GENE TARGETING STRATEGIES

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**Why Mice?**

1) Mouse is a mammal and its development, body plan, physiology, behavioural and diseases have much in common with humans

2) Almost all mouse genes (99%) have homologs in humans

3) Mouse genome supports targeted mutagenesis in specific genes by homologous recombination in ES cells, allowing genes to be altered efficiently and precisely

4) Laboratory models of human disease

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**Animal Models**

Although they do not replicate all of the features of the disease they...

1) Allow a simplified view of the complex pathology found in human diseases

2) Provide a tractable and reproducible system to identify inflammatory pathways

4) Allow testing therapeutic interventions

5) Should be characterized by simplicity of the experimental design combined with short duration of the experiment, low costs and minimal harm to animal welfare.
Reductionist approach, which involve inferring gene function from one or a small number of genes might not have sufficient power to provide significant understanding of how truly complex biological phenomena such as high re cognitive functions are mediated, particularly in

**Day 1 Gene Targeting**

- **Knockout** (deletion gene/part gene)
- **Knockin** (introduction function)

**Mutation SPECIFIC genes in embryonic stem cells**

**Day 2 Gene Transgenesis**

- **Small Transgene** (conventional Tg)
- **Big Transgene** (BAC Tg)

Injecting a transgene into fertilized eggs **RANDOMLY**

**3-Cre Recombination**

- Cre Tg animals and Applications

**4-Mouse Models a la Carte**
**Gene Targeting vs Transgenesis**

1. **Choice of genetic locus to mutate**
2. **Takes full advantage of all the resources provided by the known sequences of the mouse and human genomes**
3. **Control of how to modulate the chosen genomic locus (spatial/temporal restrictions)**
1-Gene Targeting

Produce specific mutation in ES cells

1.1 CULTURING ES CELLS
1.2 MUTATING ES CELLS
1.3 GERMLINE
1.4 STRAINS
1.5 APPLICATIONS

Gene Targeting Scheme

Mutate ES cells by Gene targeting
Produce specific mutation in mammal cells

A. Gene targeting of embryonic stem cells

Mouse blastocyst

Mouse blastocyst

Culturing ES

Mutating ES


Transfer mutation germline

Generation of gene targeted mice

Targeted ES cells are injected into blastocysts...

...which are implanted into foster mothers

Mating between chimeric mice and normal mice.

Female

Sperm

Sperm

Gene targeted mice

Normal mice
Es cells isolated from the ICM of blastocysts of 129 mouse strain

Gene Targeting Technology

1. Culturing ES Cells

- Thomas and Capecchi, Cell 1987

2. Mutating ES Cells

- Mansour et al, Nature 1988

3. Germline Transmission

- Bradley et al, Nature 1984
- Thompson et al, Nature 1989
- Wood et al, Nature 1993
- Nagy et al, PNAS 1993
Produce specific mutation in mammal cells

A. Gene targeting of embryonic stem cells

Culturing ES  Mutating ES


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

Sir Martin J. Evans  Mario R. Capecchi  Oliver Smithies
1.1 CULTURING ES CELLS

- Isolation of EC lines: Kleinsmith et al. 1964
- Culture EC cells on Feeder cells: Martin and Evans 1975
- First transgenic mouse (from EC cells): Brinster et al. 1974; Papaioannou et al., Nature 1975


Development of the Preimplantation Blastocyst in Mice from Embryonic Day 0 (E0) Through Day 5 (E5.0)
The first cleavage produces two identical cells and then divides again to produce four cells. If these cells separate, genetically identical embryos result, the basis of identical twinning. Usually, however, the cells remain together, dividing asynchronously to produce 8 cells, 16 cells, and so on. By the 16-cell stage, the compacted embryo is termed a morula. In mice, the first evidence that cells have become specialized occurs when the outer cells of the 16-cell morula divide to produce an outer rim of cells—the trophectoderm—and an inner core of cells, the inner cell mass. The cells of the inner cell mass and trophectoderm continue to divide. Information gained from the study of mouse embryos suggests that the two tissues need to interact; the inner cell mass helps maintain the ability of trophectoderm cells to divide, and the trophectoderm appears to support the continued development of the inner cell mass [32]. By embryonic day 3 (E3.0) the embryo develops a cavity called the blastocoel. It fills with a watery fluid secreted by trophodermal cells and transported in from the exterior. As a result of cavitation and the physical separation and differentiation of the trophectoderm from the inner cell mass, the morula becomes a blastocyst. Its chief structural features are the outer sphere of flattened trophoderm cells (which become the trophoblast), the small, round cells of the inner cell mass, and the fluid-filled blastocoel. By E4.0 in mice, and between 5 to 7 days postfertilization in humans, the blastocyst reaches the uterus.
The blastocyst is a hollow sphere made of approximately 150 cells and contains three distinct areas: the trophoblast, which is the surrounding outer layer that later becomes the placenta, the blastocoel, which is a fluid-filled cavity within the blastocyst, and the inner cell mass, consisting of primitive endoderm and ectoderm. Each of these compartments has its unique potential as well as limitation. Trophoblast cells are committed to the development of the trophoblast cells in the placenta. Primitive endoderm cells are capable of forming the outer layers of the yolk sac, while primitive ectoderm cells will contribute to the embryo proper. It is important to note that this potential is strictly accompanied by a limitation. Each of the three cell types of the blastocyst is restricted to the contribution listed above.
The end of the beginning for pluripotent stem cells
Peter J. Donovan and John Gearhart
Nature 414, 92-97 (1 November 2001)
**Embryonal Carcinoma Cells**

Malignant multidifferentiated tumors containing a significant population of undifferentiated cells (Embryonal carcinoma cells). EC could be propagated in culture. Individual EC cells are self-renewing, pluripotent Stem cells. Introduced in the embryo by Brinster (1974).

