Ultra-superoovervation for the CRISPR-Cas9-mediated production of gene-knockout, single-amino-acid-substituted, and floxed mice

Yoshiko Nakagawa1,*, Tetsushi Sakuma2,*, Norihisa Nishimichi3, Yasuyuki Yokosaki3,4, Noriyuki Yanaka5, Toru Takeo1, Naomi Nakagata1 and Takashi Yamamoto2

ABSTRACT

Current advances in producing genetically modified mice using genome-editing technologies have indicated the need for improvement of limiting factors including zygote collection for microinjection and their cryopreservation. Recently, we developed a novel superovulation technique using inhibin antiserum and equine chorionic gonadotropin to promote follicle growth. This method enabled the increased production of fertilized oocytes via in vitro fertilization compared with the conventional superovulation method. Here, we verify that the ultra-superoovervation technique can be used for the efficient generation of clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-mediated knockout mice by microinjection of plasmid vector or ribonucleoprotein into zygotes. We also investigated whether single-amino-acid-substituted mice and conditional knockout mice could be generated. Founder mice bearing base substitutions were generated more efficiently by co-microinjection of Cas9 protein, a guide RNA and single-stranded oligodeoxynucleotide (ssODN) than by plasmid microinjection with ssODN. The conditional allele was successfully generated by co-injection of donor DNA harboring homology arms. Using our study presents a useful method for the CRISPR-Cas9-based generation of genetically modified mice from the viewpoints of animal welfare and work efficiency.

KEY WORDS: Knockout, Floxed, CRISPR-Cas9, Zygote, Ultra-superoovervation, Inhibin antiserum and equine chorionic gonadotropin (IASe)

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Reproductive engineering techniques are essential for the efficient production and maintenance of genetically modified (GM) mice.

METHODS & TECHNIQUES

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acid-substituted mice, and floxed mice, using zygotes created by IVF via an ultra-superovulation method (Fig. 1). We generated gene-knockout mice by microinjection of an all-in-one CRISPR-Cas9 plasmid vector into zygotes, and subsequently produced the mice bearing three-base substitutions or floxed alleles by using in vitro transcribed gRNA and Cas9 protein with ssODN. This study reports an efficient method for creating various genome-edited mice using freeze-thawed zygotes created via ultra-superovulation and IVF.

RESULTS
Generation of gene-knockout mice using freeze-thawed fertilized oocytes created by IVF using an ultra-superovulation method
To examine whether freeze-thawed fertilized oocytes created by IVF using an ultra-superovulation method can be applied to the CRISPR-Cas9-mediated generation of knockout mice, the birth and mutation rates of these oocytes injected with CRISPR-Cas9 plasmid vectors were compared with oocytes previously created by IVF using the conventional superovulation method.

First, we generated fertilized oocytes by IVF using sexually matured C57BL/6 female and male mice. Female mice were treated by the ultra-superovulation method using IASe to promote follicle growth and hCG to induce ovulation. Consistent with our previous report, we collected greater numbers of oocytes compared with the conventional superovulation method (Table S1). The fertilized oocytes were cryopreserved as the stock for microinjection. Next, previously constructed and validated CRISPR-Cas9 nuclease vectors or a FokI-dCas9 vector to target the interleukin-11 (Il11) gene (Fig. 2) (Nakagawa et al., 2015) were microinjected into the pronucleus of freeze-thawed fertilized oocytes generated using ultra-superovulated sexually mature mice. Then, the surviving oocytes were transferred to pseudopregnant mice and the resultant pups were obtained. Tail lysates from pups were used for polymerase chain reaction (PCR) to amplify the targeted sequence of Il11. Each PCR product was analyzed by direct sequencing. In the previous report, we investigated the birth rate and the mutation rate of zygotes injected with these vectors. Microinjection of Cas9 nuclease vectors resulted in relatively low birth rate and high mutation rate, whereas microinjection of FokI-dCas9 vector resulted in moderate birth and mutation rates (Nakagawa et al., 2015). The birth and mutation rates of zygotes generated using the ultra-superovulation method were similar to those created by the conventional method (Table 1). To examine birth and mutation rates using zygotes from sexually immature female mice, from which greater numbers of fertilized oocytes can be collected compared with mature females, we performed microinjection and transfer using the same method. Although we confirmed that gene-knockout mice could also be created using ultra-superovulated sexually immature female mice, the birth rate was lower than using oocytes from mature females (Table 1). To decrease the toxicity, we subsequently tested microinjection of Cas9 protein and synthesized gRNA_B instead of the plasmid vector (Nuclease_B) into the zygotes generated from immature female mice. This alteration resulted in the successful production of mutant pups at the Nuclease_B locus, although no pups were born by plasmid microinjection, suggesting that the birth rate of genome-edited mice can be improved by replacing CRISPR-Cas9 vectors with Cas9 ribonucleoproteins (RNPs) (Table 1). Collectively, our ultra-superovulation technique was confirmed to be applicable to the production of knockout mice using genome-editing technology.

