Transgenics and knockouts

Dr. Derek. P. Brazil
UCD Conway Institute
UCD School Biomolecular and Biomedical Science

DMMC course
Techniques and Strategies in Molecular Medicine
TCD Dec 11th 2007

Overview

-basic concepts and terms

-how to make a knockout mouse

-tissue specific gene deletion

-recent advances (siRNA mouse)

Basic Concepts and Definitions

-a knockout is a mouse that has a deletion of both alleles of a gene in a targeted fashion (homologous recombination)

-A transgenic is a mouse typically expressing one or more copies of a cDNA that is incorporated into the genome in a random fashion

(conditional knockout mouse-Cre/loxP system)

(inducible transgenics-tet on/off system)

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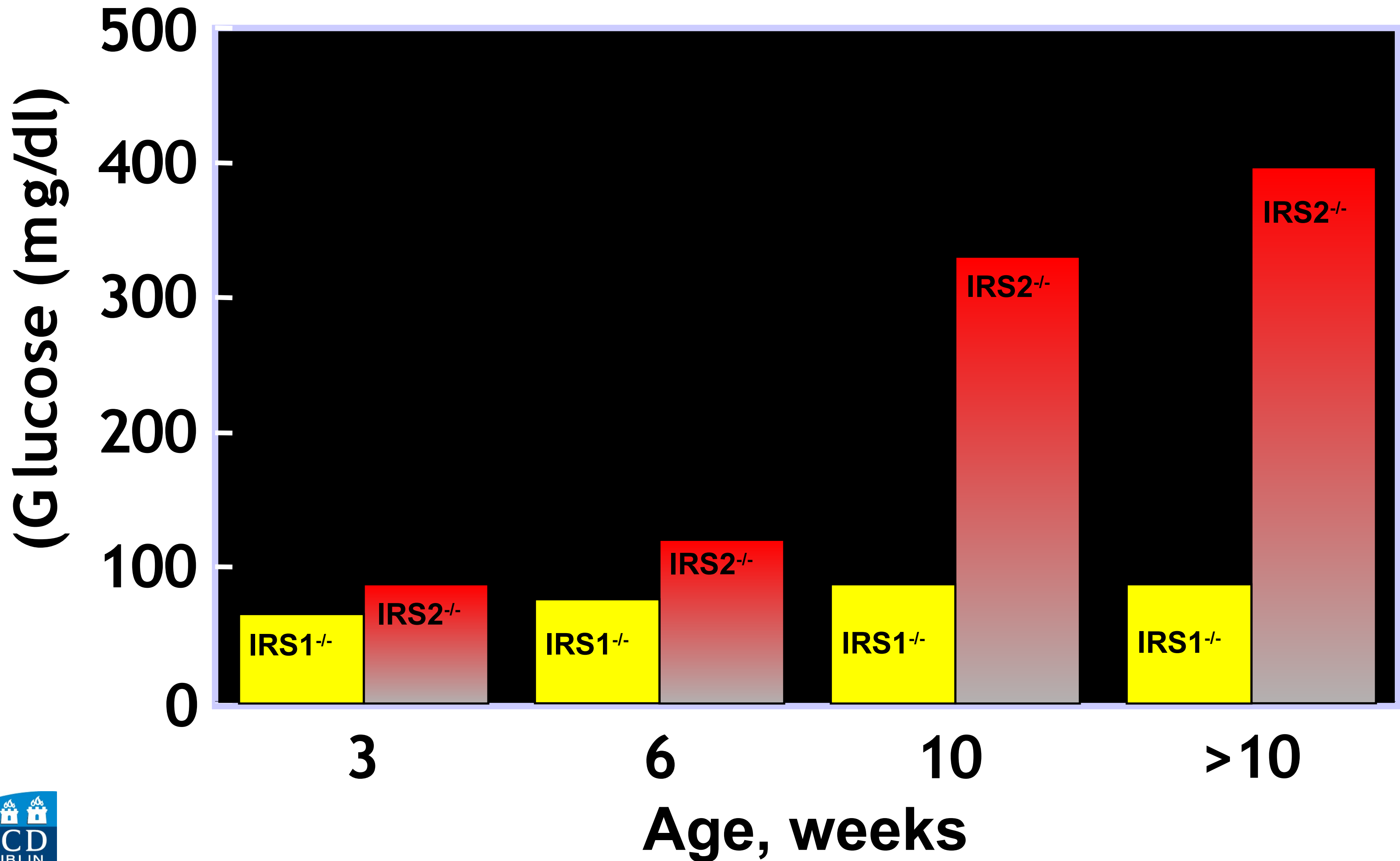
An Example of a Knockout mouse: the IRS-2 knockout



An example of a transgenic mouse: the NMDA receptor NR2B subunit

-NR2B subunit of the NMDA receptor expressed in brain

Enhanced NMDA receptor activation
Increased synaptic potentiation
Superior ability in learning and memory

Doogie

Biochemistry versus Genetics

Biochemist-reduction to principle components

Geneticist-perturbation of steps in process

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Mus Musculus (house mouse)

-mammal
-short gestation time (21 days)
-can manipulate at the embryonic stage
-embryonic stem cells maintain pluripotency in culture

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Of Mice and Men!!

Eomaia scansoria
(ancient mother climber)

75-125 million years ago
(early Cretaceous period)

Eomaia scansoria Ji et al. Ms. for *Nature* (correspondence: Luo)
Artwork Reconstruction: Mark A. Klingler/CMNH

-earliest known representative of the Eutheria lineage which gave rise to placental mammals

A short history of transgenic/knockout mice

1981 Isolation of pluripotent embryonic stem cells from mouse that remained undifferentiated in culture but retained their ability to differentiate in a suitable environment

(Martin et al, PNAS 78:7634)
(Evans and Kaufmann Nature 292:154)

1981 Ability to get foreign DNA into ES cells where they incorporated it into their genome
(Brinster RL Cell: 27:223)

1982 First demonstration of a change in mouse phenotype directly due to transgene incorporation in ES cells

(Palmiter RD Nature 30:611)

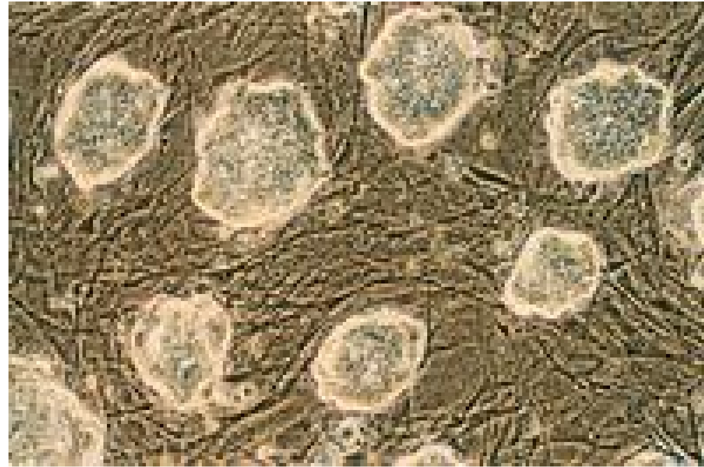
Mouse Embryonic Stem cells

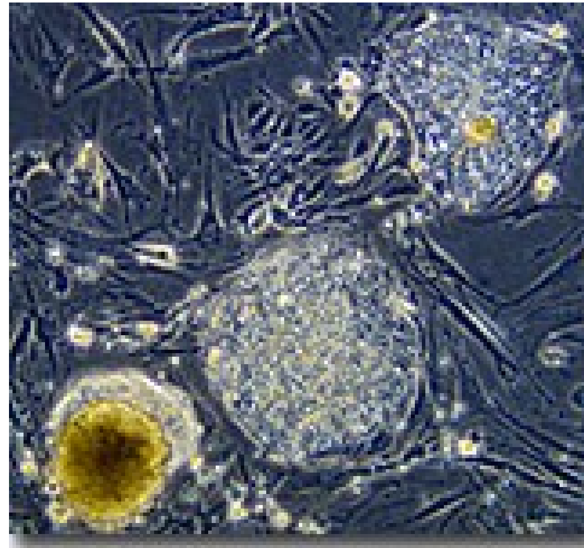
-Embryonic Stem cells (ES) are permanent cell lines isolated from mouse blastocysts which retain in culture their ability to differentiate when returned to a second host blastocyst by microinjection (Evans and Kaufmann, 1981)

-ES cells are grown in culture in the presence of leukocyte inhibitory factor (LIF) on a feeder layer of mitomycin-C treated (growth arrested) embryonic fibroblasts with antibiotic resistance

-only ES cells from mouse can contribute to the germ-line thus providing a route to transgenic animals

Mouse Embryonic Stem cells in culture





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A short history….. contd.

