GENE TARGETING STRATEGIES

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Katharina Fritsch, Mann und Maus,1991–1992, Polyester and paint, © Katharina Fritsch

Why Mice?

1) Mouse is a mammal and its development, body plan, physiology, behavious 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

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

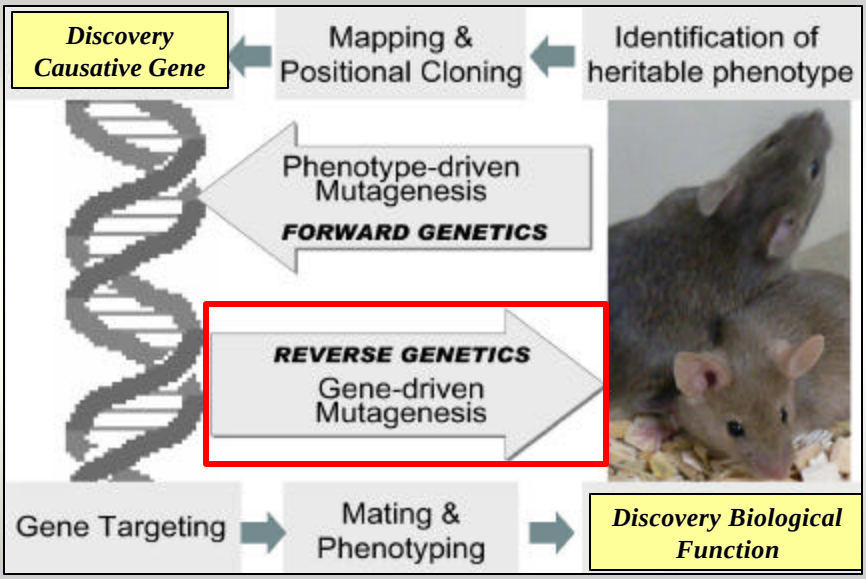
2)Provide a tractable and reproducible system to identify inflammatory pathways

4) Allow testing therapeutic interventions

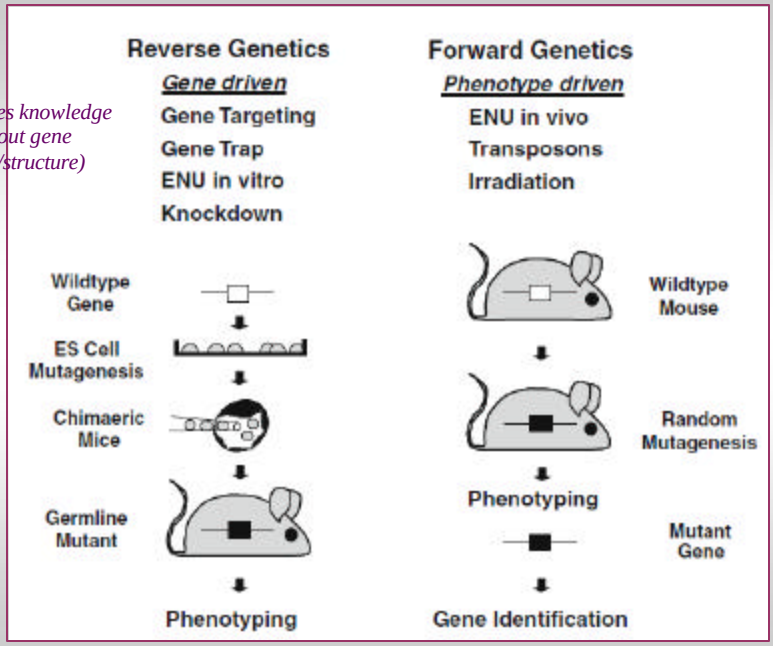
5) Should be characterized by simplicity of the experimental design combined with short duration of the experiment, low costs and minimal harm to animal welfare.

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

STUDY GENOME FUNCTION BY GENETICS



Gondo et al 2009.



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)

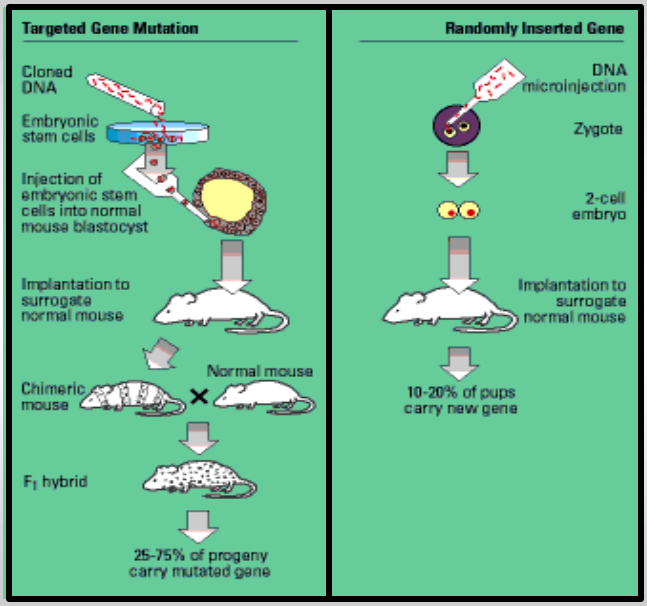
Injecting a transgene into fertilized eggs RANDOMLY

3-Cre Recombination

Cre Tg animals and Applications

4-Mouse Models a la Carte

1-Gene Targeting 2-Gene Transgenesis



Eddy M. Environ Health Perspect. 1993 Sep;101(4):298-300.

Advantages Gene Targeting vs Transgenesis

1-Choice of genetic locus to mutate

2-Takes full advantage of the all the resources provided by the known sequences of the mouse and huiman genomes

3-Control of how to modulate the chosen genomic locus (spatial/temporal restrictions)

1-Gene Targeting

Produce specific mutation in ES cells

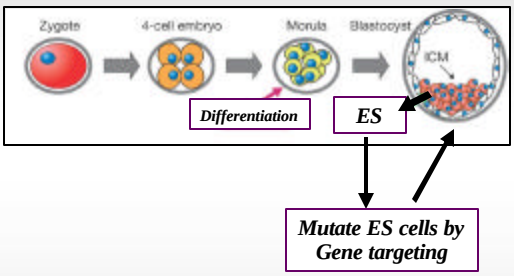
1.1 CULTURING ES CELLS
1.2 MUTATING ES CELLS

