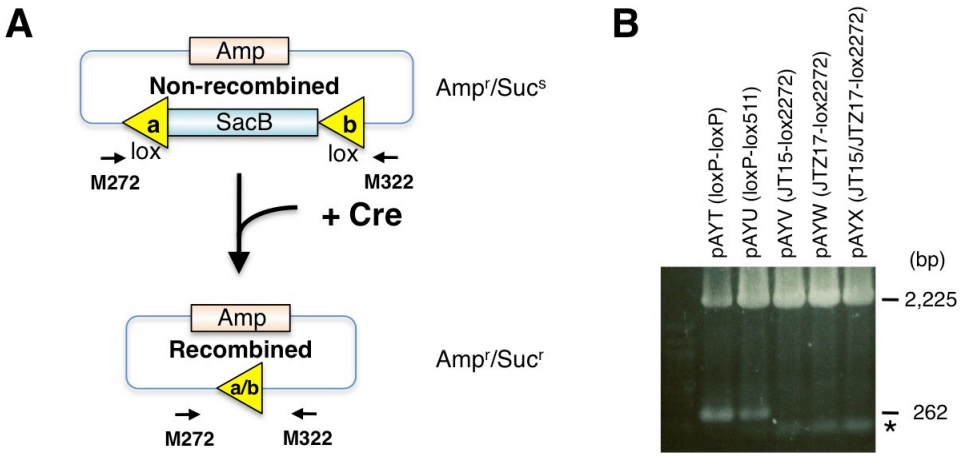
SUPPLEMENTARY MATERIAL

Supplementary Figures



C

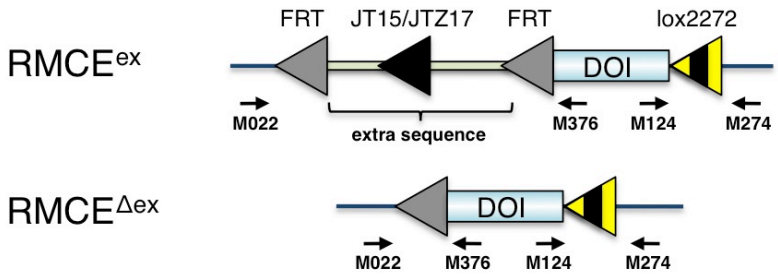
Frequencies of Cre-mediated recombination between loxP derivatives

Plasmids	loxP derivatives		Number of colonies	
	a	b	Non-recombined and Recombined Amp (1/200)	Recombined Amp/Suc (1/5)
pAYT	loxP	loxP	625	761
pAYU	loxP	lox511	732	2
pAYV	JT15	lox2272	747	0
pAYW	JTZ17	lox2272	785	0
pAYX	JT15/JTZ17	lox2272	614	0

Supplementary Figure S1. Presence or absence of Cre-mediated recombination between heterotypic lox sites. (**A**) Experimental scheme. Non-recombined plasmids containing SacB gene flanked by loxP derivatives (a and b) are ampicillin-resistant and sucrose-sensitive. When recombination takes place between two lox sites (a/b) by Cre enzyme, the resultant recombined plasmids are ampicillin-resistant and sucrose-resistant. Recombination can be detected by PCR with primer set M272 and M322 and/or selection on LB plates containing ampicillin (Amp) and sucrose (Suc).

(**B**) PCR analysis of Cre-recombination. Each plasmid was treated *in vitro* with Cre enzyme and its reaction mixture was directly subjected to PCR analysis using the primer set shown in A. 2,225 bp and 262 bp bands are amplified from non-recombined and recombined plasmids, respectively. The smaller fragments (asterisk) represent background artificial bands. (**C**) Frequencies of Cre recombination between loxP derivatives (a and b). In these experiments, 1/200 and 1/50 volumes of transformation mixtures were plated onto Amp and Amp/Suc plates, respectively, and numbers of colonies that appeared subsequently were counted. With regard to the Amp/Suc plates for pAYU, pAYV, pAYW and pAYX, only the numbers of colonies that yielded a 262-bp band by colony PCR are shown, although some background colonies (1 to 7 colonies in each plate) were also noted. With regard to Amp/Suc plates for pAYT, 16 out of the 761 obtained clones were checked by colony PCR, and all amplified the 262-bp fragment. See Materials and Methods section for experimental details.