-limitation of this technique: could not predict the location of insertion of the transgene, or how many copies were inserted

1987 Mario Capecchi developed the ability to target the insertion and deletion of specific genes in ES cells

-homologous recombination

Capecchi, Smithies, Evans: Lasker prize 2001
Nobel Prize 2006???

Medicine 2007 - Microsoft Internet Explorer

http://nobelprize.org/cgi-bin/print?from=%2Fnobel_prizes%2Fmedicine%2Flaureates%2F2007%2Findex.html

Nobelprize.org

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: Tim Roberts/PR Newswire, © HHMI

Mario R. Capecchi

1/3 of the prize

USA

University of Utah
Salt Lake City, UT, USA;
Howard Hughes Medical Institute

b. 1937
(in Italy)

Photo: The Press Association Limited

Sir Martin J. Evans

1/3 of the prize

United Kingdom

Cardiff University
Cardiff, United Kingdom

b. 1941

Photo: Scanpix/Dan Sears

Oliver Smithies

1/3 of the prize

USA

University of North Carolina at Chapel Hill
Chapel Hill, NC, USA

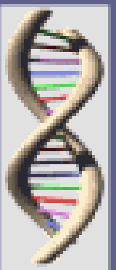
b. 1925
(in United Kingdom)

Titles, data and places given above refer to the time of the award.

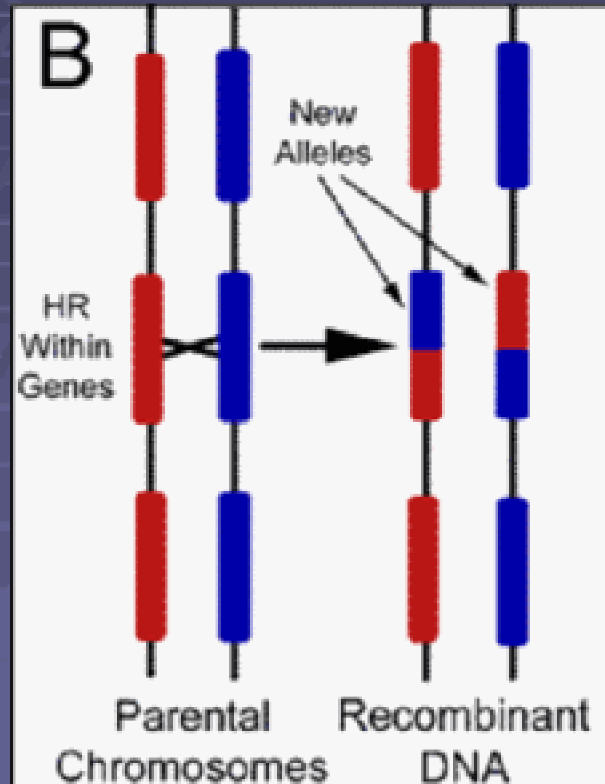
Homologous Recombination

A large group of reactions where a piece of homologous (similar) DNA is used to edit another.

- Performed during meiosis to create offspring diversity.
- Performed during mitosis to create cellular diversity.
- Repairs several types of DNA damage.
- Used to incorporate foreign DNA (genetic transformation).
- Is induced by a wide variety of environmental stresses such as nutrient deprivation, high cell density, and a great many carcinogens.
- Is induced or shut off as a programmed cell function during differentiation, and development.



New Alleles by Recombination



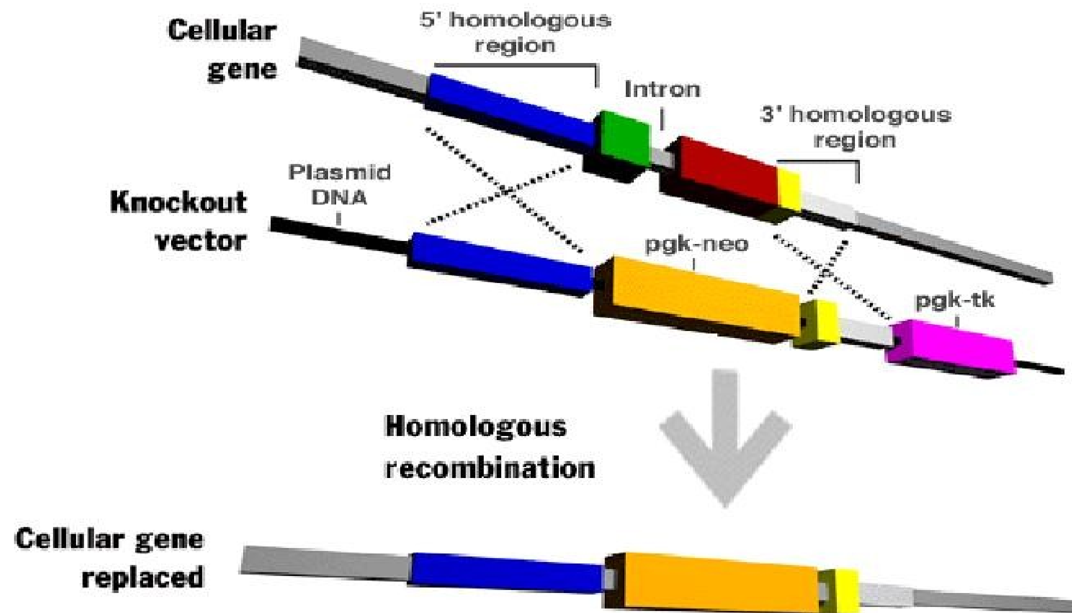
Recombination within genes can create new alleles.

Recombination is intentionally altering the genome, but the exact products remain largely unknown.

Given our limited knowledge, any alteration found to exist should be assumed the result of these reactions.

-this is why we can have different traits to either of our parents

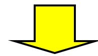
Replacement Knockout Vector



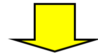
Making a knockout mouse: Where do we start?

How do we get from a piece of DNA in a test-tube to a mouse?

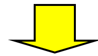
1. Vector generation



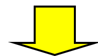
2. Electroporation of embryonic stem (ES) cells



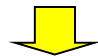
3. Selection of ES clones containing homologous recombination



4. Microinjection of targetted ES clones into blastocysts



5. Chimera generation and germline transition



6. Breeding of mice to generate knockouts

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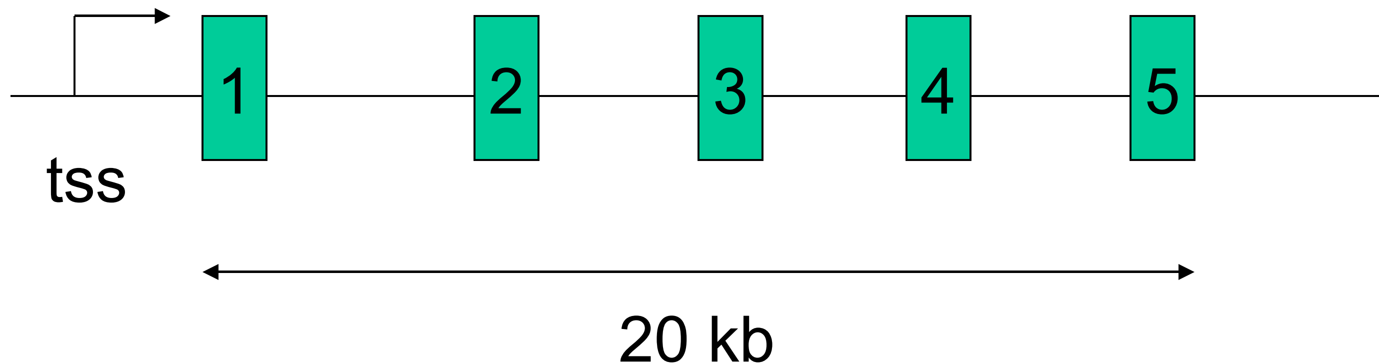
Basic Strategy to generate a plasmid vector to target a gene for deletion by homologous recombination (“knockout”)

Step 1: Determine the size and structure of your gene



Step 2: Determine what exons code for what domains in your protein (e.g. ligand binding domain, catalytic domain etc.)

