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Lasker Basic Medical Research Award

# Mouse gene targeting

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## The cultural mouse

MARTIN J. EVANS

I was inspired by biology, particularly by my experience at Cambridge in Christ's College, tutored by David Coombes and in Part II Biochemistry, where I remember in particular such luminaries as Malcolm Dixon and Don Northcote. In that year (1962–1963), a series of lectures at Cambridge by Jacques Monod burst open a new understanding for me and, together with a seminar series organized by Sidney Brenner in his rooms at King's College, inspired me with the new concepts of control of genetic readout through mRNA. I resolved to work in either plant biochemistry or developmental biology. A bout of glandular fever prevented me from taking my final examinations for which I was so eagerly preparing, and resulted in my taking a research assistantship with Elizabeth Deuchar at University College London, on *Xenopus* development. My ambition was to isolate developmentally controlled mRNA, but at that time none of the cloning tools or probes on which we now rely were available. All I could study were double-reciprocally labeled ($^{14}C$ and $^{3}H$) profiles of polyribosomes and mRNA from dissected blastula and gastrula ectoderm by sucrose density gradient centrifugation and RNA by agarose electrophoresis. In modern terms, I was looking at animal cap development in culture before induction and after commitment to either a neural or an epidermal ectoderm. At that time I saw two impediments to further progress: the difficulty of getting enough material for biochemical analysis, and the lack of any foreseeable genetics.

I sought a more 'tractable' developmental system and, at the suggestion of Robin Weiss, looked to the possibility of establishing an *in vitro* system of mammalian cell differentiation from mouse teratocarcinomas. In 1967, Leroy Stevens[1] and Barry Pierce[2] both published reviews of their formative studies. Leroy Stevens had developed a strain of mice with a high incidence of spontaneous testicular teratomas (129Sv). These teratomas contain a complex mixture of tissue types; some (teratocarcinomas) grow progressively and are serially transplantable in the inbred mouse strain. Barry Pierce, who was interested in the relationship between the tumor-forming stem cells and their non-malignant differentiated products, led a series of experiments converting the tumors to an ascites state, in which they grew as embryoid bodies, and culturing mass populations of cells from these *in vitro*. A pivotal experiment by Kleinsmith and Pierce[3] showed that these tumors could be clonally derived from a single transplanted cell, thus proving that the diverse cell and tissue types arise by differentiation from a single pluripotential stem cell line.

Leroy Stevens sent me breeding stock from his 129 inbred line and also several transplantable tumors that he had established. I established clonally derived tissue culture lines from these and demonstrated that the rounded cells (C "clump cells") depended initially on co-culture with a more flattened epithelioid (E) cell[4]. These C cells were the embryonal carcinoma (EC) stem cells from the tumor. Gail Martin joined my lab, and we were able to show that these spontaneously arising 'E-cells' could be replaced by mitotically inactivated chick or mouse fibroblasts, and that when these diminished or were withdrawn, extensive *in vitro* differentiation occurred. In every case, the differentiation proceeded through the production of a primary embryonic endoderm, and clumps of suspended cells formed recognizable embryoid bodies. Re-attachment of these to a solid surface gave rise to the most splendid and diverse differentiation, with beating cardiac muscle, nerve skin, cartilage and so on[5]. It was apparent, moreover, that they were undergoing the same first-step differentiation to an embryonic endoderm as did the inner cell mass (ICM) of a mouse embryo[6].

This likeness to the ICM was tested by experiments with Richard Gardner. I well remember transporting cells from University College London to Oxford, where he carefully introduced them into blastocysts. The chimeras we obtained demonstrated a dramatic result, with nearly every tissue of the derived mouse having contributions from the tissue culture cells[7].These cells, however, were not normal. They were derived from serially passaged tumors and had been cloned and cultured for some time. Karyotypically they were remarkably close to normal for mouse tissue culture cells, but although they had an apparently normal chromosome number they only had one X chromosome and no Y chromosome. Many of the initially normal mice later succumbed to somatic tumors (rhabdomyosarcomas, fibrosarcomas and so on), presumably as a result of the passage-derived mutational load in these cells. We and our colleagues in Oxford, as well as Francois Jacob's laboratory in Paris, tried in vain to recover a euploid XY EC cell line to obtain a perfect germline chimera, but this had to await the direct derivation of the cells from embryos rather than from tumors.

