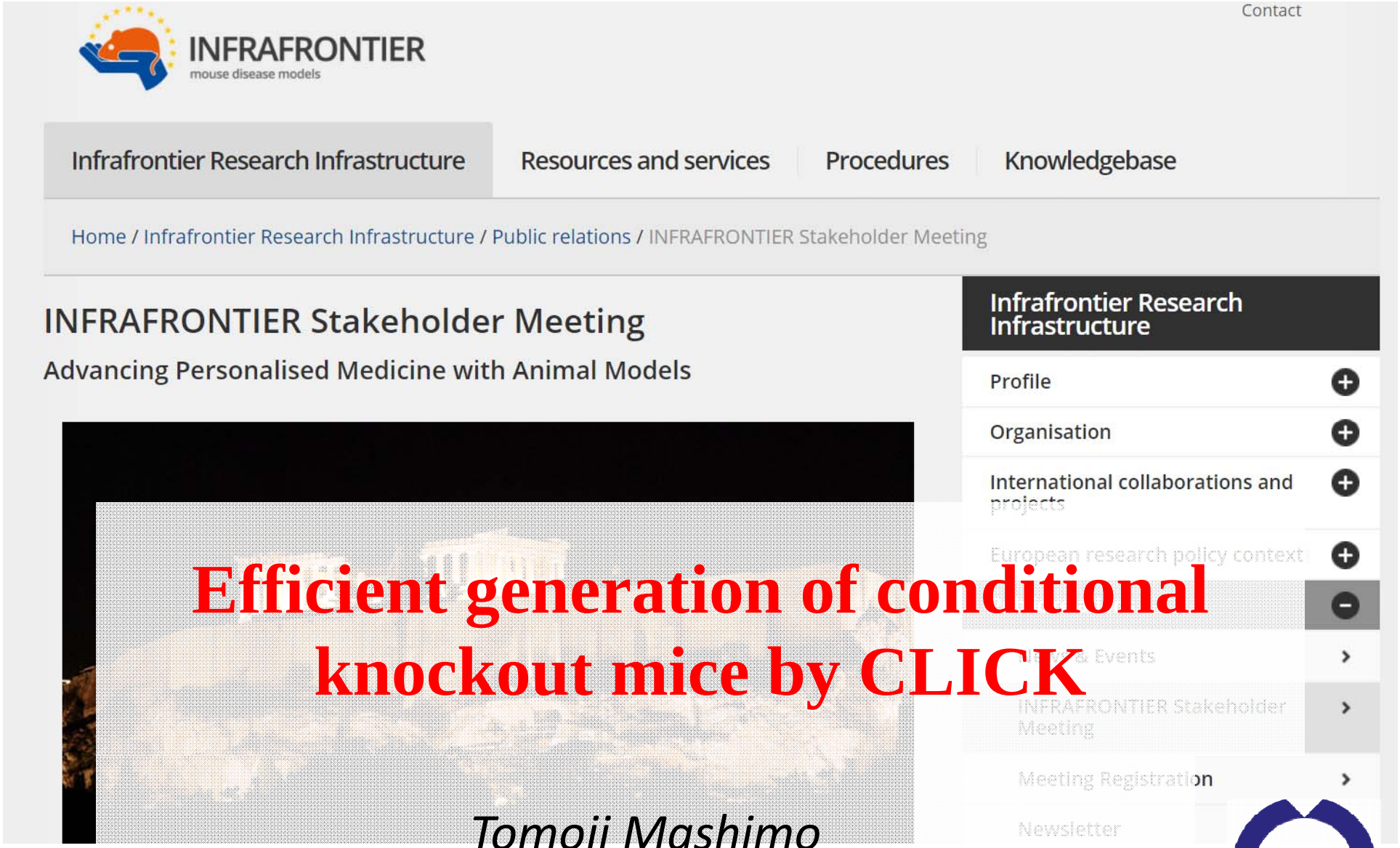

Efficient generation of conditional knockout mice by CLICK

Tomoji Mashimo

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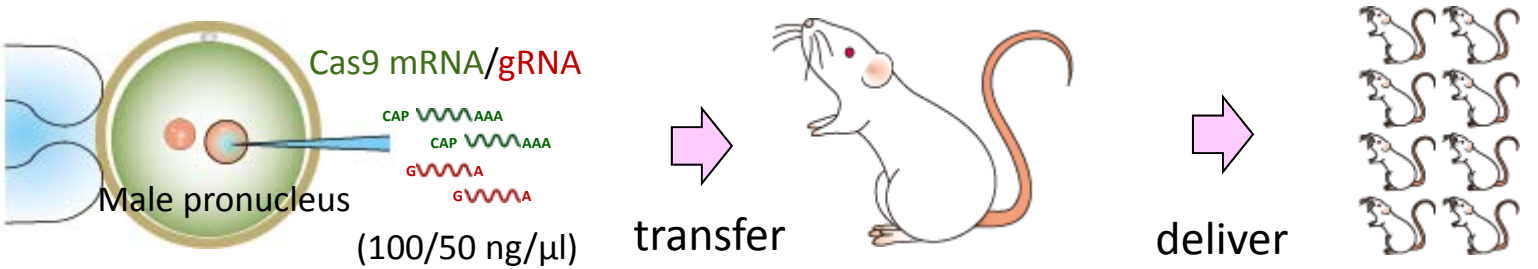
Graduate School of Medicine, Osaka University

大阪大学
OSAKA UNIVERSITY

Generation of Knockout (KO) animals by CRISPR/Cas

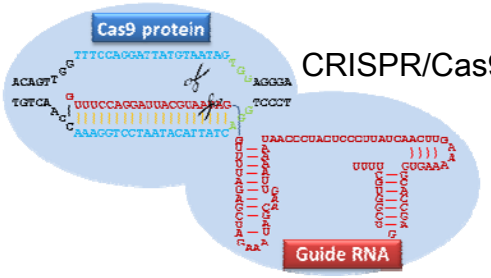


Advantage

- ✓ Knockout mice can be generated in any background strain within 2 - 4 months (ES cells: 12 - 18 months)
- ✓ Multiple genes are modified by co-injection of several gRNAs
- ✓ Co-injection of donor DNA with homology arms can provide knock-in mice
- ✓ Genome editing is available in other species, rat, pig, monkey…

（Mashimo T, *Dev Growth Differ*. 2014）

Knockout by CRIPR/Cas system



CRISPR/Cas9

transfer

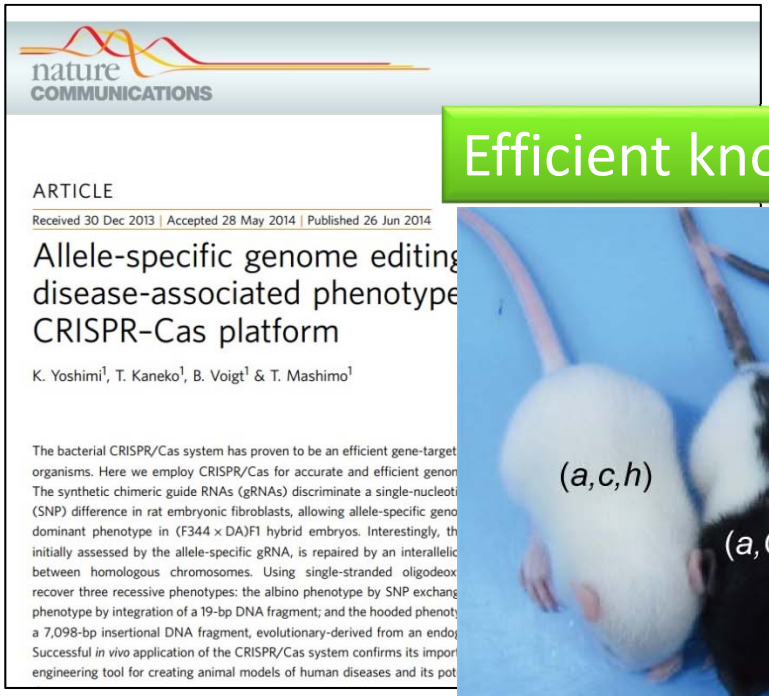
Wistar		GATCGTTTCCAGGATTATGTAATAGTCCCT
#1-8,#4-3(Ho)	+1	GGTCCCT
#2-4(Ho),#2-6 #5-3,#6-6	+1	GGTCCCT
#2-8,#4-6(Ho)	-42	-CATCA
#2-8	-41	-TAATA
#3-7(Ho),#3-8(Ho) #6-4(Ho)	-7	GTCCCT
#6-2,#6-6,#6-7(Ho)	+2	TGGTCCCT
#6-2	+2	TGGTCCCT
#6-3(Ho)	-3	GTCCCT

