Generation of Knockout, Knock-in, and Humanized Mouse Models Using the CRISPR/Cas9 Technology: Lessons Learned and Open Questions

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The CRISPR/Cas9 genome editing system has established itself as a versatile technology for inducing precise genetic alterations in a different species and has proved to have the potential to increase the efficiency and speed of producing genetically engineered models of human disease. At Taconic Biosciences, we use both in vivo strategy utilizing one-cell embryos and a complementary in vitro strategy utilizing embryonic stem (ES) cells to generate both mouse and rat models with CRISPR/Cas9. We provide a broad data-set to illustrate our experiences using these two strategies within our production pipeline.

The major advantages of in vivo gene editing using CRISPR/Cas9-based methods are the significantly reduced time frame and effort involved in establishing new mouse or rat models and the ability to utilize almost any available mouse and rat strain. The relative simplicity of this method compared to ES cell-mediated approaches facilitates the generation of founder animals with reduced costs and effort, providing an attractive alternative to homologous recombination-based approaches. We have implemented CRISPR/Cas9 technology in vitro for the accelerated generation of knockout and simple knock-in (e.g., point mutations and small tags) mice and rats and successfully generated more than 200 models in the past three years. However, the genetic modifications that can be introduced in the genome by the in vitro approach are currently limited to relatively simple allelic configuration, such as single base substitutions, gene deletion, and insertion of short sequences. To overcome this limitation, we have combined the use of CRISPR/Cas9 technology with the advantages of utilizing large targeting constructs in ES cells. The combination of the two technologies allows for the generation of large and complex alleles with the precision and efficiency provided by the CRISPR/Cas system and, importantly, the humanization of specific loci in the mouse genome by gene replacement to create relevant model for preclinical drug testing.

Gene Knockout (KO) in Embryo

- **KO by deletion**
- **Mouse Genomic Locus**
- **Targeted Allele (after CRISPR/Cas9-mediated Gene Editing)**
- **Constitutive KO-PM Allele (after CRISPR/Cas9-mediated Gene Editing)**
- **Life Born Rates of 43 Knockout Projects**

Knock-in (KI) of a Point Mutation in Embryo

- **Mouse Genomic Locus**
- **Minigene Insertion**
- **Constitutional Humanized Allele (after CRISPR/Cas9-mediated Gene Editing)**
- **Homologous Recombination Rate of 15 Humanization and Knock-in Projects**

Advantages of Humanization by Genomic Replacement

- Successful means that the humanized allele has an expression of a similar level (60% or more) of the endogenous mouse gene and the same pattern of expression as measured by RT-qPCR.
- While Minigene Insertions and Open Reading Frame (ORF) Exchanges are easy to accomplish, the lack of regulatory elements within intronic regions often compromises the expression levels and patterns.
- We recommend genomic replacements as these seem to resemble expression-levels and patterns most closely.

CRISPR-mediated KO Approach: Percentage of Modifications in Embryo

- Deletion efficiency is highly variable and dependent on gRNA sequence.
- Deletion efficiency is independent of the size of the deletion.

CRISPR-mediated KI Approach: Percentage of Modifications in Embryo

- Homologous recombination efficiency is highly variable and dependent on the gRNA sequence.

CRISPR-mediated Tag-KI Approach: Percentage of Modifications in Embryo

- Tag sequences of up to 67 nucleotides were inserted using long oligomucolides.

Analysis of Off-target Effects

- For all CRISPR/Cas9 embryos and CRISPR in ES cell projects, off-target predictions were performed according to the number and position of mismatch within the gRNA sequence.
- For 12 CRISPR in ES cell projects and 45 CRISPR in embryo projects, potential loci were analyzed by PCR and sequencing.
- Within 505 loci of 31 ES cell clones and 310 loci of 555 G1 mice, only three off-target effects were detected.
  - The first off-target in embryos with two deviations in the seed and one deviation in the non-seed region.
  - The second off-target in ES cells with two deviations in the seed and one deviation in the non-seed region.
  - The third off-target in ES cells with no deviation in the seed and two deviations in the non-seed -region (only present in one out of three validated clones).

- The off-target frequency with our setup is smaller than 0.4% and lower than the spontaneous mutation rate detected per generation or passage.

CONCLUSION

- CRISPR/Cas9 is a powerful tool that can speed up model generation from simple point mutations to large knock-ins and humanizations.
- While we can predictably control the design of each model, the efficiency of CRISPR/Cas9 strongly depends on the selected gRNA.
- Despite various prediction tool, the relation between gRNA-sequence and cleavage-efficiency remains largely unknown.

- Off-target effects are a controllable risk in our setup.

Protocol for CRISPR-mediated Gene Editing in ES Cells

- **Transfection of ES Cells to Introduce a Targeted Mutation via CRISPR/Cas9-mediated Gene Editing**
  - ES cells (e.g., C57BL/6NTac or BALB/c) were grown on a mitotically inactivated feeder layer comprised of mouse embryonic fibroblasts in ES cell culture medium, Cad9, the specific gRNAs, and the targeting vector were co-transfected into cells along with a plasmid for the expression of the respective selection cassette. One day post transfection the antibiotic was transiently added to the medium to select for transfected cells. ES cell clones were isolated as soon as they show a distinct morphology and were analyzed by PCR in a primary screen for recombination at the 5’ and 3’ side. Homologous recombinant ES cell clones were expanded and frozen in liquid nitrogen after extensive molecular validation by PCR and southern blot analysis.

Protocol for CRISPR-mediated Gene Editing in Embryo

- **Pronuclear Injection**
  - After administration of hormones, superovulated C57BL/6J females were mated with C58BL/6J males. One-cell stage fertilized embryos were isolated from the oviducts at dpc 0.5. For microinjection, the one-cell stage embryos were placed in a drop of M2 medium under mineral oil. A microinjection pipette with an approximate internal diameter of 0.5 micrometer (at tip) was used to inject the mixed nucleokid into the pronucleus of each embryo. For knockout projects, the mix contained two specific gRNA and the Cas9-protein. For knock-in projects, an oligonucleotide was injected together with the appropriate gRNA. After recovery, 25-ES injected one-cell stage embryos were transferred to one of the oviducts of 0.5 dpc, pseudo-pregnant NMRI females.

- **Founder Analysis**
  - Founder animals were genotyped for presence of the deletion or the inserted point mutation (and inserted restriction site). PCR samples per founder were subcloned and up to four clones were analyzed by DNA-sequencing.

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