*BUT* most EC line show poor differentiation potential in vitro and in vivo contribute poorly to chimeras and/or produce embryonic tumors.

After several years, they started culturing EC with **feeders**, showing that not only allowed the efficient establishment of EC culture but also increased their differentiation activity.

**Embryonic Stem cells**

They are derived directly from mouse blastocysts. Protocols for ES derivation are simple and remain unchanged to the present day. ES clones resemble EC cells in morphology, growth behavior, and marker expression. They also share the capability of forming teratocarcinomas. The most extraordinary attribute of ES is that even after extended propagation and manipulation *in vitro*, they remain capable of re-entering embryogenesis. In contrast to EC, ES behave relatively consistently in their ability to integrate into the embryo and produce viable chimeras. ES cells maintain a diploid karyotype. This is crucial because a balanced chromosome complement is necessary for meiosis. The landmark of deriving mice from cultured ES cells was reported by the Evans lab (Bradley et al, 1984).
1.1 Isolation of EC cell lines...

The concept that differentiated cells and tissues are derived from undifferentiated stem cells ("Stammzellen") was already proposed a hundred years ago [1]. However, their precise properties remained elusive for many decades. Studies of testicular teratomas showed that these tumours contain totipotent cells. In the 1950s, Leroy Stevens at the Jackson Laboratory found that mice of the 129Sv strain have a high frequency of such tumours. He showed that their cells could develop into embryoid bodies, i.e. aggregates of embryonic cells. When transplanted, such aggregates could induce solid tumours with many different cell types [2, 3]. A few years later, Kleinsmith and Pierce demonstrated that such tumours were derived from undifferentiated embryonal carcinoma cells (EC) [4].

The development of cell culture techniques permitted investigators to establish cultures of embryonal carcinoma cells (EC cells) from murine testicular teratocarcinomas. Several scientists including Martin Evans at the University of Cambridge reported on such cultures in the early 70s [5-7]. Evans obtained 129Sv mice from Stevens, established a colony of mice, and characterized the teratoma derived cells in culture [8, 9]. These embryonal carcinoma (EC) cells could be grown on feeder layers of irradiated fibroblasts. When the latter were withdrawn, extensive in vitro differentiation occurred. It proceeded through a primitive embryonic endoderm, which clumped into embryoid bodies. Attachment on a solid surface gave rise to all kinds of cell types, including skin, nerve, beating cardiac muscle, etc. This showed that the EC cells differentiated in the same way as the inner cell mass of the mouse embryo [8, 9].

Evans saw the potential in using these EC cells not only for cell culture studies but also for creating chimeric mice. In order to realise this vision, he established a collaboration with Richard Gardner in Oxford, who made injections of EC cells into blastocysts and reimplanted them into foster mice. The offspring was chimeric, with contributions from EC cells in nearly every tissue [10]. Similar findings were made by several other groups at about the same time, [11] [12]. However, chimeric mice carrying EC derived cells developed multiple tumours and could not contribute to the germ line due to karyotypic abnormalities.
**History of EC cell lines:**


1.2 Isolation of ES cell lines:

It became obvious to Evans that an alternative strategy had to be used if one were to obtain germline transmission derived from cultured embryonic stem cells. With the use of monoclonal antibodies, he characterised cell surface macromolecules of EC cells and their normal counterparts, thus identifying molecular markers of early differentiation [13]. The results suggested that normal cells with a similar phenotype as EC cells could be found and used for experiments. In 1980, Evans teamed up with the embryologist Matt Kaufman to combine cell culture and embryo manipulation. As described by Evans in a later review [14], he had intended to use haploid embryos for cell culture but prepared some diploid ones as controls. These cells were the embryonic stem cells (ES cells) that became critical for the success of gene targeting. Evans and Kaufman published their report on ES cells in a seminal paper in Nature in July, 1981 [15]. Gail Martin, a former co-worker of Evans, reported similar findings half a year later [16]. In their Nature paper, Evans and Kaufman pointed out the possibility of using ES cells for gene modification. Evans' team set up blastocyst injection techniques to test whether indeed ES cells could contribute to functional germ cells and thus be used to create a chimeric mouse. They reported successful germline transmission in 1984, in another landmark paper in Nature [17].

It's a color-enhanced electron microscope image of mouse embryonic stem cells growing on a bed of silicon nanotubes. The image was taken in the lab of Bruce Conklin at the Gladstone Institute for Cardiovascular Medicine.

http://thepluripotent.com/?tag=cirm

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**Properties of Mouse ES Cells**

1. Origin from the *ICM/epiblast*
2. Unlimited *self-renewal* capacity
3. *Pluripotent*, can generate all fetal and adult cell types in vitro and in teratoma
4. Stable diploid karyotype
5. Extrinsic suppression of differentiation by gp130 cytokine
6. Oct-4 mediated transcriptional orchestration
7. Absence of G1 cell cycle checkpoint
8. Rapid proliferation and *unique cell cycle* kinetics
9. Germline colonization and transmission
10. They are *XY*
ES cells are derived from the inner cell mass of a blastocyst

1. ES cells are derived from the inner cell mass of a blastocyst

Preparation of embryonic stem cells

These cells are used in the generation of transgenic mice. Mutated DNA is introduced into the cells, and cells are selected for the expression of the transgene.

http://stemcells.nih.gov
Self-renewal is division with maintenance of the undifferentiated state (the ability to go through numerous cycles of cell division while maintaining the undifferentiated state).

