

TIMELINE

Discovering pluripotency: 30 years of mouse embryonic stem cells

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Abstract | Embryonic stem (ES) cells are pluripotent cells isolated from an early embryo and grown as a cell line in tissue culture. Their discovery came from the conjunction of studies in human pathology, mouse genetics, early mouse embryo development, cell surface immunology and tissue culture. ES cells provided a crucial tool for manipulating mouse embryos to study mouse genetics, development and physiology. They have not only revolutionized experimental mammalian genetics but, with the advent of equivalent human ES cells, have now opened new vistas for regenerative medicine.

The initial zygote of multicellular animals proliferates and differentiates into all the cell lineages of the embryo, fetus and adult. Experimental embryology has shown that the early mouse embryo is highly regulative, and so at early stages of development there must be cells that have the potential to differentiate into a wide range of descendants. What is not necessarily self-evident is that proliferating, self-maintaining populations of pluripotent cells would exist. Evidence for the existence of such pluripotent stem cells comes from studies with mouse teratocarcinomas — transplantable, progressively growing tumours that maintain a wide variety of diversely differentiated tissues within the tumour.

In this article I shall concentrate on the ways in which the ideas and experiments came together to develop the concept that pluripotent cells from mouse teratocarcinomas

were essentially normal early embryonic cells and, following from this, that these cells might be isolated directly into tissue culture from early embryos. From these beginnings has come the practical realization of a form of completely experimental mammalian genetics and the flowering of ideas of stem cell developmental biology. In many ways this is a personal account of the flow of ideas. There are many details left unsaid, and I am not providing full bibliographies nor acknowledgements; these may be obtained from other reviews.

EC cells in mice

In 1967, LeRoy Stevens and Barry Pierce both published reviews that summarized and discussed all of the foundation work of the teratocarcinoma experimental system^{1,2} (TIMELINE). Stevens, who was a geneticist and

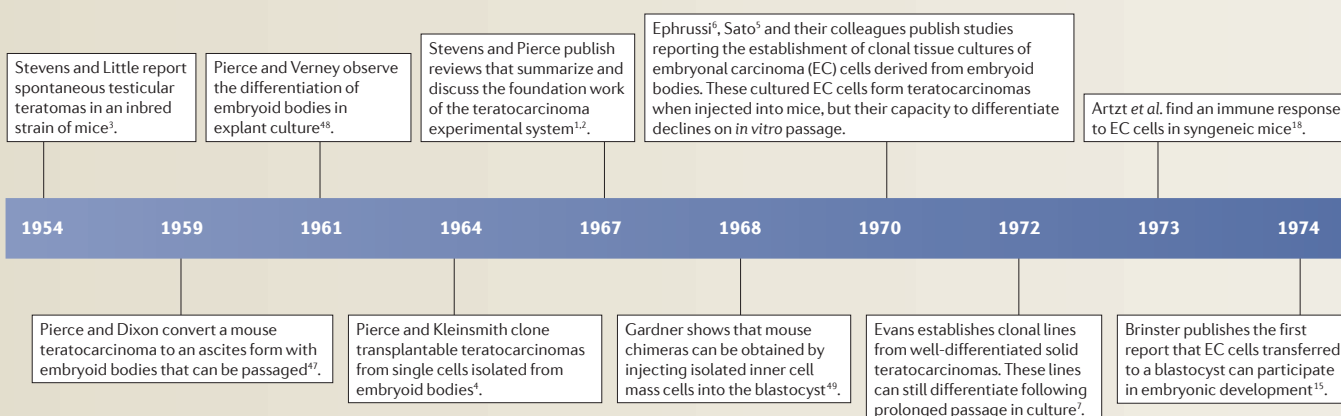
developmental biologist, reviewed his work in which he had established inbred strains of mice with a high incidence of spontaneous testicular teratocarcinomas. He had shown that these tumours were transplantable, and demonstrated their origin from primordial germ cells in fetal testes. He also showed that teratocarcinomas could be experimentally induced by the ectopic transplantation both of geminal ridges containing these primordial germ cells and of early embryos; that is, by the transplantation of sources of pluripotent cells. Prophetically, Stevens and Little, in their 1954 paper³, set the field by saying of ovarian teratomas: "Pluripotential embryonic cells appear to give rise to both rapidly differentiating cells and others which like themselves, remain undifferentiated." (BOX 1). This is the definition of an embryonic stem (ES) cell.

