The Use of Mouse Models to Study the Cytoskeleton

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Study Gene Function

- How does development happen?
- Genetic disease - when something goes wrong, where, when and how?
The Nobel Prize in Physiology or Medicine 2007

“For their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells”

Photo: U. Montan
Mario R. Capecchi
Ø 1/3 of the prize
USA
University of Utah
Salt Lake City, UT, USA; Howard Hughes Medical Institute
b. 1937
(in Italy)

Photo: U. Montan
Sir Martin J. Evans
Ø 1/3 of the prize
United Kingdom
Cardiff University
Cardiff, United Kingdom
b. 1941

Photo: U. Montan
Oliver Smithies
Ø 1/3 of the prize
USA
University of North Carolina
Chapel Hill
Chapel Hill, NC, USA
b. 1925
(in United Kingdom)

Almost any type of change can be introduced into mouse genes by gene targeting. A common change is to inactivate a gene, thereby creating a knockout “mouse”.
How to study gene function

- Gene targeting-replaces normal allele with a mutant allele. Precise incorporation of the gene to a specific site in the genome. Mice are known as knock-out or knock-in mice.
- Transgenic mouse- overexpression of a gene, normal of mutated
What is a KO/KI mouse

• A mouse in which a specific mouse gene has been genetically modified and the modification is transmitted through the germ-line.

• KO (knockout) is a modification in which the activity of the gene is eliminated (e.g. delete the gene or a key region)

• KI (knockin) is a modification in which a specific mutation(s) or rearrangement is introduced and the gene remains functional.
Why make a KO/KI mouse

• Create mouse models to study pathophysiology of disease and test therapeutic approaches to disease.

• Most useful to mimic recessive disorders (loss of function mutations). [Traditional transgenics can be used for dominant disorders].
How to make a KO mouse

- Principle is **homologous recombination**

A fragment of genomic DNA is introduced into a mammalian cell and it can locate and recombine with the endogenous homologous sequences. This type of homologous recombination is also commonly refer to as gene targeting.

It occurs in yeast, bacteria and certain viruses however it is a rare event in mammalian cells except germ cells. Transfected DNA most commonly integrates into a random chromosomal site. The relative frequency of targeted to random integration events will determine the success of generating a KO mouse.
Homologous recombination is normal when germ cells are formed.

Homologous recombination increases the genetic variability in germ cells.
How to make a KO mouse

- Principle is homologous recombination

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It occurs in yeast, bacteria and certain viruses however it is a rare event in mammalian cells except germ cells. Transfected DNA most commonly integrates into a random chromosomal site. The relative frequency of targeted to random integration events will determine the success of generating a KO mouse.
Making a knockout mouse requires

- Pluripotent embryonic stem (ES) cells

ES cells are isolated from the Inner Cell Mass of a 3.5 day old mouse embryo.
ES cells can differentiate into all the different types of cells in the body.
*In vitro* culturing of ES cells

Pluripotent ES cell colonies

Differentiated ES cell colony
Gene Targeting Strategy

1. **Blastocyst**
   - Culture in vitro

2. **Electroporate with targeting vector**

3. **Select and screen for targeted cell**

4. **Foster mother incubation**

5. **Heterozygote**

6. **Homozygote**
Knockout - How?

1. ES cells isolated from blastocyst 3.5dpc
2. DNA targeting construct into the ES cells
3. Microinjection microscope use to take up ES cells with glass syringe
4. Genetically modified ES cells injected into blastocyst
5. Blastocyst implanted into surrogate mother
Making a knockout mouse requires

- Pluripotent embryonic stem (ES) cells
- Construction of KO vector by standard cloning procedures
Standard molecular biology techniques are used to design and make the targeting DNA vector.
Making a knockout mouse requires

- Pluripotent embryonic stem (ES) cells
- Construction of KO vector by standard cloning procedures
- Introducing the KO vector into the ES cells by electroporation
- Selecting for gene targeting events
Selecting for gene replacement events

- Targeting vector contains marker genes

  **Positive selectable marker, neomycin phosphotransferase** is resistant to the antibiotic neomycin.

  **Negative selectable marker, thymidine kinase from Herpes Simplex virus.** The TK gene confers sensitivity to the chemical gancyclovir.
ES cells with this construct will grow in culture medium containing neomycin but will not survive in the presence of ganciclovir. Resistant to neomycin and sensitive to ganciclovir.