A

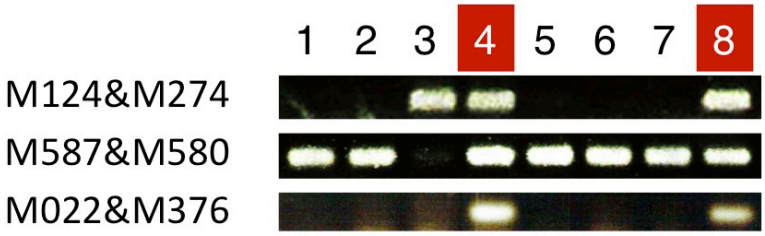


B

Deletion of extra sequences by oocyte microinjection of FLPe plasmid

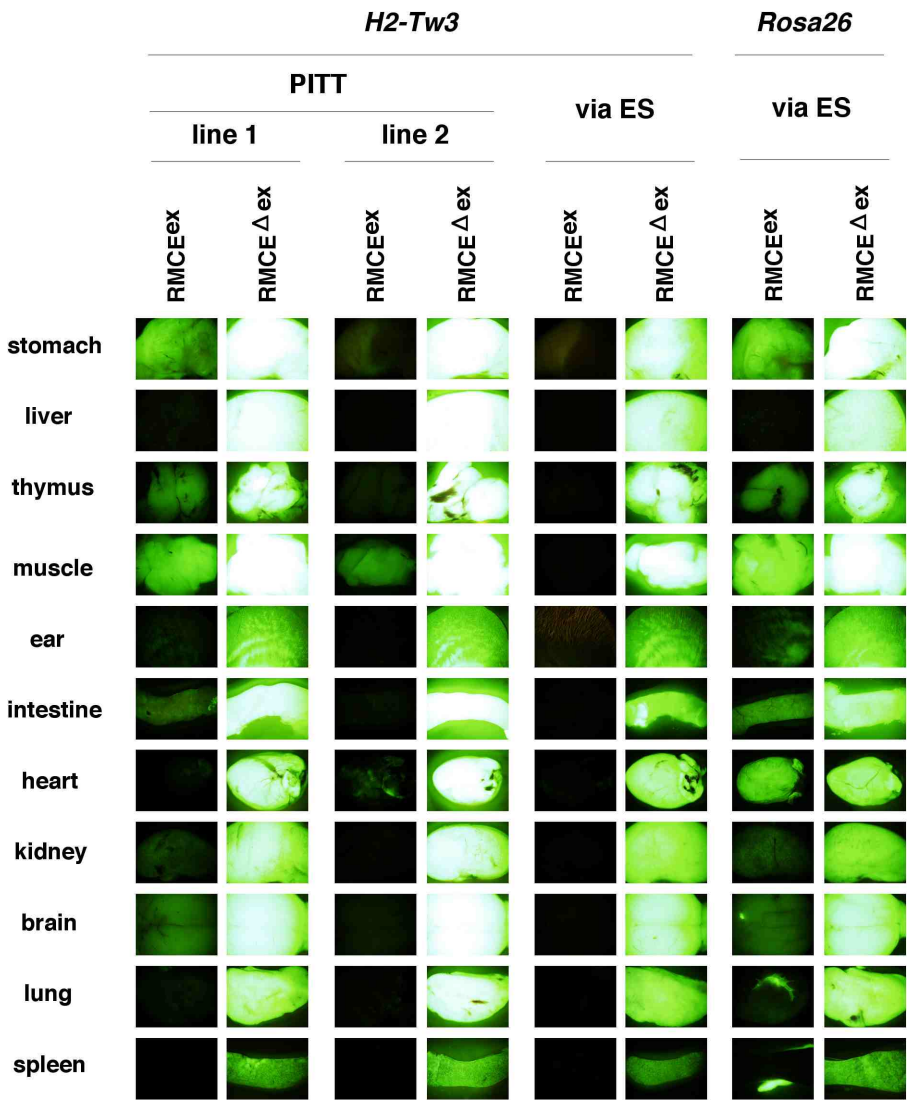
	H2-Tw3	*Rosa26*	Total
Total mice born following oocyte injection of FLPe plasmid	51	22	73
Total mice born with modified (RMCE^{ex} or RMCE$^{\Delta ex}$) allele[a]	26	12	38
FLPe recombined (RMCE$^{\Delta ex}$ allele)[b]	18	7	25
% FLPe recombined (b/a*100)	69%	58%	66%