Injected mRNA	Injected embryos	Collected cells	PCR-amplified	KO- Mutations
gRNA + Cas9	90	41 (45.6)	34 (82.9)	**14 (41.2)**

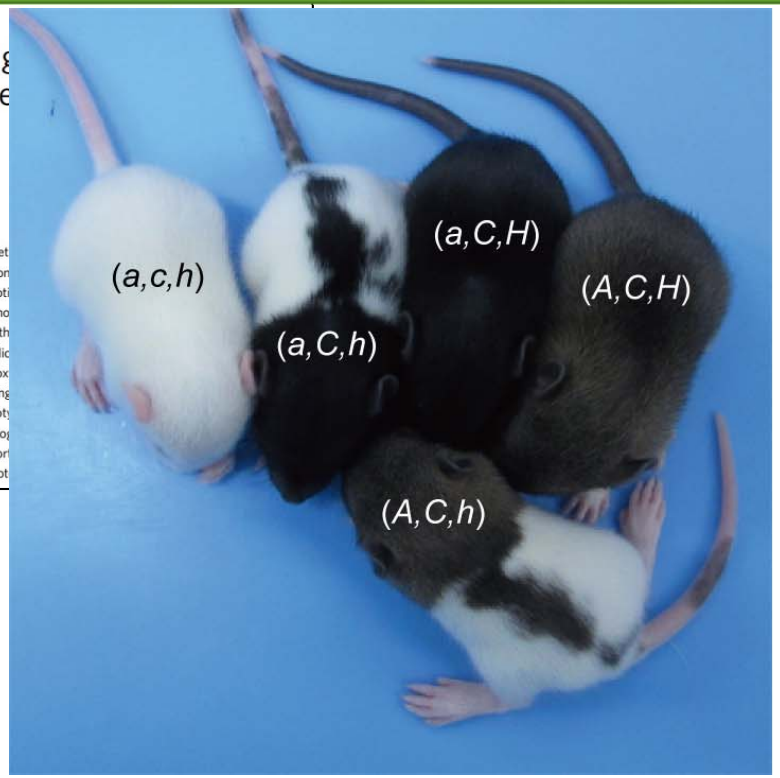
Generating KO rats is possible with CRISPR!

Allele-specific genome editing and correction of disease-associated phenotypes in rats using CRISPR/Cas platform（K Yoshimi et al. Nat Commun 2014 Jun 26;5:4240.）

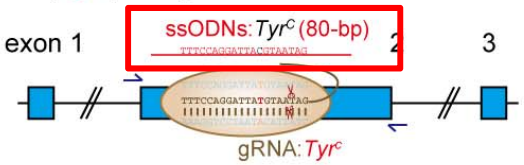
http://www.kyoto-u.ac.jp/ja/research/research_results/2014/140626_1.html



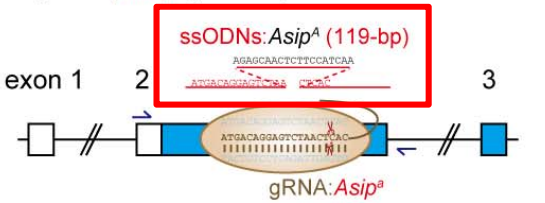
Efficient knock-ins of small DNA fragments in zygotes



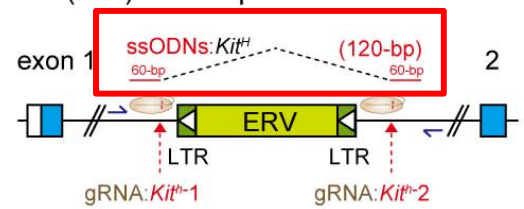
albino (*Tyr*^{c}): 1-bp missense mutation



non-*agouti* (*Asip*^{a}): 19-bp deletion mutation



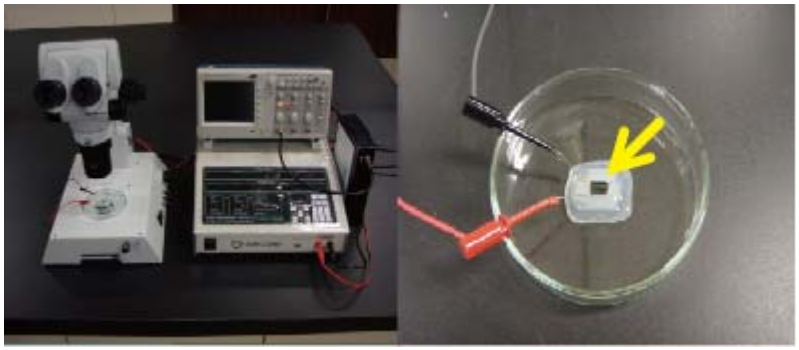
hooded (*Kit*^{h}): ~7k-bp ERV insertion

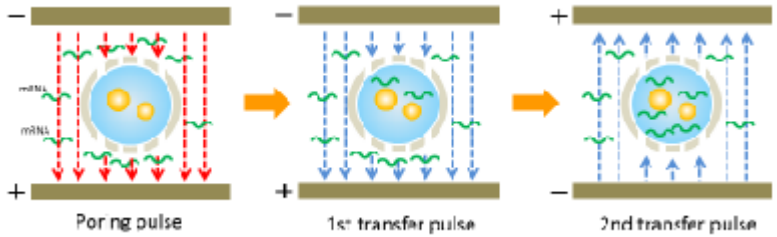


Genome engineering by 'zygote electroporation'

	Target locus	Embryos injected	Two-cell embryos (%)	Pups delivered (%)	KO (%)	KI/offspring (%)
gRNA + Cas9 (+ ssODN)	*Il2rg*	64	58 (90.6)	32 (55.2)	3 (9.4)	-
gRNA + Cas9-polyA + ssODN	*Il2rg*	60	45 (75.0)	24 (53.3)	**21 (87.5)**	**8 (33.3)**

NEPA21 electroporator





NEPAGENE ネッパジーン株式会社

- ✓ Easy transfer (without special skills)
- ✓ 5 min and low toxicity
- ✓ KO and KI with ssODN
- ✓ High-throughput engineering

Genome engineering by zygote electroporation

OPEN

SUBJECT AREAS:
EMBRYOLOGY
GENETIC ENGINEERING

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Simple knockout by electroporation of engineered endonucleases into intact rat embryos

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[1]Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan, [2]Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan.

Engineered endonucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system, provide a powerful approach for genome editing in animals. However, the microinjection of endonucleases into embryos requires a high skill level, is time consuming, and may cause

Gene Knockouts
by ZFN, TALEN, CRISPR !

netically
fields[3,4].
effector
CRISPR-
associated (Cas) system, are invaluable tools for the rapid generation of GM animals including rats[5–8]. Importantly, these new technologies provide genome-editing approaches for a wide variety of organisms that were previously inaccessible without embryonic stem (ES) cells[9–11] and induced pluripotent stem (iPS) cells[12,13]. GM animals are usually produced by microinjecting engineered endonucleases into pronuclear-stage embryos[5,6]. Although this method is now the gold standard, it requires sophisticated manual skills to prevent cell damage. Additionally, microinjection is not convenient when many cells need to be assessed simultaneously because the DNA/RNA has to be injected into embryos one by one using a micromanipulator. Electroporation is another method that introduces exogenous DNA/RNA into embryos. However, the current protocols require that the zona pellucida of the embryos is weakened by treatment with Tyrode's acid solution before electroporation for the efficient introduction of DNA[14,15]. To simplify these procedures, we introduced ZFN, TALEN, or CRISPR/Cas mRNA into intact rat embryos without weakening the zona pellucida by electroporation using the Super Electroporator NEPA 21 (NEPA GENE Co. Ltd., Chiba, Japan).