This requires cell cycle control and often maintenance of multipotency or pluripotency, depending on the stem cell. Self-renewal programs involve networks that balance proto-oncogenes (promoting self-renewal), gate-keeping tumor suppressors (limiting self-renewal), and caretaking tumor suppressors (maintaining genomic integrity). These cell-intrinsic mechanisms are regulated by cell-extrinsic signals from the niche, the microenvironment that maintains stem cells and regulates their function in tissues. In response to changing tissue demands, stem cells undergo changes in cell cycle status and developmental potential over time.

Stem cell is, in the functional definition, a cell that has the potential to regenerate tissue over a lifetime.

ES cells possess indefinite self-renewal potential


ES cells are pluripotent

ES cells are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type.

They do not contribute to the extra-embryonic membranes or the placenta. mouse ES cells cannot produce all type of cells, in particular they don’t produce trophoblast, they cannot produce a blastocyst the novo and hence are not sufficient to produce an embryo.
Simply put, stem cells are primitive cells that give rise to other types of cells. Also called progenitor cells, there are several kinds of stem cells.

**Totipotent cells** are considered the "master" cells of the body because they contain all the genetic information needed to create all the cells of the body plus the placenta, which nourishes the human embryo. Human cells have this capacity only during the first few divisions of a fertilized egg. After 3 - 4 divisions of totipotent cells, there follows a series of stages in which the cells become increasingly specialized. The next stage of division results in **pluripotent cells**, which are highly versatile and can give rise to any cell type except the cells of the placenta or other supporting tissues for the uterus. At the next stage, cells become **multipotent**, meaning they can give rise to several other cell types, but those types are limited in number. An example of multipotent cells is hematopoietic cells—blood stem cells that can develop into several types of blood cells, but cannot develop into brain cells. At the end of the long chain of cell divisions that make up the embryo are "terminally differentiated" cells—cells that are considered to be permanently committed to a specific function.
Mechanisms of stem cell self-renewal and pluripotency

The POU domain transcription factor Oct4, the SRY-related HMG-box transcription factor Sox2 are critical for the pluripotency of the inner cell mass in vivo and ES cells in culture (Nichols et al. 1998, Niwa et al. 2000). Sox2 cooperates with Oct4 to activate the expression of a number of genes that regulate pluripotency including Oct4 and Nanog (Masui et al. 2007 and references therein). The homeodomain protein Nanog is also required for the maintenance of pluripotency in the inner cell mass in vivo (Mitsui et al. 2003). The overexpression of Nanog can bypass the requirement for leukemia inhibitory factor (LIF) in maintaining mouse ES cell pluripotency in culture, and Nanog-deficient ES cells are prone to spontaneous differentiation, though Nanog is not absolutely required for the maintenance of pluripotency in ES cells under favorable culture conditions (Chambers et al. 2003, Mitsui et al. 2003). These three factors form the core of a regulatory circuit that promotes the expression of genes that maintain pluripotency while repressing genes that induce differentiation.

Intrinsic mechanisms

The POU domain transcription factor Oct4, the SRY-related HMG-box transcription factor Sox2 are critical for the pluripotency of the inner cell mass in vivo and ES cells in culture (Nichols et al. 1998, Niwa et al. 2000). Sox2 cooperates with Oct4 to activate the expression of a number of genes that regulate pluripotency including Oct4 and Nanog (Masui et al. 2007 and references therein). The homeodomain protein Nanog is also required for the maintenance of pluripotency in the inner cell mass in vivo (Mitsui et al. 2003). The overexpression of Nanog can bypass the requirement for leukemia inhibitory factor (LIF) in maintaining mouse ES cell pluripotency in culture, and Nanog-deficient ES cells are prone to spontaneous differentiation, though Nanog is not absolutely required for the maintenance of pluripotency in ES cells under favorable culture conditions (Chambers et al. 2003, Mitsui et al. 2003). These three factors form the core of a regulatory circuit that promotes the expression of genes that maintain pluripotency while repressing genes that induce differentiation.

Extrinsic mechanisms

Like other stem cells, ES cell self-renewal is also under cell-extrinsic control (Figure 1). LIF is a key factor that blocks the differentiation of mouse ES cells in culture (Williams et al. 1988). LIF binds to a heterodimer of LIF receptor and gp130, which activates JAK/Stat3 signaling (Niwa et al. 1998). The targets of the JAK/Stat3 pathway are largely unknown but have been suggested to include c-myc, a known promoter of pluripotency (Cartwright et al. 2005, Takahashi & Yamanaka 2006). Maintaining the pluripotency of ES cells also requires bone morphogenetic proteins (BMPs) that signal through SMAD proteins. SMAD signaling promotes the expression of inhibitor of differentiation (Id), helix-loop-helix domain proteins that dimerize with, and inhibit the function of, helix-loop-helix transcription factors that regulate fate determination (Ying et al. 2003). LIF/JAK/Stat3 and BMP/SMAD/Id signaling pathways work together to prevent the differentiation of ES cells in culture by inhibiting the consequences of mitogen-activated protein kinase (MAPK) signaling, which tends to promote differentiation (Ying et al. 2008). The inhibition of differentiation is key to ES cell self-renewal.
4. Unique Cell Cycle Kinetics

