The Use of Genetically-Modified Mouse Models to Study the Cytoskeleton

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Structure of the Prac

1) Talk: how to make genetically modified mice (50 min)
2) Time to answer Question a) in the Exercise (20 min)
3) Talk: example using GM mice in a research problem (30 min)
4) Time to answer Question b) in the Exercise (20 min)

ANAT3231-Cell Knockout Methods

The actin cytoskeleton together with actin binding proteins, such as tropomyosin, is an important regulator of cell function. Transgenic and knockout mice play a key role in studying the mechanism by which these structural proteins function.

a) List the key differences in the generation of transgenic and gene knock-out mice.
b) Describe how genetically modified mice have been used to study the biological function of tropomyosin. In your answer include,
   i. the tissues used to examine the function of tropomyosins
   ii. the measurements used to determine whether any phenotypic changes were observed
   iii. a model to explain how actin and changes in the levels of tropomyosin can lead to the observed phenotypic changes.

Exercise

Why Study Gene Function

- How does development happen?
- Understand the function of gene products in adults
- Genetic disease - when something goes wrong: where, when and how?
Approaches to manipulate the mouse genome

1) Transgenic
   - Random chromosomal integration of foreign DNA

2) Knockout/Knockin (Homologous Recombination)
   - Site directed disruption (Knockout) or replacement with a modified variant of a gene allele (knock-in).
   - Conditional KO/KI - tissue specific and inducible

Transgenic mice - random integration

Types of transgenics
1) Target protein may be overexpressed: excessive amounts of normal protein expressed in tissues which normally express it, e.g. GH transgenic.

2) Target protein may be ectopically expressed: protein expressed in tissues which do not normally express it.

3) Mutated protein is expressed to produce: constitutively active ("gain-of-function") or dominant negative ("loss-of-function") form of a protein or to mimic a mutation observed in a human genetic disease.

Creation of transgenic mice

Context-specific expression of the transgene

Design transgenic construct that contain transcriptional regulatory elements that direct expression to a specific cell type or developmental stage.

Example: Transgenic mouse model of human muscle disease

- Mutant skeletal actin promoter
- Mutant human skeletal actin (slow Met9Arg)
- Skeletal muscle specific
- Early stages of embryonic muscle
- Nemaline Myopathy

Creation of transgenic mice

Taken from: Strachen & Read
Human Molecular Genetics
Issues with random insertion of transgenes

- Because of the random nature of the transgene insertion, each injection of DNA into the pronucleus will result in insertion of the transgene in a different site in the genome.
- The position effect can profoundly affect the expression of both the transgene and the endogenous genes whose regulatory elements may be disrupted:
  - Transgene may disrupt the coding region of endogenous genes (insertional mutagenesis) so the phenotype of the mouse maybe due to this and not the gene that one inserted.

Variable expression with random insertion of transgenes

- The foreign DNA usually integrates as linear arrays, but the number of copies inserted is variable.

Transgenic lines

- Therefore, it is essential that lines from several different founder lines be examined before a conclusion relating a specific phenotype to transgene expression is made.
- To assess dose-response relationships between transgene expression and phenotype, it is also important to assess lines of mice which express the transgene at different levels.

What is a KO/KI mouse

- A mouse in which a selected/targeted mouse gene has been genetically modified and the modification is transmitted through the germ-line, i.e., through generations
- 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 gene with a specific mutation(s) or rearrangement is introduced into the same gene and the gene remains functional.
Why make a KO/KI mouse

- To understand the function of genes.
- Most useful to mimic recessive disorders (loss of function mutations).
- The introduced gene is regulated (expressed) in the same way as the endogenous gene: unlike transgenic mice the gene is expressed at endogenous levels and therefore maybe more physiologically relevant.

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 referred 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

- When making a KO/KI, recombination occurs between the targeting DNA construct and the DNA of the germ cell.
Making a knockout mouse requires

1. Pluripotent embryonic stem (ES) cells

ES cells can differentiate into all the different types of cells in the body

- ES cells are isolated from the Inner Cell Mass of a 3.5 day old mouse embryo.