Results

Introduction of mRNA using an electroporator. ZFN, TALEN, or CRISPR mRNA was electroporated into

(Kaneko *et al,* Sci Rep. 2014）

RESEARCH ARTICLE

Simple Genome Editing of Rodent Intact Embryos by Electroporation

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Abstract

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system is a powerful tool for genome editing in animals. Recently, new technology has been developed to genetically modify animals without using highly skilled techniques, such as pronuclear microinjection of endonucleases. Technique for animal

OPEN ACCESS

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Genome Editing of Rode
Electroporation. PLoS O
doi:10.1371/journal.pone

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Comprehensive Cancer

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Published: November 10, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Gene Knock-ins
by CRISPR with ssODN !

ve technology
ct embryos using
t study success-
ved from
deoxynucleotide
nd 88% of rats)
rful tool to pro-

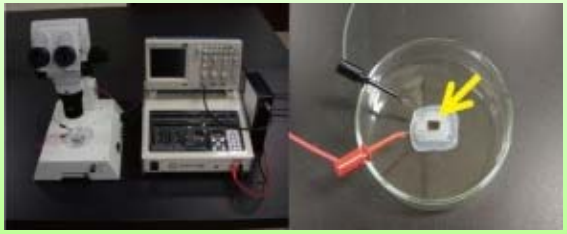
Introduction

Many types of genetically modified (GM) animals have been produced to study human diseases [1–3]. Mice and rats have been used widely as important human diseases model animals [4–6]. Engineered endonucleases, including zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system, are recently developed high-impact technologies

(Kaneko *et al,* Sci Rep. 2015）

Knock-in technologies with CRISPR/Cas

Electroporation



(Kaneko *et al.* *Sci Rep* 2014)

nsfer

Pseudopregnant rats

Founder pups

ssODNs	donor	Targeting vector
Possible	Zygote electroporation	**Impossible**
High	Efficiency	Low
Custom DNA (ssODN)	Prepare	Construct homology arms
Short (<50bp)	Insert size	Long (<10kb)

New technology for large DNA Knock-in is needed!

Protocol to make long single-stranded DNA (lssDNA)

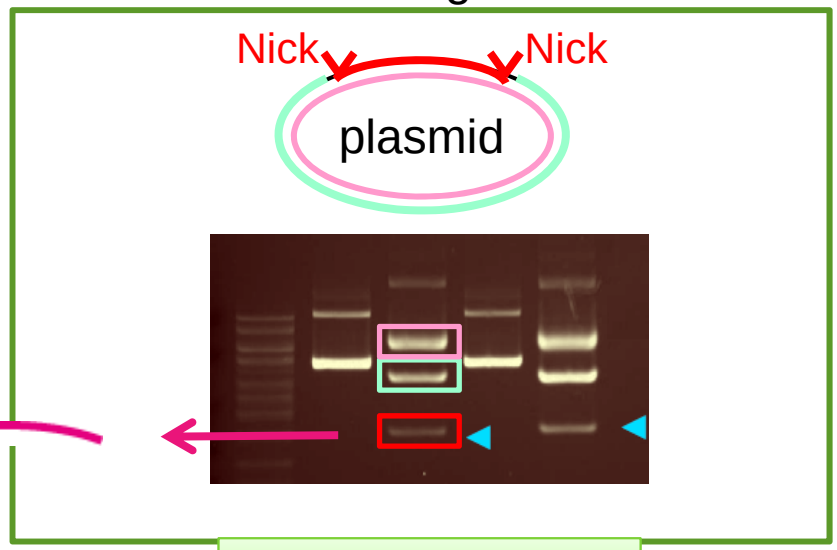
targeting sequences

nicking site nicking site

double-stranded DNA plasmids

Nicking endnucleases (1 hour)

Double nicking methods



Electrophoresis (1 hour)

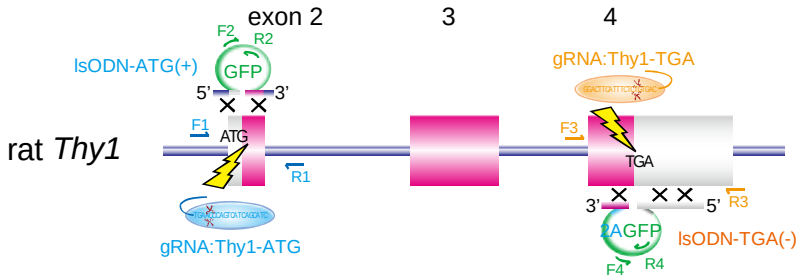
Gel extraction (1 hour)

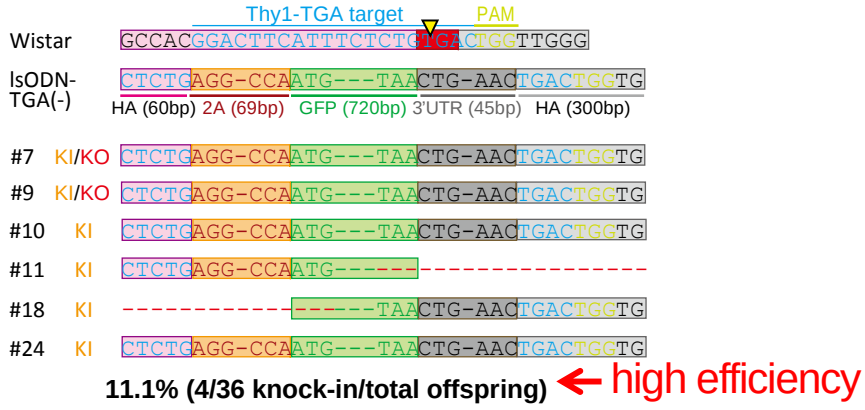
✓ ~3 kbp single-strand oligo (LssDNA) can be prepared by easy protocols

(**Yoshimi *Nat Commun* 2016**)

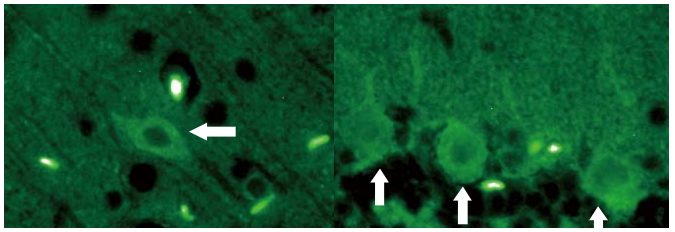
Long ssODN（lssDNA) can be used for GFP-KIs

1-hit 1-oligo (1H1O) by long ssODN (lsODN)