In 1978 I started work in the Department of Genetics at Cambridge University, and many investigations continued to show the close relationships between EC cells and early embryo epiblast. Together with Ten Fiezi, I was able to begin to determine that the main cell surface antigens on the EC cells were carbohydrate epitopes of the glycohalix[8], and Peter Stern, who had recently also moved from University College London, to Sydney Brenner's laboratory in Cambridge, produced a very useful monoclonal against a cell surface glycolipid: the Forsman antigen. The reaction of this monoclonal antibody with cells of the normal early mouse embryo allowed us to refine the apparent homology between EC cells and cells of the embryonic ectoderm before 6 days of development[9]. Robin Lovell-Badge, using what would now be called a proteomic approach—that is, two-dimensional gels of whole-protein

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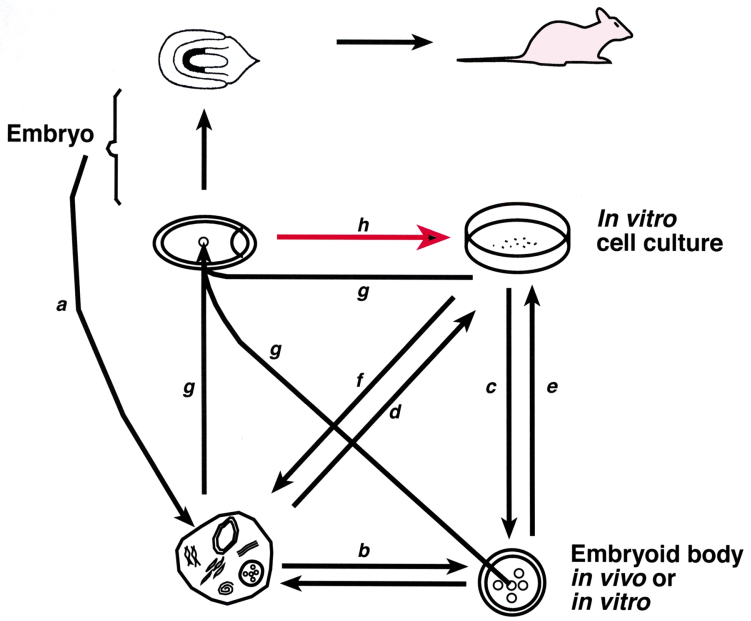


**Fig. 1** Establishment in culture of pluripotential cells from mouse embryos (reproduced from ref. 12). All of the known interrelationships of *in vivo* and *in vitro* differentiation and of derivation of EC cells via a tumor are diagrammed in black. The missing link in the network of relationships, which was provided by the experiments reported in this paper is (h) a direct derivation of the cultured stem cells from an embryo.

extracts—showed a remarkably similar protein synthetic profile in EC cells and early embryo epiblast[10]. The stage was set[11].

It was only when I met up with Matt Kaufman in 1980, however, that the breakthrough could be made. I had remained convinced of the power of a genetic approach, but the somatic cell genetic techniques we were able to use with EC cells at that time—cell hybridization and selection together with exploration of variants in differentiative capacity—were 'blunt instruments'. Matt Kaufman was making haploid mouse embryos, and I knew that I could grow cell lines from blastocysts (albeit not pluripotential lines), so we hoped that we would be able to isolate an haploid cell culture from the embryos. (In retrospect, that never proved possible; the cells always doubled up to a diploid condition during isolation in culture.)

Haploid embryos are retarded in growth and have small ICMs, but Matt had a trick to allow them to catch up. By putting them into implantation delay *in vivo,* the size of the ICM could be allowed to increase before implantation. We planned to use such implantationally delayed, haploid-derived embryos to attempt to establish a cell line, and Matt prepared some normal diploid, but delayed, embryos as controls and for me to use for practice. When I cultured these blastocysts as explants in tissue culture, using a medium that had been honed for optimum cloning efficiency of both mouse and human EC cells, I immediately noted an outgrowth of EC-like cells. These were clearly recognizable as the sought-after pluripotential cells, and they passed every test: They formed teratomas *in vivo,* and they differentiated *in vitro*. They bore the cell surface antigens that we expected. They stained strongly positive for alkaline phosphatase, were karyotypically normal and, most importantly, made splendid chimeras. At first we called them 'ED', for 'embryo-derived', and then 'EK', as a slight change from EC and as our initials (Evans–Kaufman). Gail Martin, who derived similar but slightly abnormal cells a year later, coined the term 'embryonic stem cells' or 'ES', the name that has stuck.

