Embryonic Stem Cells and their Genetic Modification

Nobel Prize in Physiology or Medicine 2007

Mitradas M Panicker

Mitradas M. Panicker is on the faculty of the National Centre for Biological Sciences, Bangalore, India. His research interests include stem cells, serotonin and its receptors and neurobiology.

The derivation of embryonic stem cells from mice and the development of techniques that allow for targeted manipulation of its genome have allowed for the generation of mice with desired mutations. This is what led to the announcement in Stockholm in 2007 – "The Nobel Assembly at Karolinska Institute today decided to award the Nobel Prize in Physiology or Medicine for 2007 jointly to Mario R Capecchi, Martin J Evans and Oliver Smithies for their discoveries of *Principles for introducing specific gene modifications in mice by the use of embryonic stem cells*". Martin J Evans was recognised for his derivation and establishment of mouse embryonic stem cells while Mario Capecchi and Oliver Smithies were honoured for techniques that allowed site-specific modification of sequences within the genome of these cells. Such cells were then used to generate animals that had their genome altered specifically and at desired locations. For this valuable body of work, all three shared the prize equally. To date, almost half of the genes in the mouse genome have been modified using their techniques. This has led to a better understanding of mammalian physiology, development and diseases.

Keywords
Nobel Prize in Physiology or Medicine, embryonic stem cells, genetic manipulation of ES cells, mouse ES cells, Human ES cells, HPRT, Mario R Capecchi, Martin J Evans, Oliver Smithies.

A combination of genetics and direct experimental manipulation is a prerequisite to understand any biological process. Genetics has been difficult for mammalian systems in particular, since short generation times and large numbers of progeny are necessary. The most effective mammalian model so far has been the laboratory mouse and this has been to a major extent due to the large variety of mutants and strains available. Considering that human genes and mouse genes bear considerable homology it is not too surprising that the findings using mouse models are being used to understand human diseases. Manipulation of mouse

embryonic stem cells affords a lot of opportunities for such studies.

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Generation of Embryonic Stem Cells

Stem cells, particularly embryonic stem cells, have been the subject of both medical and public discussion for a variety of reasons – from the promise of regeneration within the body, to providing an inexhaustible source of various cell types/tissues for repair. It is important to emphasize that these cells originated from experiments primarily designed to dissect the mechanisms of mammalian development.

What are embryonic stem cells (ES cells) or more rightly put, embryo-derived stem cells, and how they are used to generate mutant animals? ES cells are 'most often' obtained from the blastocyst stage of mammalian embryos. I say 'most often' because cells at an earlier stage have also been used to obtain ES cells. The fertilised egg, in the normal course, develops into the blastocyst, a near spherical ball of cells that has a transitory existence in development. The blastocyst has a tight layer of single cells on the outside containing within it a small group of cells at one end of a cavity. The outer layer, the trophoectoderm, comprise the cells that implant later into the uterine wall and become the placenta while the inner mass of cells – not surprisingly termed the 'inner cell mass' or ICM goes on to become the embryo and finally the animal. The inner cell mass that can be isolated mechanically or by using components of the immune system to specifically kill the trophoectodermal cells, are obtained free of the outer trophoectodermal cells and cultured. One can also provide conditions that would allow the inner cell mass to form outgrowths in culture directly from the blastocyst. In any event, these cells can be made to grow indefinitely in culture with a stable normal karyotype i.e., they self renew. More importantly, they continue to retain the property of being able to differentiate into all cell types found in the animal *in vivo* and under the right environment *in vitro.* ES cells can form mixtures of the three germ layers i.e., endoderm, ectoderm and mesoderm tissue in

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benign tumours called teratomas, when implanted in an immunosuppressed host, mimicking embryonic development to an extent. Normally, the inner cell mass has only a transitory existence but Martin Evans showed, picking his cue from the embryonal carcinoma cells present in teratocarcinomas, that these cells can be isolated and cultured indefinitely *in vitro*, if you provide the appropriate conditions, such as a feeder layer of differentiated cells [1]. He then showed that the cultured cells can be reintroduced into mouse blastocysts, which then implanted into the uteri of appropriately conditioned mice, develop into mouse pups. The introduced ES cells mix with the recipient inner mass cells and then go on to divide and differentiate into all cell types/tissues, including germ cells of the animal [2]. Such animals are termed chimeric since their tissues are composed randomly of both the inner cell mass of the blastocyst and the introduced ES cells.

