Genetic modification of the mouse

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Genetically modified mouse models

- The genome projects have provided us only with a catalogue of genes little is know regarding gene function
- Associations between disease and genes and their variants are being found yet the biological significance of the association is frequently unclear
- Genetically modified mouse models provides a powerful method of assaying gene function in the whole organism



How to study gene function

Human patients:

- Patients with gene mutations can help us understand gene function
- Human's don't make particularly willing experimental organisms
- An observational science and not an experimental one
- Genetic make-up of humans is highly variable
- Difficult to pin-point the gene responsible for the disease in the first place

Mouse patients:

- Full ability to manipulate the genome experimentally
- Easy to maintain in the laboratory breeding cycle is approximately 2 months
- Mouse and human genomes are similar in size, structure and gene complement
- Most human genes have murine counterparts
- Mutations that cause disease in human gene, generally produce comparable phentoypes when mutated in mouse
- Mice have genes that are not represented in other model organisms e.g. *C. elegans, Drosophila* genes of the immune system

What can I do with my gene of interest

- Gain of function
 - Overexpression of a gene of interest
 - Insertion of a gene of interest
- Loss of function
 - Mutation of a specific gene of interest
 - Overexpression of a mutant gene
 - RNA interference

"Transgenic mouse""Knockin mouse"

- "Knockout mouse"
- "Transgenic mouse"
- "Knockin mouse"

Mouse pre-implantation Development



- The preimplantation development of the mouse provides us a window of opportunity to genetically manipulate the mouse
- Two stages of development are frequently used
 - Fertilized oocyte
 - Blastocyst

Pronuclear Injection



Recovery of mouse zygotes

Pronuclear injection of transgene Random integration of DNA construct into the genome

Reimplantation of injected zygotes into foster mice

Potential founders for integrated transgene by PCR and Southern blot analysis

The Microinjection process



Classical transgenic constructs



Transgenic models









The problems of random integration I

- Transgenic constructs integrate randomly into the genome.
- Random insertion can cause mutagenesis.



• Position effects can alter or silence the expression pattern



The problems of random integration II



- The integration of transgenes as tandem repeats is associated with unstable expression
- An array of repetitive promoters is thought to unsettle the expression
- The resulting transgenic expression is often only present in a subset of the cells
- This phenomenon can even lead to an extinction of expression



Not every cell expresses transgene Individual transgenic mice are not directly comparable

BAC transgenes



- BAC Transgenics
 - All regulatory regions are present
 - Copy number is low
 - Large amount of insulating buffer sequence is present
- Same insertion dangers apply
 - Mapping the transgene insertion is very challenging as BACs are rarely linearised prior to injection i.e. you don't know the ends
- BACs may carry extra genes

Lines of transgenic mice



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- Due to the variation in expression, independent lines of transgenic mice must be generated, bred and phenotyped independently before conclusions can be drawn
- In an ideal situation, the variability of the strength of transgene expression can be correlated with variability in phenotype

A typical experiment with transgenic founders



- This variability means that a number of independent mouse lines must be maintained per construct
- Large animal cost of transgenic research
- Targeted manipulations via ES cells or via direct microinjection of nucleases are tackling this problem

Embryonic Stem Cells



Genetic Manipulation of ES cells

- Homologous recombination occurs at usable frequencies
- By building constructs which contain the required manipulation, these can be introduced into cells with selectable markers and cells with the required manipulation can be isolated
 - Gene Knock-out
 - Conditional (e.g. Tissue Specific Knock-out)
 - Humanization
 - Point mutation Knock-in

Homologous recombination – the traditional route to gene modification



Blastocyst Injection



Recovery of mouse blastocysts

Blastocyst injection with targeted ES cells

Reimplantation of injected blastocysts into foster mice

Pups born are "chimeras" of the host blastocysts and the injected ES cells

The Microinjection process



Chimeras

- Different coat colour makers in the injected cells and the host blastocysts allow us to distinguish the contribution of the injected stem cells
- The aim is for the stem cells to contribute to the germ cells of the chimera so that the engineered mutation is passed onto subsequent generations



"Brown" (129) ES cells injected into "black" (C57Bl/6) embryos



"Black" (C57BL/6) ES cells injected into "white" (FVB) embryos

Breeding Scheme



Conditional Manipulations



- Cre recombinase from P1 Bacteriophage catalyses the recombination between specific 34 bp sequences known as loxP sites
- If positioned appropriately within the genome, introducing Cre recombinase can lead to chromosomal deletions
- The activity of the Cre recombinase can be restricted both spatially and temporarily to fine tune the genetic ablation

Tissue specific knockouts



Molecular scissors



• Over the last 10 years, there has been a revolution in our ability to manipulate the genome in a precise and sequence dependent manner

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

- Prokaryotes have evolved a nuclease system involved in defence against invading phages and plasmids.
- Central to this system are DNA repeats called CRISPRs
- CRISPR loci consist of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage.



