Culture time of vitrified/warmed zygotes before microinjection affects the production efficiency of CRISPR-Cas9-mediated knock-in mice

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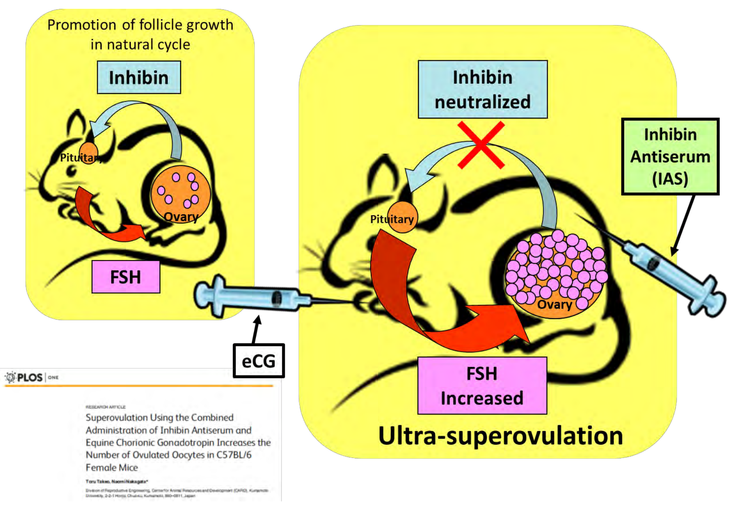
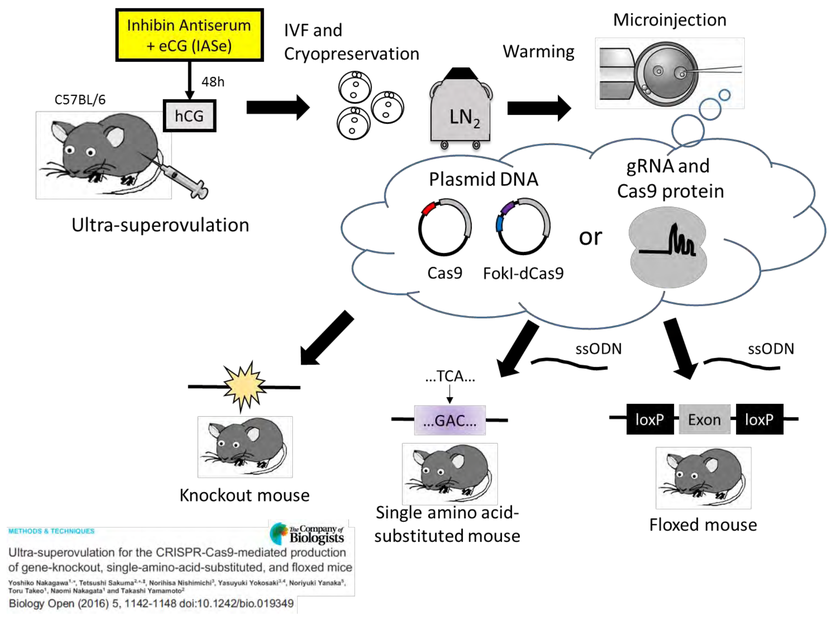
Introduction

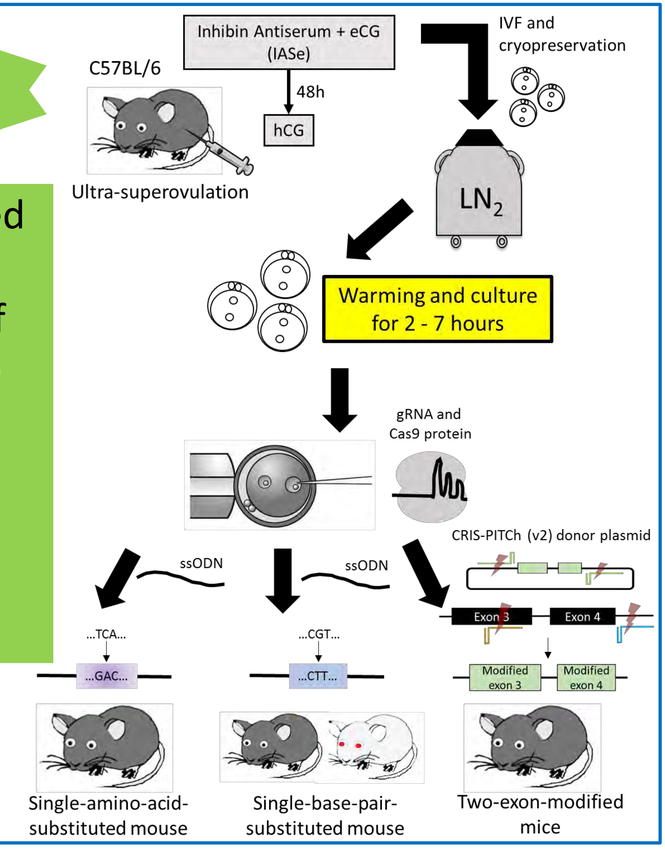
Ultra-superovulation using IAS and eCG (IASe)





