Lecture 19 - Mouse Transgenesis (AMG text pp. 214-223)

Mouse transgenesis
Two approaches have been developed to generate transgenic mice:

1) DNA microinjection into single cell embryos to produce transgenic mice containing one 
or more copies of randomly integrated expression vector DNA.

2) Use of homologous recombination at defined genomic loci to create gene knockouts 
(KOs) or replacements.

Generating transgenic mice using fertilized egg cells
Purified experimental DNA is directly injected into the male pronucleus of a fertilized 
mouse egg using a microscope-mounted manipulator and microneedle. The three essential 
instruments for embryo injection are an inverted microscope with stage magnification of 
400X, a micromanipulator-mounted negative pressure holding pipette (left) and a 
micromanipulator-mounted injection pipette (right).

The male pronucleus of a fertilized egg is injected with picoliter amounts of purified DNA 
(1ug/ml) by stabilizing the egg using the holding pipette. Success depends on the use of 
properly timed fertilized eggs and accurate injection of the male pronucleus.

Fertilized one-cell eggs for DNA microinjection are obtained from superovulating female 
mice that have been mated to a stud male. Following microinjection, 20-30 eggs are 
implanted into the oviduct of a pseudopregnant surrogate female (recently mated to a 
avasectomized male) leading to the production of ~5-8 live pups 19 days later. Transgenic 
littermates are identified by PCR analysis of tail samples performed at three weeks of age.

Mice that score positive by PCR analysis are backcrossed to non-transgenic mates to 
identify founder animals containing germline DNA integration that results in a Mendelian 
inheritance pattern of the transgene.

Cell-specific promoters can be used to direct expression of a foreign gene to tissue 
subtypes in transgenic mice. An experimental flow scheme is shown to illustrate how a 
prostate-specific promoter could be used to generate a transgenic mouse model of prostate 
cancer. Northern blot analysis is needed to identify suitable founder mice.
Characterization of prostate tissue in the offspring of founder mice would be done to 
determine how well the transgenic model mimics human prostate cancer.

What would explain the lack of gene expression of an integrated transgene in a founder 
mouse that tested positive by DNA analysis through several generations?

What could account for high levels of prostate-targeted transgene expression in kidney cells 
as observed in the P01 founder mouse in the example above (figure 8.7)?
How could you use a cell-specific transgene construct to investigate activation of a liver-specific promoter during mouse embryonic development, i.e., what would the transgene construct consist of?

Gene knockouts by homologous recombination in ES cells
Two methodological breakthroughs were required before transgenic "knockout" (KO) mice could be created that contained germline insertional loss-of-function mutations:

1) Procedures to cultivate pluripotent mouse embryonic stem (ES) cells that could be combined with non-transgenic mouse blastocysts to produce a chimeric embryo

2) The development of DNA cloning vectors and cell line screening strategies that allow the identification of transfected cells that have undergone a homologous recombination event following transfection with experimental DNA.

A pluripotent ES cell line is derived from the inner cell mass (ICM) of blastocyst embryos obtained from the uterus of a female mouse 3 days post-fertilization. ES cells are cultured on plates containing non-dividing embryonic fibroblasts called "feeder cells".

Experimental DNA is stably transfected into ES cells by electroporation or lipofection using vectors containing a selectable marker. PCR screening strategies and Southern Blotting are used to identify clonal ES sublines containing targeted gene knockouts.

Differences in the coat color of ES donor mice and blastocysts host mice are used to identify transgenic founder animals. The offspring have a mosaic coat color reflecting their chimeric genotype. Transgenic founder lines are identified as agouti offspring that arise from crossing chimeric and albino mice.

Improvements in the targeting vectors and screening strategies used to create transgenic KO mice have led to two basic strategies.

1) A gene insertion process that requires a cross-over event between two ends of the targeting vector and the genomic sequence.

2) A gene replacement strategy using vectors that contain two selectable markers that permit detection of double-cross over events in the target gene.

Gene insertion leads to disruption of the genomic gene copy due to a duplication of the targeting sequences and incorporation of the neo coding sequence.

