

## Genetic engineering in mice

Reading: 8<sup>th</sup> ed pp 92-95; 348-357. 9<sup>th</sup> ed Chp 8: 300-311

Learning Goals

Be able to:

1. Describe the properties of the mammalian blastocyst and the events that generate the inner cell mass vs. the trophoblast.
2. Compare the technique and outcome of generating transgenic mice made by injection of a cloned gene into a pronucleus at the 1-cell stage and by homologous recombination in embryonic stem cells (ES cells).
3. Explain how homologous recombination in ES cells is used to generate knock-out, knock-in, or Cre-lox mice.
4. Interpret the outcome of experiments using ES cells with specific genotypes, including being able to determine relative percentages of embryos that will have certain genotypes.
5. Design constructs and/or interpret the outcome of using certain constructs in the generation of knock-out, knock-in, or Cre-Lox mice.

**The mouse as a model system for research**

Laboratory mice have a generation time of about 8 weeks and a genome size about the same as humans, 3000 Mb (about 30 times *C. elegans* or 15 times *Drosophila*), carried on 20 chromosomes. Mice can be mutagenized with X-rays or with chemical mutagens such as ethylnitroso-urea (ENU), which tends to induce single base changes and is administered by peritoneal injection into males. Many induced or spontaneous mutations have been mapped and studied over the past 100 years, including some that result in developmental defects. However, because of the expense of maintaining large populations and mutant stocks, saturation screening for a phenotype of interest as with *Drosophila* or *C. elegans* has been only infrequently attempted. Moreover, mapping and cloning of mutationally identified genes (forward genetics) is more difficult because of the longer generation time and much larger genome size, although modern SNP mapping techniques make this easier. However, the mouse has become an indispensable model system for biomedical research. Mice are well suited to manipulation of known genes by reverse genetics, and, most important, they are mammals, closely related to humans.

## Early mouse embryonic development

*Cleavage and morula formation*

In mammals, there is apparently little or no pre-patterning of the cytoplasm and little dependence on maternally supplied proteins or mRNAs. Transcription of the embryonic genome begins early, at the 2-cell stage. There is little if any yolk (nutrients are supplied by the placenta), and early cleavages are equal. Compaction, occurring at the 8-cell stage in the mouse, forms a tight ball of cells called a morula, which is unique to mammals. Patterning of the embryo, with commitments to different cell and tissue fates, begins only later, and must depend entirely on cell signaling rather than autonomous determinants.

*Blastula formation*

At the morula stage there are two inner cells, surrounded by a sphere of outer cells connected by tight junctions. As a blastocoel cavity forms, the embryo becomes organized into the inner cell mass (ICM), which will form the embryo and some extraembryonic tissues (“membranes”), and the trophoblast or trophectoderm, the outer ring of tightly joined epithelial cells, which will form the chorion (the embryonically derived part of the placenta).

The ICM organizes into two layers corresponding to those of a chick embryo, the epiblast (embryonic tissues only) and hypoblast (extraembryonic). This stage is called the bilaminar germ disc.

{Mammalian embryos have the same set of extraembryonic membranes as chicks, with similar functions. The most notable in mammals is the chorion, since it fuses with the uterine wall to contribute to the formation of the placenta, which connects the embryo with the circulation of the mother to provide nutrients and remove waste (see slide). The amnion, derived from the hypoblast, is also important, providing a cushioned environment (the amniotic sac) for the embryo.}

#### *Chimeric mice: cells of the ICM are still pluripotent*

Embryonic cells (blastomeres) in the mouse cleavage and morula stages are totipotent. If the outer membrane (zona pellucida) is removed from each of two genetically different morula stage embryos and they are pushed together in culture, the resulting fused embryo can be reimplanted into a foster mother and will develop into a chimeric (mosaic) mouse, with some tissues derived from one embryo (set of parents) and the rest derived from the other embryo (set of parents). Such mice are technically tetraparental chimeras; they generally have equal numbers of cells derived from each of the two blastulae.

At the blastocyst stage, cells of the ICM are still pluripotent, but no longer totipotent. Although they can give rise to any of the tissues in the animal, they can no longer contribute to the trophectoderm. (Conversely, trophectoderm cells are committed to that fate and can no longer contribute to the embryo.)

Because the ICM cells are still pluripotent, unequal chimeras can be made by microinjection of dispersed ICM cells from one embryo into the blastocyst of another, where they become part of the host ICM and contribute to tissues of the embryo and the resulting mouse. This observation and the subsequent finding that ICM cells could be maintained in culture as embryonic stem cells (ES cells) led to the technologies for making transgenic mice with targeted alterations of specific genes, as well as methods for manipulation of stem cells in culture for potential tissue engineering applications. We will consider these technologies in the next two lectures.