11.1% (4/36 knock-in/total offspring) ← high efficiency



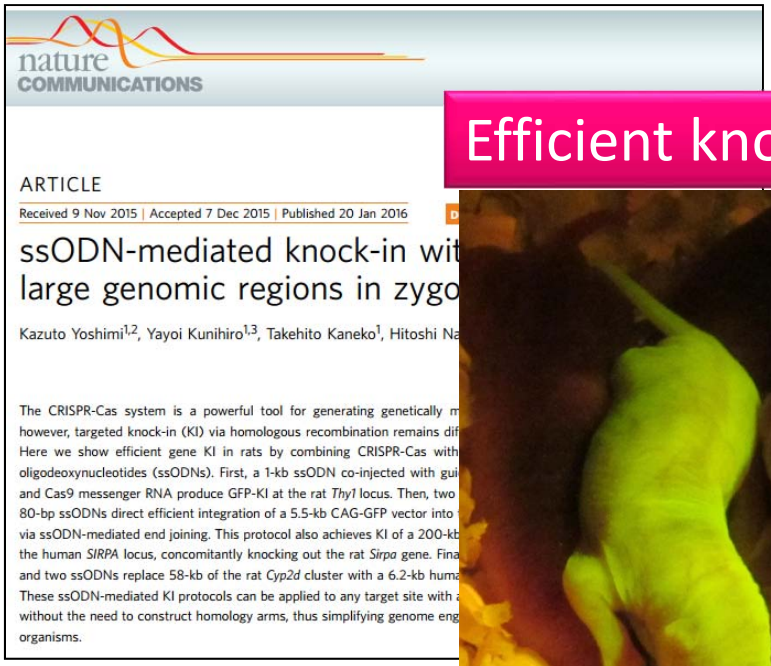
cortex cerebellum

（**Yoshimi *Nat Commun* 2016**）

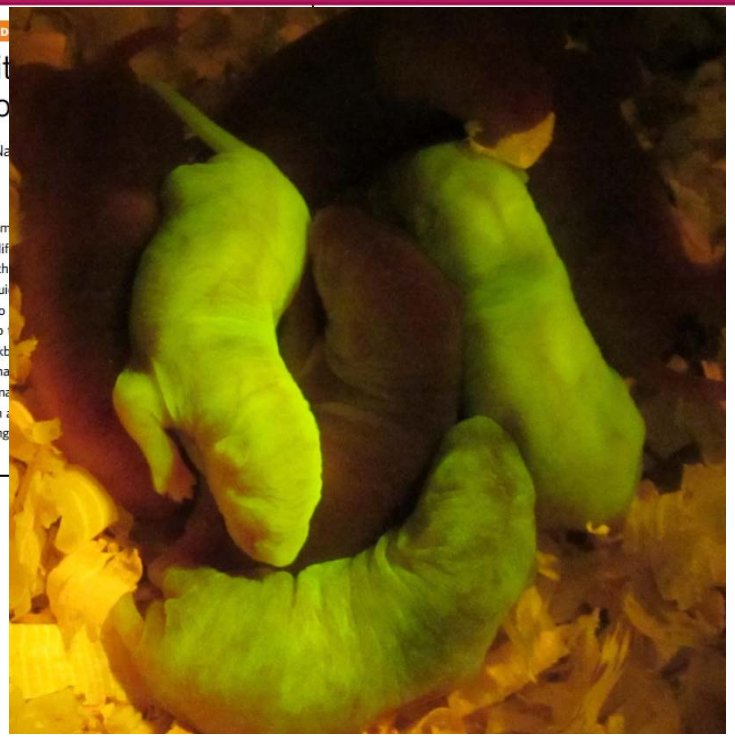
ssODN-mediated knock-in with CRISPR/Cas for large genomic regions in zygotes

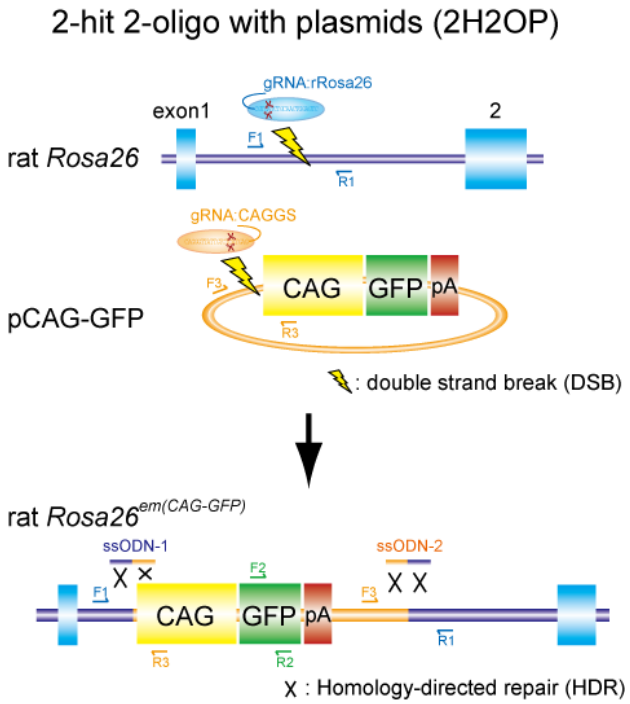
（K Yoshimi et al. Nat Commun 2016 Jan 20;7:10431.）

HP：http://resou.osaka-u.ac.jp/ja

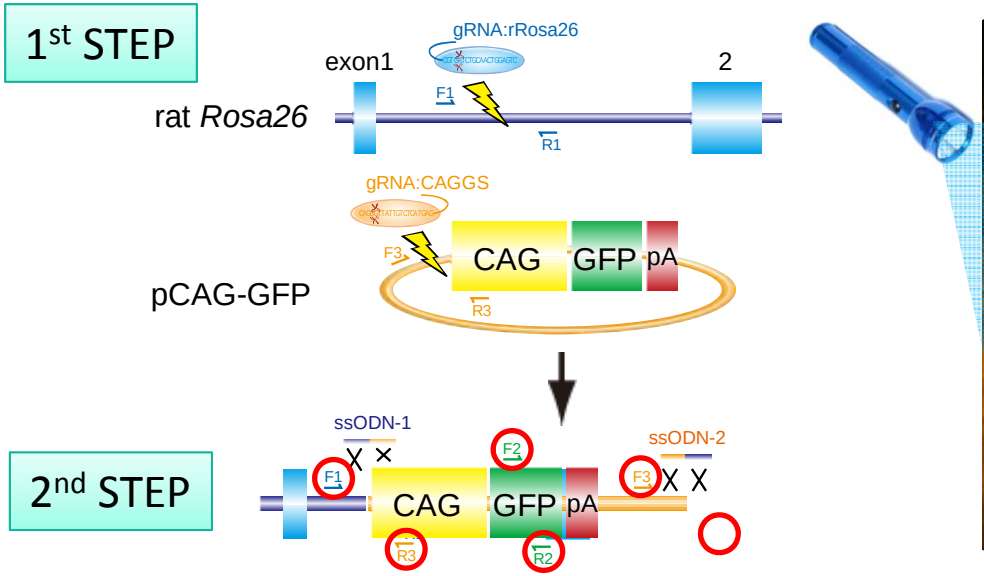


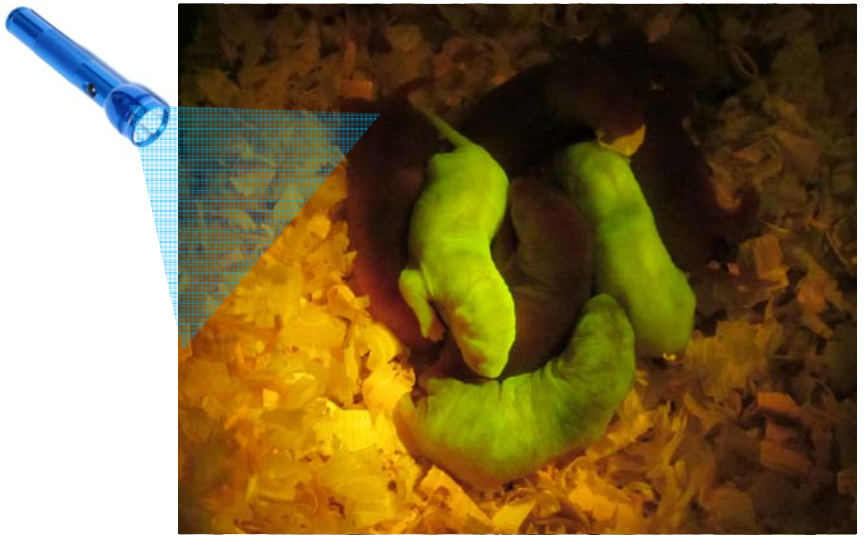
Efficient knock-in for large DNA fragments in zygotes

