Outline

• History of the laboratory mouse
• Mouse strains
• Genetic mapping
  • How do we find genes?
• Genetic Engineering
  • How do we analyze gene function?

History of Mouse Genetics

© 1970 Richard Gardner of Cambridge, U.K., performs surgery on mouse embryos, opening the way to embryo transfer, embryonic stem cell research, and transgenic mouse technology.
Before the use of microinjection, aggregation chimeras were the only way to genetically modify cells and test them during mouse development.

Routes for Introducing Genes Into Mice

• Microinjection of DNA into zygotes
  • TALEN, CRISPR
• Injection of embryos with recombinant virus
• Transfection of embryonic stem cells with cloned DNA

1982 'Supermouse' was created by Ralph Brinster (U Penn) and Richard Palmer (U of Washington).
Transgenic Mice

- Random insertion of DNA into the mouse genome
- Permits gain-of-function, dominant negative and knockdown experiments
- Allows gene regulatory elements to be tested
- Allows populations of cells to be marked with a reporter gene

Making Transgenic Mice

Transgenic mice are generated by pronuclear injection of foreign DNA into fertilized mouse oocytes and subsequent transfer into the oviduct of pseudo-pregnant foster mothers.

Microinjection

- A female is superovulated and eggs are collected
- The eggs are fertilized in vitro
- The transgene is injected into the male pronucleus using a micropipette
- Eggs with the transgenes are kept overnight in an incubator to develop to a 2-cell stage
- The eggs are then implanted into the uterus of a pseudo-pregnant female
Transgenic Mouse Issues

• No founders born
  • Technical problems with microinjection
  • Transgene function are critical for embryo development

• Germline transmission
  • F3 animals should inherit the transgene at almost 50%
  • <50% indicates founder is a mosaic (not all cells carry transgene)
  • >50% indicates integration in multiple chromosomes

• Transgene silencing and leaky expression
  • Integrated into transcriptionally inactive region
  • Or insertion in the vicinity of an endogenous enhancer or promoter
  • Insertional mutagenesis

History of Mouse Genetics

2007 Nobel Prize in Medicine and Physiology “for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells”

Making Knockout Mice

Embryonic stem (ES) cells are transfected with the targeted (mutagenized) transgene. The transgene undergoes homologous recombination with the wildtype gene. After selection, positive ES cells are introduced into blastocysts and implanted into foster mothers.
**Embryonic Stem Cell Method**

- ES cells are obtained from the inner mass of a blastocyst
- The transgene is incorporated into the ES cell by:
  - Microinjections
  - By a retrovirus
  - By electroporation
- Transgenic stem cells are grown in vitro.
- After selection they are inserted into a blastocyst and implanted into a host's uterus to develop.

---

**Selecting for the Knockout**

---

**Knock-Out Mouse Issues**

- Developmental lethality
  - Genetically altered embryos cannot grow into adult mice
- No adults means difficult to determine function in relation to health
- A gene may serve different functions at different stages of development
- Knocking out a gene may fail to produce an observable change in phenotype
- Genetic background – the entire procedure varies based on the strain from which stem cells have been derived
Transgenic versus Knockout Mice

- **Transgenic**
  - Typically expresses one or more copies of a gene (cDNA) that is integrated into the genome in a random fashion.

- **Knockout**
  - Both alleles of a gene are deleted in a targeted fashion by homologous recombination.

Vector Design

- Positive selection cassette
  - Flanked by two arms of homologous sequence to enrich recombination events.
  - Expression cassette encodes promoter and antibiotic resistance genes.

- Negative selection cassette
  - Outside of one homologous arm.
  - Used to enrich for homologous recombination events over random insertions.

Homologous Recombination Versus Random Integration

- Genes targeted by homologous recombination contain the neo<sup>R</sup> element but not HSV<sup>-</sup>tk, since the latter resides outside the sequences in the targeting vector homologous to sequences in the chromosomal gene.