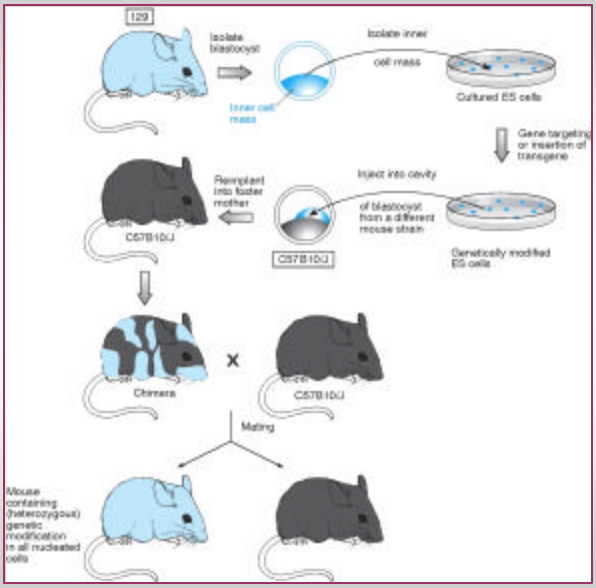
Transfer mutation germline

1.3 GERMLINE
1.4 STRAINS

1.5 APPLICATIONS

Gene Targeting Squeme

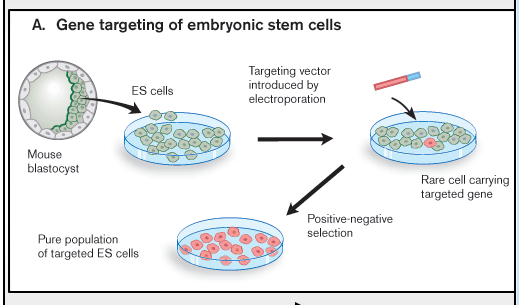




Human molecular Genetics

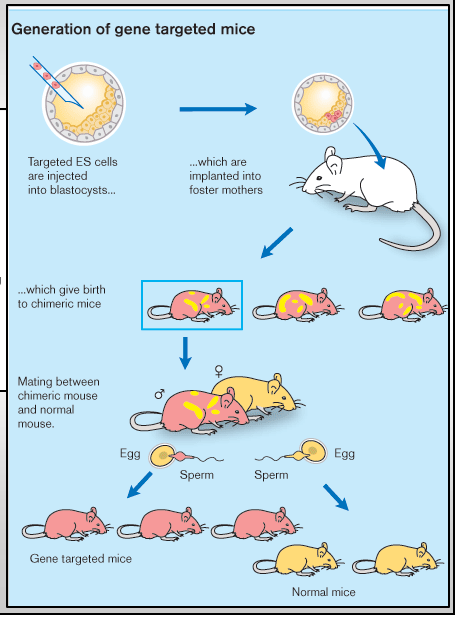
Transfer mutation germline

Produce specific mutation in mammal cells

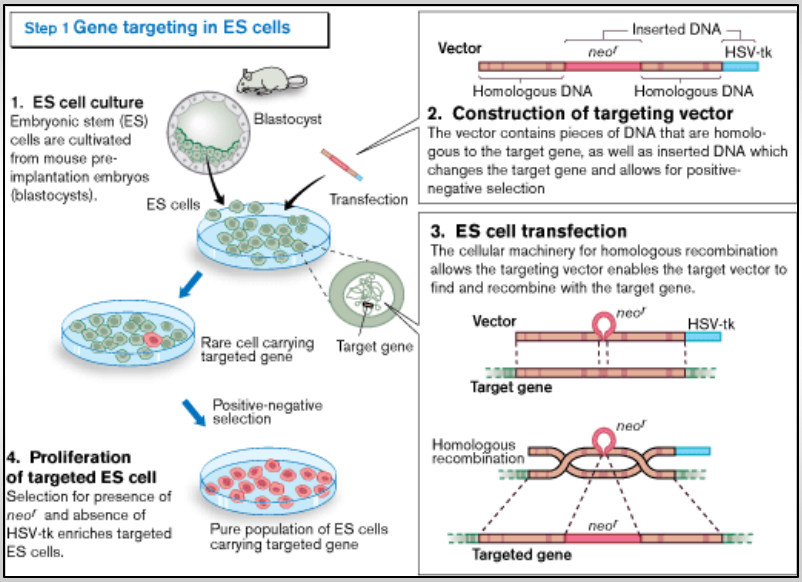


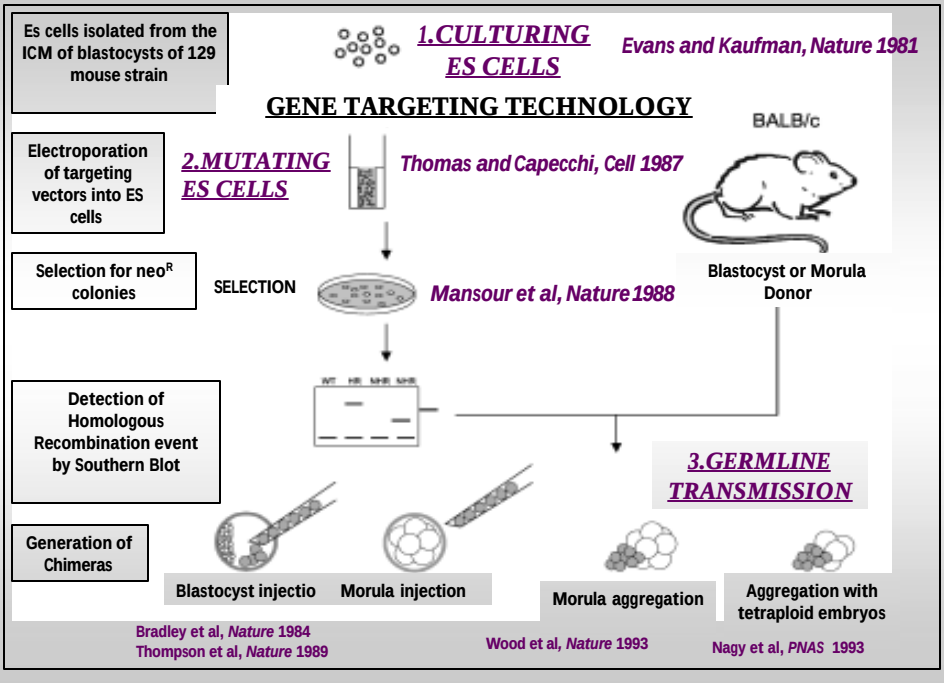
Culturing ES

Mutating ES

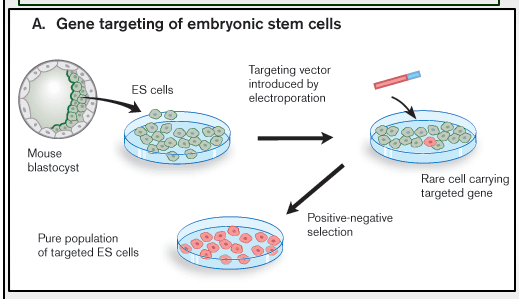


http://nobelprize.org/nobel_prizes/medicine/laureates/2007/adv.html


Step 1 Gene targeting in ES cells
1. ES cell culture
Embryonic stem (ES) cells are cultivated from mouse pre-implantation embryos (blastocysts).
Blastocyst
ES cells
Transfection
Rare cell carrying targeted gene
Target gene
Positive-negative selection
4. Proliferation of targeted ES cell
Selection for presence of neo^r and absence of HSV-tk enriches targeted ES cells.
Pure population of ES cells carrying targeted gene
Inserted DNA
Vector
neo^r
HSV-tk
Homologous DNA
Homologous DNA
2. Construction of targeting vector
The vector contains pieces of DNA that are homologous to the target gene, as well as inserted DNA which changes the target gene and allows for positive-negative selection
3. ES cell transfection
The cellular machinery for homologous recombination allows the targeting vector enables the target vector to find and recombine with the target gene.
Vector
neo^r
HSV-tk
Target gene
Homologous recombination
neo^r
neo^r
Targeted gene


Es cells isolated from the ICM of blastocysts of 129 mouse strain
1.CULTURING ES CELLS
Evans and Kaufman, Nature 1981
GENE TARGETING TECHNOLOGY
BALB/c
Electroporation of targeting vectors into ES cells
2.MUTATING ES CELLS
Thomas and Capecchi, Cell 1987
Selection for neo^R colonies
SELECTION
Mansour et al, Nature 1988
Blastocyst or Morula Donor
Detection of Homologous Recombination event by Southern Blot
3.GERMLINE TRANSMISSION
Generation of Chimeras
Blastocyst injectio
Morula injection
Morula aggregation
Aggregation with tetraploid embryos
Bradley et al, Nature 1984
Thompson et al, Nature 1989
Wood et al, Nature 1993
Nagy et al, PNAS 1993

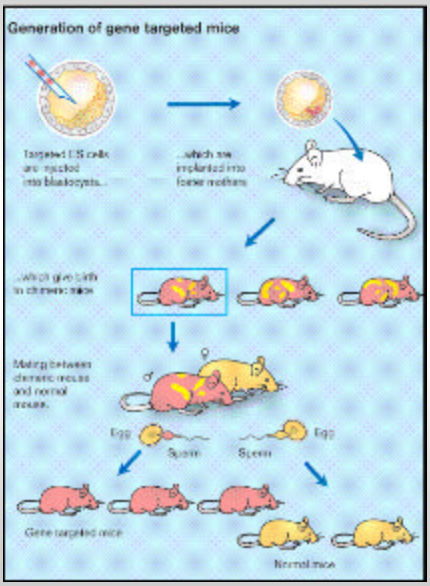
Produce specific mutation in mammal cells



Culturing ES

Mutating ES

Transfer mutation germline

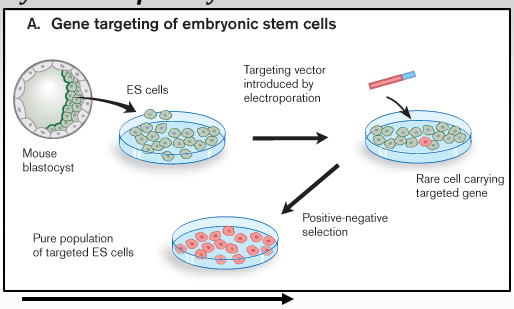


http://nobelprize.org/nobel_prizes/medicine/laureates/2007/adv.html

Nobel Prize in Medicine 2007

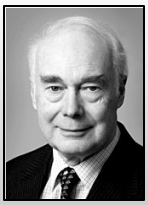
"for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells"

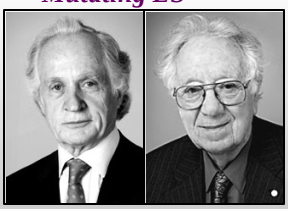
Produce specific mutation in mammal cells



Culturing ES

Mutating ES





Sir Martin J. Evans ***Mario R. Capecchi*** ***Oliver Smithies***

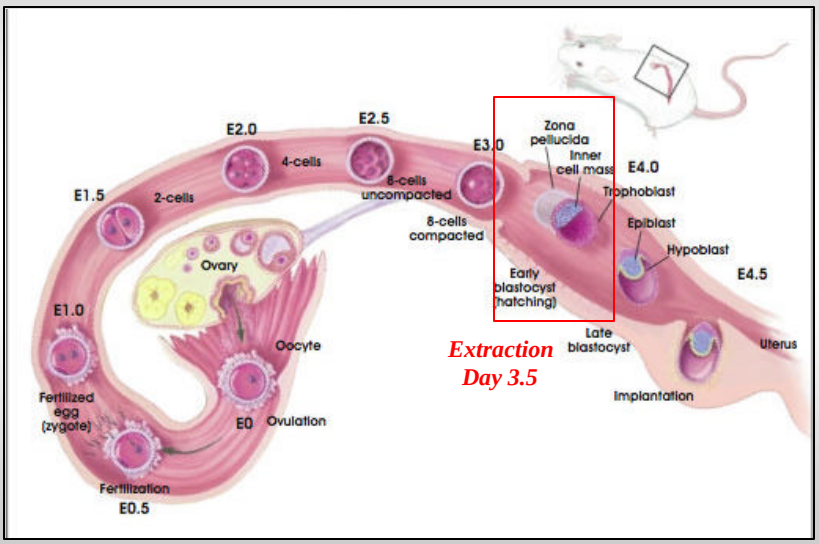
Sir Martin J. Evans

1.1 CULTURING ES CELLS

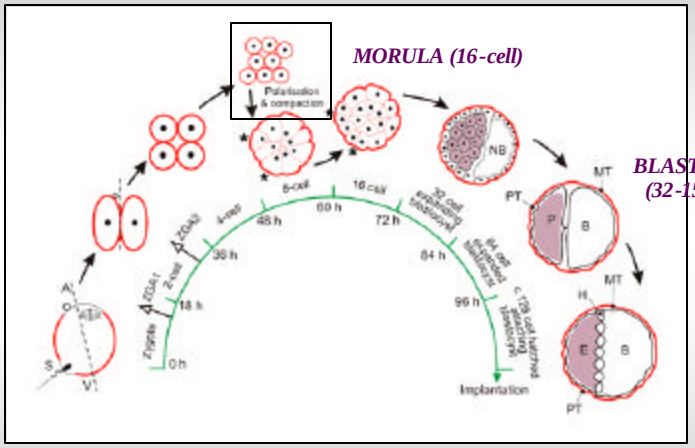
- ***Isolation of EC lines:*** *Kleinsmith et al, 1964*
- ***Culture EC cells on Feeder cells:*** *Martin and Evans 1975*
- ***First transgenic mouse (from EC cells):*** *Brinster et al 1974, Papaioannou et al, Nature 1975*

- ***Isolation of ES cell lines:*** *Evans and Kauffman (Nature,1981), Martin (PNAS,1981)*
- ***ES cells into germline by injection into Blastocysts:*** *Bradley, A. et al. 1984. Formation of germline chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309, no. 5965: 255*

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



Mouse development

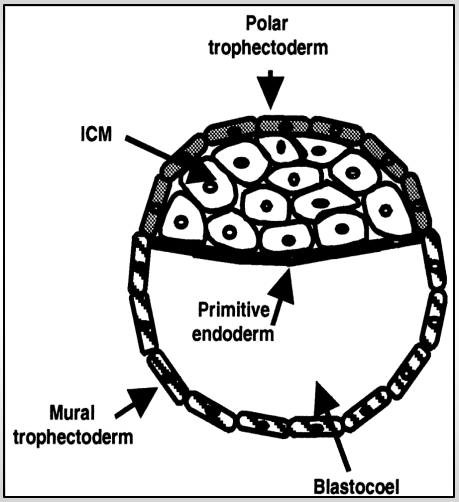


www.pdn.cam.ac.uk/staff/johnson/

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 trophectodermal 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 trophectoderm 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.