Matt and I submitted our original derivation and characterization of the ES cells to *Nature* early in 1981 and it was published in July[12]. Over the next 3 years we studied details of their establishment and maintenance and ability to form chimeras. Liz Robertson took up the challenge of determining what happened in the derivation of the ES cells from the haploid embryos, and demonstrated that the expected XX chromosome composition of the diploidized cell lines was very unstable, with either loss of one X chromosome producing XO cells or, more unexpectedly, partial deletion of one of the two X chromosomes. These deleted X chromosomes helped Sohaila Rasten to identify the site of X inactivation[13]. Allan Bradley joined me first as a final-year-project student and subsequently as a PhD student. He and Liz were most instrumental in bringing the embryo injection technology to our lab and the resulting proof of the germline capability of these cells, which we were able to report in 1983–1984 (refs. 14,15).

Having proven the germline potential of these cells, I sought to develop techniques for their mutagenesis. Richard Man, Richard Mulligan and David Baltimore published their seminal paper on packaging retroviral vectors in 1983 (ref. 16), and in October 1985 I visited the Whitehead Institute for a month of exclusive uninterrupted bench work in Mulligan's lab. We later used the techniques I had learned there to mutagenize hypoxanthine phosphoribosyltransferase; this was our first specific 'designer mutation' in the mouse[17]. When, during the stay, I received a call from Oliver Smithies, I responded that only for him would I break my work in the lab. His paper demonstrating gene targeting by homologous recombination into an endogenous locus in tissue culture cells had just appeared[18]. I took samples of the ES cell cultures to him and spent a delightful weekend in Wisconsin.

Soon after I returned to my lab in Cambridge, Mario Capecchi came for a week's visit to collect cells and

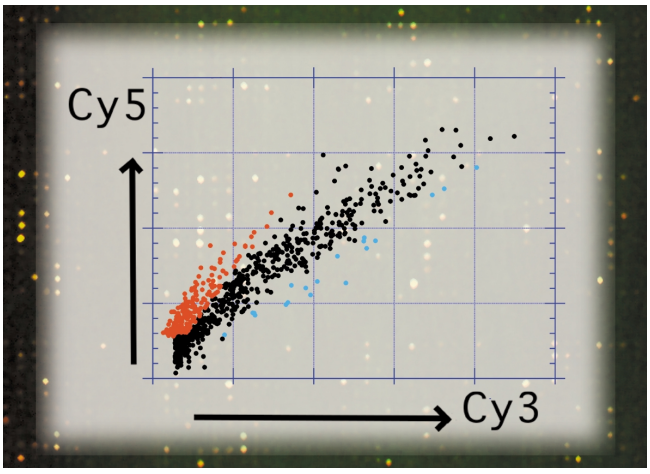


**Fig. 2** Gene expression changes associated with ES cell differentiation. Microarray analysis using the NIA 15k c-DNA set as a probe. Cy3 labelling showing RNA from undifferentiated ES cells. Cy5 labelling showing RNA from cells 18 h into differentiation into embryoid bodies. Loci showing significantly (> 2 sd) increased (red) and decreased (blue) expression. (M.J. Evans, S.M. Hunter, P. Kille and S. Turner, unpublished data.)

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learn the techniques. The rest of this story is better known. Many hundreds of specifically targeted mouse mutations have been made and the technique, although still not trivial, may now merit no more than a few lines' mention in experimental genetics papers. Almost any specific genetic change may now be generated, selected and verified in culture before being converted to the germ lines of mice, and this is the experimental genetics that is illuminating our understanding of the mammalian genome physiology and human function in health and disease.

I set out to derive a 'tractable' system for following mRNA changes coincident with embryonic cell differentiation. ES cells now provide the culture system and, at long last, methods for genome-wide monitoring of mRNA have come of age in cDNA microarrays. I am now putting the two techniques together, and results are beginning to emerge from this work (Fig. 2).

## Forty years with homologous recombination

OLIVER SMITHIES

Toolmakers—and I suspect that the three of us being honored by the Lasker Foundation fit into this category—are fortunate people. They see problems, invent tools to solve them and enjoy the solutions, which often demonstrate new principles that were not part of the original thought. As a bonus, they also enjoy the vicarious pleasure of seeing other people use the same tools to solve very different problems. Yet the invention of an effective scientific tool is rarely an isolated event; there are often many prior experiences that trigger the inventive thought, and there may be various unexpected additional problems to solve before the toolmaker can bring a nascent idea into practice.