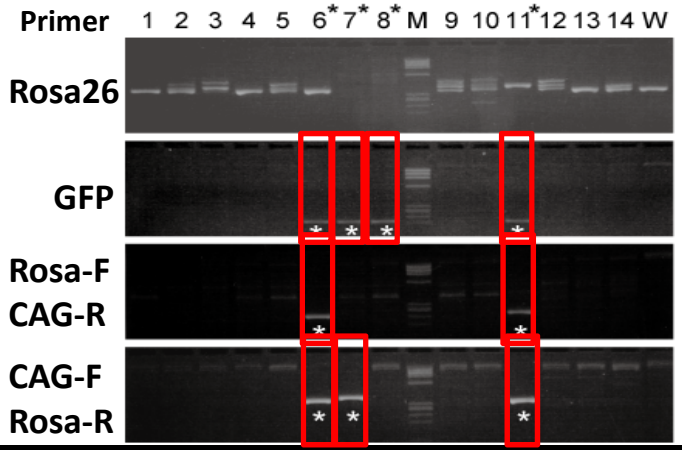


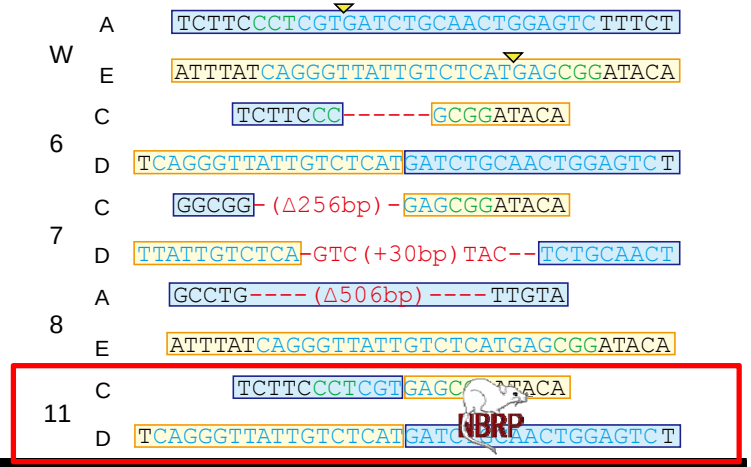


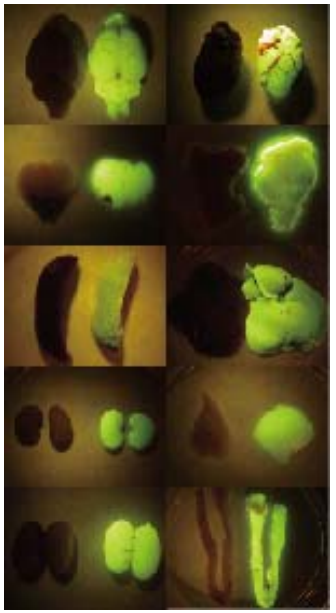
CAG-GFP Knock-in at *Rosa26* locus by 2H2OP







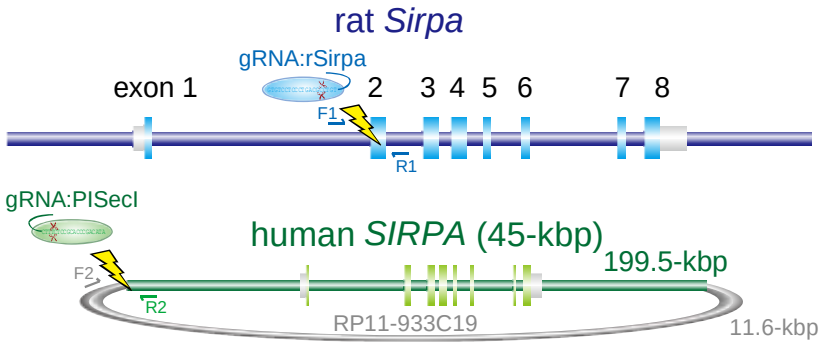




Injected RNA and DNA			Embryos injected	Two-cell Embryos (%)	Pups Delivered (%)	Knockout (%)	Knock-in (%)	KI phenotype (n, %)
gRNA	ssODN	plasmid						
rRosa26, pCAG	rRosa-pCAG, pCAG-rRosa	pCAG-GFP	119	66 (55.5)	17 (25.8)	15 (88.2)	3 (17.6)	GFP positive (n=4, 23.5%)

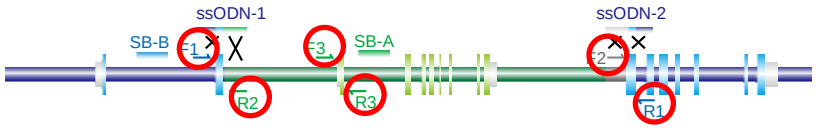
Human BAC (200-kb) Knock-in by 2H2OP

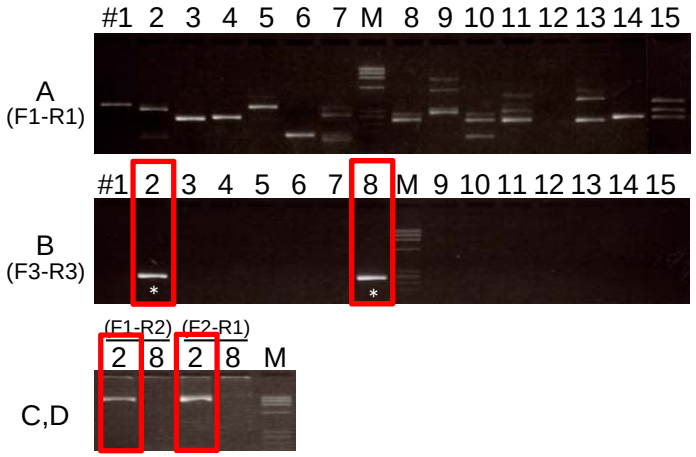
1st STEP

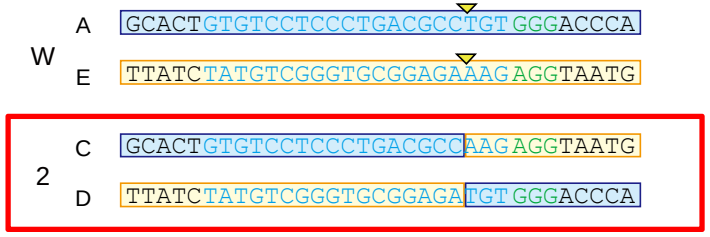


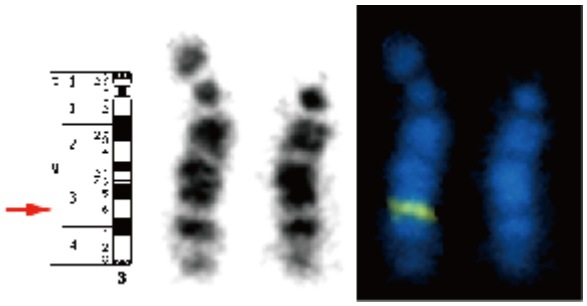
2nd STEP

rSirpa Knockout & *hSIRPA* Knock-in (humanization)