From ES Cells to Mice

Since ES cells can be cultured extensively *in vitro* and still retain their ability to contribute to all tissues in the animal, the next obvious question was to see if genetically-manipulated ES cells would do the same. And indeed they do. Since the introduced ES cells form only parts of the animal and the contribution is random, this is not as useful in comparison to an entire mouse derived from the modified ES cell. The contribution of ES cells to any particular tissue is random and also determined by the number of ES cells injected into the blastocyst. Since ES cells are often derived from a strain of mice of a particular coat colour and injected into blastocysts from mice having another coat colour – the chimeric mice can be recognised by the presence of both coat colours. As noted earlier, with some frequency, the injected i.e., manipulated ES cells also contribute to the germ cells that give rise to eggs and sperm in these animals. This is possible because if ES cells are cultured and maintained properly, they retain their karyotype i.e., they have the full and proper chromosome complement – even the genetically modified ones. These cells can therefore undergo normal meiosis and generate germ cells within the animal. Since the germ cell arises from a single diploid cell

and if it is derived from the modified ES cell, it could also carry the mutation. The progeny arising from such germ cells can be monitored from the coat colour and further analysis of the genome. This allows one to screen for animals that arise solely from ES cell-derived germ cells which subsequently carry the introduced mutation and present its effects. Once such animals are obtained, stocks can be maintained by breeding. One can also generate animals homozygous for the introduced mutation as long as it is not lethal. This technique has also been extended to introduce mutations that confer phenotypes in a temporal and spatial manner within an animal.

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Genetic Manipulation of ES Cells

Genetic manipulation of cells can be done in various ways; from growing them in mutagens to obtain random mutations, to introducing foreign DNA that is stably integrated within the genome by transfection. The latter technique had been established by Michael Wigler and Richard Axel and as one would imagine, in this case the integration is random [3]. Using random integration of retroviral DNA to inactivate genes, Martin Evans and co-workers showed that one can functionally inactivate a specific locus, if a selection for the event was possible. Evans chose the HPRT (Hypoxanthine-guanine phosphoribosyltransferase) locus in ES cells, present on the X chromosome and therefore occurring as a single locus in male ES cells, and inactivated it through viral integration. Since inactivation of HPRT could be directly selected for using 6-thioguanine, these cells could be easily identified and were transferred into blastocysts to give rise to chimeric animals [2]. Some of these cells contributed to the germline in some chimeras and the mutation could be transferred from one generation to another. This established that ES cells could be modified and could be then used to generate modified animals.

Since a more general method was desirable to modify specific loci of choice and in a defined manner, a different technique was required. A specific locus would have to be targeted, so a homologous recombination between the introduced DNA and the

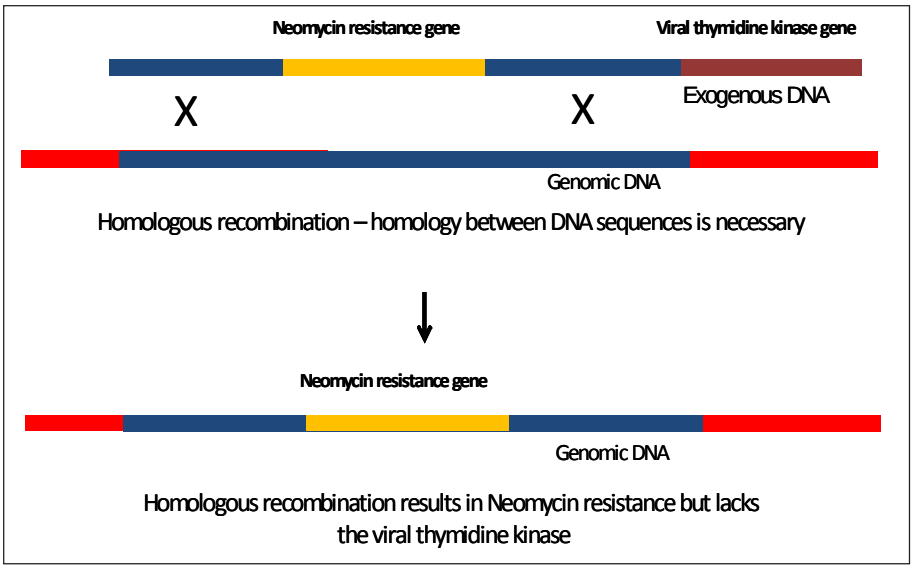
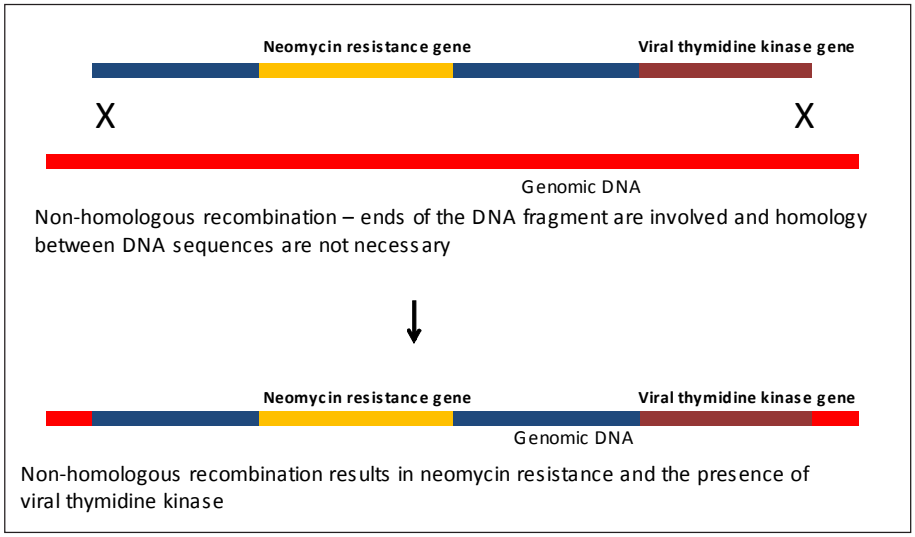