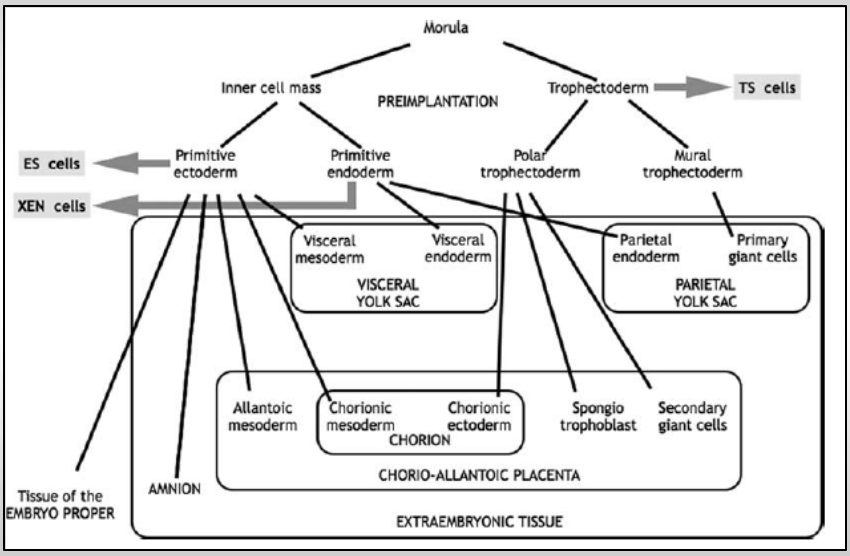
www.pdn.cam.ac.uk/staff/johnson/

Blastocyst



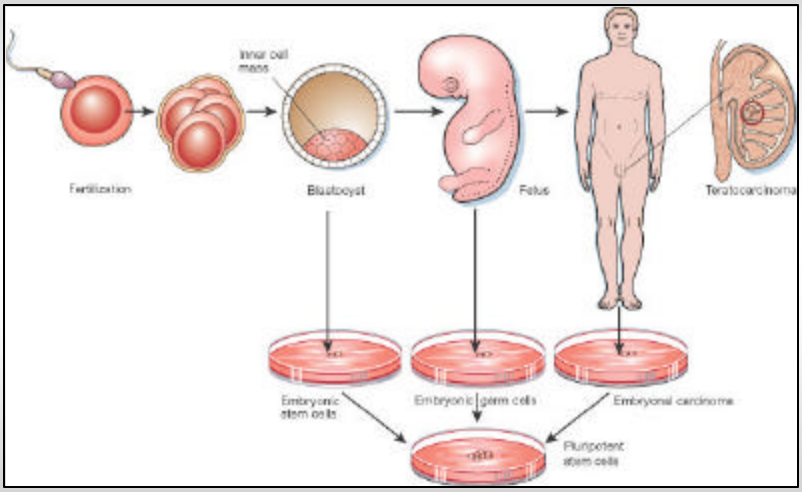
*The blastocyst is a hollow sphere made of approximately **150 cells** and contains three distinct areas: the **trophoblast**, which is the surrounding outer layer that later becomes the placenta, the **blastocoel**, which is a fluid-filled cavity within the blastocyst, and the **inner cell mass**, consisting of **primitive endoderm** and **ectoderm**. Each of these compartments has its unique potential as well as limitation. Trophectoderm 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.*

Embryo lineages

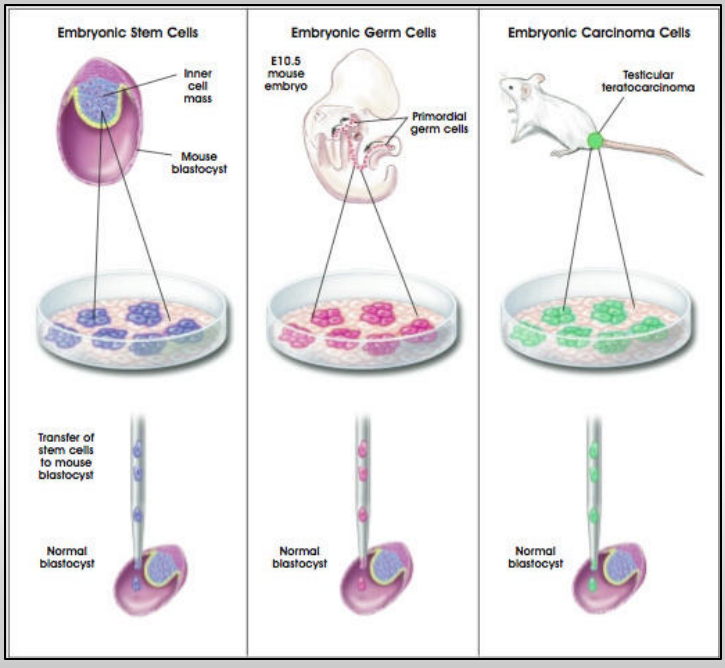


Pluripotent Stem Cells

Pluripotent Self-renewal



The end of the beginning for pluripotent stem cells
Peter J. Donovan and John Gearhart
Nature 414, 92 -97(1 November 2001)



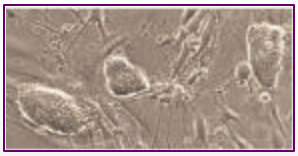
Embryonal Carcinoma Cells

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.

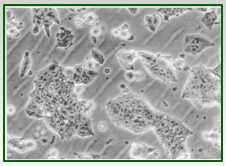
After several years, they started culturing EC with **Feeders**, showing that not only allowed the efficient establishment of EC culture but also increased their differentiation activity.

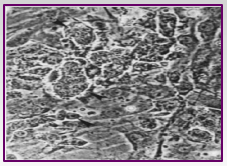
Embryonic Stem cells



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 at al, 1984).

Embryonal Carcinoma Cells *Embryonic Stem Cells*





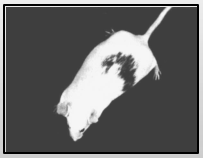
1964
Isolation EC line
Kleinsmith

1975
Culture EC line
Generation MICE
Martin and Evans
Brinster

1981
Isolation ES cells
Evans, Kauffman,
Martin

1984
Blastocyst injection
First chimeric mouse
Bradley, Evans

Low Rate of Colonization
Restricted pattern of differentiation
Many chimeras developed tumor pre/post natally



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].

1.1 Isolation of EC cell lines...

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] Askanazy M. Die Teratome nach ihrem Bau, ihrem Verlauf, ihrer Genese und im Vergleich zum experimentellen Teratoid. Verhandl Deutsch Pathol. 1907;11:39-82.
[2] Stevens LC, Little, C. C. Spontaneous testicular teratomas in an inbred strain of mice. Proc Natl Acad Sci (USA). 1954;40:1080-7.
[3] Stevens LC. Embryonic potency of embryoid bodies derived from a transplantable testicular teratoma of the mouse. Dev Biol. 1960;2:285-97.
[4] Kleinsmith LJ, Pierce, G. B. Multipotentiality of single emb ryonal carcinoma cells. Cancer Res. 1964;24:1544-52. (Isolation of EC cell lines)
[5] Rosenthaal MD, Wishnow, R. M., Sato, G.H. In vitro growth and differentiation of clonal populations of multipotential mouse cells derived from a transplantable testicular teratocarcinoma. J Natl Cancer Inst. 1970;44:1001-14.
[6] Kahan BW, Ephrussi, B. Developmental potentialities of clonal in vitro cultures of mouse testicular teratoma. J Natl Cancer Inst. 1970;44:1015-36.
[7] Evans MJ. The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratoma cells. J Embryol Exp Morphol. 1972;28:163-76.
[8] Martin GR, Evans, M.J. The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture. Cell. 1974;2:163-72.
[9] Martin GR, Evans, M.J. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc Natl Acad Sci (USA). 1975;72:1441-5. (Cell line cultures)
[10] Papaioannou VE, McBurney, M., Gardner, R.L., Evans, M.J. The fate of teratocarcinoma cells injected into early mouse embryos. Nature. 1975;258:70-3. (Tg from EC)
11] Brinster R. J Exp Med.104:1049-56. (Tg from EC)
[12] Mintz B, Illmensee K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc Natl Acad Sci U S A. 1975;72:3585-9. (Tg from EC)

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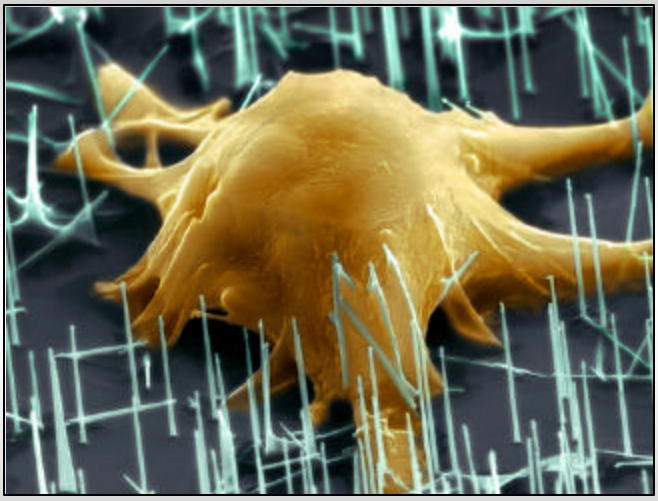
1.2 Isolation of ES cell lines:

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].