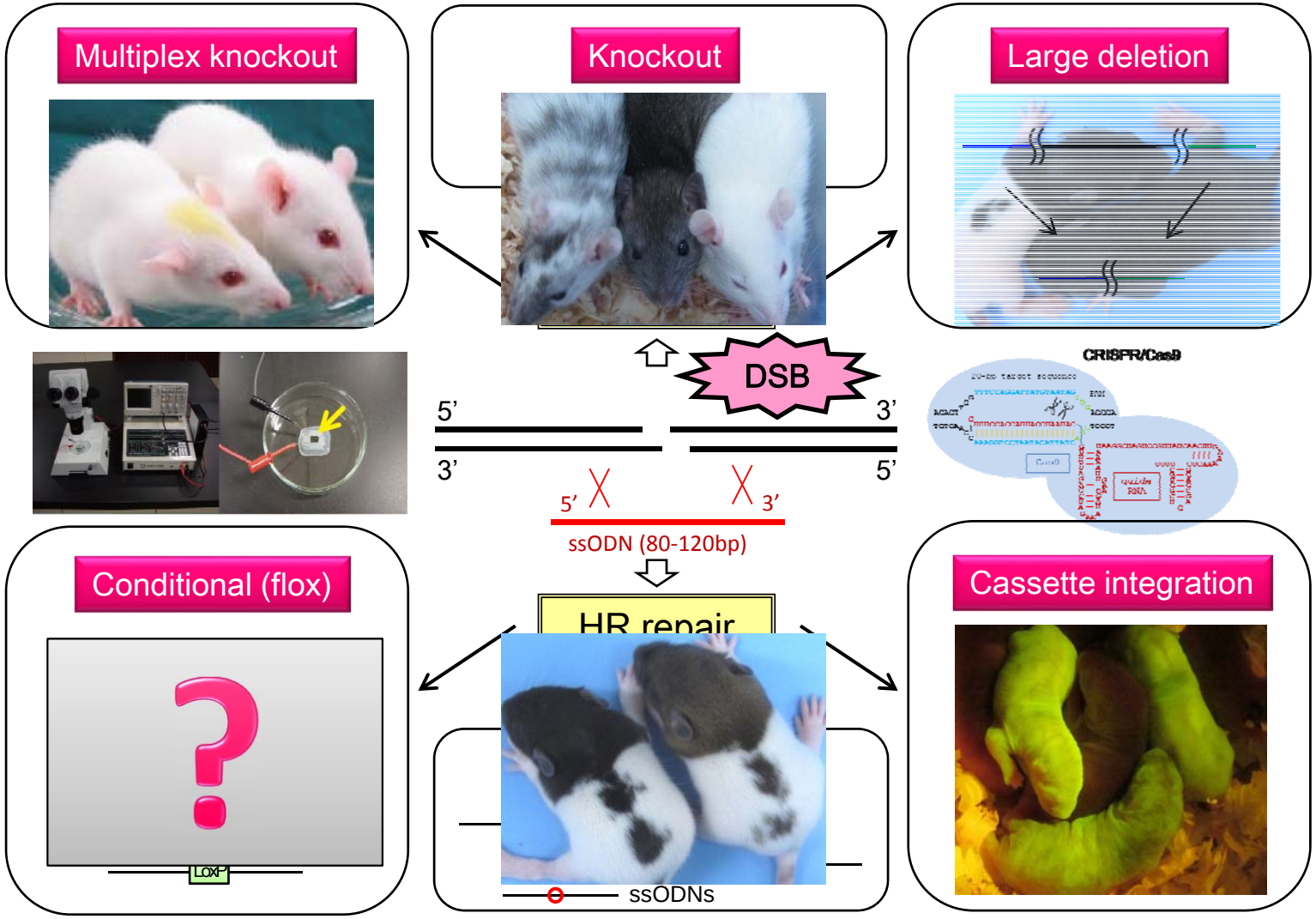






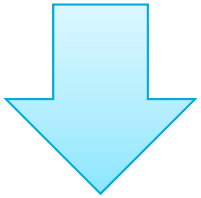


Genome engineering in mouse zygotes



Conditional knockout mice by CRISPR/Cas9 !

How can you knock-in two loxP at one allele?



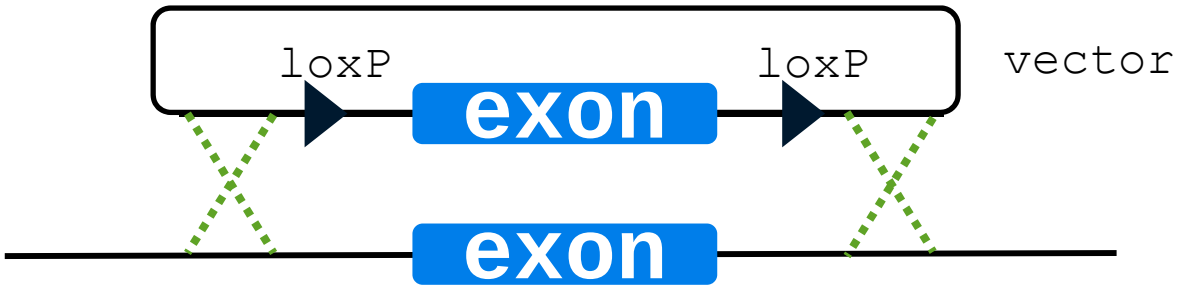
CLICK !

(CRISPR with lssDNA inserts conditional knockout alleles)

