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

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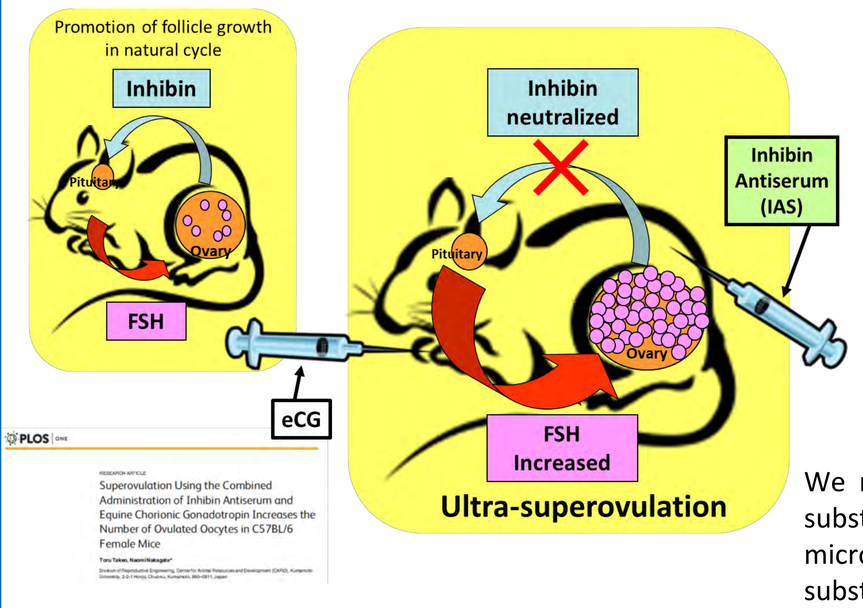
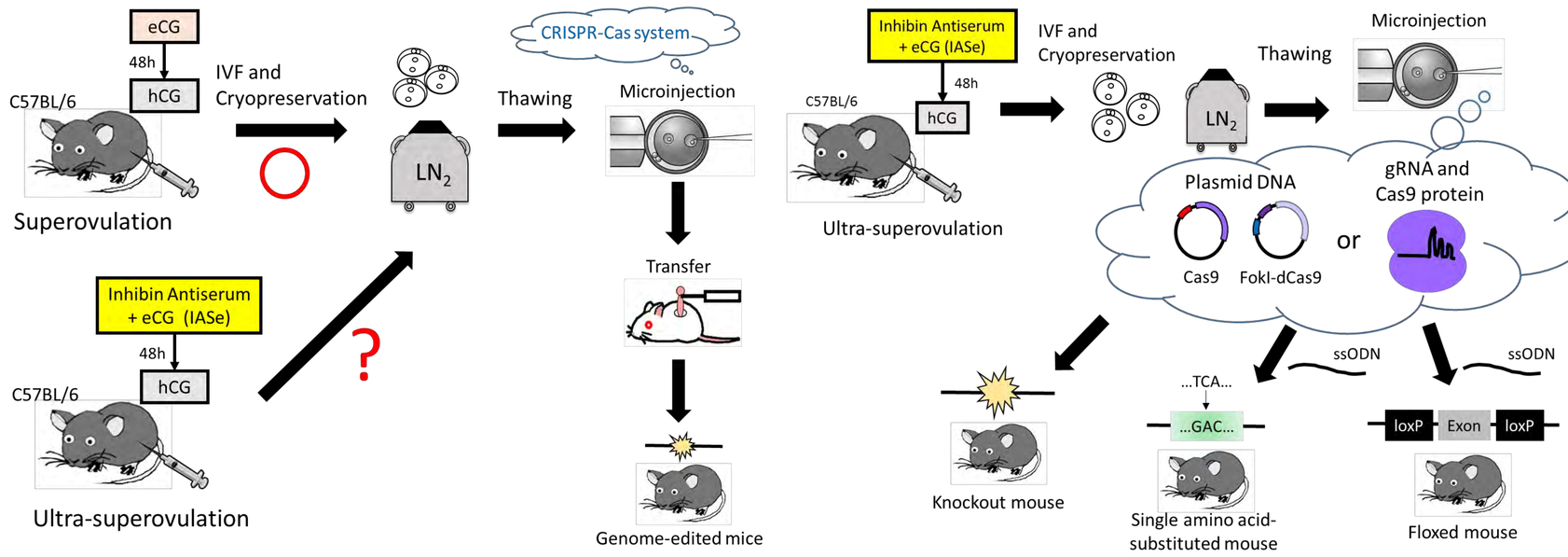
Introduction