In vitro culturing of ES cells

- Pluripotent ES cell colonies
- Differentiated ES cell colony

Knockout- How?

1. ES cells isolated from blastocyst 3.5dpc
2. ES cells cultured
3. DNA targeting construct inserted into ES cells: electroporation
4. Screen ES cells to determine the cells that have correctly integrated the targeting DNA into their genome
5. Microinjection microscope used to take up ES cells with glass micropipette
6. Genetically modified ES cells injected into blastocyst
7. Blastocyst implanted into surrogate mother
Making a knockout mouse requires

1. Pluripotent embryonic stem (ES) cells
2. Construction of KO vector by standard cloning procedures
3. Introducing the KO vector into the ES cells by electroporation
4. Selecting for gene targeting events

Construction of KO/KI DNA vector

Standard molecular biology techniques are used to design and make the targeting DNA vector so that genetic recombination will occur at the selected site.

Selecting for gene replacement events

- Targeting vector contains marker genes which select for ES cell clones that have taken up the DNA by recombinant and not by random insertion.
- 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.
7

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.

Positive and negative selection of recombinant ES cells

Making a knockout mouse requires

1) Pluripotent embryonic stem (ES) cells
2) Construction of KO vector by standard cloning procedures
3) Introducing the KO vector into the ES cells by electroporation
4) Selecting for gene targeting events
5) Screening the ES colonies for the inserted DNA

Screening the ES colonies

* To identify which ES cells accepted the KO gene
* DNA is isolated from the ES cells, run on a gel and hybridised with radioactively labelled DNA probes.

Example of Southern blot
Making a knockout mouse requires

1) Pluripotent embryonic stem (ES) cells
2) Construction of KO vector by standard cloning procedures
3) Introducing the KO vector into the ES cells by electroporation
4) Selecting for gene targeting events
5) Screening the ES colonies for the inserted DNA
6) Injecting ES cells into the blastocysts

From Targeted ES cells to producing a mouse

5. Injection of ES cells into blastocysts
   The targeted ES cells are injected into blastocysts, which then develop into mice.

6. Birth and breeding of mosaic mice
   Mosaic mice are born, which may contain both wild-type and targeted ES cell lines.

Time-line for making a knockout mouse

KO/KI Versus Transgenic mice

Gene targeting (KO/KI)

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

Disadvantage:
- Further breeding necessary to obtain non-chimeric animals

Transgenic mice

Advantage:
- Relative high rate of insertion of the injected gene into the genome
- Use for dominant disorders
- Relatively quick

Disadvantage:
- Random insertion - can lead to position effects
- Gene expressed at levels above endogenous
Use of genetically modified mice

- KO/KI mice
  - Cystic fibrosis
  - Muscular dystrophies

- Transgenic mice
  - To study dominantly acting alleles of mutated genes (nemaline myopathy)

Example: Transgenic mouse model of human muscle disease

Summary

- Gene targeting is used to delete or mutate an existing gene: KO and KI. Mice are generated by the injection of genetically modified ES cells into a blastocyst. Chimeric mice are made.

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

- Both offer valuable tools for the study of human disorders.

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 the catalyses 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.
Conditional Knockouts
Cre Recombinase Approach

- Target Gene
- LoxP sites
- Transfect into ES cells in culture
- Blastocyst injection & breeding as per normal KO

Mouse 1
Mouse 2
Target gene deleted in cells expressing Cre

By using tissue and developmental stage specific promoter that drives Cre expression can delete genes in specific tissues and at specific stages of development.

There are now inducible promotors that are only activated by drugs and these can be used with Cre to delete genes in specific tissues only when the drug is present – this allows for gene deletion in adult animals.