Floxed alleles generated by CLICK

conventional

ES

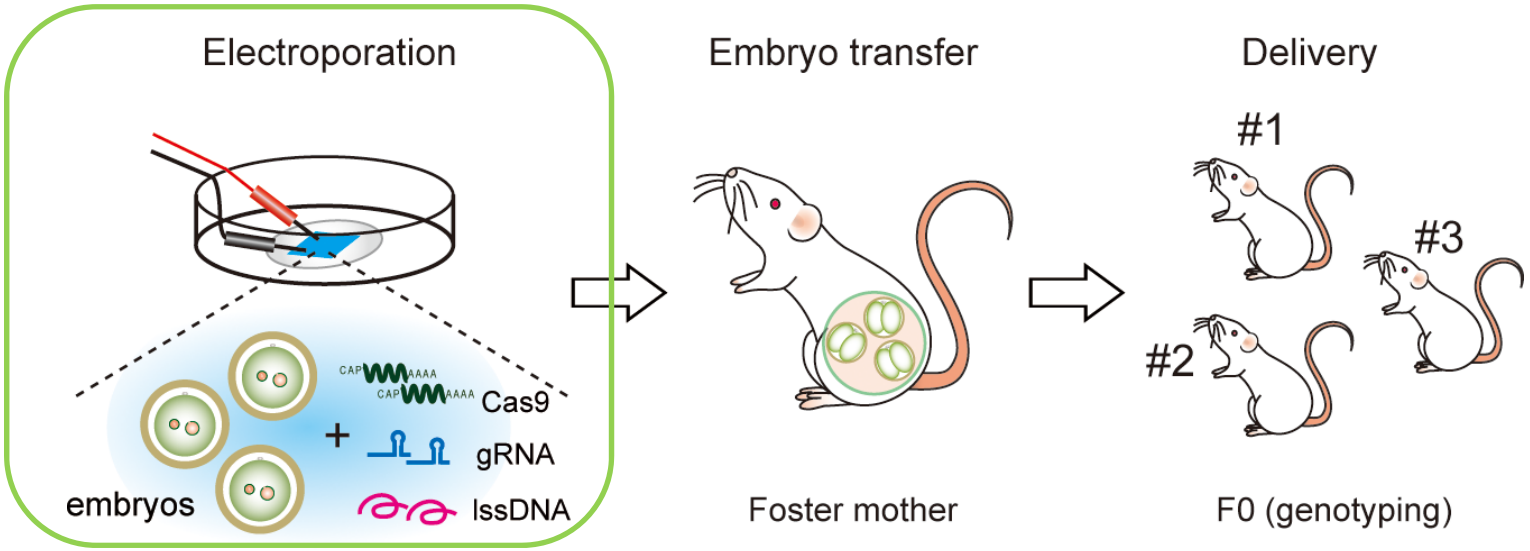


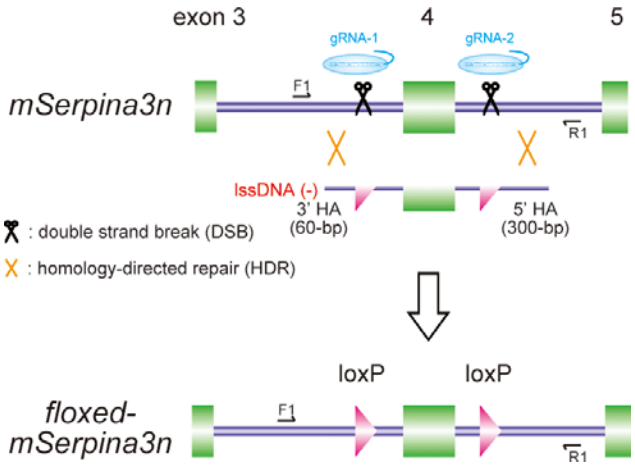
CRISPR

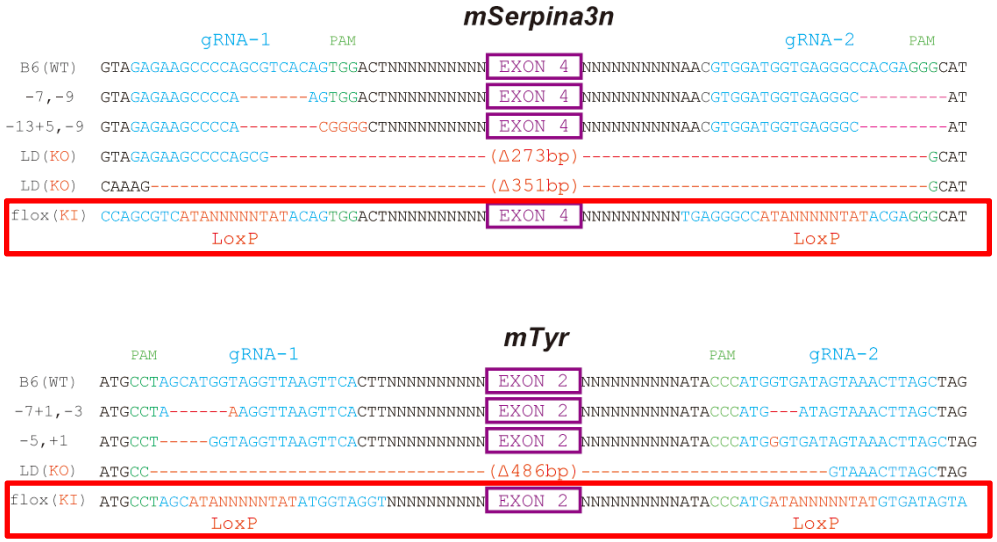
Novel method

CLICK

CLICK: CRISPR with lssDNA inducing conditional knockout alleles







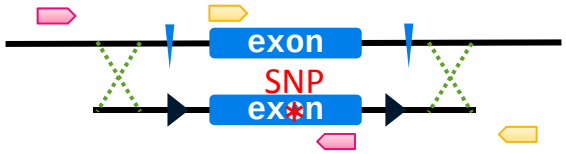
Flox rats generated by CLICK !

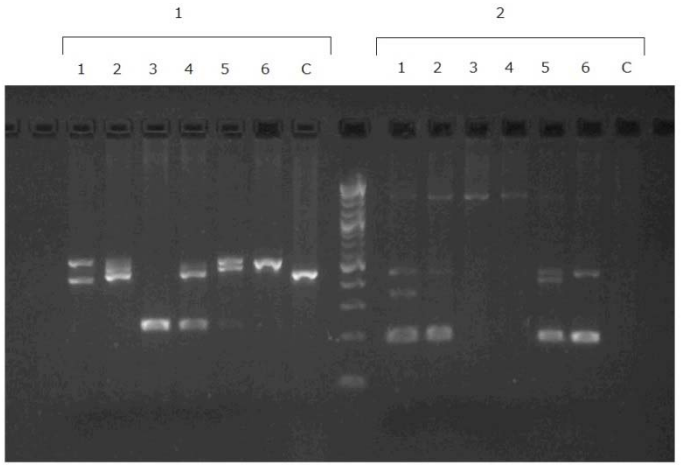
Genotyping

PCR, sequencing

Primer set 1:

Primer set 2:





1 : Vapb-exon2-Small-F + Small-R (Loxp-KI 508bp, Control 440bp)

2 : Vapb_down_loxP_F + Vapb_check_R_out (193bp)

No.	識別 (耳)	性別	遺伝子解析			
			Exon deletion	LoxP-KI Up	LoxP-KI Down	SNP mutation
1	-	M	×	○	○	○
2	R	M	×	○	○	○
3	L	M	○	×	×	×
4	RL	M	○	×	×	×
5	-	F	×	×	×	×
6	R	F	×	○	○	○

Homozygous KI of flox alleles!

Conditional knockout mice by CLICK !

Conventional (1 ～ 2 year)

ES

ES cells → injection → chimera → flox×Cre → CKO

CRISPR (ssODN)

gRNA, Cas9, ssDNA → injection → *loxP mice → injection → flox×Cre → CKO

KI mice of single loxP site

CLICK (3～6 month)

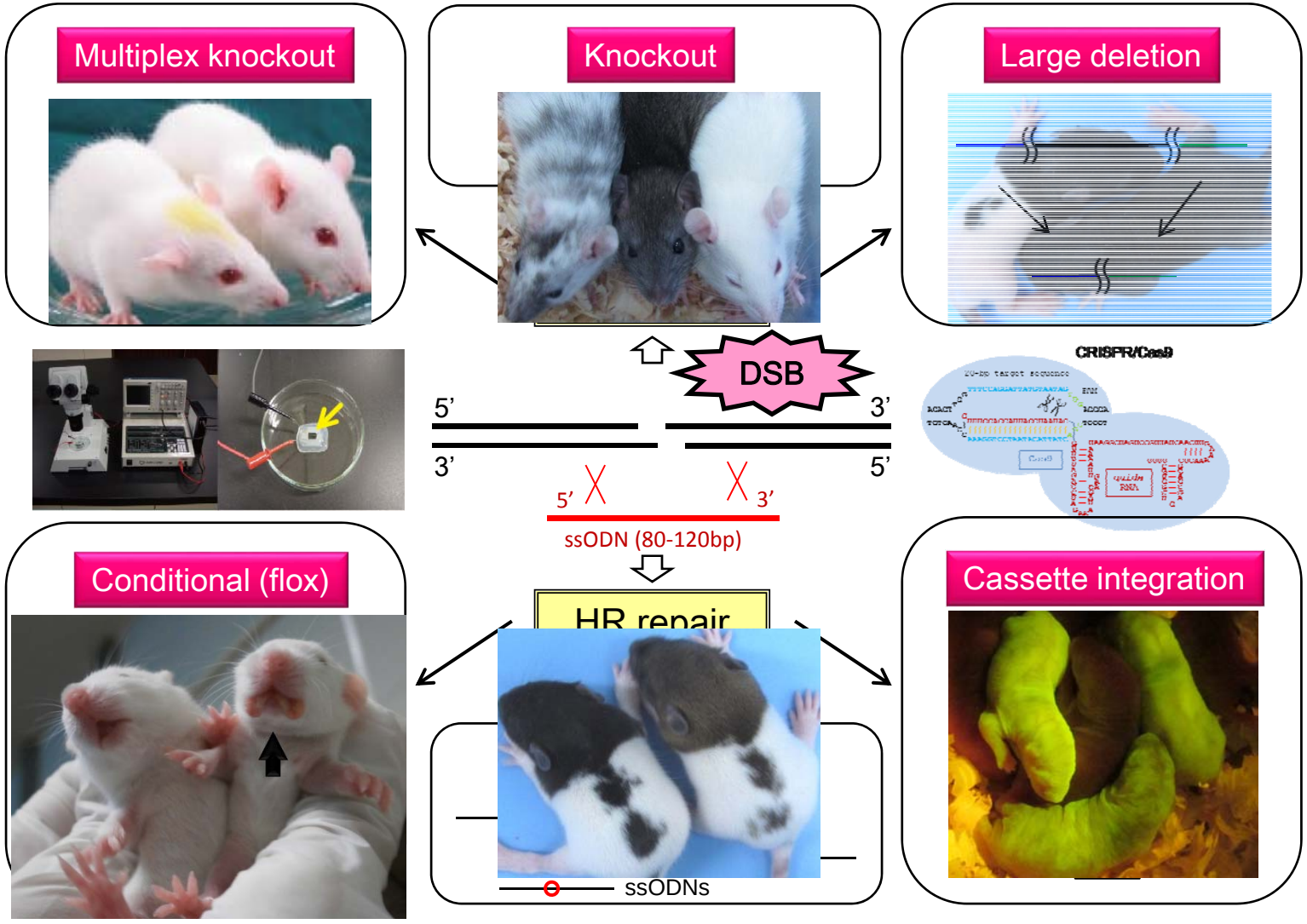
CRISPR (lssDNA)