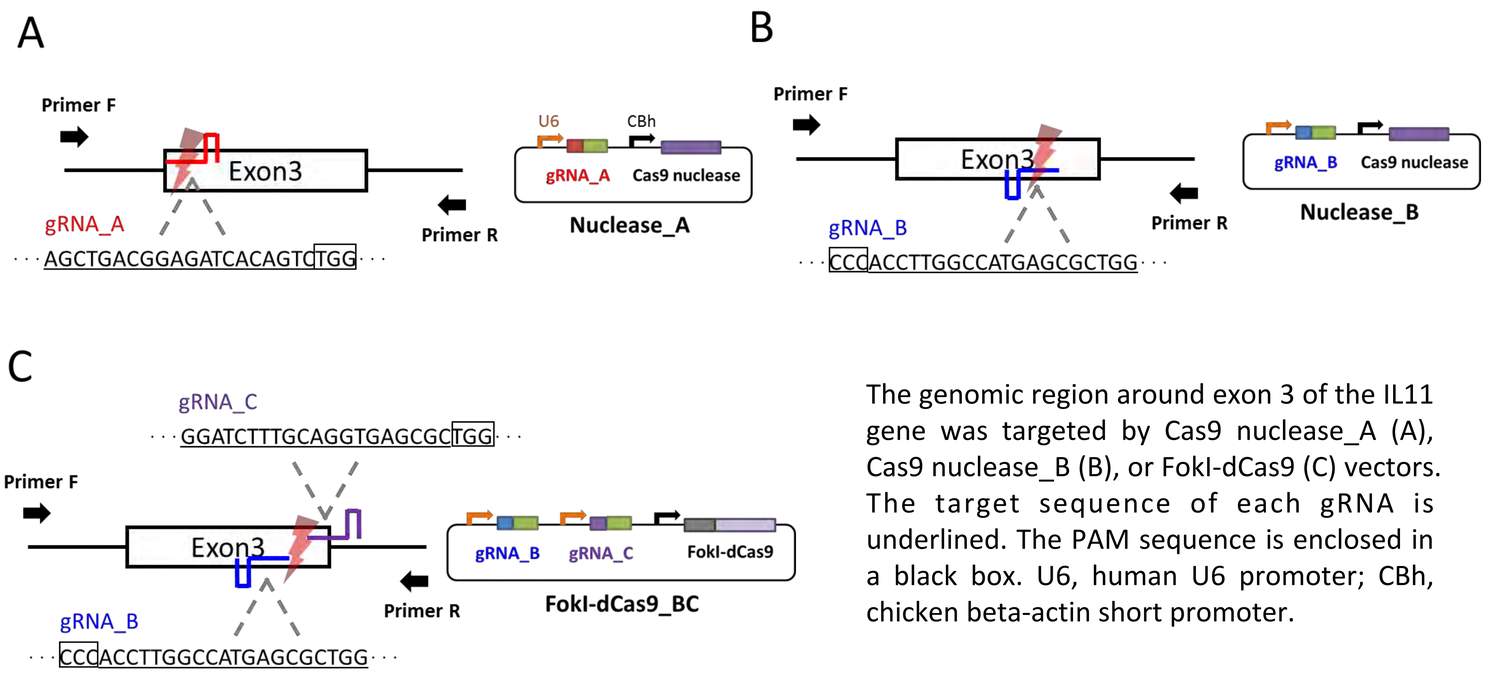
Ultra-superovulation using IAS and eCG (IASe)





We report the CRISPR-Cas9-mediated generation of various genetically modified mice, such as gene-disrupted mice, single-amino-acid-substituted mice, and floxed mice, using zygotes created by IVF via an ultra-superovulation method. 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.