- Random integration of the vector results in introduction of HSV<sup>-</sup>tk as well as neo<sup>R</sup>. Screening methods select positively for neo<sup>R</sup> clones and discard those neo<sup>R</sup> + clones that carry HSV<sup>-</sup>tk.
Issues in Interpreting Targeted Mutations

- Must be very careful to make a null allele
  - Prove that the allele is null!
- Neighboring gene effects
  - Neo cassette may influence a nearby gene
    - Remove the neo cassette to avoid selection interference and genetic ambiguity
- Unexpected phenotype
  - Lethal phenotype may result prior to the developmental stage of interest

Removing the Neo Selection Cassette

![Diagram showing the process of removing the Neo selection cassette using Cre-lox P recombination](image)

Cre-lox Tissue-Specific Knockout

- Liver-specific cre transgene
- Homozygous loxP "floxed" mouse
- Cre-lox mouse: heterozygous for GeneX conditional knockout after 1 generation
Regulated Activation/ Inactivation Using CreER Fusion Proteins
Genomic Editing

• General principle is to target a non-specific nuclease to a specific DNA sequence

• Double-stranded break will induce:
  • Error-prone non-homologous end-joining (NHEJ), which leads to variable length insertion/deletion mutations (indels)
  • Homology-directed repair (HDR), which can be used to introduce precise alternations directed by a homologous DNA template

Genome Editing Glossary

• Cas = CRISPR-associated genes
• Cas9, Csn1 = a CRISPR-associated protein containing two nuclease domains, that is programmed by small RNAs to cleave DNA
• crRNA = CRISPR RNA
• dCas9 = nuclease-deficient Cas9
• DSB = double-stranded break
• gRNA = guide RNA
• HDR = homology-directed repair
• HNH = an endonuclease domain named for characteristic histidine and asparagine residues
• Indel = insertion/deletion
• NHEJ = Non-homologous end-joining
• PAM = protospacer adjacent motif
• RuvC = an endonuclease domain named for an E. coli protein involved in DNA repair
• sgRNA = single guide RNA
• tracrRNA, trRNA = transactivating crRNA
• TALEN = transcription activator-like effector nuclease
• ZFN = zinc-finger nuclease
Zinc Finger Nucleases (ZFNs)

- Modular assembly of individual ZFNs
- Left and right target sequence with a 5nt spacer
- ZFNs microinjected as either a circular plasmid or as mRNA following in vitro transcription
- Surviving embryos transferred to foster mothers
- DNA confirmation of pups
Transcription Activator-Like Effector Nucleases (TALENs)

- Nonspecific FokI nuclease domain fused to a customizable DNA-binding domain to target a single genomic locus
- FokI nuclease functions as a dimer to cleave double stranded DNA
  - Can form unwanted dimers
  - Off-target mutagenesis is frequent
- Engineered TALEN variant exhibits equal on-target cleavage activity but tenfold lower average off-target activity

Transcription Activator-Like Effector Nucleases (TALENs)

- FokI nuclease domain
- TALEN repeats (DNA binding domain)
- Cleavage within spacer region

Transcription Activator-Like Effector Genome Editing
CRISPR/Cas9

• CRISPR = clustered regularly interspaced short palindromic repeats
• Streptococcus pyogenes SF370 type II CRISPER locus
  • Cas9 nuclease
  • Two noncoding CRISPR RNAs (crRNAs)
  • Trans-activating crRNA (tracrRNA)
  • Precursor crRNA (pre-crRNA) array containing nuclease guide
• Facilitates RNA-guided site-specific DNA cleavage
• Cas9 nucleases can be directed by short guide RNAs (gRNA) to induce precise cleavage at endogenous loci

CRISPR/Cas9

• Two critical components
  1. Cas9 nuclease
  2. Guide RNA (gRNA)
• guide RNA: protospacer/crRNA fused to a fixed trans-activating RNA (tracrRNA)
  • 20 nucleotides at the 5’ end of the gRNA direct Cas9 to a specific target DNA site using standard RNA-DNA complementarity base-pairing rules
  • Target sites must lie immediately 5’ of a PAM sequence (protospacer adjacent motif) that matches the canonical form 5’-NGG
• Cas9 nuclease activity can be directed to any DNA sequence of the form N20-NGG simply by altering the first 20 nucleotides of the gRNA to correspond to the target DNA sequence

Cas9 in vivo: Bacterial Adaptive Immunity
CRISPR/Cas9 Applications

Genome Editing Technology Issues

- Off-target modifications!
  - Does a given engineered nuclease act at genomic locations other than its intended site?
  - Critically important because unintended, off-target modifications can lead to unexpected functional consequences in both research and therapeutic contexts.

Why Mice?

- 95% of their genome is similar to our own
- Mice have short generation times and lifespans that make them easy to manage
- Variety of inbred strains and genetic backgrounds give us the opportunity to closely examine the effects of genes and their interactions
- We have the technology to manipulate their genome directly and create models for human diseases
- We have control over their environment
Outline

- History of the laboratory mouse
- Mouse strains
- Genetic mapping
  - How do we find genes?
- Genetic Engineering
  - How do we analyze gene function?