Mouse ES cells have a very short G1 phase of the cell cycle marked by little or no hypophosphorylated Rb (Burdon et al. 2002, Stead et al. 2002). The lack of Rb activity renders the cells insensitive to cyclin D–cyclin-dependent kinase (CDK) regulation and to the CDK inhibitor, p16Ink4a (Burdon et al. 2002, Savatier et al. 1996). Unlike tissue stem cells, ES cells do not undergo p53-dependent cell cycle arrest in response to DNA damage (Aladjem et al. 1998). ES cells have high levels of constitutively active CDK2-cyclin A/cyclin E, allowing rapid S phase entry (Stead et al. 2002). In contrast, when ES cells differentiate, G1 phase lengthens and the rate of cell division slows. As a result of these differences, ES cells are not subject to many of the cell cycle checkpoints that regulate tissue stem cells. Reprogramming of somatic cells to pluripotency confers similar cell cycle regulation as in mouse ES cells (Jaenisch & Young 2008), suggesting that the pluripotent state is tightly linked to the rapid and relatively unregulated cell cycle.

5. ES cells are XY

A surprising feature of mouse ES is that the great majority are 40XY. In XX Es cells as in epiblast, both XX chromosomes are active, a situation that appears to be unstable or else disadvantageous.

In any case, the XY phenotype confers advantages for establishing germline.

1) Male chimeras produce more offspring

2) XY cells can convert the indifferent genital ridge of an XX recipient embryo into testicular development. Because XX germ cells do not develop in a male gonad, this phenomenon of sex conversion results in chimeric males in which all the spermatocytes are of ES cell origin.
1- Morphologically:

- They do not form an epithelium-like layer that are either singled out, form flat colonies or prawl from a multilayered ES cell colony.
- They have a bright rim.
- They don’t appear in the same “background color” as the feeder layer.
- Differentiating ES cells do not proliferate as quickly at all and are being lost when passaging.

2- Expression of transcription factors and cell surface antigens:

- High level expression of the POU transcription factor Octamer-4 (Oct-4).
- Expression of carbohydrate antigen SSEA-1 (appears during late cleavage state of mouse embryos).
- Expression of Alkaline phosphatase.

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### Table 8.1: Comparison of Mouse, Monkey, and Human Pluripotent Stem Cells

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<tr>
<th>Marker Name</th>
<th>Mouse ESC/ES cells</th>
<th>Monkey ES cells</th>
<th>Human ES cells</th>
<th>Human iPS cells</th>
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**Key:**
- **ES cell** = Embryonic stem cell
- **iPS cell** = Induced pluripotent stem cell
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- **ES** = Embryonic stem
- **iPS** = Induced pluripotent stem
**How to Culture Mouse ES Cells**

- ES cells can be cultured and still retain their ability to contribute to all cell lineages when reintroduced into a host blastocyst.
- Need to grow feeder layers of mitotically inactive MEFS (embryonic fibroblasts, from 13-14d embryos)
- Media containing LIF
- They can grow in vitro and produce $10^9$ to $10^{10}$ cells without differentiating

**Risk factors for losing the ability to contribute to chimeras**

**Risk factors for differentiation of ES cells**

1. Inappropriate culture: High density

   - Feed
   - Split
   - Discard

   If culture is appropriate, fraction of differentiated cells is very low, and their lifespan and are continuously diluted out.
2-LIF

-LIF can be given by LIF-expressing feeders or by adding it to the media at a concentration of 10^6 Units/L.

Binding to LIFR triggers activation of STAT3 (necessary for continued proliferation of mouse ES cells).

STAT3 pathway usually promotes differentiation of other cells. G1 checkpoint does not appear to be operative.

3-Culture Manipulation

4-Fetal Calf Serum

5-Density/Quality Feeder cells

-Confluency means fibroblast bodies should fully cover the dish ground without any free spots showing. Direct adherence of ES cells to the dish is a differentiation stimulus.
-They should not be extensively layered/stacked to avoid competition for nutrients.

Signs indicative of dying MEFs cells are:

- Spindle formed, thin fibroblasts
- “Secretion” of particles from the cytoplasm, which has the aspect of little dark very small dots in proximity of the cells
- Cells with regressing cell protrusions (occurs e.g. if cells laid dry for some time on uneven shelves)

When to MMC treat EFs?

- Low density
- Correct density
- High density
**Feeder cells**

-MEFs are primary cells and have a limited life span
-They divide rapidly for about 4-5 passages (Approx. 20 divisions) and then become senescent
-They are obtained from 13-day old embryos

Depending on the number of passages, the shape (and quality) of the fibroblasts change:

-EF1: (one passage after generation) typically have big, widely stretching cell bodies. Often they form neuron-like shapes (according to their origin from embryos without liver and heart)

-EF2: resemble EF1 in appearance. Once MMC treated, they can be kept in culture for up to 14 days, which is necessary when transfecting ES cells.

-EF3 are more spindle-like. Once MMC treated, they can be kept in culture for up to one week.

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**1.2 MUTATING ES CELLS**

(Homologous Recombination)


HR in ES cells and germline transmission (First KO MICE):

-Thomas et al, Cell 1989 (HPRT)
-Thomas et al, Nature 1989 (c-abl)
-Zilstra et al, Nature 1989 (b2-microglobulin)
-Koller et al, PNAS 1989 (HPRT)
-Thompson and Capecchi, Nature 1990 (int-1 proto-oncogene)
**Genetic Recombination**

Genetic Recombination is a process by which a molecule of nucleic acid (usually DNA; but can also be RNA) is broken and then joined to a different DNA molecule.