[13] Stinnakre MG, Evans, M.J., Willison, K.R., Stern, P.L. Expression of Forssman antigen in the post-implantation mouse embryo. J Embryol Exp Morphol. 1981;61:117-31.
[14] Evans MJ. The cultural mouse. Nat Med. 2001;7:1081-3.
[15] Evans MJ, Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154-6. (Isolation of ES cells)
[16] Martin GR. Isolation of a pluripotent cell line from ealry mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci (USA). 1981;78:7634-8.
[17] Bradley A, Evans, M., Kaufman, M.H., Robertson, E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature. 1984:255-6.

Mouse ES Cell

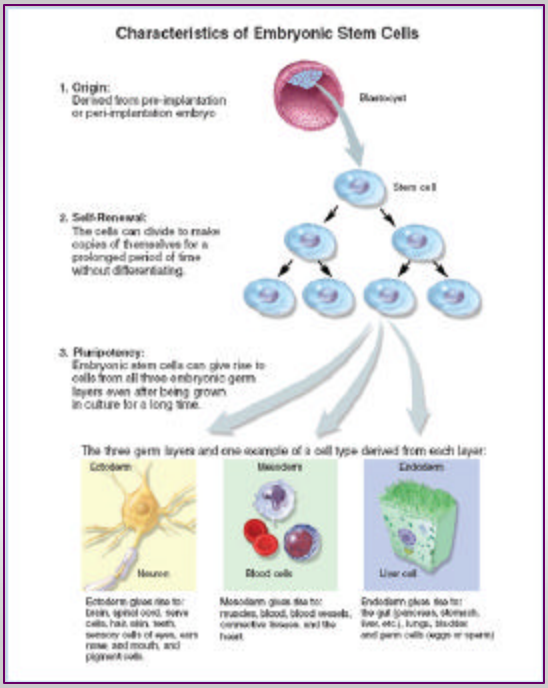


It's a color-enhanced electron microscope image of mouse embryonic stem cells growing on a bed of silicon nanotubes. The image was taken in the lab of Bruce Conklin at the Gladstone Institute for Cardiovascular Medicine.

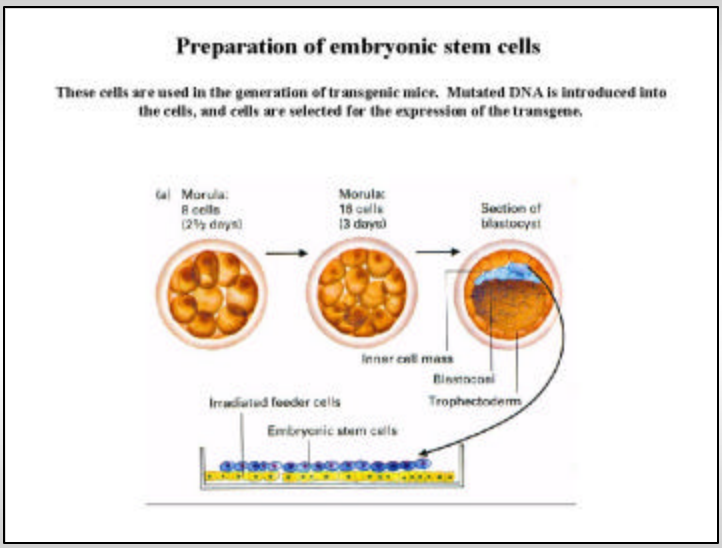
http://thepluripotent.com/?tag=cirm

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. Abcense of G1 cell cycle checkpoint
8. Rapid proliferation and ***unique cell cycle*** kinetics
9. Germline colonization and transmission
10. They are ***XY***



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



http://stemcells.nih.gov

2.ES cells possess indefinite self-renewal potential

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

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).*

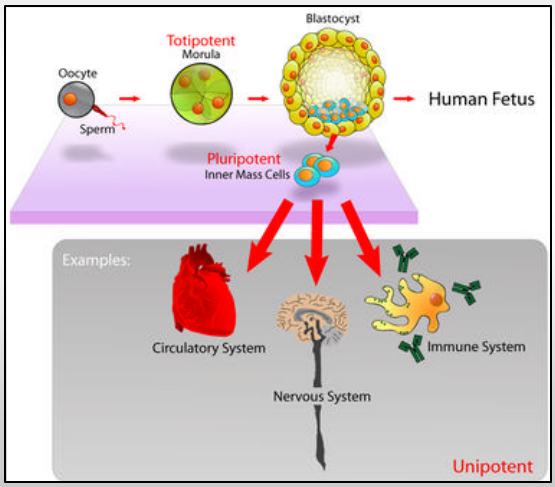
This requires cell cycle control and often maintenance of multipotency or pluripotency, depending on the stem cell.
Self-renewal programs involve networks that balance proto-oncogenes (promoting self-renewal), gate-keeping tumor suppressors (limiting self-renewal), and care-taking tumor suppressors (maintaining 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.*

Mechanisms of stem cell self-renewal.
Shenghui H, Nakada D, Morrison SJ. Annu Rev Cell Dev Biol. 2009;25:377-406.

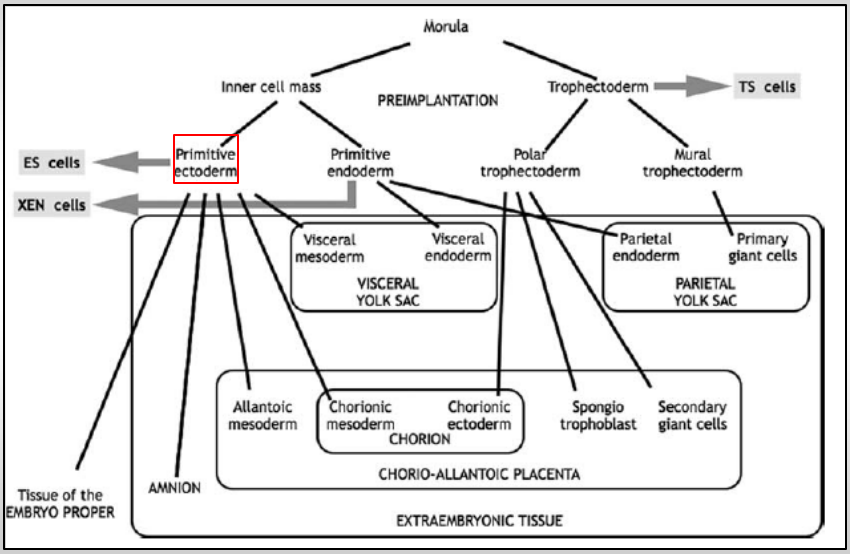
3.ES cells are pluripotent

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 trophectoderm , they cannot produce a blastocyst the novo and hence are not sufficient to produce an embryo



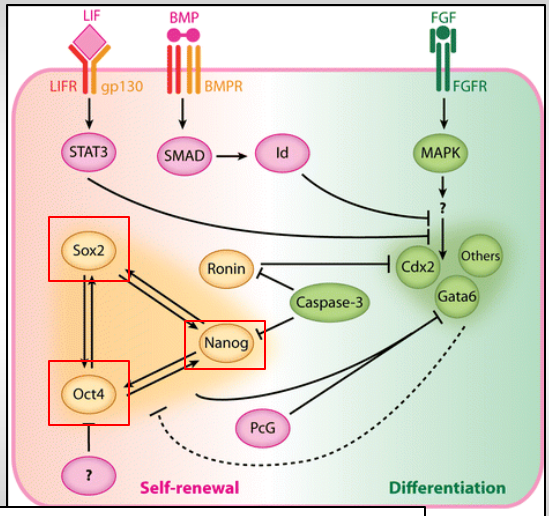
Embryo lineages



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 self-renewal and pluripotency



Mechanisms of stem cell self-renewal.
Shenghui H, Nakada D, Morrison SJ. Annu Rev Cell Dev Biol. 2009;25:377-406.

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***

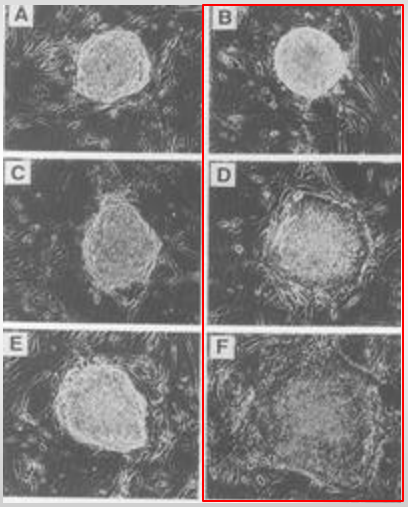
2) XY cells can convert the indifferent genital ridge of an XX recipient embryo into testicular development. Because XX germ cells do ***not develop in a male gonad, this phenomenon of sex conversion results in chimeric males in which all the spermatocytes are of ES cell origin.***

Undifferentiated state of mouse ES Cells

Undifferentiated ***Differentiated***

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***

Table B.1. Comparison of Mouse, Monkey, and Human Pluripotent Stem Cells

Marker Name	Mouse EC/ ES/EG cells	Monkey ES cells	Human ES cells	Human EG cells	Human EC cells
SSEA-1	+	–	–	+	–
SSEA-3	–	+	+	+	+
SEA-4	–	+	+	+	+
TRA-1-60	–	+	+	+	+
TRA-1-81	–	+	+	+	+
Alkaline phosphatase	+	+	+	+	+
Oct-4	+	+	+	Unknown	+
Telomerase activity	+ ES, EC	Unknown	+	Unknown	+
Feeder-cell dependent	ES, EG, some EC	Yes	Yes	Yes	Some; relatively low clonal efficiency
Factors which aid in stem cell self-renewal	LIF and other factors that act through gp130 receptor and can substitute for feeder layer	Co-culture with feeder cells; other promoting factors have not been identified	Feeder cells + serum; feeder layer + serum-free medium + bFGF	LIF, bFGF, forskolin	Unknown; low proliferative capacity
Growth characteristics *in vitro*	Form tight, rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs	Form flat, loose aggregates; can form EBs	Form rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs
Teratoma formation *in vivo*	+	+	+	–	+
Chimera formation	+	Unknown	+	–	+

KEY
ES cell – Embryonic stem cell
EG cell – Embryonic germ cell
EC cell – Embryonal carcinoma cell
SSEA – Stage-specific embryonic antigen
TRA – Tumor rejection antigen-1
LIF – Leukemia inhibitory factor
bFGF – Basic fibroblast growth factor
EB – Embryoid bodies.