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Basic Strategy to generate a plasmid vector to target a gene for deletion by homologous recombination (“knockout”)

Step 3: decide what part of the gene you want to target to generate your knockout

i) Target exon 1 (start codon)
ii) Functional domain
iii) Whole gene-may be problematic
iv) Significant portion of the gene

Step 4: get your hands on some genomic DNA from mouse

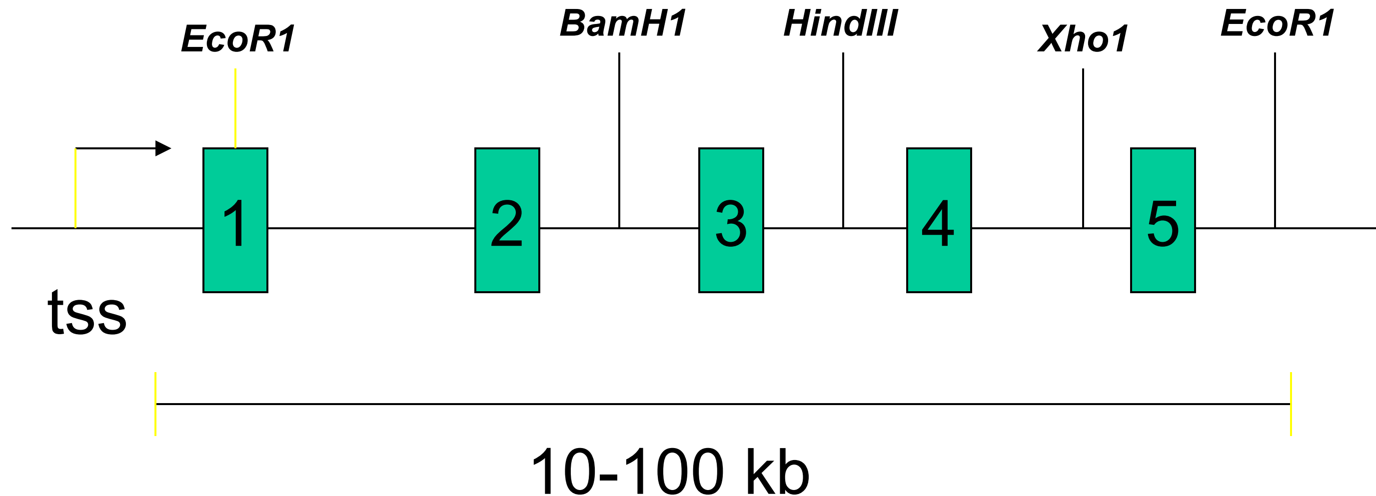
-λ phage libraries
-BAC clones (PCR screening)

→ must be isogenic with the ES cells, otherwise no recombination will occur (129 sv DNA and cells)

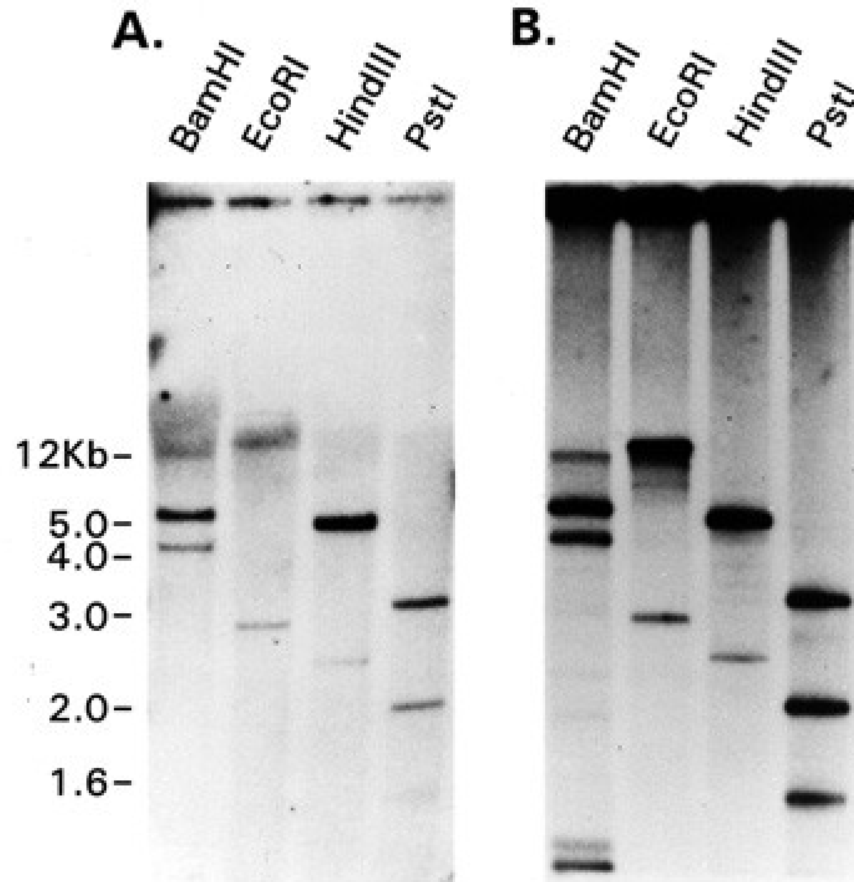
Basic Strategy to generate a plasmid vector to target a gene for deletion by homologous recombination ("knockout")

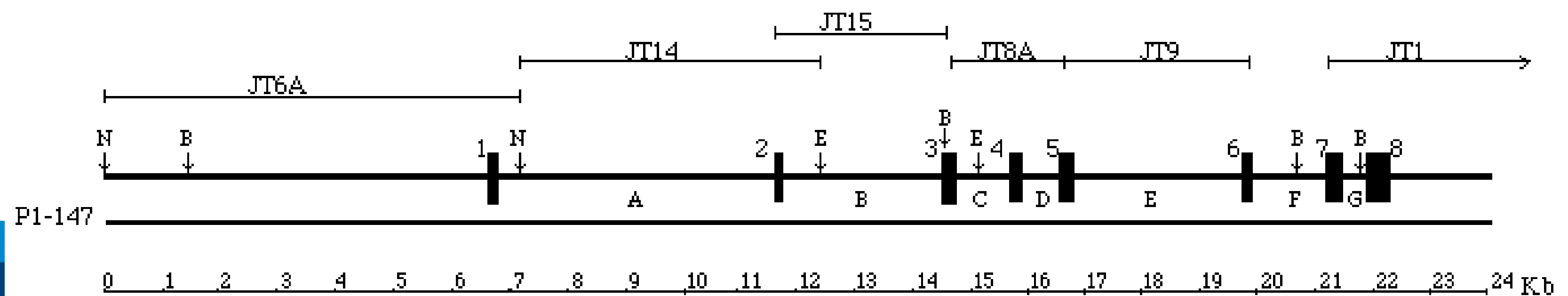
Step 5: characterise the overall gene structure using Southern blotting and PCR