Figure 1. Strategy for positive and negative selection to obtain homologous recombinants.

locus was the natural choice. Mario Capecchi had surmised from earlier work, from the "head to tail" organisation of exogenous DNA "multimers" integrated within the genome of transfected cells, that homologous recombination can occur in eukaryotic cells (*Figure* 1) [4]. He then developed vectors and more significantly a strategy that selected against non-homologous recombinants. This involved the use of viral thymidine kinase DNA outside the homologous region. Some cells may undergo non-homologous recombination through the ends of the DNA frag-

Figure 2. Non-homologus recombination is eliminated by negative selection for thymidine kinase gene.



ment and would often end up expressing the unwanted viral thymidine kinase (*Figure* 2). Such events can be eliminated using the drug gancyclovir, which is poisonous for cells expressing this enzyme – this is termed negative selection [5]. Around the same time, Oliver Smithies had also concluded from his work in trying to correct mutations in non-ES cells that homologous recombination could take place. Using 'rescuable' plasmids that contained homologous sequences, he could score for the recombinants and also clone these modified cells. Smithies, in addition, established electroporation, the introduction of exogenous DNA into cells using electrical pulses, as the method of choice to generate recombinants. He also developed a rapid and efficient Polymerase Chain Reaction (PCR)-based method to determine homologous recombination events in cells. Using these techniques, Smithies and coworkers also generated site-specific genetically-modified animals using mouse ES cells [6]. Capecchi and Smithies used the neomycin resistance gene – a gene known to function in bacteria – to directly select for all integrants i.e., positive selection. The techniques that they developed allow very infrequent events to be recognised and isolated. The technology that they introduced has come so far that it has now become near routine to target any desired gene and introduce specific deletions, insertions or point mutations within it in the mouse.

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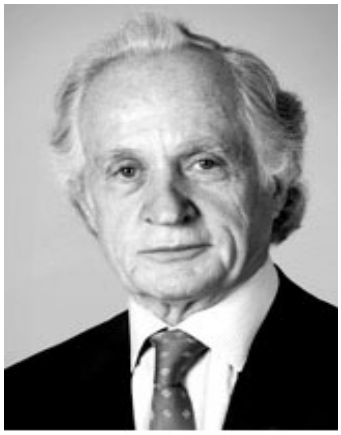
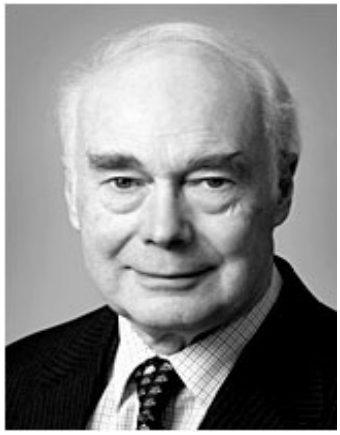
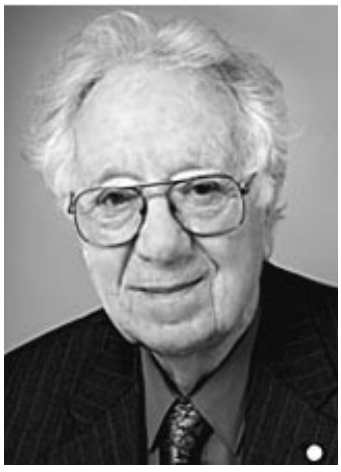
What led to all this? The work began in the late 1970s and not necessarily with these technical goals in mind. It was more of an attempt to understand the basic principles that biologists, particularly developmental biologists, grapple with – how do cells propagate, how do they differentiate and generate various cell types within complex organisms. At least this is what drove Martin J Evans to study embryonal carcinoma cells derived from teratocarcinomas – tumours that arose in the testis of mice, which often exhibited differentiated cells of all three germ layers [7]. These occurred rarely in normal mouse strains except in the 129 strain where its higher frequency made it a good model to study. Incidentally, the first ES cells were derived from this strain. Oliver Smithies was interested in the regulation/expression of