Pierce, who was a pathologist, was principally interested in the contrast between the malignant growth of tumour stem cells and the benign behaviour of their differentiated derivatives. He and his colleagues carried out a long series of experimental studies, including demonstrating that cells from the tumours could grow in tissue culture. Their single most important demonstration, however, was that transplantation of a single cell *in vivo* could result in a teratocarcinoma containing a range of differentiated tissues⁴. This unequivocally established the presence of pluripotent tumour stem cells. These cells were named embryonal carcinoma (EC) cells, following the human nomenclature.

EC cells in culture

The studies above clearly demonstrated an attractive opportunity for the isolation, from the tumour into cell culture, of

Timeline | Highlights in the history of the isolation of pluripotent cells from mice



a developmental system in which the differentiation of pluripotent cells might be studied.

Establishing EC cell cultures. In contrast to the small-scale, inaccessible mammalian embryo, cell culture could promise a tractable, scalable and manipulable experimental system. Two eminent cell biologists, Gordon Sato and Boris Ephrussi, both engaged Ph.D. students to explore the possibilities of cell culture. In 1970, these studies culminated in two publications^{5,6}; both were able to isolate mass cultures of cells from embryoid bodies (see below), to maintain them and to establish clonal cultures. Clonality is very important because, if and when differentiation was observed, it was essential to know that the process was taking place entirely *in vitro* and that it was not the result of pre-committed cell populations. The test for the maintenance of pluripotent cells in culture is to show that a clonal population can form a teratocarcinoma when reinjected into a mouse and that this tumour shows evidence of differentiation into multiple tissues. Early cultures showed maintenance of pluripotency, but the tumours only contained small areas of differentiation, and, on passage, the differentiation ability of these cultured cells diminished severely. The cultures themselves had originally been established from tumour lines that had been extensively passaged *in vivo*, and it was already understood that differentiation ability diminished with prolonged passage.

In May 1969, Stevens very generously sent me stocks of the 129 inbred mouse strain (some mice carrying transplantable teratocarcinomas), which he had derived by

Box 1 | Isolating pluripotent mouse cells: key quotes from historical papers

Stevens and Little, 1954: "We seem to be faced with a paradoxical situation in which neoplastic cells differentiate into normal-type tissue cells. Pluripotent embryonic cells appear to give rise to both rapidly differentiating cells and others which, like themselves, remain undifferentiated." (REF. 3)

Pierce and Dixon, 1959: "The mechanism of this differentiation is not known, but it is presumed to be analogous to morphogenesis of embryos, and we feel that future studies in this area must include application of the techniques and the theories of abnormal embryology." (REF. 47)

Evans, 1972: "The possibility, therefore, of obtaining a strain of cells in tissue culture which may become determined to differentiate in a variety of alternative ways is very attractive." (REF. 7)

Papaioannou *et al.*, 1975: "We have obtained unequivocal normal chimaeras following the [blastocyst] injection of pluripotential cells of two different lines." (REF. 16)

Martin and Evans, 1975: "There are similarities between the process of embryoid body formation and the early events of differentiation of the mouse embryo." (REF. 9)

Evans, 1981: "Malignant teratocarcinoma stem cells spontaneously differentiate into benign cell types and normal embryos, or primordial germ cells, are able to initiate teratocarcinoma formation at a relatively high frequency. Is it reasonable to regard this process as a malignant transformation, and cellular differentiation as a spontaneous reversion from malignancy? One alternative the teratocarcinoma stem cell is essentially a cell showing a completely normal embryonic phenotype." (REF. 13)