→ construct a roadmap of the gene of interest



Mapping gene structure using restriction digest/Southern Blot

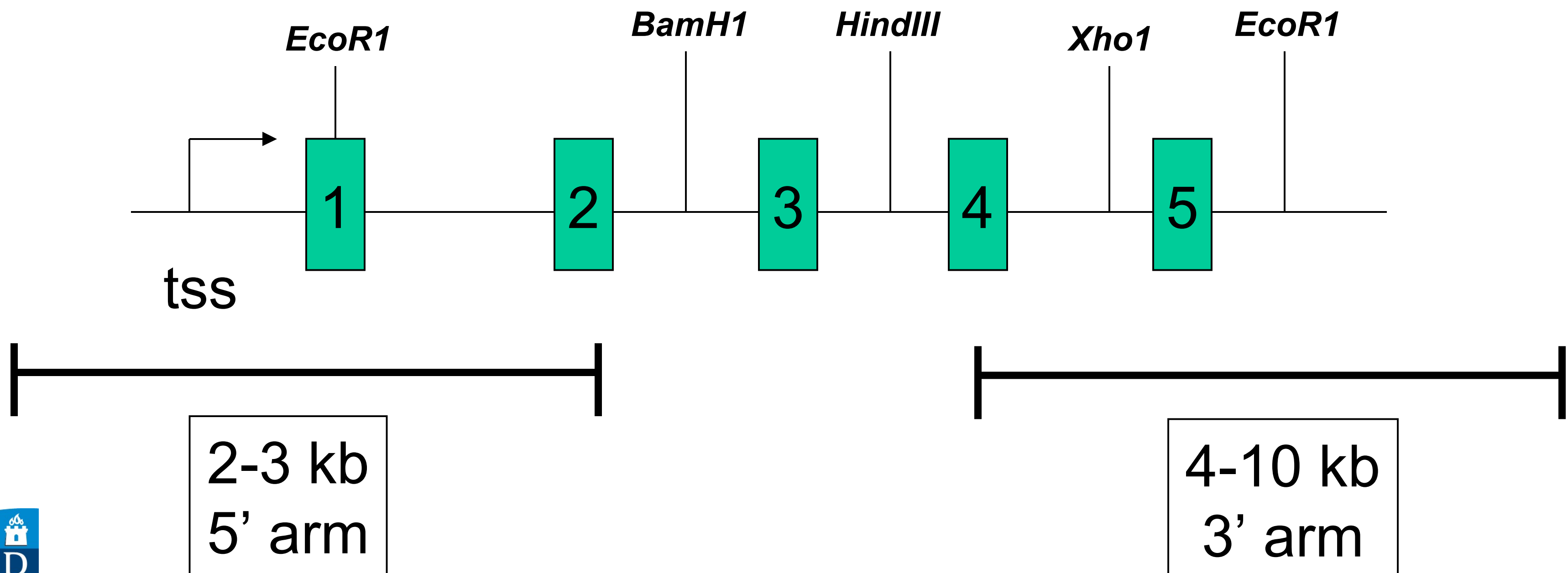




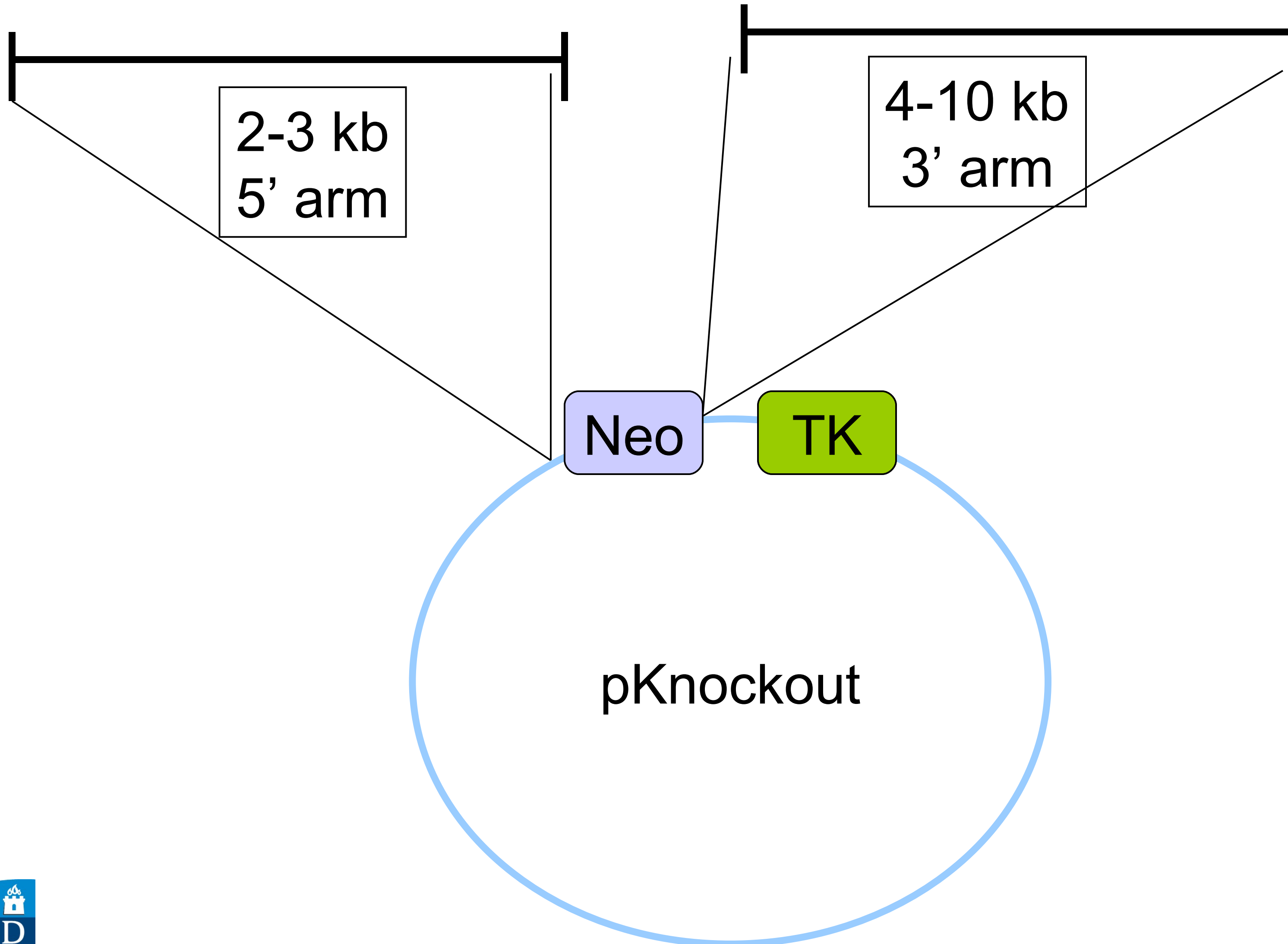
Basic Strategy to generate a plasmid vector to target a gene for deletion by homologous recombination ("knockout")

Step 6: Construction of the vector

→ e.g. to target exon 3 for deletion



Basic Strategy to generate a plasmid vector to target a gene for deletion by homologous recombination ("knockout")