C

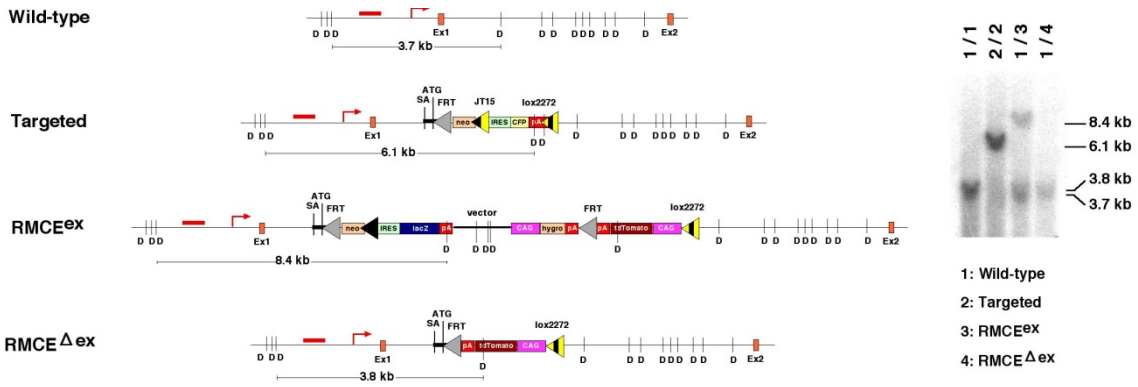


Supplementary Figure S2. In vivo FLPe recombination efficiency. (**A**) Schematic diagram of RMCE^{ex} allele and RMCE$^{\Delta ex}$ allele in the *Rosa26* locus. The extra sequence flanked by FRT in RMCE^{ex} allele can be removed by FLPe recombination, generating RMCE$^{\Delta ex}$ allele. PCR with M124 and M274 amplified the fragment from both RMCE^{ex} and RMCE$^{\Delta ex}$ alleles, while that with M022 and M376 amplified the fragment only from RMCE$^{\Delta ex}$ allele. DOI: DNA of interest. (**B**) Microinjection of FLPe plasmid into oocytes to remove the FRT-flanked extra sequence in RMCE^{ex} allele. (**C**) PCR-based typing of the progeny obtained from the cross between founder mouse and FLPe transgenic mouse. PCR with M587 and M580 amplify the FLPe