- Cas9 is a nuclease which is directed to its
- cleavage site by a guide RNA
- This guide RNA contains a 20 nt sequence which defines where the nuclease cleaves
- The cleavage site can be any 20 nt sequence, but the cleavage requires an NGG motif downstream

Microinjection of nucleases into mouse oocytes



Simple Knock-out by indel mutation



599	GCCTCA	CTGGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
598	GCCTCATGGATTCAAAGCCACTG	GGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
597b	GCCTCATGGATTCAAAGCCACTG	GGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
597a	GCCTCATGGATTCAAAGCCACTGAGG	CCT <mark>GGCCACTGGGGGCGCCATTC</mark> GCCATTAAAAGGTCCTGCTGGGCTTTT
595	GCCTCATGGATTCAAA	ACTGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
594b	GCCTCATGGATTCAAAGCCA	-TTACAGAATGGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
594a	GCCTCATGGATTCAAAGCCACTGAGG	GCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
592b	GCCTCATGGATTCAAAGCCA	TTCGCCATTAAAAGGTCCTGCTGGGCTTTT
592a	GCCTCATGGATTCAAAGCCACTGAGG	CCT <mark>GGCCACTGGGGGGCGCCATTC</mark> GCCATTAAAAGGTCCTGCTGGGCTTTT
WT	GCCTCATGGATTCAAAGCCACTGAGG	CCT <mark>GGCCACTGGGGGGCGCCATTC</mark> GCCATTAAAAGGTCCTGCTGGGCTTTT

Point mutation changes



- Codon CCA (Proline) is converted into TTA (Leucine)
- The previous codon is also mutated (synonymous) to destroy the PAM
- This manipulation also adds a novel Hpal restriction site, allowing easy genotyping



Homology Directed Repair



ATGGACCATAGTTACTCCTCCAACACC-AGTCATCCCC
ATGGACCATAGTTACTCC <mark>gtt</mark> AACACC-AGTCATCCCC
ATGGACCATAGTTACTCCTCCAACACCCAGTCATCCCC
ATGGACCATAGTTACTCC <mark>GTT</mark> AACACC-AGTCATCCCC
ATGGACCATAGTTACTCC <mark>GTT</mark> AACACC-AGTCATCCCC
ATGGACCATAGTTACTCCTCCAACACC-AGTCATCCCC
ATGGACCATAGTTACTCC <mark>GTT</mark> AACACC-AGTCATCCCC
ATGGACCATAGTTACTCCTCCCC
ATGGACCATAGTTACTCC <mark>GTT</mark> AACACC-AGTCATCCCC
ATGGACCATAGTTACTCC <mark>GTT</mark> AACACC-AGTCATCCCC
ATGGACCATAGTTACTCCTCCAACAAGTCATCCCC
ATGGACCATAGTTACTCC <mark>GTT</mark> AACACC-AGTCATCCCC
ATGGACCATAGTTACTCCTCCAACACC-AGTCATCCCC
ATGGACCATAGTTACTCC <mark>GTT</mark> AAC-CAGTCATCCCC

Larger modifications peptide tagging



Image courtesy of Ian Dykes

- A reliable strategy for introducing peptide tags to follow endogenous protein expression
- Very useful where there is no reliable antibody or where an existing antibody is unsuitable for e.g. ChIPseq applications

Larger insertions – long single-stranded DNA

- Larger insertions
 - GFP Knock-in
 - loxP flanking a criticial exon
- ssDNA can be made in the lab and used as a template following the CRISPR cut







Large chromosomal deletions





.....and inversions



CRISPR mutagenesis on a disease model background



Avoiding those lengthy breeding experiments...

- Conventionally alleles are multiplexed by breeding.
- Depending upon the number of alleles, this can be a very lengthy process as the chances of offspring inheriting all the required alleles at the correct zygosity becomes increasingly unlikely as the number of alleles increases

1st Locus

GeneA^{+/-} x GeneA^{+/-} \longrightarrow Homozygote KO in 1 generation (3 months) <u>2nd locus</u> GeneA^{-/-} x GeneB^{-/-} \longrightarrow Double KOs in 2 generations (6 months) <u>3rd locus</u> GeneA^{-/-}; GeneB^{-/-} x GeneC^{-/-} \longrightarrow Triple KOs in 2-3 generations (6-9 months)

• Since the TALEN mutagenesis method provides a fast route to achieving gene KOs, can we directly mutate complex mutant backgrounds ?

Mutagenesis directly on a pre-existing genetic model

- The technique can be applied on embryos generated from existing, well characterised, genetic models, avoiding the need for lengthy intercrosses
- Reversa model for atherosclerosis has fixed 4 homozygous genetic alleles:



WT	CATTTT <mark>TGCAGGATACAAGACCCT</mark> TTTGAAAGGGATCTCTGGGA <mark>AATTCAACAGTGGAGA</mark> GCTGGT
Reversa-171	CATTTTTGCAGGATACAAGACCCTTGGATCTCTGGGAAATTCAACAGTGGAGAGCTGGT
Reversa-752	CATTTTTGCAGGATACAAGACCCTTTTGACTGGGAAATTCAACAGTGGAGAGCTGGT
Reversa-145	CATTTTTGCAGGATACAAGACCCTTTTGAAAGGGATCTCTGGGAAATTCAACAGTGGAGAGCTGGT

New methods make it even easier to make a mouse





Simplifying things – combining genetic supply and electroporation







"Game changing" technology



Nuclease microinjection

Founders

EU/NIH Knock-out projects





AIM: A Knock-out C57BL/6 ES cell line for every annotated gene in the genome



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What's wrong with my mouse ?