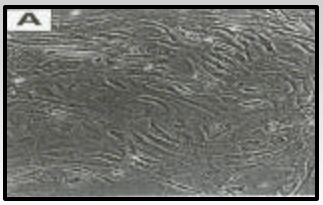
How to Culture Mouse ES Cells

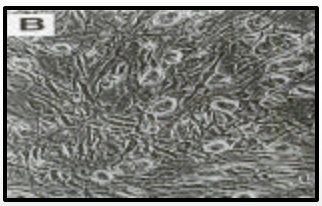
-ES cells can be cultured and still retain their ability to **contribute to all cell lineages** when reintroduced into a host blastocyst.

-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





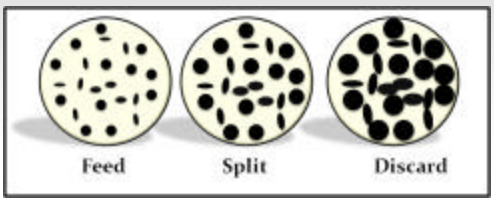
Risk factors for losing the ability to contribute to quimeras

Risk factors for differentiation of ES cells

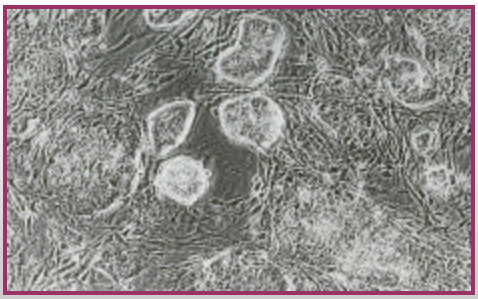
If culture is appropriate, fraction of differentiated cells is very low, and their lifespan and are continuously diluted out.

Risk factors for differentiation of ES cells

1-Innapropiate culture: High density



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



2-LIF

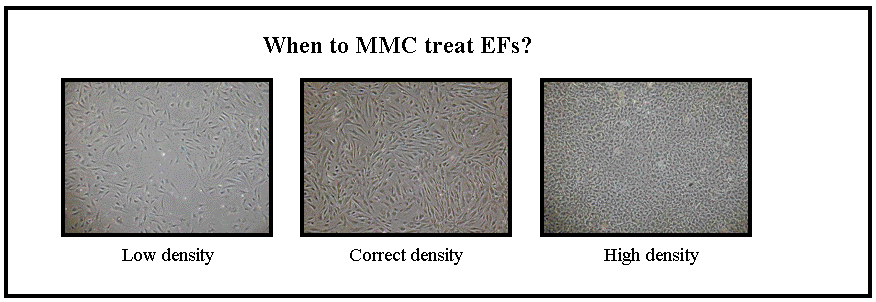
-LIF can be given by LIF-expressing feeders or by adding it to the media at a concentration of 10^6 Units/L
Binding to LIFR triggers activation of STAT3 (necessary for continued proliferation of mouse ES cells)
STAT3 pathway usually promotes differentiation of other cells.
G1 checkpoint does not appear 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)



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.

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

1.2 MUTATING ES CELLS (Homologous Recombination)

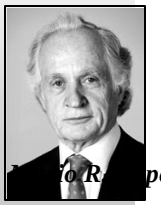
•***Mammalian cells have HR machinery:*** *Folger et al, Mol. Cell Biol. (1982)*

•***Directed Gene Targeting in MAMMALIAN CELLS:***
•Smithies et al, Nature (1985) and Thomas et al, Cell (1986) and Thomas et al Nature (1986)

•***Directed Gene Targeting in ES CELLS:*** *Thomas and Capecchi, Cell (1987); Doetschman Nature (1987)*

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

Thompson et al, Cell 1989 (HPRT)
Schwartzberg et al Science 1989 (c-abl)
Zijstra et al, Nature 1989 (b2-microglobulin)
Koller et al, PNAS 1989 (HPRT)
Thomas and Capecchi, Nature 1990 (int-1 proto-oncogene)



Mario R. Capecchi

Oliver Smithies

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.*

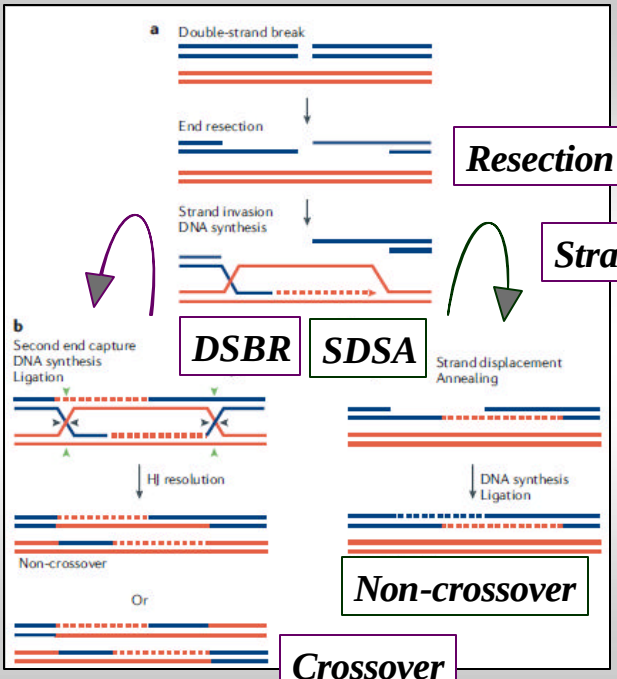
Homologous Recombination

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.*

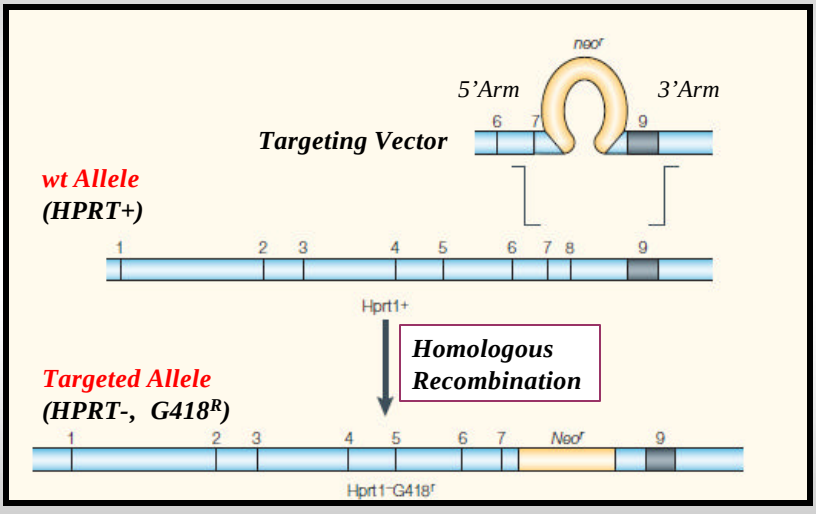
DNA repair: on-homologous end joining (DSBR): *Resolution of the exchanged DNA strands results in crossover, whereby segments of the interacting chromosomes are exchanged. Central to the DSBR model is the formation of a DNA joint molecule that harbours two* ***Holliday junctions****.*

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.*



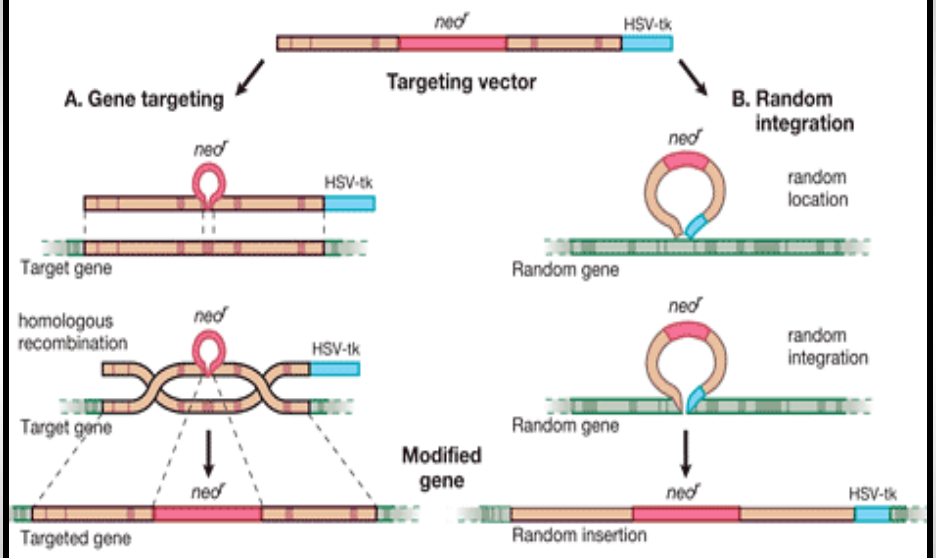
Sung et al, Nature Reviews Molecular Cell Biology 2006

Homologous Recombination in ES cells



Thoimas and Capecchi, Cell (1987), Capecchi et al Nature Reviews (2001),

HR vs Random Integration



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.

[18] Evans MJ, Bradley, A., Kuehn, M.R., Robertson, E.J. The ability of EK cells to form chimaeras after selection of clones in G418 and some observations on the integration of retroviral vector proviral DNA into EK cells. Cold Spring Harb Symp Quant Biol. 1985;50:685-9.

[19] Robertson E, Bradley, A., Kuehn, M., Evans, M. Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature. 1986;323:445-8.

[20] Gossler A, Doetschman, T., Korn, R., Serfling, E., Kemler. R. Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc Natl Acad Sci (USA). 1986;83:9065-9.

[21] Kuehn MR, Bradley A, Robertson EJ, Evans MJ. A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. Nature. 1987;326:295-8.

[22] Hooper M, Hardy K, Handyside A, Hunter S, Monk M. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature. 1987;326:292-5.

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 feasilibity 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 1,000 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 occcurs 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 1/1,000, which should be high enough to permit targeting of non-selectable genes as well.

[38] Thompson S, Clarke AR, Pow AM, Hooper ML, Melton DW. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. Cell. 1989;56:313-21.

[39] Koller BH, Hagemann LJ, Doetschman T, Hagaman JR, Huang S, Williams PJ, et al. Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. Proc Natl Acad Sci U S A. 1989;86:8927-31.

[40] Zijlstra M, Li E, Sajjadi F, Subramani S, Jaenisch R. Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. Nature. 1989;342:435-8.