Generation of knockout mice at the IL11 locus

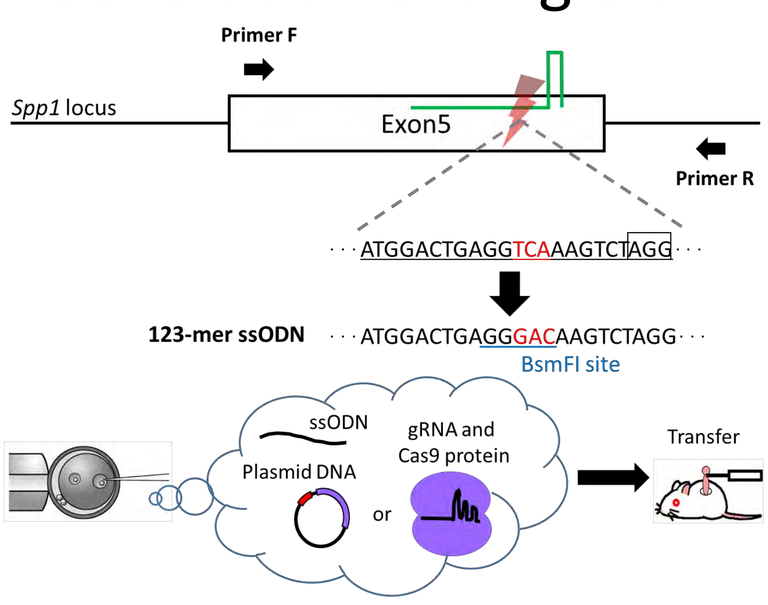


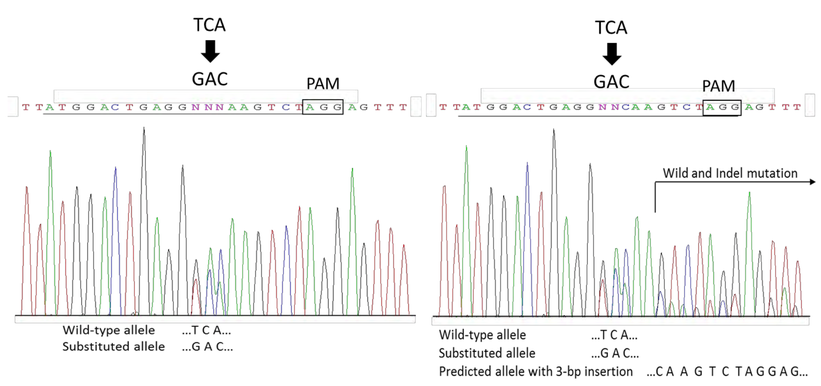
The genomic region around exon 3 of the IL11 gene was targeted by Cas9 nuclease_A (A), Cas9 nuclease_B (B), or FokI-dCas9 (C) vectors. The target sequence of each gRNA is underlined. The PAM sequence is enclosed in a black box. U6, human U6 promoter; CBh, chicken beta-actin short promoter.

Superovulation	Age in weeks	Reagent	Injected	Survived (%)	Transferred	Pups (%)	Mutants (%)
Conventional method* (eCG-hCG)	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)
		5 ng/μl FokI-dCas9 plasmid	39	35 (89.7)	34	6 (17.6)	2 (33.3)
Ultra-superovulation method (IASe-hCG)	10–13	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 (95.5)	85	6 (7.1)	5 (83.3)
		5 ng/μl FokI-dCas9 plasmid	49	43 (87.8)	43	7 (16.3)	5 (71.4)
	4–5	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)
	4	1 μM Cas9 protein and 40 ng/μl gRNA_B	42	38 (90.5)	38	4 (10.5)	4 (100)

*The results using the conventional method were quoted from the previous report (Nakagawa et al., 2015).

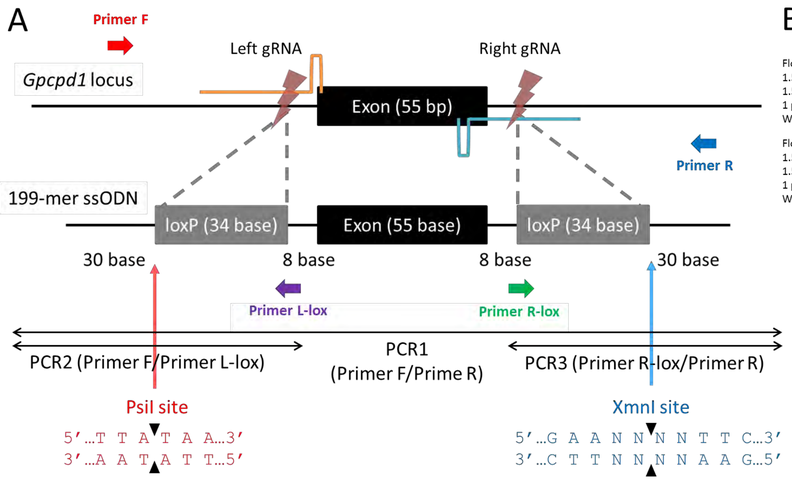
Generation of single-amino-acid-substituted mice at the Spp1 locus