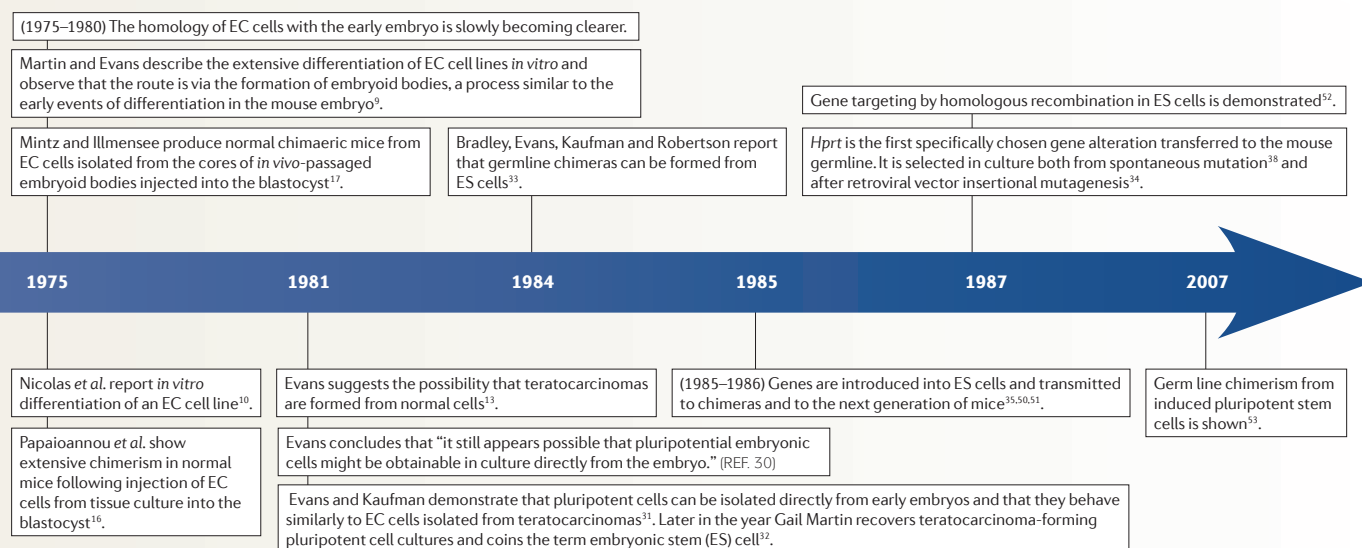
Evans and Kaufman, 1981: "We have demonstrated here that it is possible to isolate pluripotential cells directly from early embryos and that they behave in a manner equivalent to EC cells isolated from teratocarcinomas." (REF. 31)

Bradley *et al.*, 1984: "Clearly, embryo-derived stem cells seem to be particularly efficient at recolonizing the early embryo. This feature, together with the availability of XY lines such as those described here, now allows the routine construction of chimaeric males which are capable of transmitting culture-derived genomes to a potentially limitless number of offspring, and confirms our previous contention of the normality of the genome in these stem cell lines." (REF. 33)

the ectopic transplantation of early embryos. The histology of these transplantable teratocarcinomas showed them to be composed largely of well-differentiated tissues. Indeed, they were so well differentiated that it was difficult to find the stem cells of the tumour — the EC cells — in the histology sections. To facilitate cloning these into tissue culture I made the fortuitous choice to use irradiated chick fibroblasts as a feeder layer,

and was immediately successful in isolating clones of small, piling, rapidly growing cells, from which I was able to establish mass cultures. Upon reinjection to a subcutaneous site in the 129 inbred mice, these cultures, and their clonal descendants, gave rise to well-differentiated teratocarcinomas⁷.