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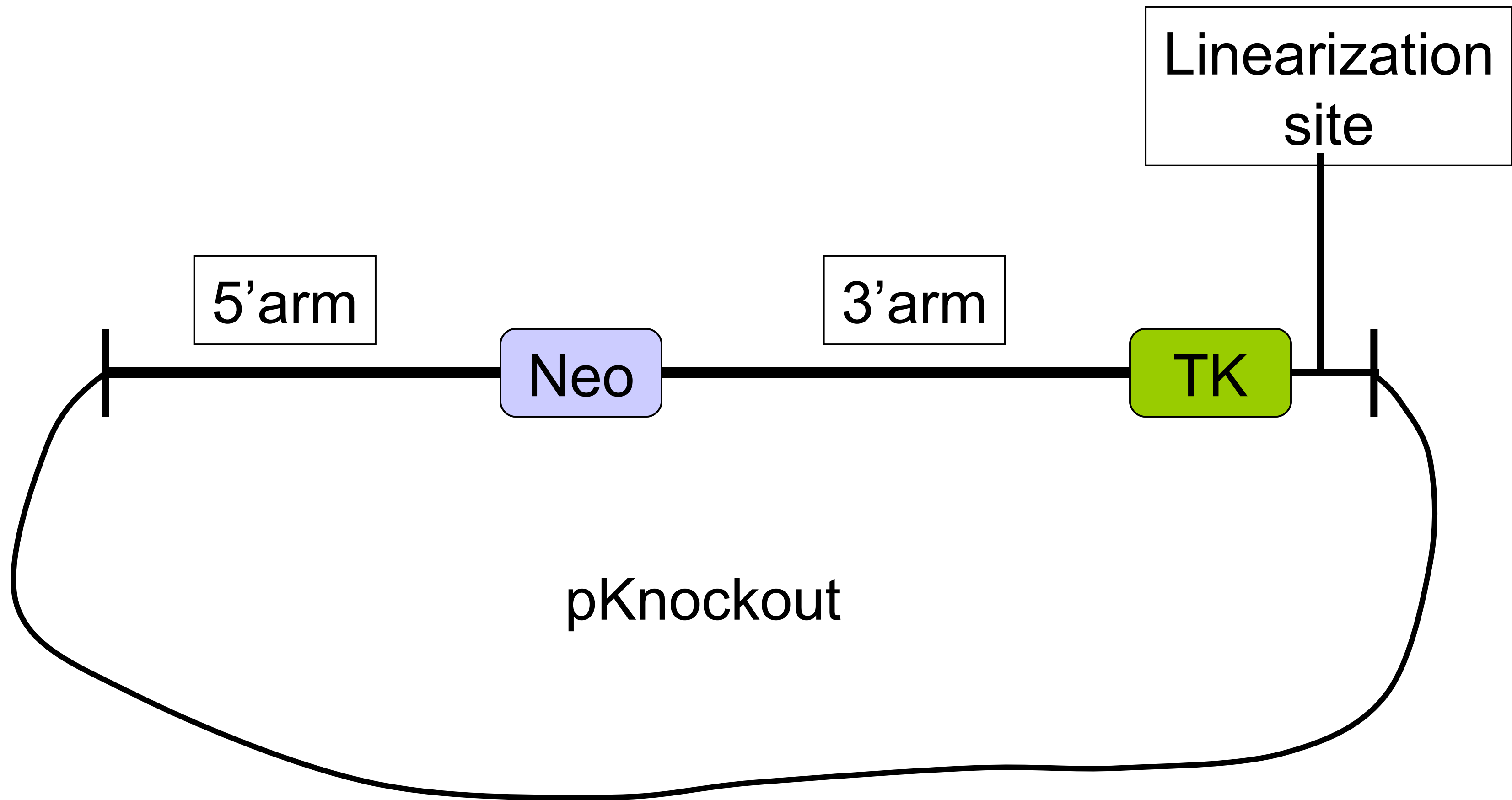
Basic Strategy to generate a plasmid vector to target a gene for deletion by homologous recombination ("knockout")



-to get to this point takes 3-6 months

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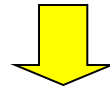
Electroporation of ES cells with plasmid vectors for generation of knockout mice

Digest and prepare 40 μg of plasmid vector containing knockout cassette



Electroporate 1-2 x 10^7 ES cells with DNA

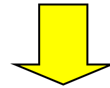
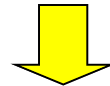
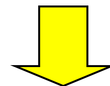


Plate cells in medium with antibiotic, change the medium every day (even Xmas!!)



Pick colonies after 10 days



Screen for homologous recombination

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How do identify ES cell colonies with homologous recombination?

Positive and Negative selection

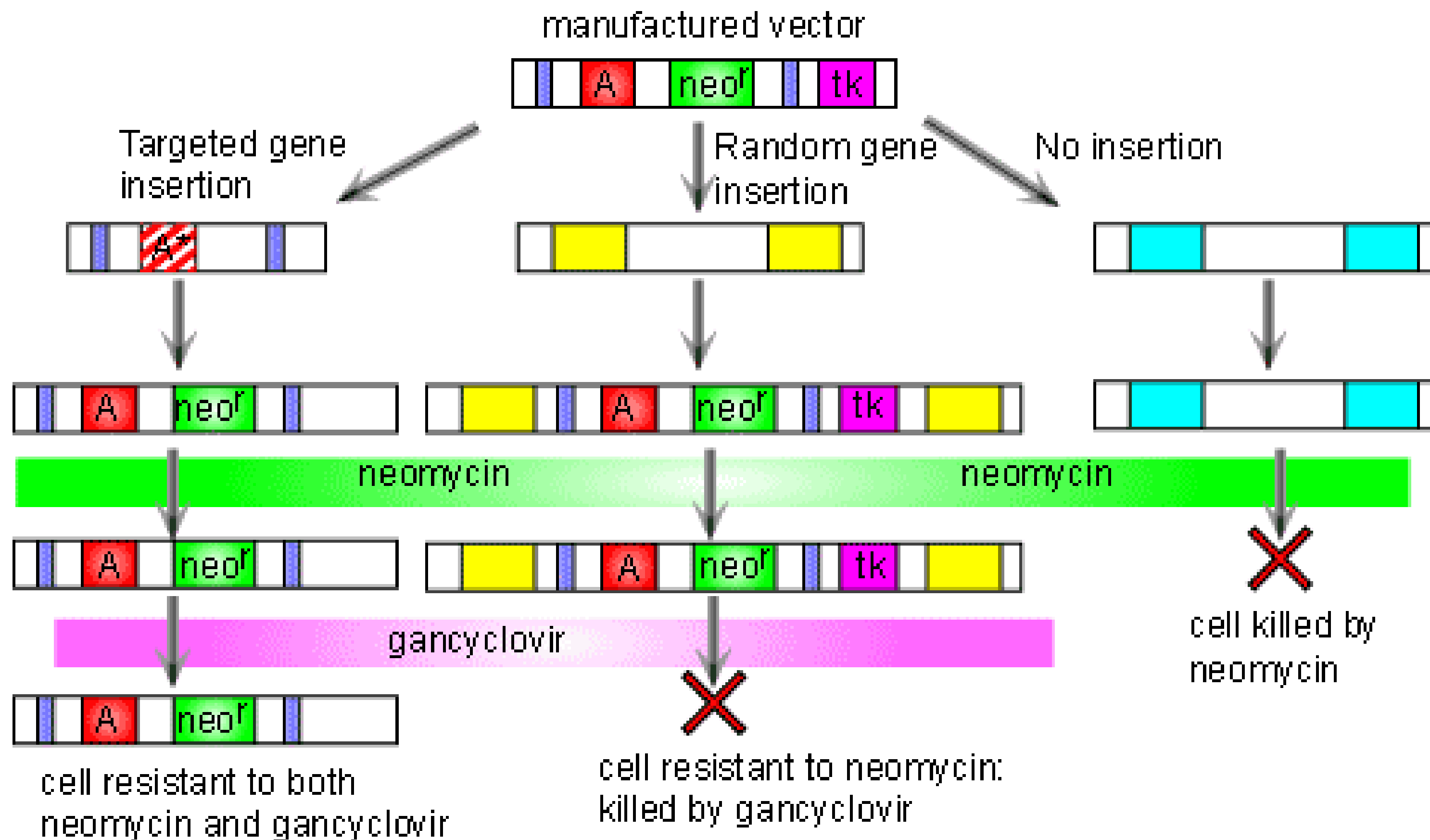
- Positive selection: cells that have undergone homologous recombination will contain the NeoR gene, and will be able to grow in the presence of G418 antibiotic

__Problem__: cells that have incorporated the targeting vector randomly into their genome will be resistant to G418

ii) Negative selection: thymidine kinase (TK) cassette is present in the targeting construct and will only be inserted into genomic DNA during random (non-homologous) recombination