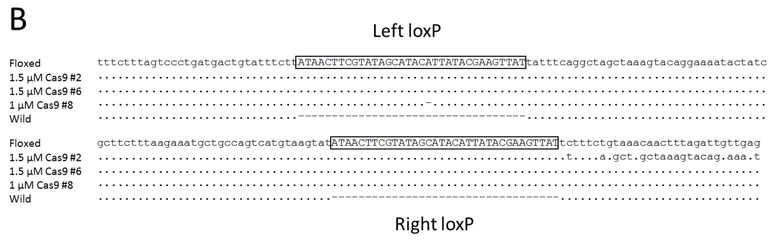




Superovulation	Age in weeks	Reagent	Injected	Survived (%)	Transferred	Pups (%)	Precise knock-in	Knock-in with indel mutation
Ultra-superovulation method (IASe-hCG)	4–5	3 ng/μl plasmid and 10 ng/μl ssODN	76	70 (92.1)	70	2 (2.9)	0	0
		5 ng/μl plasmid and 10 ng/μl ssODN	117	112 (95.7)	112	0 (0)	–	–
	11	3 ng/μl plasmid and 10 ng/μl ssODN	34	33 (97.1)	33	1 (3.0)	0	0
		5 ng/μl plasmid and 10 ng/μl ssODN	35	31 (88.6)	31	1 (3.2)	0	0
	5	0.5 μM Cas9 protein, 20 ng/μl gRNA, and 10 ng/μl ssODN	160	151 (94.4)	113	8 (7.1)	1	2
		0.75 μM Cas9 protein, 30 ng/μl gRNA, and 10 ng/μl ssODN	78	69 (88.5)	69	8 (11.6)	1	1
	10	0.5 μM Cas9 protein, 20 ng/μl gRNA, and 10 ng/μl ssODN	155	148 (95.5)	111	13 (11.7)	0	3
		0.75 μM Cas9 protein, 30 ng/μl gRNA, and 10 ng/μl ssODN	41	39 (95.1)	39	5 (12.8)	1	0

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.

Cas9 protein	gRNA	ssODN	Injected	Survived (%)	Transferred	Pups (%)	Pup No.	Left PCR	Right PCR	Left RFLP	Right RFLP	Pups positive for all analyses
2μM	L: 50ng/μl R: 30ng/μl	20ng/μl	108	103 (95.4)	103	4 (3.9)	#1–4	#1,3	#1,2,3	#1	–	–
		10ng/μl	68	66 (97.1)	49	5 (10.2)	#5–9	–	#6	–	#6	–
1.5μM	L: 40ng/μl R: 20ng/μl	20ng/μl	78	75 (96.2)	75	4 (5.3)	#1–4	#2,3,4	#1,2	#2,4	#1,2	#2 (nearly floxed)
		10ng/μl	77	75 (97.4)	75	9 (12.0)	#5–13	#6,7,13	#5,6,9,10	#6,13	#5,6	#6 (precisely floxed)
1μM	L: 25ng/μl R: 15ng/μl	20ng/μl	74	74 (100)	74	4 (5.4)	#1–4	#1,2,4	#1,4	#2	#1	–
		15ng/μl	40	39 (97.5)	39	3 (7.7)	#5–7	#5	#6	#5	–	–
		10ng/μl	74	70 (94.6)	70	8 (11.4)	#8–15	#8	#8,13	#8	#8,13	#8 (nearly floxed)

Fertilized oocytes were created by IVF using the ultra-superovulation method (IASe-hCG).
Female mice were used for IVF at 10-11 weeks of age.

In summary, our reproductive engineering techniques, especially the ultra-superovulation method, will help improve animal welfare and work efficiency when producing genetically modified mice.