Improved Efficiency of CRISPR/Cas9 Genome Editing in Rodent Models Through Optimized Microinjection Components Validation

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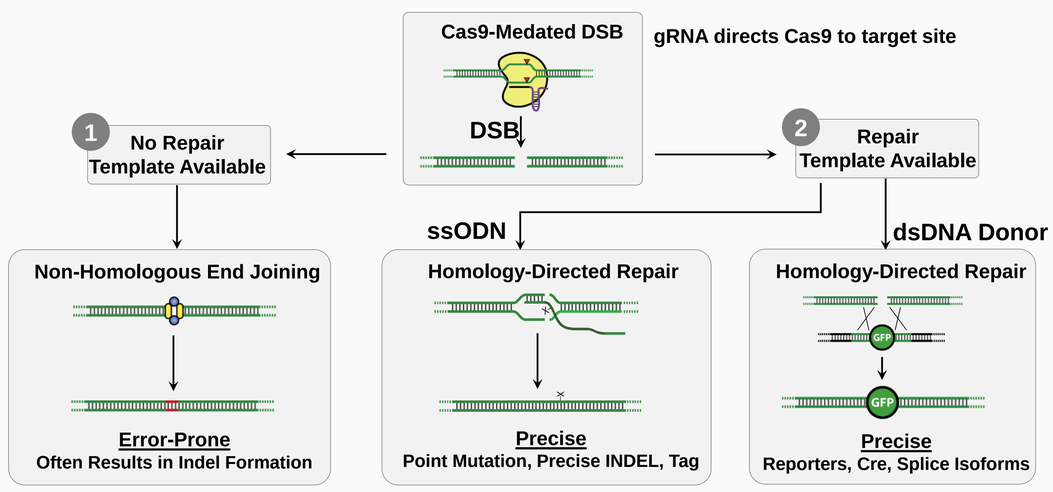
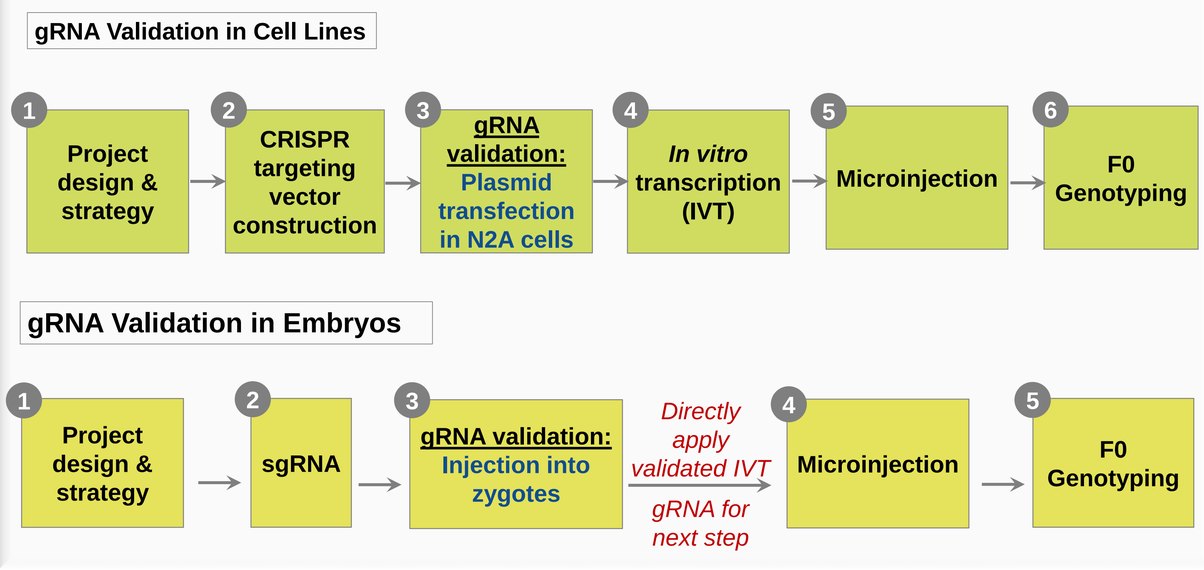
Introduction

- Animal Models are crucial to understanding genetic mechanisms *in vivo* settings.
- CRISPR/Cas9 genome editing technology has offered a highly-efficient method for the development of animal and cell models.
- CRISPR/Cas9 technology is highly precise for generating knock-in, knockout and point mutation rodent models.
- However, the efficiency and success rates vary dramatically among different laboratories due to variability in protocols, designing and construction of CRISPR elements.
- Here, we demonstrate that using gRNA validated in embryos (*in vivo*) as opposed to in cells (*in vitro*) offers a consistent and efficient outcomes of CRISPR/Cas9 technology in producing rodent models:
  - We compare efficiency of generating two mouse models using our optimized embryo-validated (blastocysts) gRNAs and cell line-validated (N2A cells) gRNAs.
  - Since application of this optimized procedure, we have been achieving consistently high efficiency in generating knockout (KO), conditional knockout (cKO), point mutation (PM) and knock-in (KI) mouse (and rat) models.