-cells containing the TK cassette (i.e. non-homologous recombinants) will die in the presence of gancyclovir

Positive-Negative screening of homologous recombinant ES cells



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Learning Objectives

-molecular biology: how to make the targetting vector

-ES cell transfection/screening

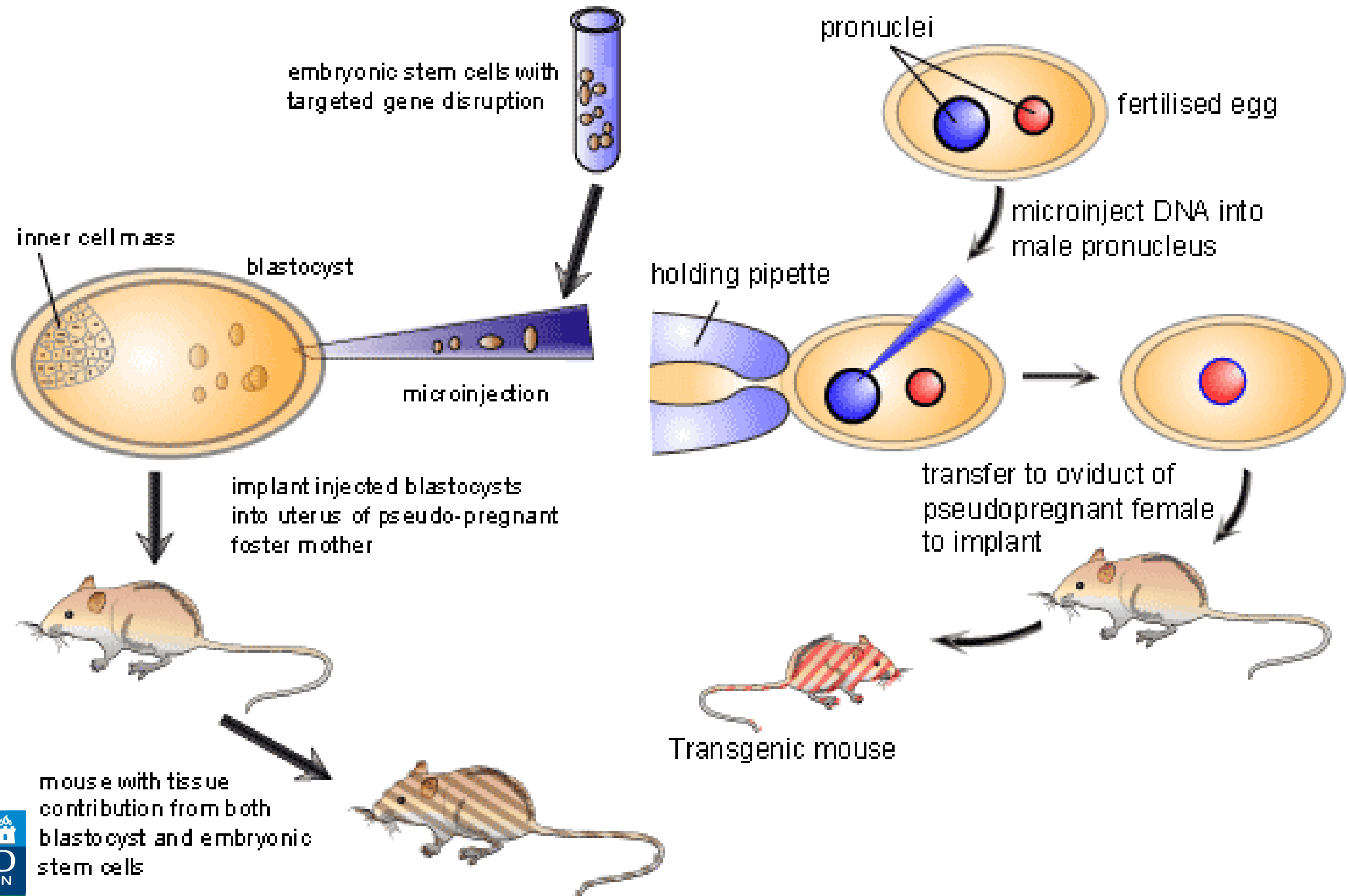
-screening strategies-positive and negative selection

-implantation of targeted ES cells

-mating strategies

-phenotype characterisation

Microinjection of targeted ES cells into mouse blastocysts



How do you know if your microinjection has worked?

-chimeric offspring will have contributions from both the parental mother (black) and the microinjected ES cells (brown 129 sv strain)

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How do you know if your gene has been targeted in the germline?

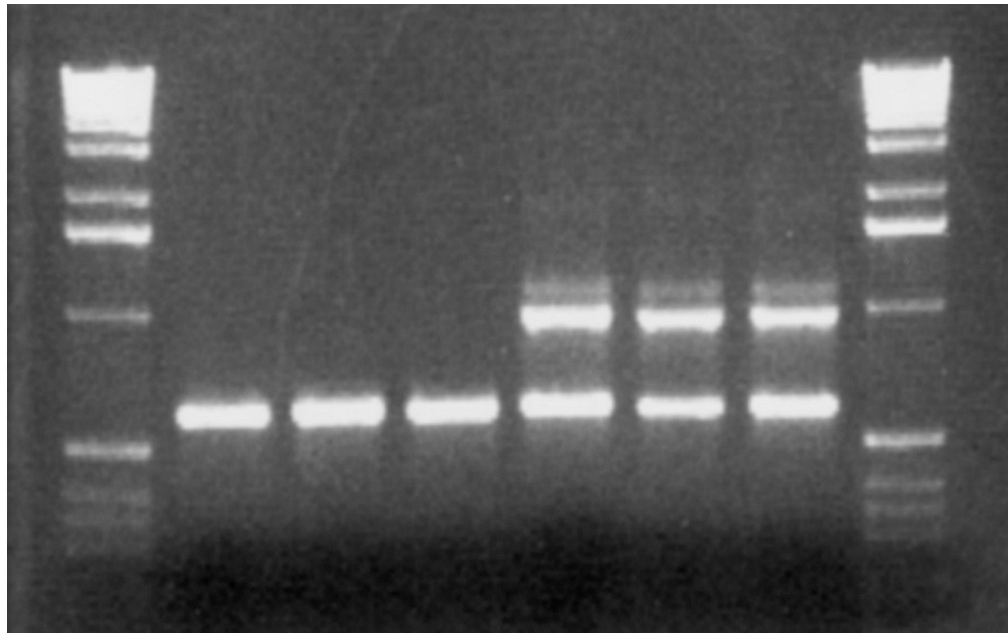
-mate chimeric male (will have both targeted and non-targeted sperm cells) with wild-type female mouse

-this F1 generation should contain both wild-type mice and mice heterozygous for the deletion of your gene

-use PCR or Southern blotting to analyse the genotype with DNA extracted from mouse tail tips

PCR genotyping on pups from CTMP chimeric crosses

Chimeric ♂ x wild-type ♀



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Generation of animals for experimental characterisation

-once you have confirmed that there is germline transmission of your targeted deletion, you can start to breed animals for phenotype characterisation

<u>Possible Phenotypes of Knockout mice</u>

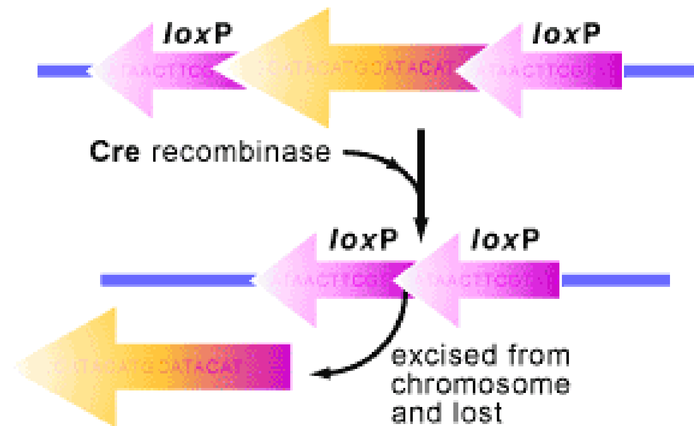
1. No phenotype (try not to cry)
2. Lethal phenotype (cancel the holiday)
3. Expected phenotype based on predictions
4. Unexpected phenotype