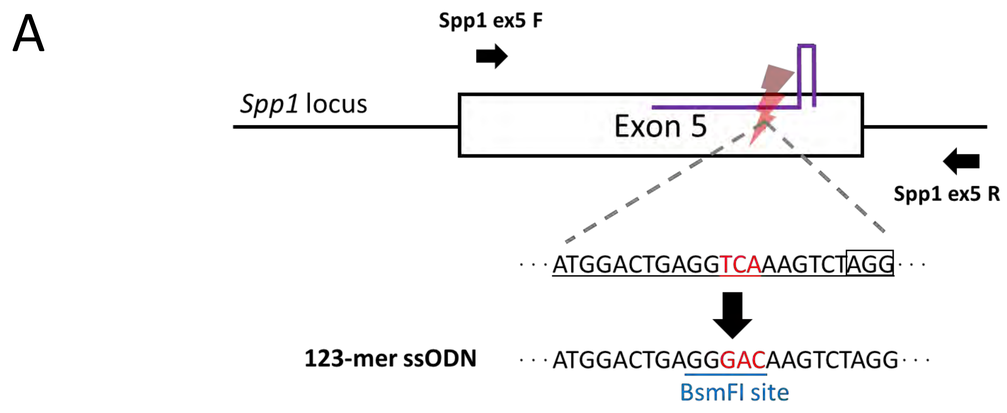
In this study

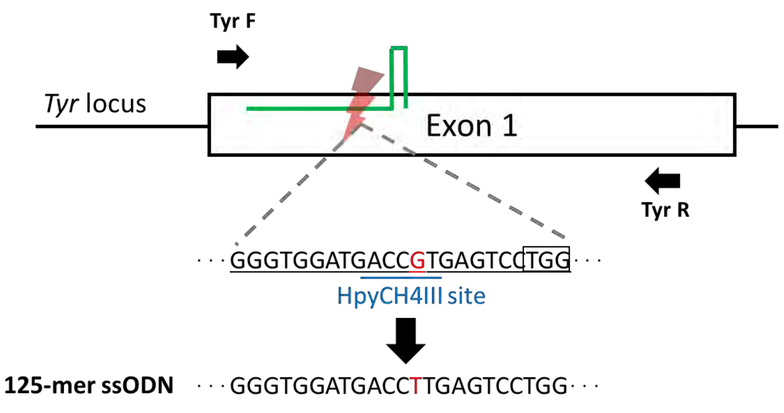
We investigated whether the culture time of zygotes before microinjection influences the efficiency of producing knock-in mice.



Reproductive engineering techniques are required for the efficient and speedy production of genetically modified mice. Recently, we have developed ultra-superovulation method to collect more oocytes from female mice than using conventional superovulation method (Takeo and Nakagata, 2015), and demonstrated that zygotes created via ultra-superovulation combined with IVF are also applicable for the production of various GM mice by direct microinjection of genome editing reagents (Nakagawa et al., 2016). We usually use vitrified/warmed fertilized oocytes created by IVF for microinjection because of work efficiency and flexible scheduling.

Generation of base-substituted mice using ssODN at the *Spp1* and *Tyr* loci





(A) Generation of single-amino-acid-substituted mice at the *Spp1* locus. A serine residue in exon 5 was replaced with an aspartic acid (TCA to GAC; red letters). A gRNA was designed to cut in close vicinity of the serine residue (underlined in black and red). An ssODN was designed to carry the three-base substitution.

(B) Generation of single-base-pair-substituted mice at the *Tyr* locus. A guanine in exon 1 was changed to thymine (red letter). An ssODN was designed to carry the one-base substitution. Black boxes indicate the PAM sequences. Arrows indicate the primer sets for PCR.

Table 1. Generation of single-amino-acid-substituted mice

CRISPR-Cas9	ssODN	Culture time	Injected	Survived (%)	Transferred	Pups (%)	KI (%)
0.75 μM Cas9 protein, 30 ng/μl gRNA	10 ng/μl	2 h	37	33 (89.2)	33	3 (9.1)	2 (66.7)
		3 h*	41	39 (95.1)	39	5 (12.8)	1 (20.0)
		5.5 h	37	36 (97.3)	35	5 (14.3)	1 (20.0)
			36	33 (91.7)	33	10 (30.3)	3 (30.0)
		6 h	36	35 (97.2)	35	4 (11.4)	1 (25.0)
			36	35 (97.2)	35	8 (22.9)	2 (25.0)
			36	33 (91.7)	33	4 (12.1)	2 (50.0)
		7 h	36	34 (94.4)	34	7 (20.6)	5 (71.4)