The Cytoskeleton

Microtubules
- organelle and vesicle transport, cell division

Intermediate filaments
- provide strength, compression resistance

Microfilaments
- cell shape, motility, cytokinesis

Answer Question a) of the Exercise: 20 min.
Microfilaments: Actin

Intermediate Filaments: Desmin

Microtubules: Tubulin

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 influence by actin binding proteins.

Organization of actin filaments within cells

Classes of actin binding protein:
- Cross-linking proteins (in cell cortex)
- Nucleation protein
- Monomer-sequestering protein
- Motor proteins
- Side binding proteins
- Capping (end-blocking) protein
- Nucleating protein
- Actin-activated protease
- Actin-stabilising protease

Organisation
- Membranes
- Contractile bundles in the cytoplasm (astrocytes linked to focal contacts - muscle)
- Actin filaments during cell division

Participation
- Cell shape
- Cell adhesion
- Cell motility
- Vesicle transport
- Exocytosis
- Endocytosis
- Golgi function
- Cytokinesis
- Membrane function
Classes of actin binding proteins

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

Tropomyosin (Tm)

- filamentous protein
- Forms dimers
- Dimers interact head-to-tail to form a polymer
- Tm polymer interacts along the length of the actin filament
- Provides stability and regulates the binding of other actin binding protein to the actin filament

Different Tm isoforms regulate actin filaments by recruiting different actin binding proteins

- Stable microfilaments
- Higher actin filament turnover
- Shorter filaments

Using knockout and transgenic mice to understand the function of Tm5NM1 in vivo

- Tm5NM1/2 knockout
  - Tm5NM1/2 eliminated in all tissues
- hTm5NM1 transgenic (Tg)
  - Tm5NM1 overexpressed in most tissues
Phenotypes in the Tm5NM1/2 KO & hTm5NM1 Tg mouse

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<thead>
<tr>
<th>Parameter</th>
<th>Affected/Unaffected</th>
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<tbody>
<tr>
<td>Organ Weights (Fat, brain, kidneys)</td>
<td>↓ KO ↑ Tg</td>
</tr>
<tr>
<td>Adipocyte Proliferation</td>
<td>↓ KO ↑ Tg</td>
</tr>
<tr>
<td>Body Weight</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Histopathology</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Food Intake</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Food Utilisation &amp; Metabolism</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Glucose Uptake/Insulin Secretion</td>
<td>? KO ↑ Tg</td>
</tr>
</tbody>
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But in Tm5NM1/2 KO mice glucose clearance is not increased

Compensation from other Tms may be responsible for lack of an effect on glucose clearance in Tm5NM1 KO mice
Glucose homeostasis - coordinated by many organs and tissues of the body

Adapted from: Rosen & Spiegelman, 2006

Insulin-stimulated glucose uptake is increased in Tm5NM1 transgenic mice

Glucose Uptake – adipose tissue

The obligatory step in insulin-stimulated glucose uptake is translocation of the glucose transporter (GLUT4) to the plasma membrane

Glucose Transport Pathway (muscle and fat)

From: Ramm & James, 2005

Components of GLUT4 trafficking increased in Tg mice

Data based on Western blotting

Adapted from: Zaid et al., 2008
Western blots showing components of GLUT4 trafficking increased in Tg mice

From adipose tissue

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>Tg</th>
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<tr>
<td>Myo1c</td>
<td></td>
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<td>Sec 8</td>
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Can Tm5NM1 increase in actin filaments in the mouse tissues?

Tm5NM1 increases filamentous actin in adipose tissue

F-actin staining
Key points from this part of the lecture

Overexpression of Tm isoforms achieved by the generation of genetically modified mice allow us to conclude that:

- Tm isoforms regulate actin filament function in vivo
- Tm5NM1 specifically regulates glucose uptake via altering the ratio of G- to F-actin

Looking for keen Honours Students!

Supervisors:
Dr. Anthony Kee (a.kee@unsw.edu.au)
&
Professors Edna Hardeman & Peter Gunning

Projects:
Utilizing GM mouse models to understand the functions of actin cytoskeleton in vivo.