[41] Thomas KR, Capecchi, M.R. Targeted disruption of the murine int-1 protooncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature. 1990;346:847-50.

31] Folger KR, Wong, E.A., Wahl, G., Capecchi, M.R. Patterns of integration of DNA microinjected into cultured mammalian cells: Evidence for homologous recombination between injected plasmid DNA molecules. Mol Cell Biol. 1982;2:1372-87.
[32] Capecchi MR. Generating mice with targeted mutations. Nat Med. 2001;7:1086-90.
[33] Smithies O, Connell, G.E., Dixon, G.H. Chromosomal rearrang ements and the evolution of haptoglobin genes. Nature. 1962;196:232-6.
[34] Slightorn JL, Blechl, A.E., Smithies, O. Human fetal Gg and Ag globin genes: Complete nucleotide sequences suggest that DNA can be exchanged between t hese duplicated genes. Cell. 1980;21:627-38.
[35] Doetschman T, Gregg, R.G., Maeda, N., Hooper, M.L., Melton, D.W., Thompson, S., Smithies, O. Targeted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature. 1987;330:576-8.
[36] Thomas KR, Capecchi, M.R. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell. 1987;51:503-12.

Gene Targeting

1981
HR in Mammalian cells
Folger, Capecchi,

1987
Gene targeting in ES cells
Correcting mutant HPRT gene Smithies

1986
Gene Targeting in Mammalian Cells
Capecchi, 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

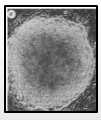
Gene Targeting in ES cells: correcting HPRT

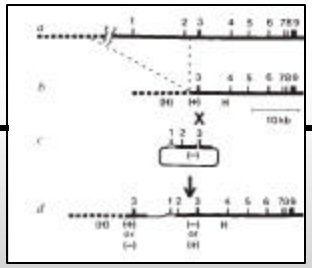
1987

Nature. 1987 Dec 10-16;330(6148):576-8.
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.

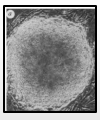
Correcting mutant HPRT gene

HPRT$^-$ ES cell line





HPRT$^+$ ES cell line



HAT-sensitive

HAT-resistant

Gene Targeting in ES cells: mutating HPRT

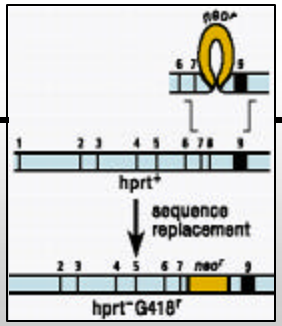
1987

Cell. 1987 Nov 6;51(3):503-12.
Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells.
Thomas KR, Capecchi MR.

Mutating HPRT gene

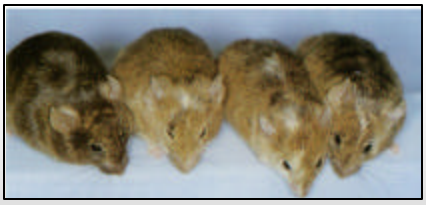
HPRT^{+} ES cell line → ***HPRT^{-} ES cell line***

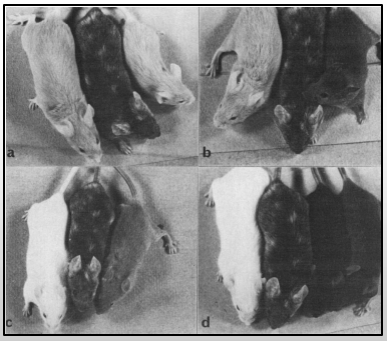
6-TG-sensitive → ***6-TG-resistant***



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)

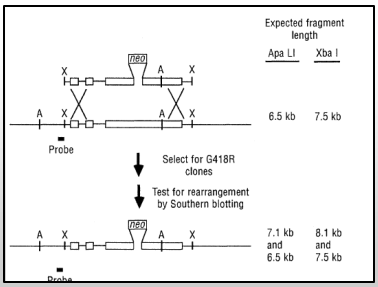


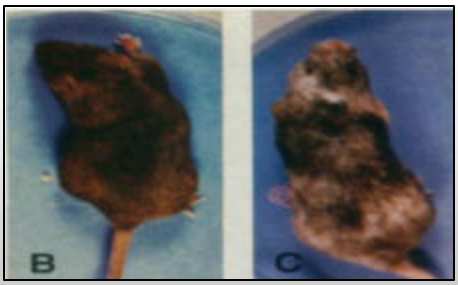


HR in ES cells and germline transmission of non-selectable genes

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

1-Positive and Negative selection *(Thomas and Capecchi et al Nature (1986), Mortensen et al PNAS (1991), Van Deursen et al, Nuc. Acid. Res (1992), Mansour et al, PNAS (1990), Yagi, PNAS (1999))*

2-Homology length of arms. *(Thomas et al, Cell (1987), Deng et al, Mol. Cel. Biol. (1992), Hasty et al, Mol. Cel. Biol. (1992))*

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

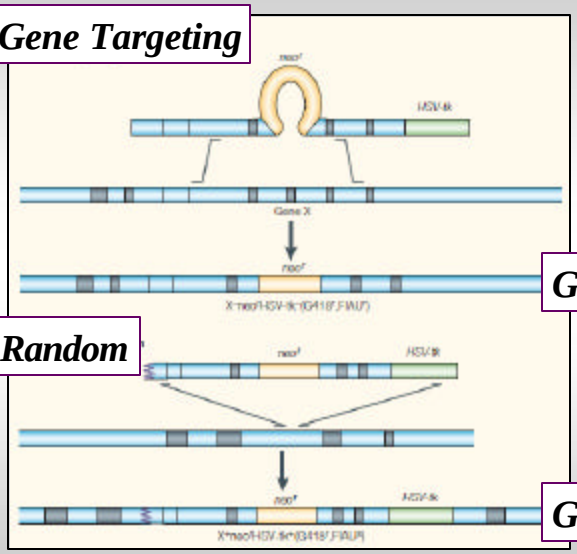
4-Stretches of DNA deletion *(Zhang et al, Mol. Cel. Biol. (1992))*

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*

$G418^R$ $FIAU^R$

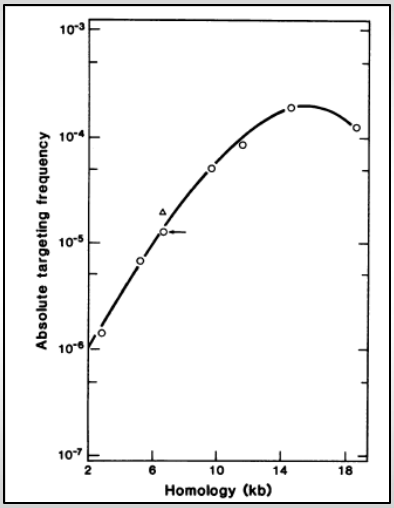
Random

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

$G418^R$ $FIAU^S$

(Thomas and Capecchi et al Nature (1986), Mortensen et al PNAS (1991), Van Deursen et al, Nuc. Acid. Res (1992), (Mansour et al, PNAS (1990), Yagi, PNAS (1999))

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.*

(Thomas et al, Cell (1987), Deng et al, Mol. Cel. Biol. (1992), Hasty et al, Mol. Cel. Biol. (1992))

3-Isogenecity

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

(Van Deursen et al, Nuc. Acid. Res (1992), te Riele et al, PNAS (1992)).

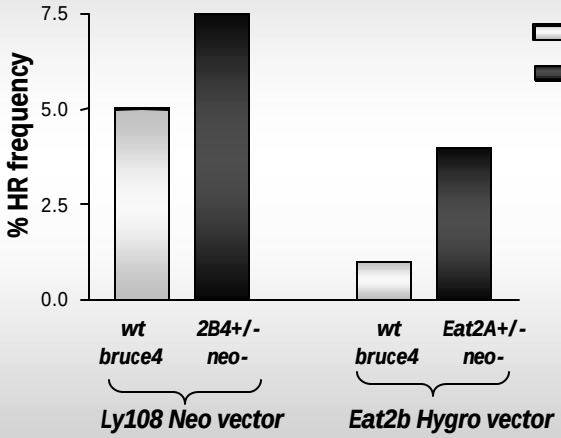
4-Maximal deletion size

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

(Zhang et al, Mol. Cel. Biol. (1992))

5-Previous targeting events

Increased %HR frequency in previously targeted cells

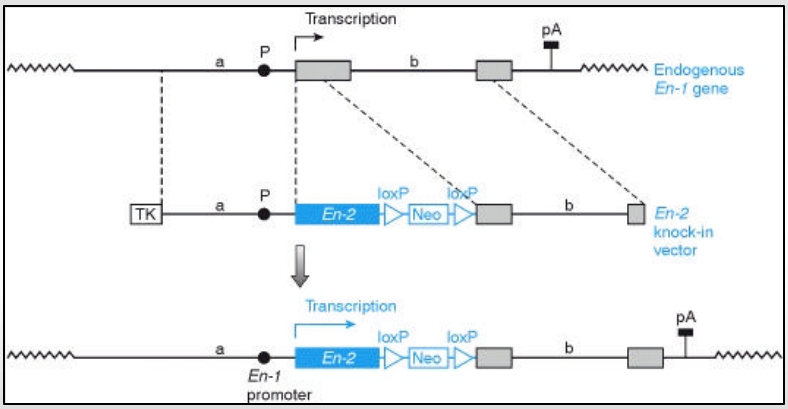


1-Increase when using both **Hygro and Neo** vectors
2-Increase even if events are separated by **almost 400KB**

(Calpe, Wang et al, Unpublished)

Knockins

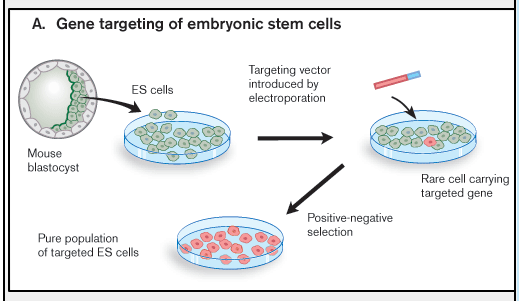
Deletion of En1 gene but addition of En2

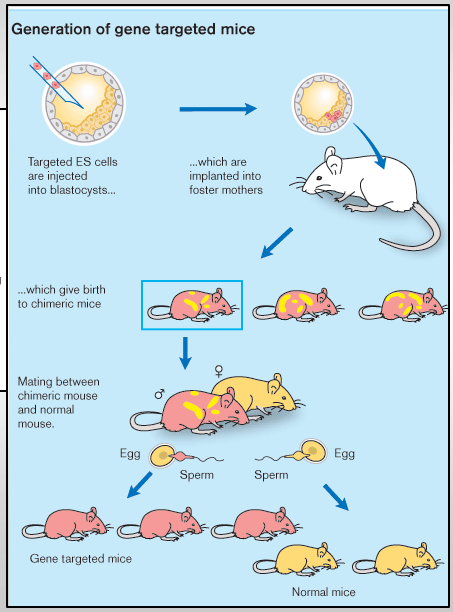


Science. 1995 Aug 4;269(5224):679-82.
Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2.
Hanks M, Wurst W, Anson-Cartwright L, Auerbach AB, Joyner AL.