### **Transgenic mice**

#### Introduction of cloned genes.

The mouse became a much more useful organism for developmental geneticists with the advent of techniques for introducing cloned DNA sequences into the germ line. DNA microinjected into one of the pronuclei of a mouse egg just after fertilization usually integrates prior to first cleavage *at one apparently random chromosomal site*. If the embryo is reimplanted into a foster mother, all cells of the resulting animal carry the integrated sequences, which are generally present in multiple copies arranged in tandem head-to-tail arrays. Integrated genes are generally expressed, although the level tends to vary from one transgenic mouse to another, presumably due to position effects of the integration site (what can cause position effects?). However, intact genes are often expressed with relatively normal temporal and tissue specificity.

#### Uses of mice with randomly inserted transgenes

- *Reporter constructs (lacZ or GFP)*: If the transgene includes its normal regulatory elements, the reporter will show where the gene is expressed, just as in flies and worms.
- *Transgenic rescue: "gene therapy" for a defective gene*. Transgenic animals carrying a normal copy of the gene (or the homologous gene from another species) as a transgene can be bred into a mutant background and tested for rescue of the mutant phenotype. Rescue by a gene from another species is strong evidence for functional homology.
- *Introducing any engineered gene from another species, such as the Cre recombinase under control of a tissue specific promoter*. See explanation of the Cre-Lox technique at the end of these notes.
- *Artificial regulation of expression*: Mixing and matching of genes and regulatory elements allows excess production of a normal RNA, its production in a tissue where it isn't normally made, or the production of an mRNA encoding something abnormal. Thus, dominant (or dominant negative) mutations can be created, and the phenotype of the resulting animals studied as a means of analyzing gene function *in vivo*. In a classical example, which provided proof of principal in the early days of transgenics, a transgene containing the mouse

growth hormone gene was fused to regulatory elements of the gene for metallothionein (MT). MT is an inducible detoxification protein that binds strongly to and protects the animal against heavy metal ions; it is normally induced and synthesized at high levels in liver in response to heavy metals in the diet. Feeding these transgenic mice small amounts of heavy metals caused them to produce high levels of growth hormone, bypassing the normal controls on growth and resulting in very big mice, about twice the normal size!

### Targeted mutagenesis by homologous recombination

The most useful transgenic technology in mice, and one with many possible broader applications, has been targeted gene replacement by homologous recombination (HR). This technique allows “reverse genetic” manipulations of known genes *in their normal chromosomal location* in the animal, including both gene knock-outs and gene modifications (“knock-ins”). Achievement of homologous recombination with transgenes depended on the finding that ES cells could be cultured *in vitro* and then added back to the ICM of a recipient blastocyst, where they would contribute to all tissues of the resulting fetus (including the germ line) when the embryo was reimplanted to allow development. DNA introduced into mammalian cells tends to integrate randomly into chromosomal locations wherever single strand breaks (nicks) in the DNA happen to be present. Integration by HR occurs more rarely and must be screened or selected for, which is impractical to do with whole embryos. However, ES cells in culture can be transfected with the desired transgene, selected for transgene incorporation using an appropriate marker, and tested for homologous recombination before reintroducing into host blastocysts and implanting in a pseudopregnant foster mother to produce a chimeric mouse. Since the introduced ES cells have the potential to contribute to all tissues of the animal, some of these chimeras will have transgenic cells in the germ line, making them useful for further breeding experiments. These chimeras are generally made and identified as described below.

### Making “knock-out” mutant mice

- 1) ES cells from mice homozygous for the dominant coat color gene *black* are cultured and transfected with a linear DNA construct carrying the desired transgene, a selectable marker such as the bacterial neomycin resistance (*neoR*) gene that can be selected for (using neomycin or G418), and often at one end a herpes viral thymidine kinase (TK) gene that can be selected against (using the anti-herpes drug gancyclovir). Cells are first selected for incorporation of the transgene (conferring neomycin resistance) using G418. Then they are counterselected for homologous recombination (leading to loss of the TK gene) using gancyclovir. The selected colonies of cells are then screened by PCR to verify insertion by homologous recombination. (What will be the genotype of these cells at the *black* locus? At the transgene locus?).
- 2) Cells that pass these tests are then introduced by injection into blastocysts from a white (i.e. *black -/-*) strain of mice, and these embryos are implanted into pseudopregnant mothers. Chimeric progeny mice (identified by black and white patched coat) are checked for presence of the transgene by PCR and crossed to white mice, to determine whether their germ lines include transgenic (*black -/-*) cells.
- 3) Some of the Black progeny from these crosses (*black +/-*), will be heterozygous for the transgene (what fraction?), and can be identified by PCR analysis.
- 4) To determine the effect of the new mutation, heterozygous mice are interbred and their progeny analyzed for a phenotype. If the initial transgenic construct was engineered to contain an inactive form or deletion of the gene (i.e. a null allele), the resulting homozygous transgenic animal is called a "knockout" mutant.