Once the cultures were well established, the addition of the irradiated chick fibroblast feeder cells was discontinued. It was



clear, however, that they were still mixed cultures of small, actively growing clump-forming cells (C cells) and of other flattening, more epithelioid cells termed E cells; this formed a balanced culture system. The E cells were contact inhibited and stopped growing on confluence, but the C cell colonies continued to grow. Thus, at passage there were ten times or more C cells than E cells, but on re-plating the C cells were severely diminished. Re-cloning by dilution did little to resolve this issue of a mixed culture. Both cell types had a near-normal karyotype, although the E cells rapidly became aneuploid when cloned out of the mixture. It was hypothesized (retrospectively, probably correctly) that the E cells were derived by differentiation from the C cells. But, this wasn't a simple transition; essentially the C cells could survive only in mixed cultures⁸.

Differentiating EC cells in culture. Gail Martin resolved to explore whether E cells were derived by the differentiation of C cells by using, for the feeder layer, an inactivated 3T3-like cell line that was negative for hypoxanthine-guanine phosphoribosyl-transferase (HPRT; also known as HGPRT) (known as STO cells). By doing so, she was able to distinguish the wild-type teratoma-derived cells from the feeder cells by their incorporation of radiolabelled hypoxanthine. She also verified the cloning by manually isolating single cells. In this way she obtained pure populations of C cells, the EC cells maintained by passage onto new feeder layers. When the feeder cells were removed, the culture stopped growing well, and there was extensive cell death; after a crisis and upon refeeding, however, layers of differentiated cells appeared. Most dramatically, these included spontaneously beating cardiac myocytes and long nerve processes⁹. A similar observation was reported by Jean-François Nicolas and colleagues¹⁰. At the same time, we observed that isolated colonies of EC cells, originally established on feeder layers, became differentiated when they were re-fed *in situ* so that the feeder cells died off. This second technique, which did not involve extensive cell death, led to magnificent differentiation; histological sections of the cultures showed tissue organization not dissimilar to that found in well-differentiated teratomas¹¹.

Studying EC cells in culture

Clearly the mechanisms by which differentiation was initiated were now open to direct observation in tissue culture.

Differentiation occurs through an embryo-like route. A major breakthrough in our understanding of EC cell differentiation was the observation that, in the differentiating mass cultures, recognizable 'simple' embryoid bodies were formed. Embryoid bodies had been recognized previously as the form in which teratocarcinomas could grow as an ascites. They had been observed in two forms: as simple embryoid bodies, comprising a core of EC cells surrounded by a shell of primary extra-embryonic endodermal cells; and in a cystic form, which contained a more complex mixture of early embryonic cell types. The primary extra-embryonic endoderm cells secrete a hypertrophied basement membrane (Reichert's membrane), which separates them from the inner EC cells.

EC cell cultures from tumour cell lines that, over prolonged passage, have become incapable of differentiation (nullipotent) are unable to form embryoid bodies. Some EC cultures that give rise to relatively poorly differentiating tumours on reinjection into mice and do not differentiate extensively *in vitro* can form only simple embryoid bodies. The embryoid bodies formed by normal, well-differentiating EC cell cultures progress to form cystic embryoid bodies *in vitro*. Thus, the first step of differentiation of EC cells is into primary extra-embryonic endoderm. The differentiation seen in the mass cultures is the result of the formation of embryoid bodies and the subsequent reattachment to the tissue culture surface. Extremely good differentiation may be obtained by deliberately forming simple embryoid bodies and then allowing them to reattach to the plastic surface. The first-appearing differentiated cell types seen during the differentiation of attached colonies were also invariably extra-embryonic endoderm.

Richard Gardner brought us the information, concomitantly shown by Janet Rossant¹², that an isolated inner cell mass (ICM) from a mouse blastocyst formed a complete outer layer of extra-embryonic endoderm. This revealed that the first steps of differentiation of EC cells were exactly the same as those undertaken by the ICM of a normal mouse embryo (FIG. 1). This was the basis of a very important insight (see below).

Are EC cells normal early embryo cells?