Generation of single-amino-acid-substituted mice
To investigate further whether gene knock-in mice can be generated using freeze-thawed zygotes created via ultra-superovulation and IVF, as well as the production of gene-knockout mice, we aimed to generate founder mice harboring a three-base substitution in the secreted phosphoprotein 1 (Spp1) gene. The three-base substitution in Spp1 gene was designed to encode thrombin cleavage-
incompetent mutant osteopontin protein (Nishimichi et al., 2011). To knock-in these substitutions precisely, we designed a gRNA on the target locus as well as the ssODN carrying the intended substitutions (Fig. 3A).

We initially tested the microinjection of CRISPR-Cas9 nuclease vector and ssODN, followed by single blastocyst assays for the validation of gene knock-in. The survivingzygotes were cultured for 3.5 days, and restriction fragment length polymorphism (RFLP) and direct sequencing analyses were conducted. We confirmed correct three-base substitution without any insertion and deletion (indel) mutations in one blastocyst (Fig. S1, Table S2). To generate the founder mice, we subsequently performed microinjection of CRISPR-Cas9 nuclease vector and ssODN, and then survivingzygotes were transferred to pseudopregnant mice. We used the oocytes collected from immature or mature female mice treated with ultra-superovulation for IVF. The birth rates were low and no pups with the three-base substitution were detected by RFLP analysis and direct sequencing analyses were conducted. We confirmed knock-in of the three-base substitution in three pups by RFLP analysis and direct sequencing of PCR amplicons. Together with the knock-in rate were significantly improved (Table 2). The precise integration of only one loxP sequence and/or integration with indel mutations, as well as the difficulty of performing single blastocyst assays because of the small amount of template genome available. To ensure the exact genotype, the six PCR1 products from blastocysts #1, #11, #14, #19, #21, and #27 were analyzed by direct sequencing. Consequently, we confirmed the nearly correct integration of ssODN in one blastocyst (Fig. S4), correct integration of the right loxP in four blastocysts (#11, #14, #21, and #27) and nearly correct integration of the right loxP in one blastocyst (#1). No loxP insertion or indel mutation was observed at the left target site in the six blastocysts except for #19, suggesting that the left gRNA has a lower cleavage activity compared with the right gRNA.