**Homologous Recombination:** occurs between similar molecules of DNA. Common method of DNA repair (mitosis) in both bacteria and eukaryotes. In eukaryotes, recombination occurs in meiosis as a way of facilitating chromosomal crossover.

**Non-homologous end joining:** dissimilar molecules of DNA.

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**Homologous Recombination**

Resection: after a double-strand break occurs, sections of DNA around the break on the 5' end of the damaged chromosome are removed.

Strand invasion: an overhanging 3' end of the damaged chromosome which invade an homologous chromosome to copy genetic information into the donor chromosome.

DNA repair: on-homologous end joining (DSBR): Resolution of the exchanged DNA strands results in crossover, whereby segments of the interacting chromosomes are exchanged. Central to the DSBR model is the formation of a DNA joint molecule that harbours two Holliday junctions.

Crossover (SDSA): The SDSA model is similar to the DSBR model in the initial steps of DSB-end processing and invasion into a homologous chromosome, but instead of capturing the second end of the DSB into the recombination intermediate, the invading strand is displaced after repair synthesis and reanneals with the single-stranded tail on the other DSB end. SDSA probably also accounts for those meiotic DSBR events that do not give rise to crossovers25.
Homologous Recombination in ES cells


Sung et al., Nature Reviews Molecular Cell Biology 2006
The next step was to determine whether ES cells could be used to introduce genetic material into the germline. Evans and his co-workers infected ES cells with a recombinant retrovirus before injecting them into blastocysts [18]. Retroviral DNA was identified in the founders and transmitted to the F1 offspring, demonstrating introduction of the foreign DNA into the mouse germline [19]. In October, 1986, Evans et al. reported their findings in Nature and concluded that "cultured embryonic cells provide an efficient means for the production of transgenic animals" [19]. In December of that year, another laboratory reported germline transmission of a neomycin resistance gene that they had introduced into ES cells by retroviral infection [20].

Evans now took the important step of introducing a mutant form of a specific, endogenous gene into the mouse genome. He and his co-workers transferred a mutant gene for hypoxanthine phosphoribosyltransferase (HPRT), which is defective in Lesch-Nyhan syndrome, an X-linked monogenic defect of purine metabolism [21]. Several copies of the mutated HPRT gene were introduced into the genome of the ES cells by retroviral infection in culture. Mutated ES cells were injected into blastocysts and contributed to chimeras. The mutations were transmitted germline and identified in the male offspring as loss of HPRT activity. In a paper published in Nature back-to-back with the one from Evans' lab, Hooper et al in Edinburgh reported germline transmission of another mutated HPRT gene, a spontaneous deletion mutation in ES cells [22]. For the first time, models of human disease had been created by genetic manipulation of ES cells.


Homologous Recombination

A series of careful experiments were performed, which unequivocally demonstrated that head-to-tail concatemers were generated by homologous recombination [31]. This, in turn, provided evidence that mammalian somatic cells possess an efficient enzymatic machinery for mediating homologous recombination. If this machinery could be harnessed to accomplish homologous recombination between a newly introduced DNA molecule and the same DNA sequence in the recipient cell's genome, any cellular gene could be mutated. Capecchi now submitted a grant proposal to the U.S. National Institutes of Health to test the feasibility of gene targeting in mammalian cells. It was rejected since the reviewers considered it extremely unlikely that the introduced DNA would find its matching sequence within the host genome (cited by Capecchi in a later review [32])!

Capecchi decided to continue working on homologous recombination in spite of being turned down by NIH. He generated recipient cell lines that carried a defective neomycin resistance gene (neor) and was able to repair it by introducing a functional neor gene [23]. Correction occurred at a relatively high frequency (in one cell per 1000 injected cells), making it likely that homologous recombination could be used to manipulate genes of the mammalian genome.

In parallel with Capecchi's work, Oliver Smithies had developed the concept that homologous recombination might be used to repair mutated genes. As early as the 1960s he had already established that an allelic variant of haptoglobin had occurred through recombinatorial events [33]. Later on, he cloned human fetal globin genes and concluded that G? and A? had arisen through a process involving homologous recombination [34]. He devised a stepwise selection procedure for recovering targeted cells carrying modified genes. The strategy was successful and he reported in a landmark paper in the September 19, 1985 issue of Nature the successful integration by homologous recombination of a plasmid into the chromosomal ß-globin gene of human erythroleukaemia cells [24].
By 1985, Capecchi had shown that homologous recombination occurs with high frequency in mammalian cells and Smithies had used homologous recombination to insert a plasmid DNA sequence into a chromosomal gene of a human cell. However, all this work was carried out in cell culture. Could homologous recombination be used to target genes in the germline and obtain strains of genetically modified animals? Both Capecchi and Smithies had heard of Martin Evans' ES cells and decided to give them a try. With the help of Evans, they both set up ES cell culture for use in homologous recombination experiments.

Smithies first used homologous recombination to correct a mutant HPRT gene in cultured ES cells [35]. For this purpose, an ES cell line was used that carried a deletion mutation; this cell line had previously been used for production of mutant mice. The HPRT gene was repaired with a plasmid carrying the missing promoter and first 2 exons and Smithies showed that treated cells survived and grew in HAT selection medium, which requires HPRT enzyme activity. Smithies and his co-authors concluded that “This modification of a chosen gene in pluripotent ES cells demonstrates the feasibility of this route to manipulate mammalian genomes in predetermined ways” [35].

