCAC-3′.

For RNA synthesis:

T7-Sf-1-gRNA1 5′-TTAATACGACTCACTATAGGTGGGCAGGAGCAGTCT-GTC-3′, T7-Sf-1-gRNA2 5′-TTAATACGACTCACTATAGGTGCGTGCTGA-TCGAATGCG-3′, T7-Sf-1-gRNA3 5′-TTAATACGACTCACTATAGGG-GGTGGCCGGAACAAGTTT-3′, T7-SOX9-K396R gRNA1 5′-TTAATACGA-CTCACTATAGGCCTGGCTCGCTGCTCAGCG-3′, T7-SOX9-K396R gRNA2 5′-TTAATACGACTCACTATAGGCAGCGAACGCACATCAAGA-3′, gRNA rev template for RNA synthesis 5′-AAAAGCACCGACTCGGTGCC-3′, T7-hCAS9 5′-TAATACGACTCACTATAGGGAGAATGGACAAGAAGTACTCCATTGG-3′, hCAS9-rev 5′-TCACACCTTCCTCTTCTTC-3′.

For genotyping: Sox9 exon 3 fw 5′-ACCAATACTTGCCACCCAAC-3′, Sox9 exon 3 rev 5′-CGGCTGCGTGACTGTAGTAG-3′, Sf-1 exon 4 fw 5′-TGGGGAATG-GTATAAGCGTGTG-3′, Sf-1 exon 4 rev 5′-CGTGCAGGCTAGGGGGTAAC-3′.

For the off-target analysis:

SOX9-K396R gRNA2 OT1 fw 5′-TACACACCCGAGTCCCTTTC-3′, SOX9-K396R gRNA2 OT1 rev 5′-ACCAAACAACACGGCCTTAG-3′, SOX9-K396R gRNA2 OT2 fw 5′-ATCTGACTTGGCGTGGAAAC-3′, SOX9-K396R gRNA2 OT2 rev 5′-GACCAGGACCTGTCGTCAAT-3′, SOX9-K396R gRNA2 OT3 fw 5′-GCC-AAGAGGAGGTAGCAGTG-3′, SOX9-K396R gRNA2 OT3 rev 5′-CACATCCC-CATAGGAAATGG-3′, SOX9-K396R gRNA2 OT4 fw 5′-GTCTCTTGGCCTC-TGCAATC-3′, SOX9-K396R gRNA2 OT4 rev 5′-AGCTCCACCCACAGAG-AGAA-3′, SOX9-K396R gRNA2 OT5 fw 5′-AGACAGAGCTGCTGCAAACA-3′, SOX9-K396R gRNA2 OT5 rev 5′-TGTCAACTACCGCACATGGT-3′, Sf-1 gRNA1 OT1 fw 5′-AGCAGAGAAGCAGGAGCAAG-3′, Sf-1 gRNA1 OT1 rev 5′-CCA-TCCCAAACTCAGCTGTT-3′, Sf-1 gRNA1 OT2 fw 5′-CTTGACTGCTTCTG-GGAAGG-3′, Sf-1 gRNA1 OT2 rev 5′-GCCCACCTGTGCCTTATCTA-3′, Sf-1 gRNA1 OT3 fw 5′-GACCATTGGAGGGCAAACTA-3′, Sf-1 gRNA1 OT3 rev 5′-GGCCAGGCTATAAACACCAA-3′, Sf-1 gRNA1 OT4 fw 5′-AGTGTGAGG-TCCCTGTTTGG-3′, Sf-1 gRNA1 OT4 rev 5′-AGGCTTAGGGATCTGGCATT-3′, Sf-1 gRNA1 OT5 fw 5′-CTTGCCTTTTCTCTGCCATC-3′, Sf-1 gRNA1 OT5 rev 5′-GCAGCCTGAAGGAAATGAAG-3′, Sf-1 gRNA2 OT1 fw 5′-TGGGAGAGT-TCCCTGATTTG-3′, Sf-1 gRNA2 OT1 rev 5′-GTCTCCAAACCCACCAGAAA-3′, Sf-1 gRNA2 OT2 fw 5′-ACTCCCTGGGACACTGTCTG-3′, Sf-1 gRNA2 OT2 rev 5′-TGCAAGAGTCCACACTACGG-3′, Sf-1 gRNA2 OT3 fw 5′-TTCCTAAGT-TGGCTCGCAGT-3′, Sf-1 gRNA2 OT3 rev 5′-CTTGGGTTTTTCATGGGCTA-3′, Sf-1 gRNA2 OT4 fw 5′-GATTAAAGGCGTGTGCCACT-3′, Sf-1 gRNA2 OT4 rev 5′-TCCCAGCCACATTCATGTTA-3′, Sf-1 gRNA3 OT1 fw 5′-GCACCGTCA-AGGAGAAAGAG-3′, Sf-1 gRNA3 OT1 rev 5′-GTTCCCCAGTCCTTCAACAA-3′, Sf-1 gRNA3 OT2 fw 5′-CACCAATTCAGCGACATCAG-3′, Sf-1 gRNA3 OT2 rev 5′-AAAGATGCTTGCACCTTGCT-3′, Sf-1 gRNA3 OT3 fw 5′-TGAGTCCAGC-CTCATCACAG-3′, Sf-1 gRNA3 OT3 rev 5′-CCCTGTCTCCAAACACCCTA-3′, Sf-1 gRNA3 OT4 fw 5′-GCTTGTCATGCTCAGTTCCA-3′, Sf-1 gRNA3 OT4 rev 5′-GACCTTTGGAAGAGCAGTCG-3′, Sf-1 gRNA3 OT5 fw 5′-TCCAGGAGACC-TGTTGCTCT-3′, Sf-1 gRNA3 OT5 rev 5′-AGGCTCCAAGACAGTGTGGT-3′.

Statistical analysis. Statistical analysis to assess the differences of the frequency of mutations and substitutions among experiments 1–4 (Table 1) was performed using the χ^2 test. We hypothesized that the expected frequency (calculated as total number of mice with mutated (or substituted) allele/total number of genotyped mice) is same among 4 experiments and esamined with χ^2 test.

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Author contributions

M. Inui designed the substitution of *Sox9* and M. Igarashi. and M.F. defined the position of *Sf-1* substitution. A.K. constructed gRNA vectors. M.T. performed the microinjection. M. Inui, M.M. and S.Y. carried out the genotyping and the sequence analysis. M. Inui, H.A. and S.T. designed the project and wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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