Dr Mario Capecchi was born in Verona, Italy, in 1937. He had an extremely traumatic childhood during the war and barely survived illness and malnutrition on the streets of Italy as his mother was sent to the concentration camp. He migrated to the United States with his mother shortly after the war with the support of his uncle. His formal education started only after arriving in the USA in 1946. He obtained his BS degree in Physics and Chemistry from Antioch College and his PhD was from Harvard University in biophysics under Dr James D Watson. He later became a member of the faculty in the Department of Biochemistry at Harvard University. Since 1973 he has been a Professor at the University of Utah. Along with Smithies and Evans, he won the 2001 Lasker award and continues to work on mammalian development. Mario Capecchi's life is a shining example of the undying human spirit.



Sir Martin J Evans is from the United Kingdom and is presently at the Cardiff University, Cardiff, United Kingdom. Born in 1941, he obtained his BA in Biochemistry from Christ College, University of Cambridge followed by an MA, as well as a DSc. His PhD was from University College, London in 1969. A fellow of the Royal Society, he was knighted in 2004. He has taught at University College, London and the University of Cambridge. His early work with Matthew Kaufman was on teratocarcinomas which then led to the isolation of mouse embryonic stem cells, which was carried out at Cambridge in 1981. This led to an explosion in targeted mouse genetics carried on by a talented group of students and postdoctoral fellows who trained under him. He moved to Cardiff in 1999 where he continues to explore the relationships between embryonic stem cells and the normal embryo in addition to generating murine models of human diseases.



Dr Oliver Smithies is the Excellence Professor at the University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. Presently a US citizen, he was born in the United Kingdom in 1925 and is the oldest of the trio that won the 2007 Nobel prize. Along with his co-winners, he also won the Lasker award in 2001 for Basic Medical Research. He obtained his BA. MA, and DPhil degrees from Oxford University in biochemistry and physiology. His postdoctoral research was in physical chemistry and one of his early notable achievements was inventing starch gel electrophoresis to separate proteins – a forerunner of the currently widely used acrylamide gel electrophoresis. He continues to be active in the laboratory, often working full seven days of the week. His contribution to targeted mutations began when he was in his late fifties and unlike most senior scientists he works at the bench as well.

globin genes and that led him to homologous recombination [8], while Mario Capecchi was interested in gene expression, suppression of mutations and eventually turned to homologous recombination to study development [9]. The establishment of mouse ES cells and its modification has, not surprisingly, led them to newer areas in developmental biology and disease models. It is also instructive to note that along with their work a few other groups had also succeeded in one or the other aspects of this work around the same time [10, 11, 12]. For example, Gail Martin, at the University of California at San Francisco (and also a former postdoctoral associate of Martin Evans) reported the isolation of mouse ES cells very shortly after Martin Evans and Matt Kaufman did in 1981. She also showed that these cells could form teratomas and numerous differentiated cell types – something that is now standard for establishing the pluoripotency of ES cells where chimeras are not possible like human ES cells [13]. Success with mouse ES cells encouraged other groups to pursue the isolation of primate ES cells, particularly human ES cells, which was achieved in 1998 by James A Thompson [14]. More recently, a number of groups, notably the one led by Shinyo Yamanaka have even successfully converted human somatic cells i.e., fibroblasts into ES-like cells [15]. This should lead to individual-specific ES cells.

To hear about the discovery and the process of discovery from the scientists themselves is often enlightening and rewarding[1]. The Nobel Lectures given by the awardees every year at Stockholm is available in the printed form. This year's lectures have been made available over the internet as videos[2]. They are truly worth watching and I strongly encourage the reader to do so. The laureates provide one with a 'benchside' perspective, with actual laboratory notebook pages sometimes included and successfully convey the excitement of the discovery process and a historical as well as a long-term perspective.

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Address for Correspondence
Mitradas M Panicker
National Centre for Biological Sciences (TIFR)
PO Box 6501
GKVK Campus
UAS Bellary Road
Bangalore 560 065, India
Email: panic@ncbs.res.in