Capecchi’s team also chose the HPRT gene for their early studies. Standard methods were available for selectively growing cells with functional HPRT enzymes and had already been used for several years for selection of mutants, hybridoma cells in monoclonal antibody production etc. Thomas and Capecchi [36] introduced a neomycin resistance gene into an exon of the HPRT gene in ES cells and showed that clones of transfected cells had lost HPRT but gained neoR activity. They concluded in their Cell paper that “It is hoped that this combination of using ES cells as the recipient cell line and site-specific mutagenesis achieved by gene targeting will provide the means for generating mice of any desired genotype.”

It was important to proceed from the “model gene” HPRT to a general strategy that would allow targeting of genes whose function cannot be selected for in cell culture. Thomas and Capecchi [36] had pointed out that the frequency of homologous recombination vs random integration was 1:1000, which should be high enough to permit targeting of non-selectable genes as well.


[34] Slightorn JL, Blechl, A.E., Smithies, O. Human fetal Qq and Ag globin genes: Complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. Cell. 1980;21:27-38.


Gene Targeting

1981
HR in Mammalian cells
Folger, Capecchi

1986
Gene Targeting in Mammalian Cells
Capecchi, Smithies

1987
Gene targeting in ES cells
Correcting mutant HPRT gene
Smithies

1987
Gene targeting in ES cells
Mutating HPRT gene
Capecchi, Thomas

1989
First KO mice from gene targeting in ES cells

Manipulating the HPRT gene, a selectable gene

1-The Hprt gene encompasses over 33 kb of DNA and contains 9 exons that encode 1307 nucleotides of mRNA (Melton et al., 1984).

2-HPRT is located in the X Chromosome and ES cell lines are usually XY, so that only a single HPRT locus has to be disrupted to yield HPRT- ES cell lines .

3-The drug 6-TG kills cells with a functional HPRT

4-Loss of HPRT renders the cells sensitive to HAT media

Gene Targeting in ES cells: correcting HPRT

1987
Targetted correction of a mutant HPRT gene in mouse embryonic stem cells.
Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S, Smithies O.

Correcting mutant HPRT gene
**Gene Targeting in ES cells: mutating HPRT**

**1987**


Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells.

Thomas KR, Capecchi MR.

**Mutating HPRT gene**

HPRT$^+$ ES cell line  
6-TG-sensitive  

HPRT$^-$ ES cell line  
6-TG-resistant

**HR in ES cells and germline transmission of selectable genes**

**First KO MICE:**

Thompson et al, Cell 1989 (HPRT)
Koller et al, PNAS 1989 (HPRT)
**HR in ES cells and germline transmission of non-selectable genes**

*First KO MICE:*

Schwartzberg *et al.* Science 1989 (c-abl)
Zilstra *et al.*, *Nature* 1989 (b2-microglobulin)
Thomas and Capecchi, *Nature* 1990 (int-1 proto-oncogene)

**HR efficiency in ES cells**

*HR frequency*

Homologous events/Total events
(homologous/total analyzed colonies)
(total of 80% have been reported)

*Absolute HR frequency*

Homologous events/Total number cells transfected
(homologous/total analyzed colonies)
Gene Targeting of non-selectable genes
Factors affecting HR efficiency in ES cells


3-Isogenicity if construct (strain) (Van Deursen et al., Nuc. Acid. Res (1992), te Riele et al., PNAS (1992)).

4-Stretches of DNA deletion (Zhang et al., Mol. Cel. Biol. (1992))

5-Previous Targeting events (Calpe, Wang et al. Unpublished)

6- Other: targeted locus itself, vector design, and the status of cellular HR machinery, efficiency DNA delivery,

1-Positive-negative selection

Gene Targeting

Positive selection: Selects recipient cells that have incorporated targeting vector anywhere

Negative selection: Selects eliminates cells that have incorporated at non-homologous sites

2-Homology Length Arms

Exponential relationship between the total length of homology and the targeting frequency when the homologous DNA ranges from 2 to 14Kb. The targeting frequency at the Hprt locus as a function of the extent of homology between the targeting vector and the endogenous target.


3-Isogenecity

Using isogenic DNA is 25-fold more effective that with a non-isogenic vector


4-Maximal deletion size

The maximal size of deletion which can be made through one targeting step is around 19Kb.

**5-Previous targeting events**

Increased %HR frequency in previously targeted cells

![Bar chart showing increased %HR frequency for wt Bruce4 and previously targeted Bruce4 with Ly108 Neo vector and Eat2b Hygro vector.]

1. Increase when using both Hygro and Neo vectors
2. Increase even if events are separated by almost 400KB

(Calpe, Wang et al, Unpublished)

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**Knockins**

Deletion of En1 gene but addition of En2

![Diagram showing the deletion of En1 gene and addition of En2 with a TK promoter and loxP sites.]

Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2.
Hanks M, Wurst W, Anson-Carter J, Auerbach AR, Joyner AL.
Produce specific mutation in mammal cells

A. Gene targeting of embryonic stem cells

Culturing ES

Mutating ES

Transfer mutation germline

Generation of gene targeted mice

1.3 GERMLINE

  - Labosky et al, 1994
- Tetraploid embryos: Nagy et al, PNAS 1993
- 129Sv BLASTOCYST are more efficient than B6: Brook et al, PNAS 1997
- COISOGENIC BLASTOCYSTS are better in supporting the development and germline transmission:
  - Schuster-Gossler et al, Biotechniques 2001
  - Auerbach et al, Biotechniques 2000
- RAG-2 Blastocysts complementation: Chen et al, PNAS 1993

Transferring mutation through the germline

Bradley et al, Nature 1984
Thompson et al, Nature 1989
Wood et al, Nature 1993
Nagy et al, PNAS 1993
1-Blastocyst injection

(from the Transgenic/Gene Targeting Facility of Oregon Health & Science University)

Development of the Preimplantation Blastocyst in Mice from Embryonic Day 0 (E0) Through Day 5 (E5.0)

Extraction
**Blastocyst**

The blastocyst is a hollow sphere made of approximately 150 cells and contains three distinct areas: the trophoblast, which is the surrounding outer layer that later becomes the placenta, the blastocoel, which is a fluid-filled cavity within the blastocyst, and the inner cell mass, also known as the embryoblast, which can become the embryo proper.

**Steps**

1-**Injection ES cells**

In a conventional blastocyst (3.5 day) injection ES cells are injected into the blastocoel by piercing the trophectoderm at a cell-cell junction. The merged fluorescent/bright-field photomicrograph shows a real example in which ES cells that express green fluorescent protein (eGFP) from the Gt(ROSA)26Sor locus promoter were injected into a blastocyst. The injected ES cells mingle with the preexisting cells of the inner cell mass (ICM). The injected ES cells compete with the host ICM of the blastocyst to yield F0 Chimeras.

2-**Embryo reimplantation**

The inner cell mass could also be oriented at the top of the field of view. The embryo is aligned to avoid damage of the inner cell mass. Note that the injection tip is aligned in opposition to a joint in the trophoblast layer. Attempts to inject blastocysts through a thick part of the trophoblast layer are often unsuccessful. The tip may not penetrate fully and the blastocyst can collapse before cells are introduced into the cavity.

(A) A transfer pipet loaded for reimplantation. Air bubbles surround the blastocyst and act as markers that can be seen during surgery to ensure that the embryos are expelled into the uterus. (B) Proper location of the skin incision for access to both uterine horns from a single site. Two incisions would be necessary to reimplant embryos in both horns. (C) Isolated uterine horn ready for puncture with a 25-G needle. The uterus is secured by a clamp attached to the ovarian fat pad. Blunt forceps are used to grasp the uterus near the oviduct junction as the tissue is punctured and the transfer pipet is inserted. The uterus should be held gently, to avoid damage.
Morphology of embryonic stem cell colonies. The colonies remain composed of a homogenous population of stem cells. Stem cells are comparably small and are tightly packed within the colony. Note the smooth outline of the colony of densely packed cells. (b) Single cell suspension of ES cells for injection. (c) The injection needle is used to collect ES cells. (d) Blastocyst. Arrowheads mark the junctions of trophoblast cells. (e) Immobilize the blastocyst on a holding pipette so that the ICM is positioned at either 12 o'clock or 6 o'clock. The tip of the injection needle is brought into the same focal plane as the equator/midpoint of the blastocyst. (f) With a swift movement, the needle is introduced into the blastocoel of the blastocyst. (g) The cells are released slowly into the cavity. (h) After releasing the injected blastocyst from the holding capillary the blastocyst will collapse and the ES cells (star) will come into contact with the ICM.

**Step 2: From gene targeted ES cells to gene targeted mice**

5. **Injection of ES cells into blastocysts**
   - The targeted ES cells are injected into blastocysts... where they engraft and form a mosaic with the cells of the inner cell mass from which the embryo develops... The injected blastocysts are implanted into a surrogate mother where they develop into mosaic embryos.

6. **Birth and breeding of mosaic mice**
   - The mosaic mice mate with normal mice to produce both gene-targeted and normal offspring.

© The Wellcome Trust | Image Credits: Annika Richter
**2-Tetraploid embryo aggregation**

ES cells are aggregated with stage embryos, followed by culture to blastocyst stage.

1-Generating tetraploid embryo by electrofusing the cells of a two-cell stage embryo

2-Aggregation of ES cells

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**Comparison methods**

**Blastocyst injection**

1-Individual cells are selected, offering a way to control best ES cells that hopefully will give germline transmission

2-Technically less demanding and expensive

**Embryo Aggregation**

1-F0 generation mice are almost completely ES cell-derived and thus immediately available for phenotypic analyses.

1-It requires certain low-passage hybrid strain ES cell lines
2-Fails to yield viable mice when ES cells from inbred strains are used.
3-The resulting F0 mice, which can possess up to 2% host contamination, exhibit poor viability and have other abnormalities (such as changes in growth rates and body weight).

1-High Skills
2-Expensive to establish in a laboratory
Risk factors for losing the ability to contribute to quimeras

1-Prolonged cultured periods (chromosome abnormalities i.e gain or loss.): KARYOTYPE

2-Stress due to poor culture conditions

3-Differentiation

1.4 CHOICE OF MOUSE STRAINS FOR GENE TARGETING

Since 1955 (King et al, Genetics): Differences in fertility between different strains.

The responses are observed in B6, 129, C3h or DBA.
Th2 are observed in Balb/c mice.
Neurobiological differences between strains.

Knockout can lead to different phenotypes depending on STRAINS
Choice of mouse strains for gene targeting

*Choice of mouse strain is critical:*
  - Genomic DNA library/BAC clone for targeting vector
  - The Embryonic Stem Cell
  - Recipient Embryos
  - Foster mother
  - Strain in which the chimaeric mice are mated

Contribute to the genetic composition of the targeted mouse

Why Strain is an IMPORTANT determinant for the mutant phenotype?