### Generation of floxed mice

Based on the results of Il11 and Spp1 targeting, we chose microinjection of Cas9 RNP instead of CRISPR-Cas9 vector to generate the founder mice. According to the validation using blastocysts, we increased the concentration of the left gRNA compared with that of the right gRNA to balance the different cleavage activity of these two gRNAs. We microinjected the left and right gRNAs together with the Cas9 protein and ssODN into zygotes generated via the ultra-superovulation method, under several concentration conditions. We then transferred the survivingzygotes to pseudopregnant mice. As summarized in Table 3, higher concentrations of ssODN resulted in a reduction of the birth rate. Tail lysates of all pups were analyzed using the PCRs and XmnI-RFLP (Figs S2B, S3A). In PCR3 and XmnI-RFLP, however, four (#11, #14, #25, and #27) and six (#1, #11, #14, #19, #21, and #27) blastocysts were estimated to be positive, respectively (Fig. S2C, Fig. S3B). These discordances might be caused by various patterns of partial integrations, such as the integration of only one loxP sequence and/or integration with indel mutations, as well as the difficulty of performing single blastocyst assays because of the small amount of template genome available. To ensure the exact genotype, the six PCR1 products from blastocysts #1, #11, #14, #19, #21, and #27 were analyzed by direct sequencing. Consequently, we confirmed the nearly correct integration of ssODN in one blastocyst (Fig. S4), correct integration of the right loxP in four blastocysts (#11, #14, #21, and #27) and nearly correct integration of the right loxP in one blastocyst (#1). No loxP insertion or indel mutation was observed at the left target site in the six blastocysts except for #19, suggesting that the left gRNA has a lower cleavage activity compared with the right gRNA.

### Design and validation of two loxP insertions

We designed a novel strategy for the one-step generation of floxed mice by utilizing double-cut CRISPR-Cas9 and a single ssODN donor. To perform a proof-of-concept experiment of this strategy, we constructed an all-in-one CRISPR-Cas9 vector expressing two gRNAs to target the both regions outside a 55-bp exon in Il11 (Okazaki et al., 2010) (Fig. 4A). The ssODN was used for targeting. By using Cas9 RNPs, both the birth rate and the knock-in rate were significantly improved (Table 2). The precise knock-in of the three-base substitution was confirmed in three pups by RFLP analysis and direct sequencing of PCR amplicons (Table 2, Fig. 3B). In six pups, the three-base substitution was detected with indel mutations (Table 2, Fig. 3C). Together with the Il11 results, we confirmed increased birth rate and knockout/knock-in rates by using Cas9 RNPs.

| Superovulation Method | Age in weeks | Reagent | Injected | Survived (%) | Transferred | Pups (%) | Mutants (%) |
|-----------------------|--------------|---------|----------|--------------|-------------|----------|-------------|
| Conventional method*  | 8–12         | 5 ng/µl nuclease_A plasmid | 32       | 25 (78.1) | 25          | 3 (12.0) | 3 (100)     |
|                       |              | 5 ng/µl nuclease_B plasmid | 34       | 22 (64.7) | 22          | 2 (9.1)  | 2 (100)     |
| Ultra-superoovulation | 10–13        | 5 ng/µl FokI-dCas9 plasmid | 39       | 35 (89.7) | 34          | 6 (17.6) | 2 (33.3)    |
| method (IASe-hCG)    | 4–5          | 5 ng/µl nuclease_A plasmid | 90       | 80 (88.9) | 40          | 4 (10.0) | 2 (50.0)    |
|                       |              | 5 ng/µl nuclease_B plasmid | 133      | 127 (95.6) | 85          | 6 (7.1)  | 5 (83.3)    |
|                       |              | 5 ng/µl FokI-dCas9 plasmid | 49       | 43 (87.8) | 43          | 7 (16.3) | 5 (71.4)    |
|                       |              | 5 ng/µl nuclease_A plasmid | 112      | 104 (92.8) | 53          | 3 (5.7)  | 3 (100)     |
|                       |              | 5 ng/µl nuclease_B plasmid | 64       | 62 (96.9) | 62          | 0 (0)    | –           |
|                       |              | 5 ng/µl FokI-dCas9 plasmid | 68       | 63 (92.6) | 49          | 2 (4.1)  | 1 (50.0)    |
|                       |              | 1 µM Cas9 protein and 40 ng/µl gRNA_B | 42       | 38 (90.5) | 38          | 4 (10.5) | 4 (100)     |

*Results using the conventional method were from a previous report (Nakagawa et al., 2015).