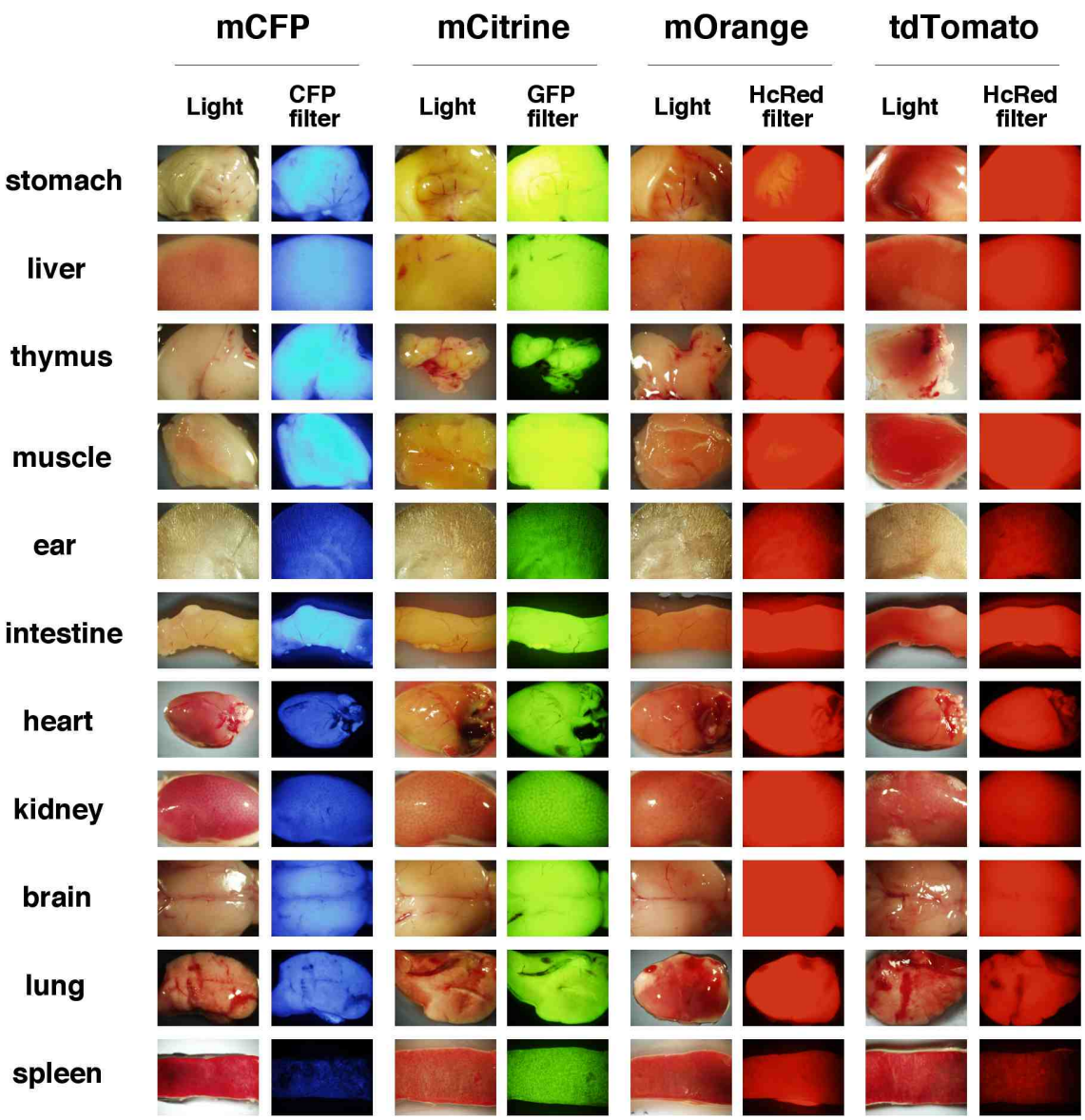
transgene. The progeny 4 and 8 indicated in red harbor both the targeted transgene and FLPe transgene.