- Since 1955 (King et al, Genetics): Differences in fertility between different strains.
- Th1 responses are observed in B6,129, C3h or DBA.
- Th2 are observed in Balb/c mice.
- Neurobiological differences between strains.
**ISOGENIC DNA for gene targeting**

- Use of isogenic DNA has been reported to improve the gene targeting frequencies in some homologous recombination studies, reflecting the extent of polymorphisms between any two strains.
- Genomic DNA libraries from most commonly used inbred strains such as 129, Balb/c, B6 and DBA are commercially available.
- BAC clones from 129 and B6 are also available.
- If ISOGENIC DNA is not available, it may be possible to COMPENSATE for the polymorphisms by using LARGER constructs to increase the regions of homology and hence the overall chances of homologous recombination.

**2-ES cells**

- Majority of ES cell lines that are available for use in gene targeting have been derived from substrains of the 129 mice. (capacity of this strain to generate ES cell lines that have the availability to contribute to germline transmission after extensive manipulation in culture)

- Brook et al, PNAS 1997: 129Sv ES cells are more efficient to generate chimeras
- Kawase et al, Int. J. Dev. Biology 1994: ES cell lines are most easily established from 129Sv
- Two C57BL/6-derived ES cells have been described, the BL/6-III and Bruce4 (TNF, MHCIIAa...) and also Balb/c.
- Even Es cells from MRL mice, spontaneously develops a generalized autoimmune disease with features similar to systemic lupus erythematosus.

### Strains to be considered

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruce4 (C57BL/6)</td>
<td>MHC class II Aa, CD3 $\gamma$, $\delta$</td>
<td>Koentgen et al. (1993), Malissen et al. (1993)</td>
</tr>
<tr>
<td>BL/6-III (C57BL/6)</td>
<td>Ig $\epsilon$, Perforin, CD23, PBGD, IL-5</td>
<td>Zou et al. (1993), Kägi et al. (1994), Yu et al. (1994), Lindberg et al. (1996), Kopf et al. (1996)</td>
</tr>
<tr>
<td>BALB/c-I</td>
<td>IL-4, IL-4R $\alpha$</td>
<td>Noben-Truth et al. (1996), Mohrs et al. (1999)</td>
</tr>
<tr>
<td>DBA-252 (DBA/1)</td>
<td>FLAP</td>
<td>Roach et al. (1995)</td>
</tr>
<tr>
<td>MRL</td>
<td>Ep2</td>
<td>Goulet et al. (1997)</td>
</tr>
</tbody>
</table>

Ledermann B, Exp. Physio. 2000

### C57BL/6-, BALB/c-, DBA/1- and MRL-derived ES cell lines used for gene targeting

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gene</th>
<th>Reference</th>
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</table>
**3- Recipient embryos**

Don’t contribute to the genetic background of the final mouse

- Although the host embryo does not contribute to the genetic background of the final KO mouse, the combination of the strain of mouse from which the ES cells and the strain of mouse from which the host embryos are derived is critical for the ability of ES cells to generate germline chimeras. This is postulated because of the relative growth properties of the ES cells and the host blastocyst inner cell mass. B6 and Balb/c appear to have similar growth properties.

**129Sv ES CELLS → B6 Embryos**

Schwartzberg et al., Nature 1989

Frequency of chimera was equal in all backgrounds, but degree of ES contribution to the coat and the rate of germline transmission was higher with B6 embryos.

**B6 ES CELLS → BALB/c Embryos**

Lemcet et al., Nucleic Acids Res. 1997

Optimized methods for the quality and quantity.

Seong et al., Trends Genetics 2004

Low chimersim ratio but High Germline transmission

- Low yield of embryos per mouse
- Delayed embryonic development

**Albino B6 Embryos**

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<table>
<thead>
<tr>
<th>Injection</th>
<th>Pups</th>
<th>Mate to</th>
<th>You want</th>
<th>Don’t want</th>
</tr>
</thead>
<tbody>
<tr>
<td>129 ES cells into B6 blast</td>
<td>Chimera</td>
<td>129</td>
<td>129</td>
<td>B6</td>
</tr>
<tr>
<td>B6 ES cells into Balb/c blast</td>
<td>Chimera</td>
<td>B6</td>
<td>B6</td>
<td>129</td>
</tr>
</tbody>
</table>
TO KNOCKOUT in 129 or in C57BL/6: that is the question

<table>
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<tr>
<th>129SV ES CELLS → B6 Embryos</th>
<th>B6 ES CELLS → BALB/C Embryos</th>
</tr>
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<tbody>
<tr>
<td>Difficulty of making non-129 ES cells</td>
<td>Proportion of chimerism is lower in BALB/c Blastocyst (more injections required)</td>
</tr>
<tr>
<td>Isogenic DNA: than B6 Genomic DNA available from BAC clones, while 129 have to be identified by library screening</td>
<td>Low yield of embryos with BALB/c mice</td>
</tr>
<tr>
<td>Similar homology frequencies using identical targeting vectors</td>
<td>Low sex ratio</td>
</tr>
<tr>
<td>ES cell contribution to the coat is lower</td>
<td>High germline transmission (higher number of pups because fertility is higher) even for low level chimeras (10%)</td>
</tr>
</tbody>
</table>
| Avoid Background genotype | }