Gene replacement vectors contain both positive (neo-resistance) and negative (HSV tk) marker genes that are used to identify drug-resistant ES cells containing a predicted double cross-over event in the target locus. Ganciclovir (GC) is a suicide nucleotide analog that is phosphorylated by the HSV tk gene product, but is not a substrate for mammalian thymidine kinases.
The frequency of homologous recombination is \( \sim 1\% \), based on comparing the number of G418-resistant colonies to the number of G418- and ganciclovir-resistant colonies. This corresponds to an overall homologous recombination frequency of \( \sim 10^{-5} \), when considering the total number of ES cells transfected.

Factors influencing the frequency of homologous recombination are:

1) the length and arrangement of homologous sequences in the targeting construct.

2) the extent of homology between genomic sequences contained in the targeting vector and the target gene.

3) the chromosomal location of the gene target.

Transgenic mouse models of human diseases
Mice have 19 autosomal chromosomes and one sex-linked chromosome, many of which contain large segments of DNA that are highly conserved between mouse and humans. One of the goals of mouse transgenesis, is to use molecular genetic approaches to create better mouse models of human diseases that are based on known genetic lesions.

A good place to start is the National Center for Biotechnology Information (NCBI) genomic biology page. Extensive lists of transgenic mouse models are available from Jackson Labs in Bar Harbor, Maine.

- What attribute of the agouti coat color mutation facilitates its use as a marker in mouse transgenesis using ES cell technology, i.e., is agouti a loss or gain of function mutation in the parental mouse blastocysts?

- Design a PCR primer pair that could be used to identify homologous recombination of a gene replacement vector containing the neo-resistance gene.

- What is the most likely explanation of no overt phenotype in a homozygous mouse KO, i.e., the KO mouse is normal despite loss of expression of the targeted gene?

- How is it possible to maintain a KO strain of mice in which the gene disruption is lethal?
Lab Practicum 8:
Creating cell-specific gene knockouts in transgenic mice

Based on the recent isolation of the hypothetical mouse gene, bean pole (bpl), a physiology graduate student proposes experiments to test her hypothesis that bpl is required for hormonal signaling in the brain to induce normal feeding behaviors. Since another lab reported that bpl knockout mice are embryonic lethal, for reasons that are obviously unrelated to feeding behavior, the graduate student intends to use the Cre-loxP recombination system to target a bpl exon deletion to the hippocampus region of the mouse brain.

The Cre-loxP recombination system can be used to create transgenic mice with cell-specific deletions of the hypothetical bpl gene. Gene targeting of the bpl gene in ES cells with a loxP replacement vector will be used to generate a heterozygous flox-bpl founder mouse that can be crossed to a homozygous CaMKII-Cre mouse (Cre protein expression is restricted to the brain by the CaM kinase II promoter).

Available Information and Reagents
1. A Cre recombinase transgenic mouse strain has been obtained that specifically expresses Cre in the forebrain as a result of developmental activation of the calcium-calmodulin-dependent kinase II (CaMKII) promoter linked to the Cre expression cassette. This mouse strain has already been shown to direct hippocampus-specific deletion of a loxP-flanked target gene.

2. A bpl targeting vector has been constructed using portions of a 10 kb genomic segment of the mouse bpl gene. This replacement vector contains a loxP site in intron 1 and another downstream of exon 3. In addition, a neo and HSVtk gene are included in the vector to facilitate genetic selection of ES cells containing homologous recombination at the bpl locus. Transgenic mice will be generated and a homozygous flox-bpl offspring will be mated to the CaMKII-Cre mouse.

3. An eating behavior assay has been developed that measures the amount of food eaten, the frequency of eating, and water consumption over a 24 hour period. These quantitative measurements can be combined to give a single value for called the “Blimpie” Quotient (BQ).

Basic Strategy
The only two functional units required for in vivo targeted DNA deletion with the Cre-loxP system are,

1) expression of the P1 Cre recombinase gene, often times by a cell-specific or regulated promoter

2) an integrated DNA segment that is flanked by direct repeat copies of the 34 bp P1 DNA sequence called loxP; loxP-targeted DNA is said to be “floxed”.

The illustration below shows how the bpl-loxP targeting vector could be used to create a homozygous transgenic flox-bpl founder mouse that can be bred with the CaMKII-Cre mouse to produce offspring with one or both bpl genes inactivated in pyramidal cells of the CA1 hippocampal region. The bpl genotype (+/+, +/-, -/-) and phenotype (fat, thin,
starving) is depicted of these cre/flox-bpl heterozygous offspring, and of offspring arising from a cross between cre/flox-bpl heterozygous siblings.