Cas9-Mediated Genome Engineering



Experimental Design for Embryo-Validation & Cell Line-Validation of gRNAs



Comparing Efficiency of Point Mutation Model Generation Using Cell Line Vs. Embryo Validation Methods

Gene: Mutation	Cell Line Validation				Embryo Validation			
	# Injections	# Total mice born	# Positive founders	% Mod. Efficiency	# Injections	# Total mice born	# Positive founders	% Mod. Efficiency
Autism gene: A350V	3	97	1	1.0%	1	33	13	39.3%
Neuronal gene: S338A	4	73	0	0.0%	1	34	15	44.1%

- % Modification efficiency = # of positive founders/ # of pups born
- % Modification efficiency in projects using cell line validated gRNAs is significantly lower compared to projects using embryo-validated gRNAs

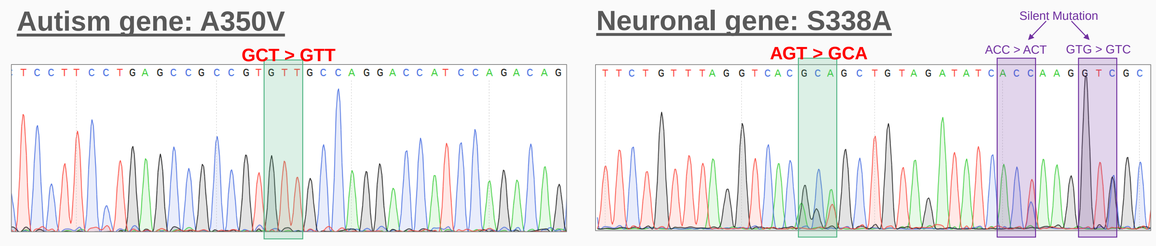


Figure. Sequence chromatogram of representative founder mice generated using embryo-validation gRNAs

CRISPR/Cas9-Mediated Gene Editing in Mouse Models Using Embryo-Validated gRNAs

Models	# Embryo Injections	# Embryo Transfers (%)	# Pups Born (%)	# Positive Founders (%)
KO*	144	83 (57.6)	17 (20.5)	9 (52.9)
PM	156	126 (80.8)	37 (29.4)	11(29.7)
cKO-1st LoxP	120	88 (73.3)	26 (29.5)	13 (50.0)
cKO-2nd LoxP (retargeting**)	213	147 (69.0)	29 (19.7)	6 (20.7)***
KI	256	178 (69.5)	33 (18.5)	8 (24.2)

* Knockout (KO) models are generated using a donor-free targeting scheme.

** Conditional knockout (cKO) mouse models are generated by inserting two LoxP sequences using a sequential targeting strategy: (1) The 1st LoxP site is inserted either upstream (5') or downstream (3') of the region that needs to be floxed; (2) The single LoxP+ mice thus generated are used to produce zygotes for re-targeting and inserting the 2nd LoxP; (3) If heterozygous LoxPs are present at both locations, it is necessary to breed the founders with WT mouse to confirm the LoxPs are on the same allele.

*** Efficiency of generating cKO mice with two LoxP sites inserted at targeting site (double positive)

Comparing gRNA Validation in N2A Cells Vs. Blastocysts

Criteria	N2A Cells	Embryos/ Blastocysts
gRNA used for validation	Plasmid	*In vitro* transcribed sgRNA
gRNA used for injection	sgRNA (different from validation)	sgRNA (same as validation)
Background mouse	Mouse neuroblastoma cells	Same as final mouse model
Validation timeline	~4-6 weeks	1-2 weeks
Correlation between validation and final mouse model	No direct correlation because of different types of cells	Direct correlation resulting in ~100% gRNA/Cas9 modification
Work procedure	More steps needed: build plasmid	Fewer steps
# Injections	Unpredictable; More injections needed	Highly predictable
Efficiency	Lower efficiency	Higher efficiency

Conclusions and Discussion

- Validation of gRNAs is a key parameter for successful genome modification in mouse (and rat) models when using the CRISPR/Cas9 technology.
- Validation of gRNA should be done in the embryos/blastocyst isolated from the same strain in which the desired animal model is to be generated.
- For generating the final animal model, successful gRNAs should be in the same form as that used for the validation assay. This embryo-based validation method also serves as a quality control step for the reagents being used for microinjection.
- With embryo-validation there is a strong correlation between gRNA activity and modification efficiency: higher gRNA activity results in higher efficiency of genome modification.
- Validation of gRNA in blastocysts is a fast and efficient method to provide consistent and higher efficiencies for genetically engineering mouse models.