Table 1. Generation of Il11 mutants using freeze-thawed fertilized oocytes created via IVF

[Table 1 image]

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analyses, while they were negative for both the PCR2 and PsiI-RFLP analyses (four pups) or for the PsiI-RFLP analysis (one pup). Among 37 pups analyzed, three pups, including two injected with 1.5 µM and one pup with 1 µM Cas9, were positive for all analyses, which were identified as potential floxed mice (Table 3). The PCR products from these pups were cloned and analyzed by DNA sequencing. Of these, one pup was identified with a precisely floxed allele, and the other two pups carried nearly correct integrations (Fig. 4B).

DISCUSSION
We have previously shown that reproductive engineering techniques were useful for creating knockout mice using a genome-editing technique (Nakagawa et al., 2014, 2015). In this study, we showed that approximately two and three times the number of fertilized oocytes could be generated from sexually matured and immature C57BL/6 female mice, respectively, by IVF using our ultra-supervoluation method compared with a conventional supervoluation method, consistent with a previous report (Takeo and Nakagata, 2015). To clarify its applicability in the generation of genome-edited mice, we demonstrated the successful production of gene-disrupted, single-amino-acid-substituted, and floxed mice by microinjecting the CRISPR-Cas9 plasmid vectors or RNPs with or without ssODN donors.

We initially aimed to generate various genome-edited mice by microinjecting an all-in-one CRISPR-Cas9 plasmid vector in the C57BL/6 strain because of its simple handling. However, the birth rates of Cas9 nuclease plasmid-injected zygotes were low, similar to other reports using inbred strains (Li et al., 2013; Horii et al., 2014), when compared with B6D2F1 hybrid mice (Mashiko et al., 2013). To solve this problem, we demonstrated that the use of Cas9 protein and synthesized gRNA boosted the birth rate, leading to the successful generation of various genome-edited mice.

Recent achievements using a long double-stranded plasmid donor together with CRISPR-Cas9 enabled the one-step generation of floxed mice (Lee and Lloyd, 2014). In our study, a long ssODN was...
used instead of the plasmid donor to avoid the labor of donor construction and lower the risk of random integration, and the successful generation of the intended mouse was demonstrated. Unfortunately, three pups containing precise or slightly mutated floxed alleles died within about 2 weeks after birth. This might be explained because they were mosaic founders containing the conditional allele with some other alleles, such as alleles harboring short indel mutations and exon-skipped alleles, which might be

Table 2. Generation of Spp1-modified mice

| Age in weeks | CRISPR-Cas9 | ssODN | Injected | Survived (%) | Transferred | Pups (%) | Precise knock-in | Knock-in with indel mutation |
|--------------|-------------|-------|----------|--------------|-------------|----------|-----------------|-----------------------------|
| 4–5          | 3 ng/µl plasmid | 10 ng/µl | 76 | 70 (92.1) | 70 | 2 (2.9) | 0 | 0 |
| 11           | 3 ng/µl plasmid | 10 ng/µl | 117 | 112 (95.7) | 112 | 0 (0) | – | – |
| 5            | 0.5 µM Cas9 protein and 20 ng/µl gRNA | 10 ng/µl | 160 | 151 (94.4) | 113 | 8 (7.1) | 1 | 2 |
|              | 0.75 µM Cas9 protein and 30 ng/µl gRNA | 10 ng/µl | 78 | 69 (88.5) | 69 | 8 (11.6) | 1 | 1 |
| 10           | 0.5 µM Cas9 protein and 20 ng/µl gRNA | 10 ng/µl | 155 | 148 (95.5) | 111 | 13 (11.7) | 0 | 3 |
|              | 0.75 µM Cas9 protein and 30 ng/µl gRNA | 10 ng/µl | 41 | 39 (95.1) | 39 | 5 (12.8) | 1 | 0 |

Fertilized oocytes were created by IVF using the ultra-supersovulation method (IASe-hCG).