Conditional or tissue-specific knockout mice

-if your complete/whole body knockout is lethal, then try to target specific tissues for deletion

-Cre/lox system of gene deletion

-Cre gene is a DNA recombinase that cuts DNA at *loxP* sites (34 bases) and excises intervening sequence and ligates DNA back together



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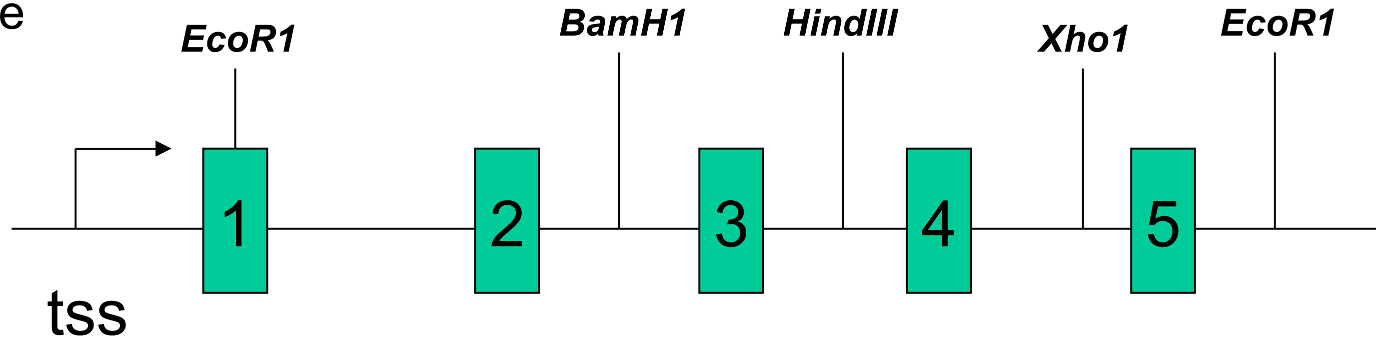
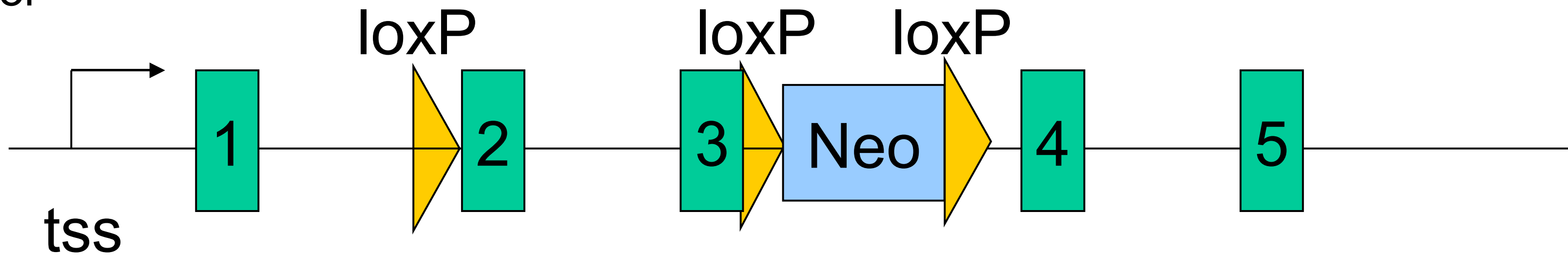
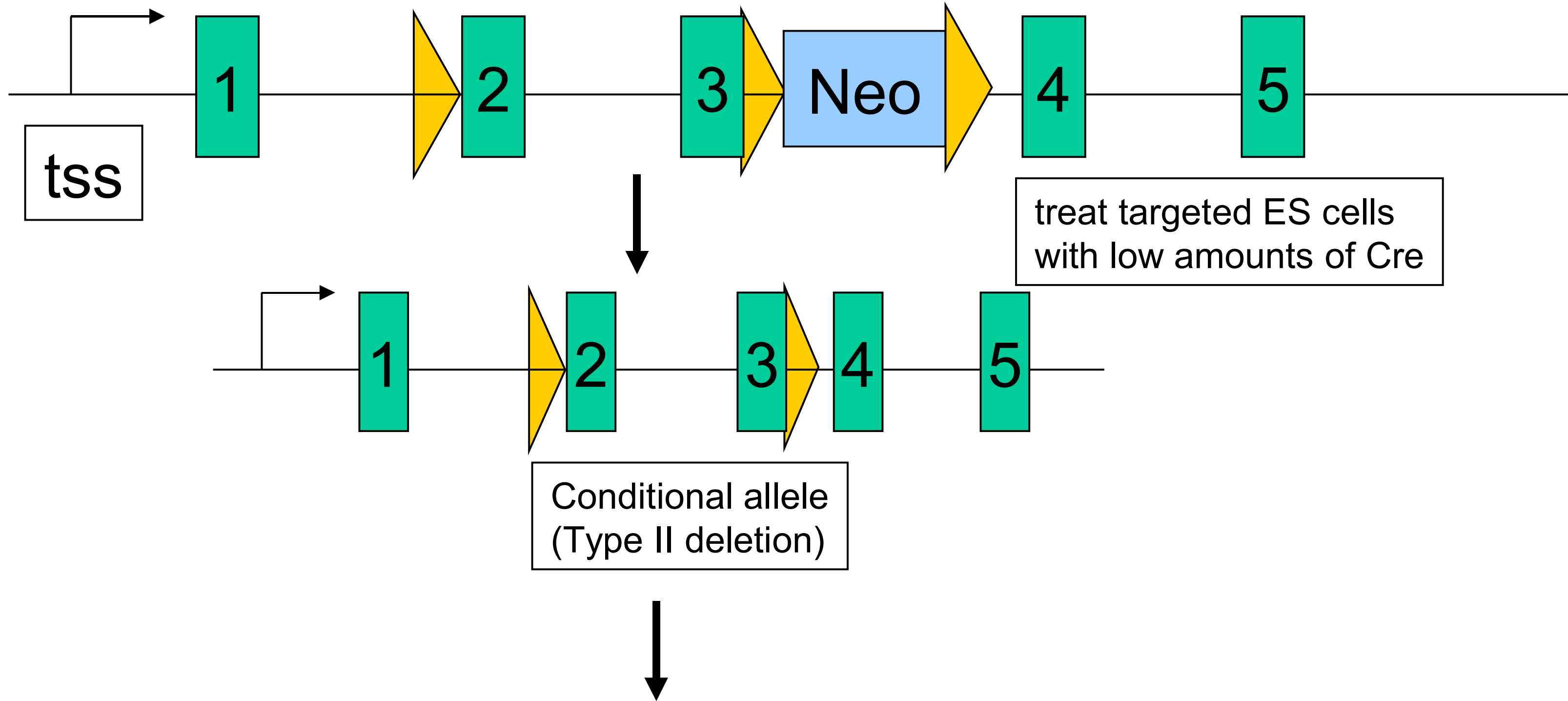
Cre/lox system for tissue-specific gene deletion (conditional knockouts)





Cre/lox system for tissue-specific gene deletion (conditional knockouts)



use these ES cells for blastocyst injections to generate heterozygous mice with a single allele of your target gene "floxed"