The chain of events leading to my contributions to the use of homologous recombination to modify genes in the mouse genome began over 40 years ago as an unplanned consequence of my somewhat serendipitous invention in the 1950s of an earlier tool—high-resolution gel electrophoresis—to solve a completely non-genetic problem. On 26 October 1954, during final pre-publication tests of my starch-gel electrophoresis system (the immediate forerunner of one of molecular biologists' primary tools, polyacrylamide gel electrophoresis), I ran a sample of serum from a female. My notebook (Fig. 1) has the entry that the pattern was "Most odd—many extra components." For about a week I enjoyed the misconception that I had discovered a new way of telling males from females. But this 'sexy' hypothesis soon gave way to the idea that "hereditary factors may determine the serum groups"[19] and, with the help of Norma Ford Walker, who began my education as a geneticist, this was soon proven correct[20]. The field of normal human protein polymorphic variants was seeded!

The hereditary variations we had discovered proved to be in the hemoglobin-binding serum protein haptoglobin, and their details were worked out during a happy collaboration between George E. Connell, Gordon H. Dixon and me in the early 1960s. The haptoglobin alleles *Hp1F* (fast) and *Hp1S* (slow) encoded polypeptides differing by two amino acids, but the third allele, *Hp2,* seemed to be a tandem joining together of sequences from *Hp1F* with sequences from *Hp1S*. The then-chairman of my department at the University of Wisconsin, James F. Crow, on being asked how the *Hp2* allele might have arisen, directed me to the *Bar* locus in *Drosophila* with its fascinating history of repeated 'mutations' resulting from unequal crossing over[21]. This led us to hypothesize that the *Hp2* allele was formed by a unique non-homologous recombinational event that joined the end of *Hp1F* to the beginning of *Hp1S* (ref. 22). *Hp2* therefore contained a small intragenic tandem duplication. The Bar gene in *Drosophila* is also a unique tandem duplication, but it is large enough to be visible when the fly salivary chromosomes are under the microscope. Yet the consequences of the tandemly repeated sequences in *Bar* and in *Hp* are completely comparable. In both cases, subsequent predictable unequal homologous crossing over events occur, which generate a new triplicate product and regenerate the singleton: B–B × B–B leads to B–B–B + B.

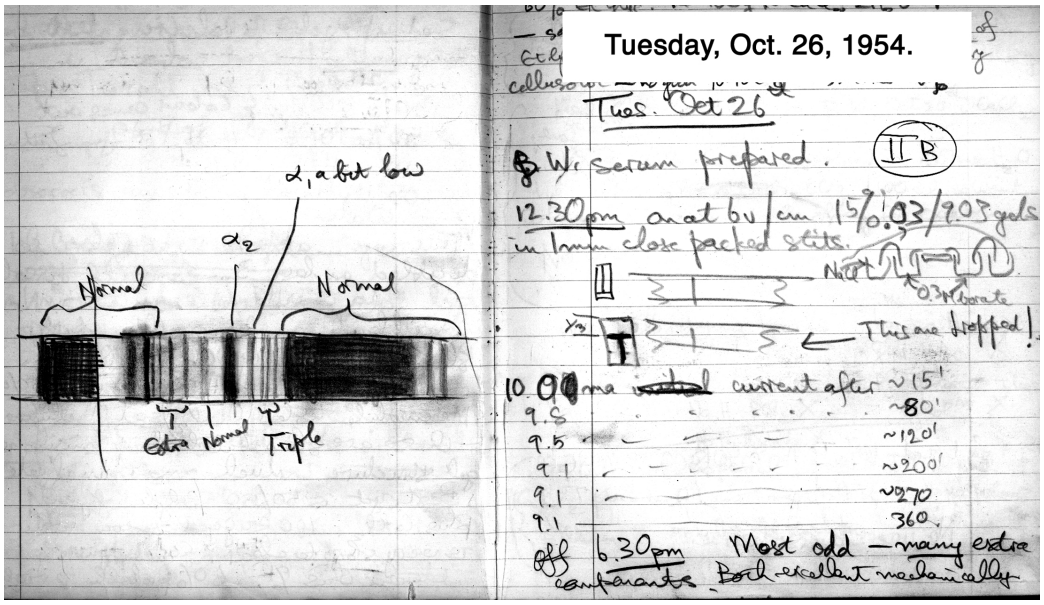


**Fig. 1** Pages 97 & 98 from Smithies' lab notebook "Physical IV", 1954.

I found the predictability of homologous recombination seductive, and enjoyed enormously hypothesizing that antibody variability might be achieved by homologous recombination between tandemly arranged sequences[23]. The hypothesis turned out to be incorrect in mammals, but was remarkably close to being correct in chickens. Homologous recombination reappeared in my experimental science in the early days of cloning human genes when we were determining the nucleotide sequences of the two

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