### “Knock-ins”

Using the HR technology, *any* sequence can be introduced into a genetic locus, including variants of the original sequence. The term "knock-in" refers to replacement of a normal gene not with a deleted gene, as in a knock-out, but with an altered form of the original, or with a different gene. The *neoR* gene can be placed in an intron, downstream, or removed after isolation of ES cells having the targeted mutation so that it does not interfere with subsequent function of the knocked-in gene.

One powerful example is the use of knock-ins to test for functional homologies (or differences) between related genes. For example, it has been shown that the protein coding sequences of *myogenin* and *Myf-5* (both basic HLH transcription factors in the same gene family) are functionally equivalent. If the coding sequences at the *myogenin* locus are replaced by those of *Myf-5* in a mouse's genome, myogenesis is normal. A similar experiment was done with the mammalian homologs of *Drosophila engrailed*, *En-1* and *En-2* (these are both homeodomain transcription factors). It was found that knocking *En-1* into the *En-2* locus gave a normal mouse (*En-1* null mutants die at birth with a large portion of their mid- and hindbrain missing). Think about it - is this surprising? What does it suggest about multigene families of developmental regulators in vertebrates?

Knock-ins can also be used to introduce essentially any desired mutation into the mouse. This application has been particularly useful in creating mouse models for human genetic diseases, where the molecular lesion in a human disease allele is known. For example, a point mutation in a gene known to cause Alzheimer's, or any other disease, can be introduced into the mouse, often creating a mouse model for the disease. This illustrates the remarkable flexibility of mouse reverse genetics with present state-of-the-art technology: literally *any* desired mutation can be generated!

### **Tissue-specific knock-outs: "Cre-lox"**

If a null mutation causes embryonic lethality, it is not possible to directly observe the consequences of that mutation in an adult animal. If a gene is expressed in multiple tissues or at multiple times during development, it will often be desirable to create animals mutant in only a specific tissue in order to ask where and when gene function is required. This can be done using the Cre-lox system, named for a bacteriophage site-specific recombinase (*Cre*) and the 34-bp tandem repeat sequences (*lox*) that it recognizes. Neither of these two sequences are normally found in mouse; their functions are simply borrowed to create transgenic mice whose DNA will essentially be reorganized under control of the Cre recombinase. When the Cre enzyme is created, it binds to the *lox* sequences, mediating an excision of the DNA between the two lox sites. To use this system, generally, two strains of mice are generated. Using HR, an exon of the gene of interest is replaced with a modified exon flanked by two *lox* sequences. Because there is normally no *Cre* recombinase activity in mice, the gene flanked by lox will be unaffected most of the time. This is the "lox" mouse. Then, another strain of mice is generated that is transgenic for the *Cre* gene. A construct containing *Cre* driven by a carefully selected promoter is used to generate this transgenic mouse (the "cre" mouse). Now, if the two strains of mice are mated together, the Cre recombinase will be made under the control of whatever was chosen as its promoter, and only in those tissues where Cre is generated, will the gene of interest be removed through cutting out the Lox sites. Interbreeding of such progeny mice to produce mice that carry the tissue-specific *Cre* gene and are homozygous for the mutant transgene allows analysis phenotypes resulting from loss of the gene in just that tissue. Similar experiments can be done using a stage-specific promoter for *Cre*, to ask about the gene's function at different stages in development. Technically, a single strain of mice could be produced from the beginning containing both the cre and the lox sites. However, because the generation of such mice is complicated and expensive, groups of scientists often create different lines of transgenic Cre mice that will express Cre in different tissues. Then the scientists can share the lines of mice to generate a tissue-specific knockout of their particular gene of interest.

This potentially very powerful technique can also be used to investigate the functional role of individual regulatory elements (by deleting them in a particular tissue), to selectively turn on a target gene by deleting a negatively acting regulatory element (silencer element), or simply to "clean up" a transgenic organism by removing marker genes (e.g. *neoR*) that were introduced during its construction.

### **Review Questions**

- 1) What are some reasons that transgenic animals might be useful for experimental analysis?
- 2) What are the methods for introducing transgenes into the mouse germ line, advantages and disadvantages of various methods, and some applications of transgenic technology?

- 3) What is the difference between a chimeric and a transgenic animal?
- 4) What is homologous recombination (HR)? How are ES cells carrying a targeted knockout or knock-in mutation of any cloned gene obtained?
- 5) What are the steps in obtaining a homozygous mutant mouse (or embryo) for analysis following targeted knockout of a gene in ES cells.
- 6) Why do you need to test mice of a particular phenotype (coat color) in the process of generating chimerica and transgenic mice rather than relying only on their color to predict their genotype?
- 7) How is this procedure used to make a mosaic mouse with Cre-lox technology?
- 7) What kinds of questions can you answer using the Cre-lox technique as opposed to a straight knock-out?
- 8) What is a knock-in, and why would making one be valuable?