Fig. 4. Generation of floxed mice at the Gpcpd1 locus. (A) Schematic illustration to generate a conditional allele at the Gpcpd1 locus. A 55-bp exon was targeted by two gRNA, which were designed to cut both ends of the exon. A single ssODN was designed to carry the two loxP sequences. Bold arrows indicate primers for PCR. Thin arrows indicate the recognition sites of restriction enzymes for the RFLP analysis. (B) Sequencing analysis of subcloned PCR1 products from three pups that were positive for all analyses. The intended floxed allele is shown at the top (Floxed) with loxP sequences (enclosed in black boxes). The wild-type allele is shown at the bottom. Dots indicate the same bases as the floxed sequence.
Table 3. Generation of floxed mice

| Age in weeks | Cas9 protein | gRNA | ssODN | Injected | Survived (%) | Transferred | Pups (%) | Pups positive for all analyses |
|--------------|--------------|------|-------|----------|--------------|-------------|---------|-------------------------------|
| 10–11        | 2 µM         | L: 50 ng/µl | 20 ng/µl | 108 | 103 (95.4) | 103 | 4 (3.9) | 0 |
|              |              | R: 30 ng/µl | 10 ng/µl | 68 | 66 (97.1) | 49 | 5 (10.2) | 0 |
|              | 1.5 µM       | L: 40 ng/µl | 20 ng/µl | 78 | 75 (96.2) | 75 | 4 (5.3) | (nearly floxed) |
|              |              | R: 20 ng/µl | 10 ng/µl | 77 | 75 (97.4) | 75 | 9 (12.0) | (precisely floxed) |
|              | 1 µM         | L: 25 ng/µl | 20 ng/µl | 74 | 74 (100) | 74 | 4 (5.4) | 0 |
|              |              | R: 15 ng/µl | 15 ng/µl | 40 | 39 (97.5) | 39 | 3 (7.7) | 0 |
|              |              | 10 ng/µl | 74 | 70 (94.6) | 70 | 8 (11.4) | (nearly floxed) |

Fertilized oocytes were created by IVF using the ultra-superovalulation method (IASe-hCG).

Microinjection
Each CRISPR-Cas9-plasmid DNA was diluted in DNase-free PBS, and then injected into the pronucleus of fertilized oocytes. For the microinjection of RNPs, in vitro transcribed gRNA(s) and Cas9 protein were mixed with or without ssODN in 0.1 TE buffer (Aida et al., 2015). The concentration of plasmid DNA, ssODN, and RNP is described in Tables 1, 2, and 3. Single blastocyst assay to validate the generation of the floxed mice. The injected oocytes were cultured in potassium simplex optimized medium with amino acids (KSOM-AA), prepared as previously described (Lawitts and Biggers, 1993), at 37°C in 5% CO2 and 95% humidified air for about 1 h. Surviving oocytes were transferred to the oviducts of pseudopregnant ICR female mice.

Single blastocyst assay
The single blastocyst assay was performed according to a previous report (Nakagawa et al., 2015). To detect the targeted Sppl gene, PCR was performed using KOD FX (Toyobo, Osaka, Japan) with the Sppl F and R primers listed in Table S3 under the following conditions: 95°C for 1 min, followed by 38 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 30 s. Each PCR product was subjected to automatic electrophoresis using MultiNA (Shimadzu Corporation, Kyoto, Japan). The PCR products were purified and analyzed by RFLP analyses using BsmFI (New England Biolabs Japan), then the PCR products identified as positive were analyzed by direct sequencing using an ABI 3130 Genetic Analyzer (Life Technologies). To detect the targeted Gpcpd1 gene, PCR was performed with the F and R primers listed in Table S3 under the following conditions: 95°C for 1 min, followed by 39 cycles of 98°C for 10 s, 60°C for 30 s, 68°C for 45 s. PCR2 or PCR3 were performed with the F and L-lox primers or R-lox and R primers listed in Table S3 under the following conditions: 95°C for 1 min, followed by 39 cycles of 98°C for 10 s, 57°C for 30 s, and 68°C for 25 s. Each PCR product was subjected to automatic electrophoresis using MultiNA. The PCR1 products were purified and analyzed by RFLP analyses using PsiI (New England Biolabs Japan) or Xmnl (New England Biolabs Japan), then the PCR1 products identified as positive in all analyses were analyzed by direct sequencing.