**Prospective**

Based on what is known about other signaling pathways in the brain, and the sequence homology of bpl with other membrane receptors, one future research direction would be to design a binding assay that could be used in a functional expression cDNA cloning scheme to isolated transcripts encoding bpl binding proteins. This could be done using a modified yeast two-hybrid assay to study peptide hormone-receptor interactions, similar to what has been done with insulin-like growth factor 1 (IGF-1) and its receptor (chapter 5). Other research directions to take include, finding the human bpl (hbpl) homologue using either a low stringency cDNA library screening approach (chapter 5) or a GENBANK database homology search (chapter 9). With the hbpl gene in hand, it would be important to determine if any of the previously described human eating disorders are due to defects in hbpl signaling. This could be done using molecular genetic probes, for example PCR genotyping assays (chapter 6), or by using bpl antibodies as immunocytological probes.

What is the advantage of the Cre-lox system for generating KO mice over the standard gene replacement strategy?

Why doesn't the Cre recombinase protein cause DNA damage in mouse tissues in which it is constitutively expressed, e.g., in the brain of CaMKII-Cre founder mice as described in the lab practicum?

What is the significance of the partial phenotype in the heterozygous mouse with regard to the type of genetic mutation that has been created?

How important is quantitating the eating behavior assay to understanding the role of bpl in behavior?

What might it mean if the bpl KO mouse displayed only a partial reduction in weight with only a minimal difference in eating behavior?

**Brown and Blue All Over**

**Background**

A researcher in the basic science unit of a Cardiovascular Research Center recently identified a novel calcium-activated potassium channel as a possible contributor to cerebral chronic high blood pressure and migraine headaches. The clue came from human genetic studies of an isolated population of island-dwelling people that had suffered for generations from chronic cerebral high blood pressure and migraine headaches. Standard gene mapping using SNP analysis of DNA samples from living individuals, and from samples taken from the remains of their deceased relatives, led the investigator to identify a previously uncharacterized human gene on chromosome 11 as the candidate disease
gene. He tentatively named the gene the Arterial Migraine Grinder (AMG) gene. Using mouse-human mapping data and genomic library screening methods, he isolated a 10 kb region of genomic DNA corresponding to the mouse AMG gene. The researcher hypothesized that the mouse AMG gene is required for maintaining basal blood pressure levels and that inactivation of the AMG gene in KO mice would lead to elevated cerebral blood pressure and mouse migraines.

**Research Strategy**

To directly test his hypothesis, the researcher cloned portions of the mouse AMG gene into a Gene Replacement vector for use in a homologous recombination knockout (KO) strategy to inactivate the gene. The pKO-AMG plasmid vector contained the bacterial beta-galactosidase gene which he used to create an AMG-LacZ gene as an in-frame fusion to exon 2 sequences. Following standard gene replacement methods, he generated a founder mouse strain from brown agouti ES cells and bred the mice to homozygosity. Southern blot analysis and in situ cytochemistry was used to confirm that this mouse did not express the normal AMG protein, and moreover, that Lac Z enzyme activity could be detected in cerebral arterial cells in the same pattern as he had observed using anti-AMG antibodies (data not shown). With these mice in hand, he set out to collect physiological data to support or refute his primary hypothesis.

Flowscheme depicting the strategy used to generate the AMG KO mouse that expressed the AMG-LacZ fusion protein in cerebral arteries. Portions of a BamHI-KpnI genomic segment of the mouse AMG gene that were used to construct pKO-AMG are shown as red lines. The position of unique restriction sites and the four AMG exons are also shown.

The pKO-AMG gene replacement plasmid was constructed using the indicated AMG genomic segments (red lines in figure above). The plasmid was linearized at the unique BamHI site prior to electroporation into agouti-derived ES cells. Using the template below, identify the arrows representing non-mouse genes and draw in all the AMG exons in the correct orientation.

What functional element is represented by the "??" in the pKO-AMG plasmid?

List one advantage, and one disadvantage, of constructing a LacZ fusion gene in a gene replacement KO strategy?

Would the BlueScript KS plasmid vector be a good source for the LacZ gene sequences used in pKO-AMG? Explain.

What type of physiological data would the researcher need to collect using the KO mouse strain he developed to support his hypothesis that the AMG gene was responsible for elevated cerebral blood pressure and migraine headaches?