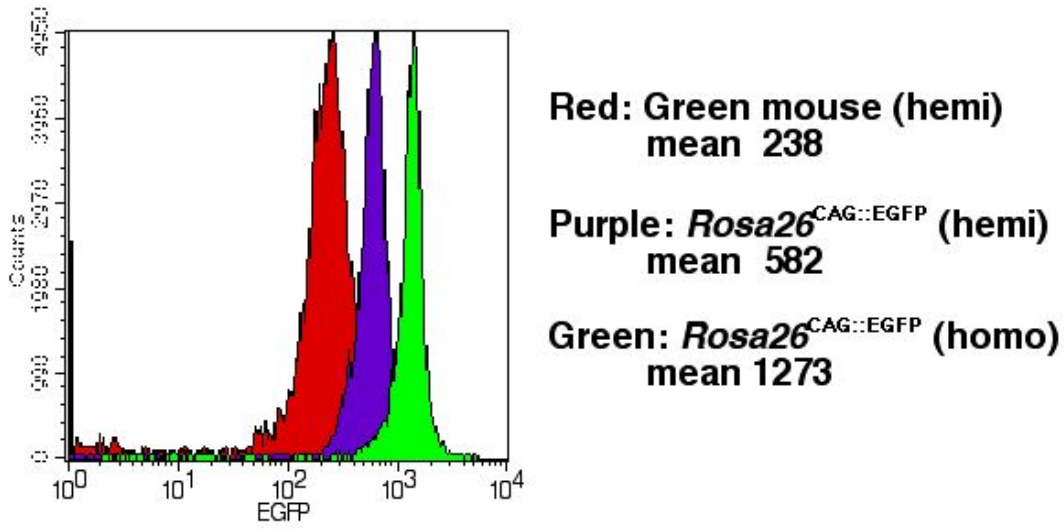
Supplementary Figure S3. EGFP fluorescence in tissues of "CAG-EGFP-polyA" transgenic mice generated via RMCE. Expression of EGFP gene was silenced (weak and mosaic) in mice with RMCE^{ex} allele. After removal of the extra sequences by FLPe from mice with RMCE^{ex} allele, all mice with RMCE$^{\Delta ex}$ allele exhibited ubiquitous, stable and strong EGFP expression. With regard to mice generated by PITT, two transgenic lines (lines 1 and 2) derived from different founder mice are shown.



Supplementary Figure S4. Examples of mice containing alleles obtained in each step of our method. From top to bottom rows: "wild-type (WT) allele" of *Rosa26* locus, "targeted allele" containing genomically tagged sites, "RMCE^{ex} allele" in which pAOM donor plasmid is introduced by PITT and "RMCE$^{\Delta ex}$ allele" without extra sequence. The genotype of each allele was analyzed by genomic southern blotting after digestion with *Dra*I using the probe marked in red bar. The 8.4 kb fragment from the RMCE^{ex} allele indicates correct Cre-mediated recombination at only JT15/JTZ17 site but not at lox2272/lox2272 site. In this RMCE^{ex} mouse, correct recombination at lox2272/lox2272 site was confirmed by PCR that amplified the junction region. D: *Dra*I site.



Supplementary Figure S5. Ubiquitous and strong fluorescence expression in tissues of "CAG-fluorescent gene-polyA" transgenic mice generated via PITT. In all transgenic mice, the transgene is located at the *Rosa26* locus with a single copy configuration.



Supplementary Figure S6. EGFP fluorescence intensities in spleen cells. Fluorescence intensities were compared between *Rosa26*$^{CAG::EGFP}$ mice (hemizygote and homozygote) and commercially available EGFP transgenic mouse (green mouse; hemizygote). The spleen cells from *Rosa26*$^{CAG::EGFP}$ mice exhibited brighter EGFP fluorescence compared with the green mouse.

Supplementary Tables

Supplementary Table S1. Primers used in the present study.