Until these insights were obtained, EC cells, which had been derived from malignant tumours (highly malignant in the case of the human testicular tumours), were

considered to be grossly abnormal cells that could become entrained into more normal behaviour by going through the process of differentiation (as the differentiated cells were non-malignant). We now saw that the EC cells were behaving as normal early embryonic cells; the onset of differentiation was not random, stochastic or malignant, but followed the normal pathways of development. This was part of the beginning of the realization that these cells, which had hitherto been regarded as abnormal cancer cells with the peculiar property of being able to differentiate, were essentially early embryonic cells out of context¹³. The realization that EC cells are homologous to normal early embryonic cells was also reached through findings from experimental embryological studies of early mouse development, the use of cell surface antibody markers of cell lineage and, dramatically, the finding that EC cells could incorporate into a mouse blastocyst and take part in the development of the whole animal as a chimera.

EC cells can make chimaeric mice. The establishment of restrictions of cell fate and of cell lineages in the early mouse embryo was being actively explored in the early 1970s and gave us insights into the existence of a population of cells in the ICM that had the potential to form the entire animal. One central experimental platform was the ability to form chimaeric embryos by transferring cells isolated from the ICM into the blastocoel cavity of a carrier embryo. An excellent retrospective review is provided by Richard Gardner¹⁴, with whom I initiated such experiments with cultured EC cells. The previous year, in 1974, Ralph Brinster¹⁵ had reported evidence that EC cells isolated from embryoid bodies *in vivo* might be able to colonize a mouse embryo, but he had very low chimerism and little in the way of definitive markers. We obtained some magnificent heavily chimaeric mice, most tissues of which proved to have substantial contributions from the injected tissue-cultured EC cells¹⁶. Beatrice Mintz and Karl Illmensee¹⁷ also reported good normal chimeras from EC cells taken from the cores of embryoid bodies cultured *in vivo*. Some chimaeric mice bore teratocarcinomas at birth and others developed tumours of a differentiated cell type later in life. None of the chimaeric mice made with cell culture lines proved to be germline chimaeric, but this was not surprising, as the EC cells (after tumour and culture passage) were not karyotypically normal.

