Class 22: CRISPR-Cas9 genome engineering

Reading assignment

- Horii T, Arai Y, Yamazaki M, Morita S, Kimura M, Itoh M, Abe Y, Hatada I. Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. Sci Rep. 2014 4:4513

Classroom activity (limit 45 minutes)

1. What aspect(s) of the biology of CRISPR-Cas9 made the mode of delivery of of the complex into mice an issue that needed to be addressed? What gene did they attempt to alter?

   The two components of the complex are produced in the cytoplasm (Cas9) and the nucleus (sgRNA) and need to come together in the nucleus as a complex to target the DNA. Injecting them into either the pronucleus or the cytoplasm produces a problem for one of the two components. Also, introducing DNA into the pronucleus could result in it being inserted into the genome, which was not desired. The gene they targeted was Tet1, encoding a DNA demethylase.

2. How did the authors demonstrate that the guide RNA they designed for this experiment was specific? What experiment did they use to show that the mutation had been introduced into the target gene? What efficiency did they achieve?

   They showed that the authentic guide RNA but not a control RNA could target the desired gene. The sgRNA targeted a position in the gene that includes a SacI restriction site (GAGCTC) immediately adjacent to the PAM motif. Most mutations were expected to result in loss of all or part of that site, resulting in loss of cleavage by SacI. They showed that in 55% of the cases the site was lost.

3. What three modes of delivery of the CRISPR-Cas9 complex did the authors test? What are the important differences among the delivery methods?

   The three delivery methods were (1) DNA encoding the sgRNA and Cas9 into the pronucleus, (2) injection of RNA encoding these products in to the pronucleus and (3) injection of this RNA into the cytoplasm. In the first case the DNAs must be transcribed and, for Cas9, translated. For the second, the Cas9 RNA must move to the cytoplasm to be translated. In the third, the sgRNA must move to the nucleus along with the Cas9 translated in the cytoplasm..

4. How did the various methods of delivery affect the survival of the injected pups during development? How did the authors explain the greater effect of some methods compared to others?

For the exon 4 experiment, the two methods of pronuclear injection produced fewer pups (20-30%); the third method of RNA into the cytoplasm produced 2 to 3-times as many (60%). The second and third methods produced exclusively or a large majority of double mutant pups. The authors suspect that injection into the pronucleus is the problem for viability presumably because the injection frequently damages the pronucleus.

5. What was the phenotype of the double mutant pups in this experiment? What percentage of the successfully homozygous mutants had that phenotype?

The double mutant phenotype is pups that are slightly smaller than the wild type or the heterozygote. All of the successful homozygotes had the phenotype.

6. What types of mutations did the authors observe? How did they explain the fact that so many of the mutations were identical in structure given that all the mutants were created independently?

Most of the mutants were 9 bp deletions, mostly identical, along with some small insertions and a longer deletion. The identical mutants occurred by removal of a 9 bp region between microrepeats of the sequence 5'-TCA-3' or 5'-GG-3'. The authors attribute the frequent identity to a deletion involving the repeated sequences.

7. How do the authors explain the high efficiency of cytoplasmic delivery of CRISPR RNA and Cas9 given that the site of action of the complex is in the nucleus? Why do you think that so many of the mice were homozygous for the mutation, eliminating the need to cross mutant mice to generate homozygotes from two heterozygous parents?

The authors suggest that the sgRNA and Cas9 either are transported across the nuclear membrane or that they enter after the breakdown of the pronuclear membrane. In the exon 4 experiment, the second and third methods produced essentially all double mutant pups. The first method of DNA into the pronucleus produced only 20% doubled mutants. Apparently the efficiency of the second and third method is so high that the CRISPR complexes could quantitatively mutate both chromosomes