ES cells with this construct will grow in culture medium containing neomycin and ganciclovir. Resistant to neomycin and ganciclovir.
Positive and negative selection of recombinant ES cells

Recombinants with random insertion

Treat with neomycin (positive selection)

Ganciclovir kills the recombinants with random insertion

Non recombinants

Recombinants with gene-targeted insertion

Treat with ganciclovir (negative selection)
Making a knockout mouse requires

- Pluripotent embryonic stem (ES) cells
- Construction of KO vector by standard cloning procedures
- Introducing the KO vector into the ES cells by electroporation
- Selecting for gene targeting events
- Screening the ES colonies
To identify which ES cells accepted the KO gene, DNA is isolated from the ES cells, cut with restriction enzymes, run on a gel and hybridised with radioactively labelled DNA probes. This is to test for the organisation of the target gene.

Example of Southern blot
Making a knockout mouse requires

- Pluripotent embryonic stem (ES) cells
- Construction of KO vector by standard cloning procedures
- Introducing the KO vector into the ES cells by electroporation
- Selecting for gene targeting events
- Screening the ES colonies
- Injecting the KO cells into blastocysts.
ES cells from a brown mouse

Blastocyst from a white mouse

Surgically transfer embryo into pseudopregnant mouse

Homozygous white progeny

Chimeric progeny

Mate chimeric mice to homozygous white mice

Black progeny develop from germ-line cells derived from ES cells and are heterozygous for disrupted gene X
Making a knockout mouse requires

- Pluripotent embryonic stem (ES) cells
- Construction of KO vector by standard cloning procedures
- Introducing the KO vector into the ES cells by electroporation
- Selecting for gene targeting events
- Screening the ES colonies
- Injecting the KO cells into blastocysts. The progeny will be a chimera consisting of both KO and wild type cells
- Hopefully some KO cells will contribute to the germ line. Heterozygous and homozygous progeny for the KO construct can be generated and analysed for phenotypic alterations
Screening of Mice

Injection of ES cells

Genetically modified ES cells are injected into blastocysts...

...where they mix with the inner cell mass, which will later develop into an embryo.

Mosaic of cells

Blastocysts with modified ES cells are implanted in a surrogate mother. There they develop into mosaic embryos, because the blastocyst contains both modified and normal cells.

Mosaic mouse pup

The pups that are born have mottled fur. This is because cells from the original blastocyst carry a gene that gives yellow fur, whereas the injected ES cells, in addition to the targeted genetic modification, carry a gene that gives dark fur.
(2-24 months) isolate genomic clone and characterize
(modify construct to) (months) mutate and allow double selection
(2 days) electroporate into ES cells
(2 weeks) select G418
Gancyclovir R ES clones
(1-52 weeks) screen extensively to identify homologous recombinants, PCR, Southern blots
(2 days) culture targeted ES cell clones

- day 1 inject ES cells into C57BL/6 blastocysts
- week 3 pups born
- week 5 chimeric offspring identified by coat color
- tail biopsies, Southern blot to confirm
- week 11 outcross male chimeras
- week 16 Southern blot to determine germ line transmission
- week 22 breed germ-line chimeras
- week 27 2 week old pups
  ++, +/−, −/−, −/−

Homologous recombination gene targeting in mice
Transgenic Mouse - How?

- Injection pipette containing DNA construct
- Holding pipette
- Oocyte (0.5 days old)
- Male & female pronuclei
Transfer injected embryos into oviduct of pseudopregnant foster mother.

Test DNA of pups to find out which pups have a successfully integrated transgene.
microinject purified DNA into male pronucleus of fertilized mouse egg
implant injected eggs in oviduct of pseudopregnant foster mother

pups born

isolate DNA from tails and analyze by PCR or southern blot for transgene

mice transferred to care of investigator

transgenic mice ready to be outcrossed

pronuclear injection to make transgenic mice
KO/KI Versus Transgenic mice

Gene targeting – Advantage: Specific insertion of gene at specific location or removal of specific gene (knockout). Mimic recessive disorders (loss of function mutations).

Disadvantage: Low level of ES cells with wanted gene inserted. Further breeding necessary to obtain non-chimeric homozygotic animal.

Transgenic mice – Advantage: Relative high rate of insertion of the injected gene into the genome. Use for dominant disorders.

Disadvantage: Random insertion- can lead to position effects.
Use of genetically modified mice

- **KO/KI mice**
  - Cystic fibrosis
  - Familial hypertrophic cardiomyopathy (cardiac muscle disorders)

- **Transgenic mice**
  - To study dominantly acting alleles of tumor-causing genes (cancer)
Gene targeting is used to delete or mutate an existing gene. KO and KI. Mice are generated by the injection into a blastocyst of genetically modified ES cells. Chimeric mice are made.