Analysis of pups
Tail lysates of pups were prepared by an alkaline lysis method and PCR was performed using KOD FX with each primer sets. For the analysis of Il11 mutants, the IL11 F and R primers listed in Table S3 were used according to a previous report (Nakagawa et al., 2015). Each PCR product was analyzed by direct sequencing. For the analysis of Sppl gene, PCR was carried out...
with 37 cycles, and then the products were analyzed as described in the same way as the single blastocyst assay. For the analysis of Gpca1 gene, three kinds of PCR were carried out with 37 cycles, and then the products were analyzed as described in the same way as the single blastocyst assay. The PCR1 products harboring floxed mutations were subcloned into a pTA2 vector using Target Clone -Plus- (Toyobo). Each subcloned vector was PCR1 products harboring floxed mutations were subcloned into a pTA2 vector using Target Clone -Plus- (Toyobo). Each subcloned vector was analyzed as described in the same way as the single blastocyst assay. The

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Y.N. designed the study, carried out the mouse experiments and analyses, and wrote the manuscript. T.S. designed the study, constructed CRISPR-Cas9 vectors, synthesized gRNAs, and wrote the manuscript. N.Ni., Y.Y., N.Y., and T.T. provided instructions. N.Na. and T.Y. supervised the work. All authors read and approved the manuscript.