gRNA, Cas9, lssDNA → CLICK → flox×Cre → CKO

Cre-expressing embryos

gRNA, Cas9, lssDNA → CLICK → CKO

Genome engineering in mouse zygotes

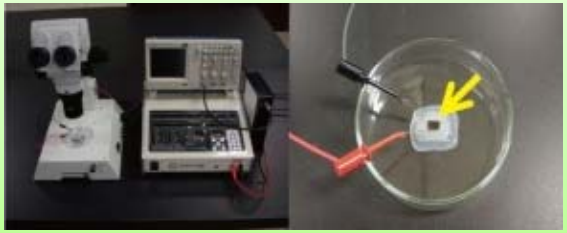


（**Miyasaka** ***under review***）

Genome engineering in mouse zygote !

	Knockout	ssODN Knock-in	CLICK !	2H2OP Knock-in
Goal	Knockout gene	SNP Tag, LoxP ～50 bp	Flox alleles	GFP、Cre BAC、Replace ～200 Kbp
Repair	NHEJ	SDSA	SDSA	NHEJ + SDSA
Efficiency	60-90%	20-40%	10-20%	5-30%
Advantage	◎ E ◎ M ○ Ar △ Inj		ise ple ne	○ Simple ◎ Large DNA △Precise ?

Electroporation



(Kaneko *et al.* *Sci Rep* 2014)

Yoshimi 2016 Nat Commun

Severe combined immunodeficiency (SCID) Rats & Rabbits

Rag2 & Il2rg KO rats

SCID **F344**

NBRP-Rat

www.anim.med.kyoto-u.ac.jp/nbr

Foxn1 or Il2rg KO rabbits

FOXN1_KO_F0-12

KITAYAMA LABES CO.,LTD.

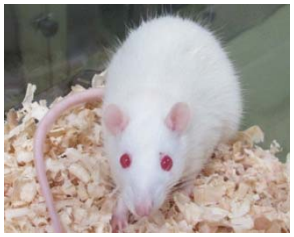
www.oyc.co.jp/company/group.html

Human cell or tissue xenograft models (Humanization) !

Humanized rats

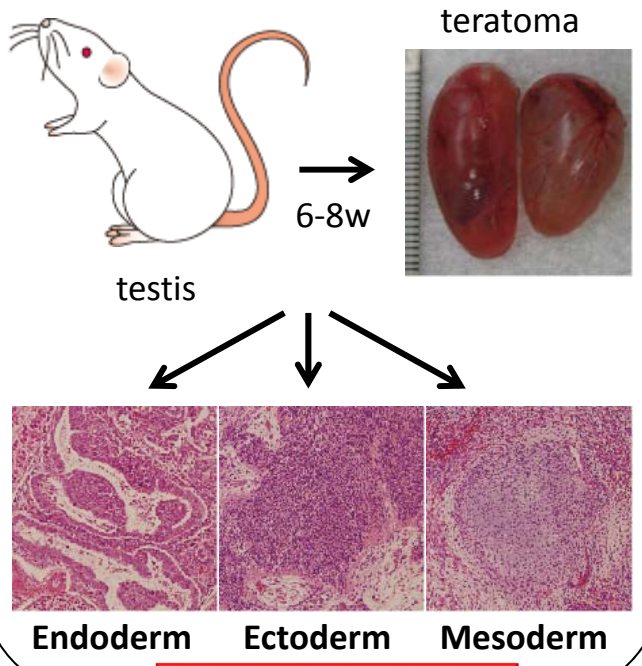
Humanized animals:

Mice carrying human cells, tissues, and/or organs.
Immunodeficient animals are often used as recipients.



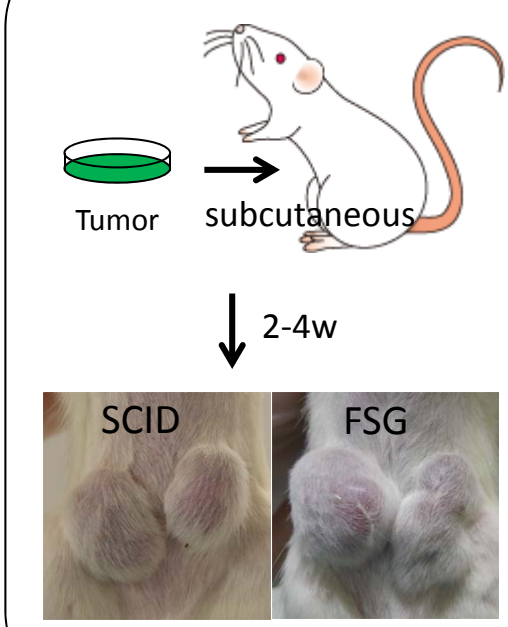
FSG rats

iPS cells



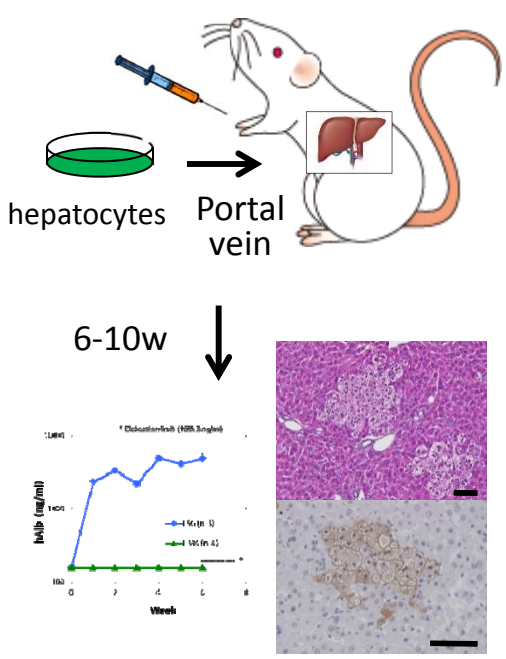
Teratoma Assay !

Tumor cells



Tumor-bearing !

Hepatocytes



Liver humanized !

(Mashimo *et al.* Cell Reports, 13 Sep 2012)

Genome Editing Research and Development (R&D) Center

▶アクセス ▶お問い合わせ

大阪大学大学院医学系研究科附属共同研
ゲノム編集センター

HOME センター長のご挨拶 センターについて 受託解析サービス部門 モデル動物開発部門 研究紹介

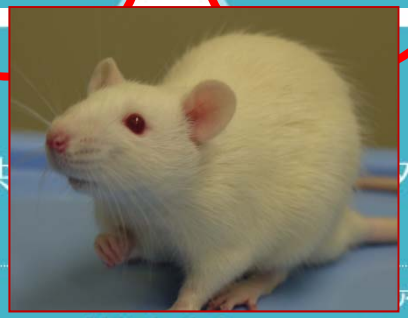
大阪大学大学院医学系研究科共同研附属 ゲノム編集センター

Genome Editing Research and Development Center (GERDC) launched in 14th December 2016

http://www2.med.osaka-u.ac.jp/gerdc/

受託解析
サービス部門

モデル動物
開発部門



http://www.iexas-osaka-u.jp/index.html

Acknowledge