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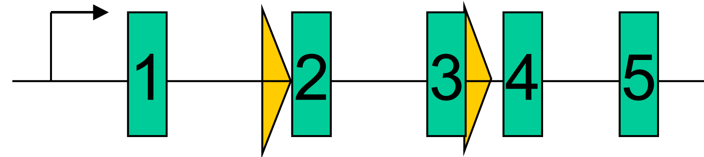
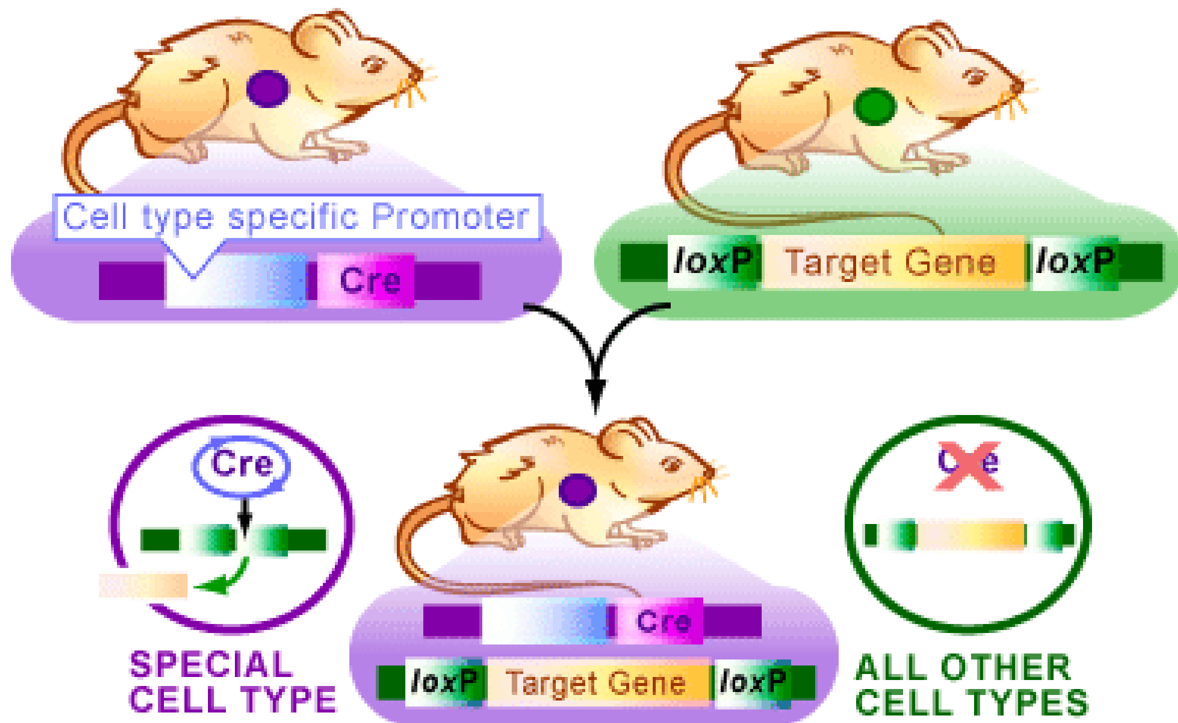
Cre/lox system for tissue-specific gene deletion (conditional knockouts)

Cre-mice commercially available





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Cre/lox system for tissue-specific gene deletion (conditional knockouts)

Examples of Cre mice

Neurons
Liver
Muscle
Adipocyte
Endothelium
Heart

-slight caveat: expression of Cre is not always confined to one tissue

Don’t Panic!!!!

-European Mouse Mutant Archive (EMMA)
-NIH Consortium

-make mice lacking every single gene (15 % are needed For development)

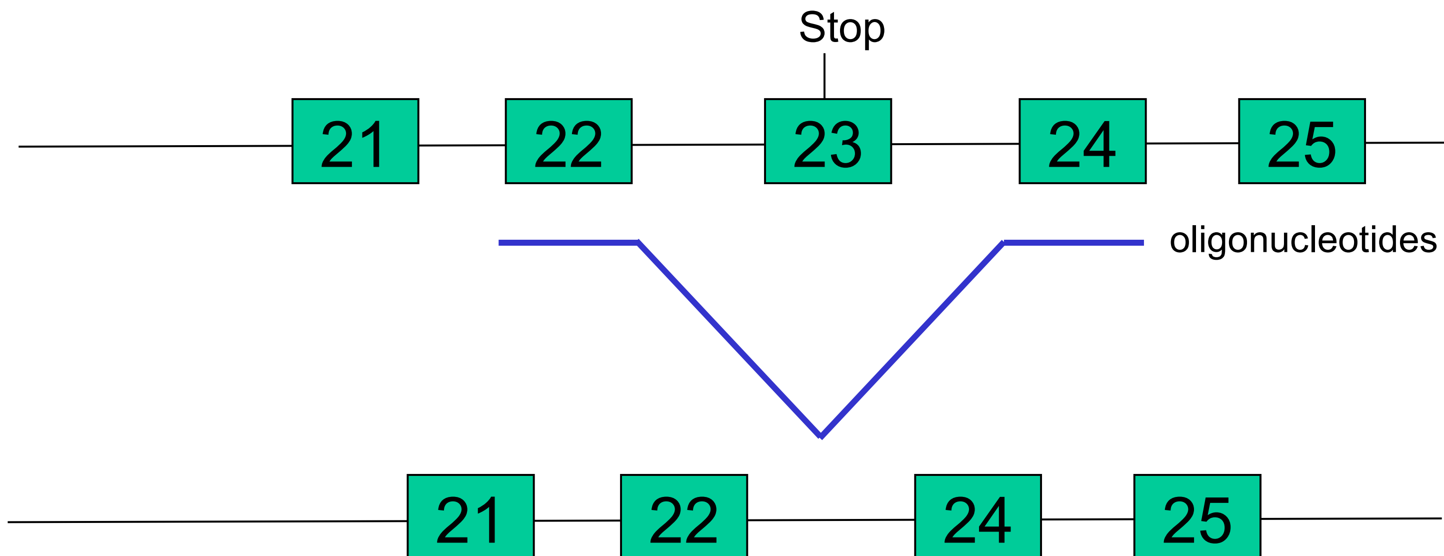
-make 10 or more type of mutant per gene

-within the next 5-10 years

Spliceomouse and Duchenne Muscular Dystrophy

-exon skipping to "bypass" disease-causing mutations

Muscular Dystrophy: premature termination of dystrophin



-expression of shortened dystrophin
-partial restoration of function

Gene therapy?

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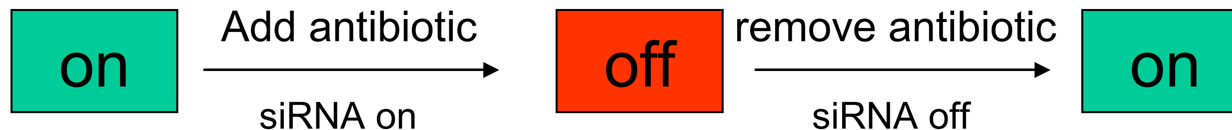
In vivo siRNA expression in mice

-more relevant pharmacologically?

-takes 3-4 months to make an inducible RNAi mouse

-targetted transgenesis into “neutral” chromosomal loci by homologous recombination e.g. Rosa 26



-large numbers of mice available (>100)

-90 % concordance btw siRNA mice and knockout mice!

-drawback? “off target” effects

http://www.genscript.com/sirna_invivo.html

Overview

-mouse embryonic stem cells can be cultured in an undifferentiated state, and retain their pluripotent status

-exogenous DNA can be stably inserted into ES cells both randomly (transgenics) and targeted (homologous recombination)

-homologous recombination allows for targeted deletion of genes

-germline transmission permits the propagation of gene deletion from generation to generation

-Cre/LoxP system allows the tissue specific deletion of genes in individual tissues

Further reading

-www.bioteach.ubc.ca/Molecular Biology

-www.labs.roslin.ac.uk/mcwhir   ES Cells

The Scientist 16:13
The Scientist 14:15 General review on knockouts
BioEssays 20:200-208 (Conditional knockouts)
Genesis 32:49-62 (Conditional knockouts)

The gene knockout Facts book: Tak Mak (Academic press)