Transgenic mice are used to study overexpression of a gene product. Mice are generated by DNA microinjection of fertilized oocytes. Results in random integration of the DNA.

Both offer a valuable tool for the study of human disorders. i.e. Cystic fibrosis—cause by mutation of 1 or more of 4 genes. Use of knockin mutation approach.
KO/KI

DNA

Embryonic stem (ES) cells

Select for cells expressing desired gene

Inject transformed ES cells into inner cell mass

Blastocyst

Inner cell mass

Implant in uterus

Foster mother

Implant in uterus

Test offspring for presence of gene

Mate heterozygous offspring to produce homozygous transgenic strain

Transgenic

Pronuclei

Desired gene (with vector)

Fertilized egg
Transgenesis

- Preparation of the transgene clone the DNA of interest
- Microinjection: mate egg donors to males, collect fertilized eggs, microinject the construct into fertilized egg, transfer eggs to pseudo-pregnant female, genotype newborn pups to identify the founders
- Breeding and analysis: mate transgenic founders, genotype newborn F1 generation
- Gene product is overexpressed

Gene-Targeting

- Preparation of the transgene clone the DNA of interest
- Electroporation of ES cells and selection: prepare ES cells in culture, electroporation of ES cells with targeting construct, pick drug-resistant ES clones, Southern or PCR screening of targeting event,
- Microinjection: mate eggs donors to males, collect blastocysts, microinject targeted ES cells into blastocyst, transfer blastocysts to pseudo-pregnant female, genotype newborn chimeric pups
- Breeding and analysis: mate chimeric mice, genotype KO/KI heterozygous cross-breed heterozygous to obtain homozygous
- Gene product is missing or mutated
Advances in gene targeting

• Ability to inactivate a gene at a specific time and in a specific tissue.

• Conditional gene targeting is achieved with the use of the Cre/lox system. Cre recombinase is an enzyme that catalyzes sequence-specific recombination between two 34 base pair repeats (LoxP sites). The result of this recombination is deletion of the DNA between the LoxP sites.
LoxP Mouse

Exon1  Exon2  Exon3
LoxP  LoxP

All cells carry endogenous gene X with LoxP sites flanking exon2

Cre Mouse

Cell-type-specific cre promoter

All cells carry cre transgene mouse is heterozygous for gene X knockout

LoxP-Cre mouse: all cells carry one copy of loxP-modified gene X, one copy of gene X knockout, and cre genes

Cells not expressing Cre

Gene function is normal

Cre-expressing cells

Cre protein

Gene function is disrupted
The Cytoskeleton

- **Microfilaments**
  - cell shape, motility, cytokinesis

- **Intermediate filaments**
  - provide strength, compression resistance

- **Microtubules**
  - organelle and vesicle transport, cell division
Microfilaments

Intermediate Filaments

Microtubules
Actin monomers (G actin) with the aid of actin binding proteins can polymerize to form actin filaments (F actin).

Within cells an equilibrium exists between monomeric and filamentous actin. This equilibrium is influenced by actin binding proteins.
Organization of actin filaments within cells

Organisation

Participation

cell shape
cell adhesion
cell motility
vesicle transport
endocytosis
exocytosis
golgi function
cytokinesis
membrane function

- microvilli
- contractile bundles in the cytoplasm (stress fibers linked to focal contacts) = muscle
- lamellipodia (sheetlike)
- filopodia (fingerlike)
- ruffles
- contractile ring during cell division
Classes of actin binding proteins

- nucleation protein
- severing protein
- cross-linking protein (in cell cortex)
- capping (end-blocking) protein
- monomer-sequestering protein
- side binding proteins
- motor proteins

Actin monomers
Actin filaments
Bundling protein (in filopodia)
Classes of actin binding proteins

- Nucleation protein
- Cross-linking protein (in cell cortex)
- Severing protein
- Side binding proteins
- Capping (end-blocking) protein
- Motor proteins
- Monomer-sequestering protein
- Bundling protein (in filopodia)

Actin filaments connected to actin monomers and tropomyosin.
The Tropomyosin family

- Tropomyosin is an actin filament stabilizing protein
- In mammals 4 genes code for tropomyosin protein
- Due to alternative splicing >40 tropomyosin isoforms (variants) found in mammals
- Tropomyosin proteins share a high degree of homology
Tropomyosin