Transfer mutation germline

Produce specific mutation in mammal cells





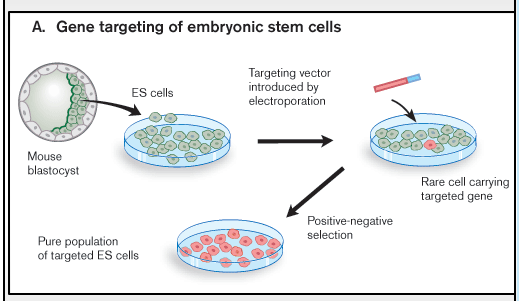
Culturing ES

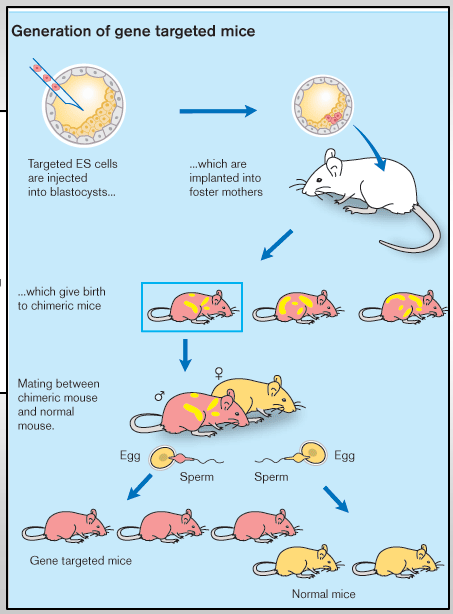
Mutating ES

http://nobelprize.org/nobel_prizes/medicine/laureates/2007/adv.html

Transfer mutation germline

Produce specific mutation in mammal cells





Culturing ES

Mutating ES

http://nobelprize.org/nobel_prizes/medicine/laureates/2007/adv.html

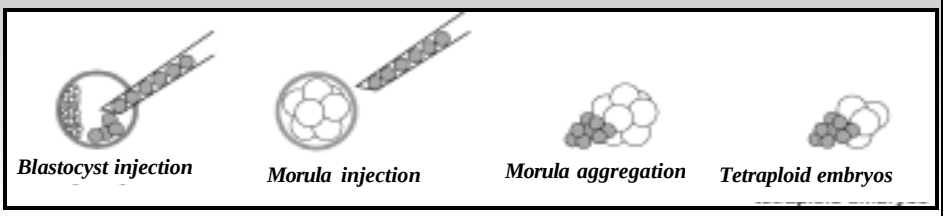
1.3 GERMLINE

Andreas Nagy

- *Blastocyst injection: Bradley et al, Nature 1984*
- *Morula aggregation: Wood et al, Nature 1993*
- *Labosky et al, 1994*
- *Tetraploid embryos: Nagy et al, PNAS 1993*
- *Wang et al, Nature 1997*
- *129Sv BLASTOCYST are more efficient than B6: Brook et al, PNAS 1997*
- *COISOGENIC BLASTOCYSTS are better in supporting the development and germline trasnmission:*
  - *Schuster-Gossler at el, Biotechniques 2001*
  - *Auerbach et al, Biotechniques 2000*
- *RAG-2 Blastocysts complementation: Chen et al, PNAS 1993*

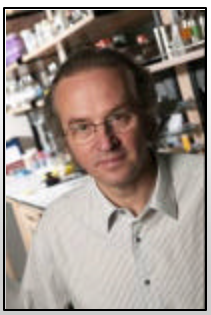
Allan Bradley

Transferring mutation through the germline

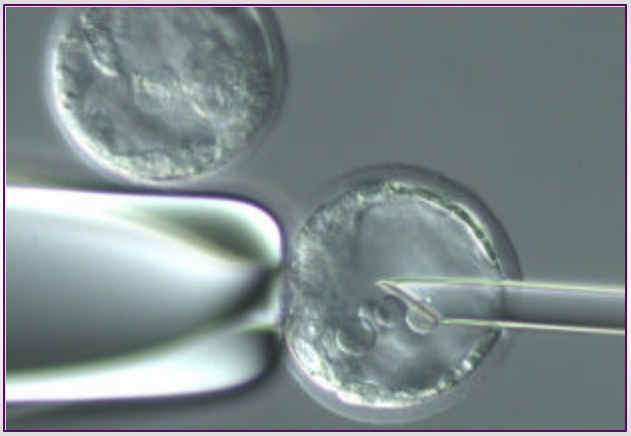


Bradley et al, Nature 1984
Thompson et al, Nature 1989

Wood et al, Nature 1993
Nagy et al, PNAS 1993

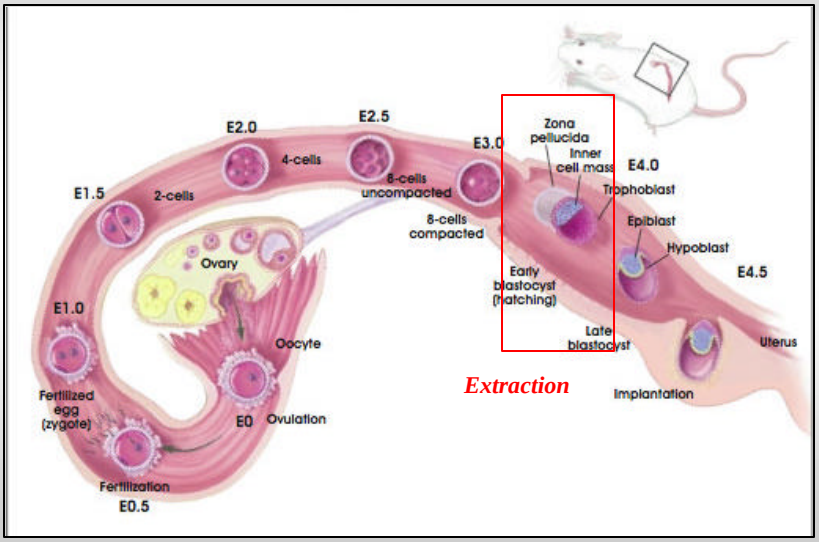


1-Blastocyst injection

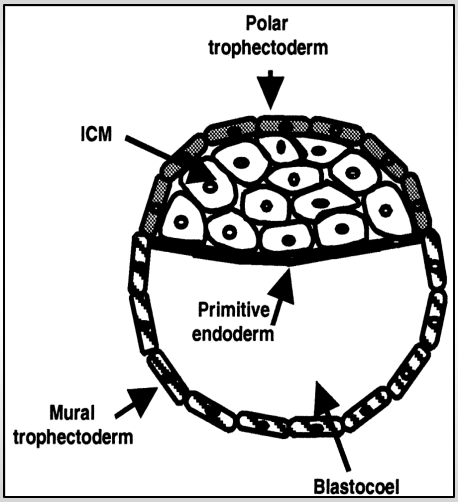


(from the Transgenic/Gene Targeting Facility of Oregon Health & Science University)

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

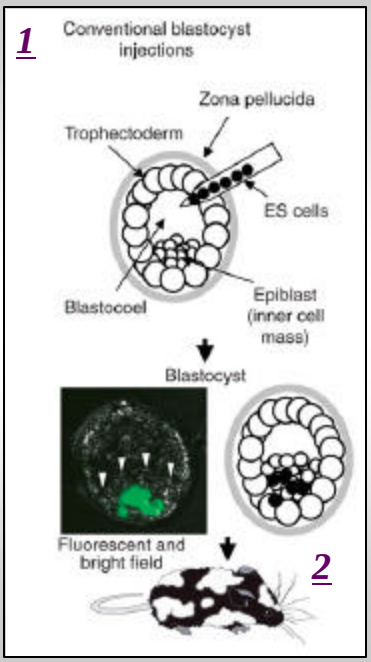


Blastocyst



*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



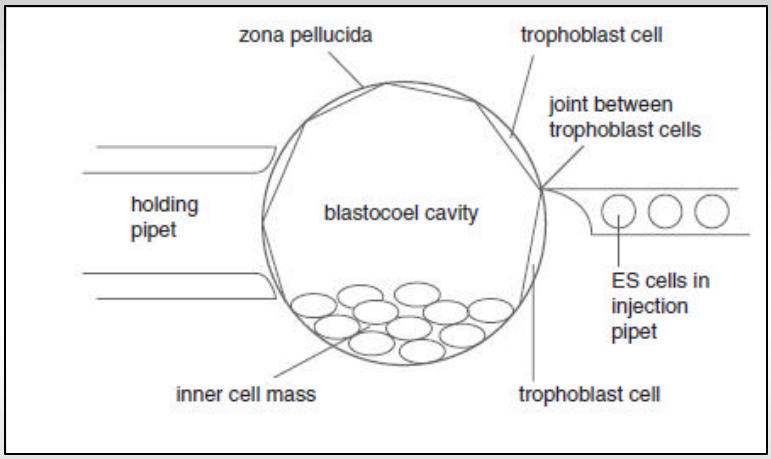
1-Injection ES cells

In a conventional blastocyst (3.5day) 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

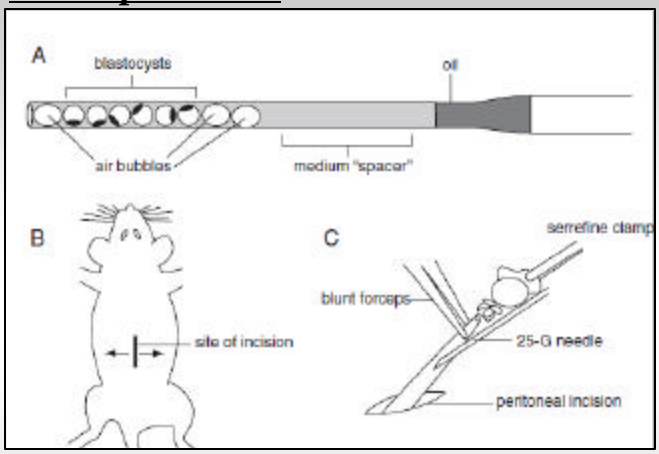
Poueymirou et al 2007. Nature Biotech

1-Injection ES cells



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.