#142	AAGAAGACAGGGCCAGGTTT
#143	ATGGTCCTGCTGGAGTTCGT
#145	TATGAAACAGCCCCCTGCT
#166	ACCCGTGATATTGCTGAAGAG
#169	TAAAAGGATTTGCAGACTACGG
M022	TGAGCGGCTGCGGGGCGGGTGCAA
M024	CCTAAAGAAGAGGCTGTGCTTTGG
M025	ACCAGCTACCGGAGCAAGAAG
M026	GGTGGTGCAGATGAACTTCAG
M053	GGCGCGCCTCAGAGAGCCTCGGCTAGGTAG
M054	GGCGCGCCTACGCCAACCAAAACACGC
M055	GGCGCGCCGCACGTTTCCGACTTGAGTTG
M056	GGCGCGCCAAGACTGGAGTTGCAGATCACG
M057	GGCGCGCCTTCTGGGCAGGCTTAAAGG
M058	GGCGCGCCAGCTACAGCCTCGATTTGTGG
M059	GGCGCGCCGTGTTGTTGAGCCACTGAGAATG
M060	GGCGCGCCGTCACTGACCATCATGCCTCTG
M124	CGTAAGTTATGTAACGCGGAACTC
M132	CATTTGTGGGCTGTTTACCAAC
M153	GCTCTAGACCCTGAGTTATAAGTCCTCAAG
M154	CGGAATTCCTTGGTTTCTTGCAGTATG
M155	GGCGCGCCTGTGCTTGTATGAATGTCCATGTAC
M159	GCAAGGCGATTAAGTTGGGTAAC
M194	AAGAAGGCACATGGCTGAATATC
M195	CATCAAGGAAACCCTGGACTACTG
M272	CAGGAAACAGCTATGACC
M273	TCAGTAAGGGAGCTGCAGTGG
M274	CGATGGAAAATACTCCGAGGC
M322	TGCGCAACTGTTGGGAAG
M372	GTTGCTGGTGAAGACGTTACAC
M373	GAAAAGAGACACCGAACCACAC
M374	GCTTGTTATGCTGACAAGTGTGA
M375	GAGGCTAGAAGCTGGTGTTAATTG
M376	TGCATTCTAGTTGTGGTTTGTCC
M393	GGCGCGCCACTGTGGCGTGTGAGGAGAC
M394	GGCGCGCCTGCAGGCAAGGACAGCTTC
M395	GGCGCGCCTCATGTTAGGGAAATCCGAAG
M396	GGCGCGCCTCTCCACTGCATTCCAGACC
M397	GGCGCGCCAACACAGCCTGAGTTCGGTC
M398	GGCGCGCCTCCAGCACCTCTACCACATG
M399	GGCGCGCCTATGGGTCCAAACTGCCTTG
M400	GGCGCGCCGTGAATGTGTGTCTGTGCCTG
M401	GTACATAGCTGTGGGCTACC
M402	CCATCATAGCCATGCTGCTC
M515	GCTGTTTCACTGGTTATGCG
M581	GCGAGTTGATAGCTGGCTG
M730	GATAACTTCGTATAGCATACATTATACGAAGTTATACGTCCACATATACCTGCCG
M731	GAATTATTCGTATAGCATACATTATACGAAGTTATACGTCCACATATACCTGCCG
M732	GATAACTTCGTATAGCATACATTATAGCAATTTATACGTCCACATATACCTGCCG
M733	GAATTATTCGTATAGCATACATTATAGCAATTTATACGTCCACATATACCTGCCG
M734	GATAACTTCGTATAATGTATGCTATACGAAGTTATGAAAAGTGCCACCTGACGTC
M735	GATAACTTCGTATAATGTATACTATACGAAGTTATGAAAAGTGCCACCTGACGTC
M736	GATAACTTCGTATAAAGTATCCTATACGAAGTTATGAAAAGTGCCACCTGACGTC

Supplementary Table S2. Targeted transgenesis by RMCE in embryonic stem cells

Exp.	Plasmid transfected	Promoter-less reporter gene	Locus	Method	Colonies examined	Correct integration	Efficiency (%)
1	pAJK	lacZ	*H2-Tw3*	electroporation	48	11	22.9
2	pA748	EGFP	*H2-Tw3*	electroporation	72	19	26.4
3	pA617	EYFP-nuc	*H2-Tw3*	electroporation	96	37	38.5
4	pA617	EYFP-nuc	*H2-Tw3*	lipofectamine 2000	24	9	37.5
5	pAJK	lacZ	*H2-Tw3*	lipofectamine 2000	564	116	20.6
6	pAMF	lacZ	*H2-Tw3*	lipofectamine 2000	427	141	33.0
7	pAMF	lacZ	*Rosa26*	lipofectamine 2000	358	84	23.5
8	pAKB	lacZ	*Rosa26*	lipofectamine 2000	367	123	33.5
9	pAJK	lacZ	*Rosa26*	lipofectamine 2000	560	136	24.3
10	pA748	EGFP	*H2-Tw3*	lipofectamine 2000	24	24	**100.0**
11	pA748	EGFP	*Rosa26*	lipofectamine 2000	24	24	**100.0**

In all experiments, transfected cells were selected with hygromycin. The presence of clones with correct integrations was checked by PCR that amplified the junction regions (for experiment [exp.] 1 - 4), or by the X-gal staining (for exp. 5 - 9). Regarding exp. 10 and 11, EGFP-positive and hygromycin-resistance clones were picked and checked by PCR.