• Tropomyosin Expression
  – Ubiquitously expressed, found in both muscle and non-muscle cells
  – Muscle expresses 5 Tm isoforms the remaining isoforms are found in non-muscle cells
  – A restricted repertoire of Tm isoforms is expressed by different cell types

• Tropomyosin Function
  – In muscle, Tm plays a very important role in muscle contraction
  – In non-muscle cells the role of Tm is not well understood
Non-muscle Tm isoforms sort to distinct subcellular compartments

Hughes et al., 2004
Schevzov et al., 2005
Percival et al., 2004
Temm-Grove et al., 2004
Sachezov et al., 2005

in vitro

in vivo
Altered actin cytoskeleton in cancer cells

Normal (fibroblasts)

Normal repertoire of Tm isoform expression

Transformed (HeLa)

Altered repertoire of Tm isoform expression
Neurons have distinct subcellular compartments
  cell body
dendrites
growth cones

Primary embryonic neuron cultured in vitro for 5 days
Different tropomyosin isoforms sort to different subcellular compartments within neurons.
In order to evaluate the role of different tropomyosin isoforms in determining the morphology of neurons, tropomyosin Transgenic mice were generated.

- Tm3 was chosen as an isoform that is not present in neurons.
- Tm5NM1 was chosen as an isoform that is present in neurons and found in the growth cone.
Preparation of primary embryonic neurons

16.5 day embryo

Brain is isolated

Tissue is dissociated

Single cell suspension is plated

Neurons cultured \textit{in vitro} for 1 day

Process outgrowth occurs
Overexpression of Tm5 results in enlarged growth cones

- Primary embryonic neurons were isolated from Tm5 transgenic 16.5 day old embryos
- Cultured in vitro for 5 days
- Immunofluorescence stained with actin antibody in green

Overexpression of Tm3 and Tm5 leads to distinct neuronal morphogenesis.
Tm3 impacts on dendrites and Tm5 effects both dendrites and axonal branching.

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<th>Total axon length (μm)</th>
<th>Control</th>
<th>Tm3</th>
<th>Tm5</th>
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We propose that the overexpression of Tm5 within the growth cone leads to enlarge growth cones. The motility of these large growth cones is now altered, they may pause for longer allowing for new branches to be formed.
Construct use to generated Tm5 knockout mice
Overexpression or elimination of products from the γTm gene both impact on growth cone sizes of cultured cortical neurons

Cortical neurons were prepared from 16.5 days old mouse embryos, cultured in vitro for 1 or 5 days as indicated and analyzed for changes in growth cone area

**Overexpression of Tm5**

**Elimination of Tm5**

\[ \text{Growth cone area (μm}^2\text{)} \]

- **1d growth cones**
- **5d growth cones**

\[ \text{control} \quad \text{hTm5NM1} \]

\[ \text{wt} \quad \text{γ9d } \]

\[ \text{Growth cone Area [μm}^2\text{]} \]

\[ \text{1d growth cones} \]

**Scheyzov et al., 2005**
Why do changes in the expression of Tm5 within the growth cone lead to changes in the size of the growth cone?
Organization of actin filaments and microtubules in a neuronal growth cone
Method:
• Neurons were isolated from the Tm5 transgenic mice and control mice
• Both Tm5 and control neurons were culture in vitro together
• Neurons were immunofluorescence stained with phalloidin (detects actin filaments) and an antibody that detects the transgenic Tm5 protein
• The largest growth cone was chosen per cell
• The intensity of phalloidin staining was measured

Changes in the levels of the actin filament stabilizing protein, Tm5, leads to an increase in actin filaments and ultimately impacts on the morphology of the growth cones.
What other phenotypes have been observed in the Tm3 transgenic mice?

Tm3 is an isoform not normally expressed in neurons.

Western blot on brain protein
Spinning behavior in the Tm3 mice
The Cerebellum

http://digitalcortexmedia.com/images/cerebellum_cell_layer.jpg

Tm3 mice show loss of Purkinje neurons
• Hair cells are the sensory cells of the inner ear
• Perform essential function in hearing and balance

We propose that altering the Tm composition within this hair cells leads to the observed spinning behavior.
Key points from the lecture

- Multiple Tm isoforms are present within a cell
- Tms sort to distinct subcellular compartments
- The distinct sorting of Tms leads to the generation of distinct population of actin filaments with functionally distinct properties

Over or under expression of Tm isoforms achieved by the generation of genetically modified mice allow us to conclude that:

- The expression of Tm isoforms is important for the establishment of neuronal cell structure and viability