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References
Aida, T., Chiy, K., Usami, T., Ishikubo, H., Imashiki, R., Wada, Y., Tanaka, K. F., Sakuma, T., Yamamoto, T. and Tanaka, K. (2015). Cloning-free CRISPR-Cas9 system facilitates functional cassette knock-in in mice. Genome Biol. 16, 87.
Chu, V. T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K. and Kühn, R. (2015). Increasing the efficiency of homology-directed repair for CRISPR/Cas9-induced precise gene editing in mammalian cells. Nat. Biotechnol. 33, 543-546.
Han, Y., Silvano, O. J., Christie, C. K., Cheng, A. W. and Miano, J. M. (2015). CRISPR-Cas9 genome editing of a single regulatory element nearly abolishes target gene expression in mice—brief report. Arterioscler. Thromb. Vasc. Biol. 35, 312-315.
Hashimoto, M. and Takemoto, T. (2015). Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing. Sci. Rep. 5, 11135, Erratum in: Sci. Rep. 5, 12685.
Ho, Y., Wigglesworth, K., Eppig, J. J. and Schultz, R. M. (1995). Preimplantation development of mouse embryos in KSMO: augmentation by amino acids and analysis of gene expression. Mol. Reprod. Dev. 41, 232-238.
Horii, T., Arai, Y., Yamazaki, M., Morita, S., Horii, T., Arai, Y., Yamazaki, M., Morita, S., Kimura, M., Itoh, M., Abe, Y. and Hatada, I. (2014). Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. Sci. Rep. 4, 4513.
Inui, M., Miyado, M., Igarashi, M., Tamano, M., Kubo, A., Yamashita, S., Nakamura, Y., Matsunaga, H., Tsuichiyama, S., Ishizuka, Y. and et al. (2013). Applications of cryopreserved unfertilized mouse oocytes for in vitro fertilization. Cryobiology 67, 188-192.
Nakagawa, Y., Sakuma, T., Nakagata, N., Yamashita, S., Takeda, N., Ohmuraya, M. and Yamamoto, T. (2014). Application of oocyte cryopreservation technology in TALEN-mediated mouse genome editing. Exp. Anim. 63, 349-355.
Nakagawa, Y., Sakuma, T., Nakagata, N., Ohmuraya, M. and Yamamoto, T. (2015). Production of knockout mice by DNA micronjection of various CRISPR/Cas9 vectors into freeze-thawed fertilized oocytes. BMC Biotechnol. 15, 33.
Nakao, K., Nakagata, N. and Katsuki, M. (1997). Simple and efficient vitrification procedure for cryopreservation of mouse embryos. Exp. Anim. 46, 231-234.
Nakao, H., Harada, T., Nakao, K., Kiyonari, H., Inoue, K., Furuta, Y. and Aiba, A. (2016). A possible aid in targeted insertion of large DNA elements by CRISPR/Cas in mouse zygotes. Genetics 6, 5-77.
Nishishita, N., Hayashita-Kushiyama, H., Chen, C., Matsuda, H., Sheppard, D. and Yokosaki, Y. (2011). Osteopontin undergoes polymerization in vivo and gains chemotactic activity for neutrophils mediated by integrin alpha1beta1. J. Biol. Chem. 286, 11170-11178.
Okazaki, Y., Ohshima, N., Yoshizawa, I., Kamei, Y., Marigött, S., Okamoto, K., Maeda, M., Nogusa, Y., Fujikyo, Y., Izumi, T. et al. (2010). A novel glycoporphosphodiester phosphodiesterase, GDE5, controls skeletal muscle development via a non-enzymatic mechanism. J. Biol. Chem. 285, 27652-27663.
Qin, W., Dion, S. L., Kutny, P. M., Zhang, Y., Cheng, A. W., Willette, N. L., Malhotra, A., Geurts, A. M., Chen, Y.-G. and Wang, H. (2015). Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of oligonucleotides with TALEN and CRISPR/Cas9 nucleases. Cell Rep. 14, 2293-2272.
Sakuma, T., Nishikawa, A., Kume, S., Chayama, K. and Yamamoto, T. (2014). Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system. Sci. Rep. 4, 5400.
Sakurai, T., Kamiyoshi, A., Kawate, H., Mori, C., Watanabe, S., Tanaka, M., Uetake, R., Sato, M. and Shindo, T. (2016). A non-inheritable maternal Cas9-based multiple-gene-editing system. Sci. Rep. 6, 20011.
Singh, P., Schiment, J. C. and Bolcun-Filas, E. (2015). A mouse geneticist’s practical guide to CRISPR applications. Genetics 199, 1-15.
Song, J., Yang, D., Xu, J., Zhu, T., Chen, Y. E. and Zhang, J. (2016). RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. Nat. Commun. 7, 10548.
Takeo, T. and Nakagata, N. (2015). Superoovulation using the combined administration of inhibin antisera and equine chorionic gonadotropin increases the number of ovulated oocytes in C57BL/6J female mice. PLoS ONE 10, e0123300.
Tsuchiya, K., Minami, Y., Umemura, Y., Watanabe, H., Ono, D., Nakamura, T., Takahashi, T., Honma, S., Kondoh, G. and Matsuhi, T. et al. (2015). Disruption of MeCP2 attenuates circadian rhythm in CRISPR/Cas9-based Rett syndrome model mouse. Genes Cells 20, 992-1005.
Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F. and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910-920.
Wang, L., Shao, Y., Guan, Y., Li, L., Wu, L., Chen, F., Liu, M., Chen, H., Ma, Y., X. et al. (2015). Large genomic fragment deletion and functional gene cassette knock-in via Cas9 protein mediated genome editing in one-cell rodent embryos. Sci. Rep. 5, 17517.
Yang, H., Wang, H., Shivalila, C. S., Cheng, A. W., Shi, L. and Jaenisch, R. (2013). One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154, 1370-1379.
Yoshimi, K., Kunihiro, Y., Kaneko, T., Nagahara, H., Voigt, B. and Mashimo, T. (2016). ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. Nat. Commun. 7, 10431.