Supplementary Table S3. Survival rates of embryos after injection of Cre expression plasmid.

Concentration of Cre plasmid (ng/µl)	Eggs injected	Normal[a]	2-cell (%)[b]	4-cell (%)[b]	8-cell (%)[b]	Morula (%)[b]	Blastocyst (%)[b]
10	25	22	17 (77)	15 (68)	9 (41)	9 (41)	7 (32)
5	25	23	23 (100)	23 (100)	20 (87)	19 (83)	17 (74)
2.5	25	25	25 (100)	25 (100)	25 (100)	25 (100)	20 (80)
1	20	19	19 (100)	19 (100)	19 (100)	19 (100)	17 (89)
control (no injection)	10	-	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)

[a] Number of embryos free of abnormalities just after injection.

[b] (Number of normal embryos) / a * 100

Supplementary Table S4. Survival rates of embryos after injection of donor plasmid and Cre expression plasmid.

Concentration of plasmids	Eggs injected	Normal[a]	2-cell (%)[b]	4-cell (%)[b]	8-cell (%)[b]	Morula (%)[b]
Cre 5 ng/μl	25	25	23 (92)	23 (92)	18 (72)	18 (72)
Cre 5 ng/μl + pAOF 5 ng/μl	25	20	15 (75)	14 (70)	14 (70)	14 (70)
Cre 5 ng/μl + pAOF 10 ng/μl	25	23	18 (78)	17 (74)	17 (74)	10 (43)
Cre 5 ng/μl + pAOF 20 ng/μl	25	20	10 (50)	10 (50)	8 (40)	6 (30)
pAOF 20 ng/μl	25	22	21 (95)	18 (82)	18 (82)	18 (82)
injection buffer only	10	10	10 (100)	10 (100)	10 (100)	10 (100)
no injection	10	10	10 (100)	10 (100)	10 (100)	9 (90)

[a] Number of embryos free of abnormalities just after injection

[b] (Number of normal embryos) / a * 100

Supplementary Table S5. Frequency of germline transmission from individual founders.

Founder mice	Plasmid from which transgene was derived	Locus	Sex	Frequency of germline transmission (%)[a]
1	pAMF	*H2-Tw3*	male	4/7 (57)
2	pAMF	*H2-Tw3*	female	15/24 (63)
3	pAMG	*Rosa26*	female	sterile
4	pAMJ	*Rosa26*	male	6/17 (35)
5	pANQ	*Rosa26*	female	8/20 (40)
6	pAOB	*Rosa26*	male	0/7 (0)
7	pAOF	*Rosa26*	female	1/23 (4)
8	pAOF	*Rosa26*	female	8/16 (50)
9	pAOK	*Rosa26*	female	5/8 (63)
10	pAOL	*Rosa26*	male	0/10 (0)
11	pAOL	*Rosa26*	female	2/5 (40)
12	pAOL	*Rosa26*	male	2/19 (11)
13	pAOL	*Rosa26*	male	2/17 (12)
14	pAOM	*Rosa26*	male	8/14 (57)
15	pAOM	*Rosa26*	male	5/20 (25)
16	pAOM	*Rosa26*	female	2/8 (25)
17	pAOT	*Rosa26*	female	3/9 (33)
18	pAOT	*Rosa26*	female	3/13 (23)
19	pAOT	*Rosa26*	female	1/8 (13)
20	pAOU	*Rosa26*	female	3/10 (30)
21	pAOU	*Rosa26*	female	6/13 (46)

Germline transmission was analyzed by PCR using genomic DNA isolated from the ears of progeny mice as template.

[a] Number of F1 progeny with germline transmission / total F1 mice born