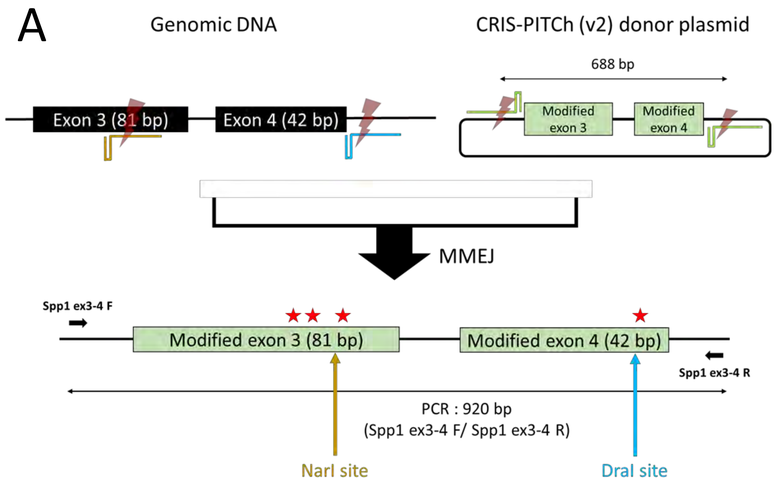
*Result of 3 h was from a previous report (Nakagawa et al., 2016).

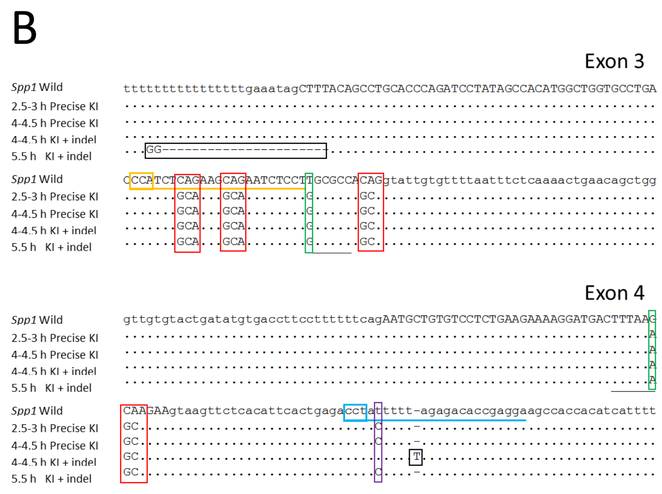
Table 2. Generation of single-base-pair-substituted mice

CRISPR-Cas9	ssODN	Culture time	Injected	Survived (%)	Transferred	Pups (%)	KI (%)
0.5 μM Cas9 protein, 20 ng/μl gRNA	10 ng/μl	2 h	74	74 (100)	70	4 (5.7)	3 (75.0)
		2.5 h	65	63 (96.9)	62	3 (4.8)	2 (66.7)
		4.5 h	65	64 (98.5)	64	8 (12.5)	1 (12.5)
		6 h	80	75 (93.8)	75	11 (14.7)	1 (9.1)
		6.5-7 h	140	117 (83.6)	117	13 (11.1)	4 (30.8)

For base substitutions using an ssODN, we could generate knock-in mice from zygotes cultured for any length of time (between 2 and 7 h) but with high efficiency for short and long culture times and low efficiency for intermediate culture times.

Generation of two-exon-modified mice with CRIS-PITCh (v2) system





(A) Schematic illustration to generate a PITCHed allele at the *Spp1* locus, mediated by CRIS-PITCh (v2) system. Four glutamine residues in exons 3 and 4 (three in exon 3 and one in exon 4) were replaced with alanine residues. Two gene-specific gRNAs were designed within exon 3 and downstream of exon 4. A PITCh donor plasmid was designed to carry the substituted sequences encoding four alanine residues, silent mutations for RFLP analysis (from T to G in exon 3 and from G to A in exon 4) and point mutation (from T to C) in intron region. Yellow and blue arrows indicate the recognition sites of restriction enzymes for the RFLP analyses. (B) Sequencing analysis of subcloned PCR products from pups harboring knock-in and nearly knock-in allele with indel mutation.

Table 3. Generation of two exon-modified mice at the *Spp1* locus

CRISPR-Cas9	PITCh vector	Culture time	Injected	Survived (%)	Transferred	Pups (%)	Precise KI (%)	KI with indel mutation (%)
1 μM Cas9 protein, 40 ng/μl gRNA	5 ng/μl	2.5-3 h	75	66 (88.0)	66	5 (7.6)	3 (60.0)	0 (0)
		4-4.5 h	74	66 (89.2)	66	6 (9.1)	1 (16.7)	1 (16.7)
		5.5 h	70	65 (92.9)	65	10 (15.4)	0 (0)	1 (10.0)
		6 h	70	68 (97.1)	68	10 (14.7)	0 (0)	0 (0)
			76	71 (93.4)	71	7 (9.9)	0 (0)	0 (0)
		7 h	75	73 (97.3)	73	5 (6.8)	0 (0)	0 (0)

The shorter culture times resulted in high knock-in efficiency with the PITCh strategy, which is consistent with the ssODN knock-in results, whereas longer culture times did not result in successful knock-in, unlike the ssODN knock-in.

Different production efficiencies of knock-in mice were observed by changing the timing of microinjection. Our study provides useful information for the CRISPR-Cas9-based generation of knock-in mice.

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