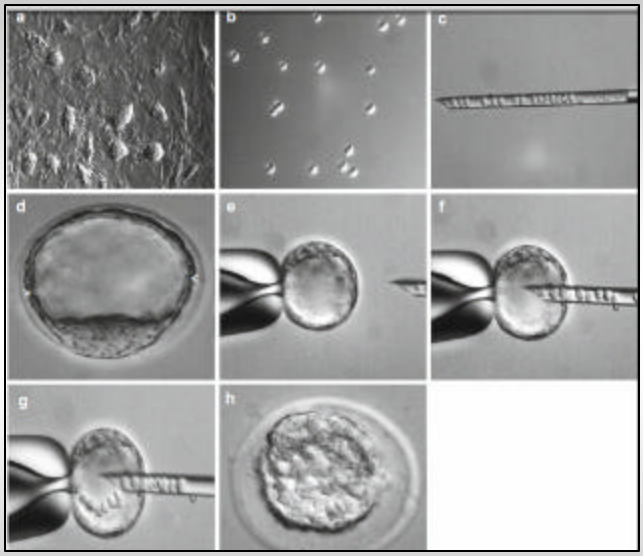
2-Reimplantation



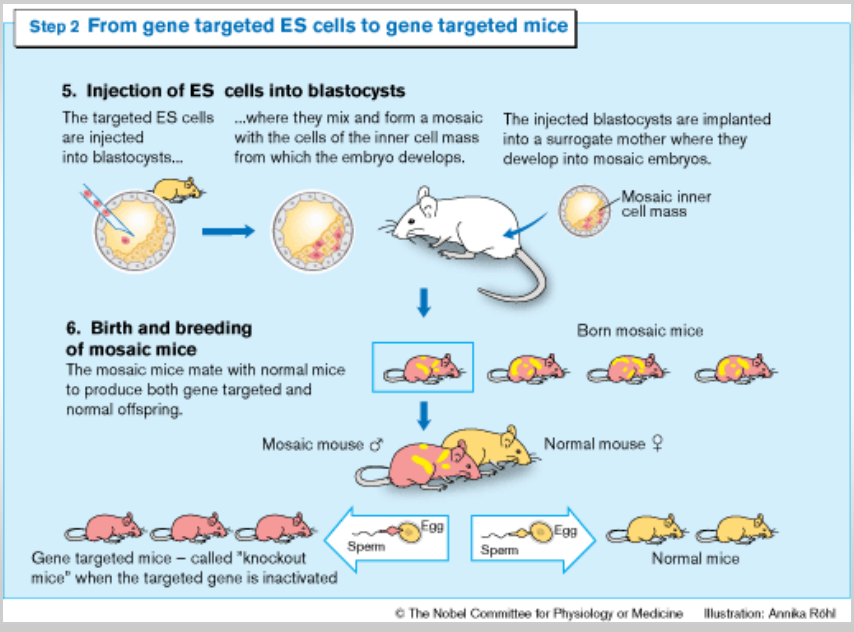
Diagrams of various aspects of the reimplantation procedure

(**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.

ES cell injection into the blastocoel of a blastocyst



(a) 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 .

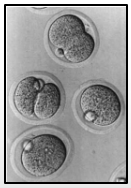


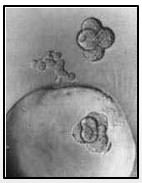
2-Tetraploid embryo aggregation

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





11.Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. & Roder, J.C. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc. Natl. Acad. Sci. USA 90, 8424–8428 (1993).

12.Eggan, K. et al. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc. Natl. Acad. Sci. USA 98, 6209–6214 (2001).

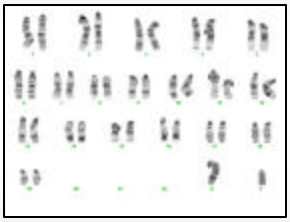
13.Eakin, G.S., Hadjantonakis, A.K., Papaioannou, V.E. & Behringer, R.R. Developmental potential and behavior of tetraploid cells in the mouse embryo. Dev Biol (2005).

Comparison methods

Blastocyst injection	***Embryo Aggregation***
1-Individual cells are selected, offering a way to control best Es cells that hopefully will give germline transmission	***1-F0 generation mice are almost completely ES cell–derived and thus immediately available for phenotypic analyses.*** ***2-Technically less demanding and expensive***
1-High Skills ***2-Expensive to establish in a laboratory***	***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).***

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

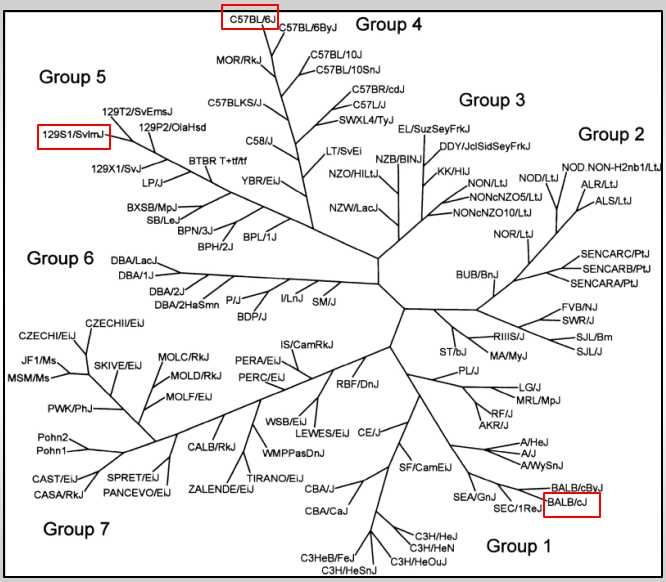
1.4 CHOICE OF MOUSE STRAINS FOR GENE TARGETING

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

Th1 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***

Mouse family tree



Genome Res. 2004 14: 1806-1811
Petko M. Petkov, Yueming Ding, Megan A. Cassell, et al.
An Efficient SNP System for Mouse Genome Scanning and Elucidating Strain Relationships

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.

Strains to be considered

1-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.

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

Cell line	Gene	Reference
Bruce 4 (C57BL/6)	MHC class II Aa CD3ε/γ	Koentgen *et al.* (1993) Malissen *et al.* (1993)
BL/6-III (C57BL/6)	Igκ Perforin CD23 PBGD IL-5	Zou *et al.* (1993) Kägi *et al.* (1994) Yu *et al.* (1994) Lindberg *et al.* (1996) Kopf *et al.* (1996)
BALB/c-I	IL-4 IL-4Rα	Noben-Trauth *et al.* (1996) Mohrs *et al.* (1999)
DBA-252 (DBA/1)	FLAP	Roach *et al.* (1995)
MRL	Ep2	Goulet *et al.* (1997)

Ledermann B, *Exp. Physio.* 2000

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 to 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**

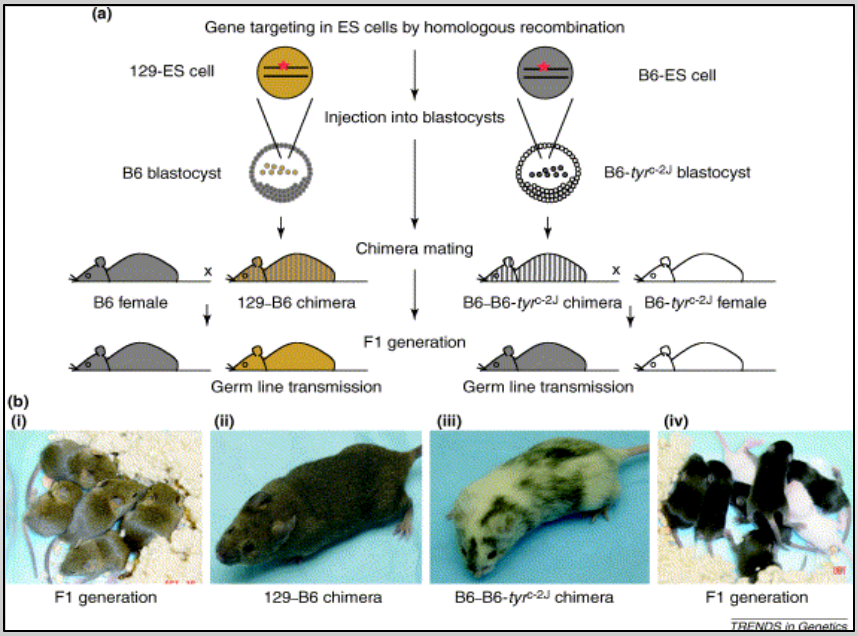
- Low yield of embryos per mouse
- Delayed embryonic development

Lemckert et al, Nucleic Acids Res. 1997
Optimized methods for the qualitty and quantity

Seong et al, Trends. Genetics 2004
Low chimersim ratio but High Germline trasnmission

Albino B6 Embryos

Injection	Pups	Mate to	You want	Don't want
129 ES cells into B6 blast	Chimera		129	
B6 ES cells into Balb/c blast	Chimera		B6	



TO KNOCKOUT in 129 or in C57BL/6: that is the question

129SV ES CELLS → B6 Embryos **B6 ES CELLS → BALB/C Embryos**

Difficulty of making non-129 Es cells

Isogenic DNA: than B6 Genomic DNA available from BAC clones, while 129 have o be identified by library screening

Similar homology frequencies using identical targeting vectors

Proportion of chimerism is lower in BALB/c Blastocyst (more injections required)

Low yield of embryos with BALB/c mice

Low sex ratio

ES cell contribution to the coat is lower

High germline trasnmission (higher number of pups because fertility is higher) even for low level chimeras (10%)

Avoid Background genotype