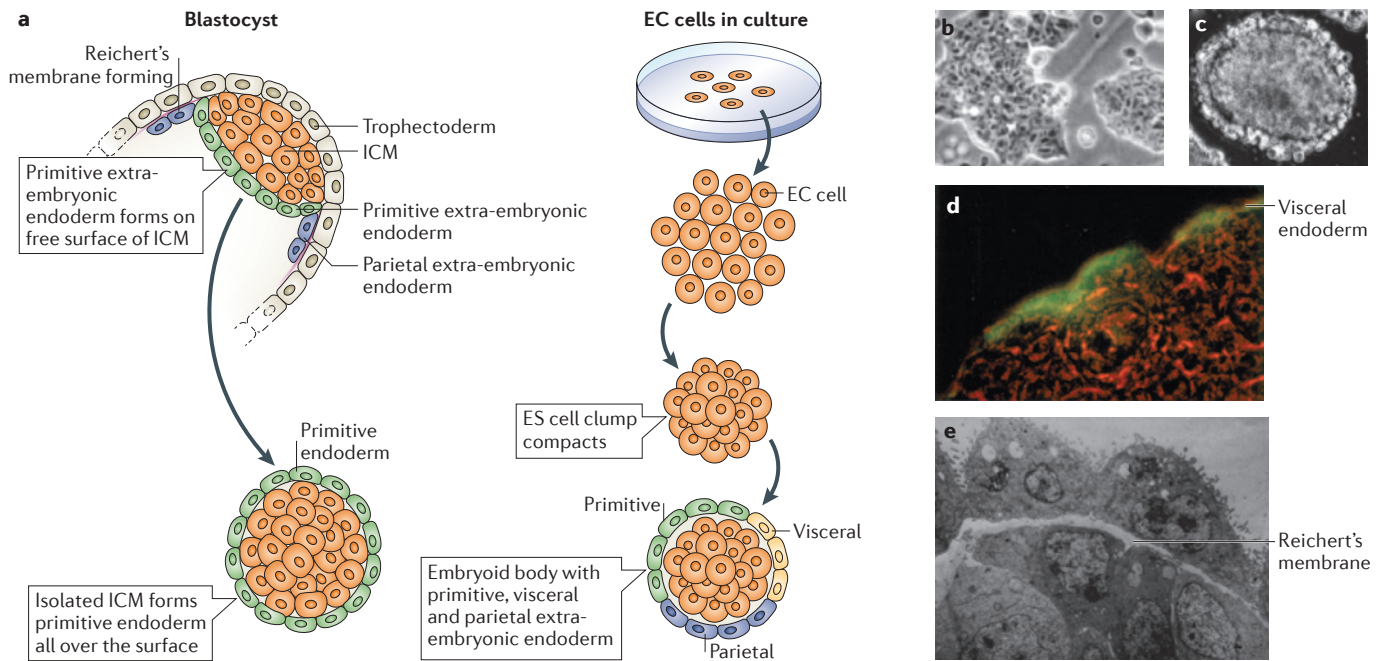


Figure 1 | Similarity between the differentiation of the embryo inner cell mass and a cluster of EC cells. a | A layer of cells delaminates from the inner cell mass (ICM) of the blastocyst. As the descendants of these cells will contribute only to the extra-embryonic tissues, they are termed the primitive extra-embryonic endoderm. Perhaps a better terminology borrowed from the chick embryo would be 'hypoblast'. These cells migrate around the inside of the trophoblast, where they make a hypertrophied basement membrane known as Reichert's membrane. These cells are the extra-embryonic parietal endoderm. Other cells from the lineage of the hypoblast will form part of the yolk sac; these are secretory and make

large amounts of α -fetoprotein (the extra-embryonic visceral endoderm). An ICM isolated from a blastocyst initially forms primitive endoderm all over the surface¹². Extra-embryonic endoderm of all types (primitive, visceral and parietal) is found in embryoid bodies made from embryonal carcinoma (EC) cells, highlighting the similarity of EC cell differentiation to that of cells of the ICM. **b** | Image of EC cells in culture. **c** | Image of an embryo. **d** | Immunofluorescent detection of α -fetoprotein (green) in the visceral endoderm of an embryoid body. **e** | Electron micrograph of the edge of an embryoid body. Image in part **e** is reproduced, with permission, from REF. 9.

Markers of EC cell lineage. Sometime in early 1973, Salvador Luria visited my laboratory, bringing the news that work in the Pasteur laboratory had found that it was possible to raise an immune reaction to EC cells in syngeneic mice¹⁸. These studies had used F9, a strain of EC cells that showed little or no differentiation. Luria proposed that we immunize our mice with our strain of EC cells, which showed extensive differentiation *in vivo*. This might change the differentiation seen *in vivo* and allow an immune dissection of the pathways of differentiation. Mice immunized with irradiated EC cells did indeed produce sera that reacted strongly against the EC cell surface, but upon challenge with live EC cells, these mice readily produced well-differentiated tumours that were identical to those observed in control animals. Although the original hypothesis was unproductive, the antiserum (which we termed anti-C) obtained from the syngeneic immunized mice proved to be a very valuable reagent. It was an immunoglobulin M (IgM) and could be used as a cell surface immunofluorescent labelling reagent. The syngeneic anti-F9 antiserum was mainly used in assays

using adsorption followed by immunocytotoxicity, and hence it could not be used to study the very limited number of cells available in early embryos. Davor Solter and Barbara Knowles¹⁹ used a mouse immunized with F9 cells to prepare a monoclonal antibody with reactivity equivalent to the anti-C and anti-F9. This antibody, called stage-specific embryonic antigen 1 (SSEA1), has subsequently proved to be a very useful reagent and was eventually shown to be specific for an epitope of the cell surface carbohydrate glycohalix²⁰. Furthermore, Ten Feizi and colleagues²¹ had characterized a series of human naturally occurring monoclonal autoantibodies, the I and i series, and Peter Stern and colleagues^{22,23} introduced anti-THY1 and Forssman antigens. The main message from these studies was that EC cells have a cell surface phenotype that matches early embryonic cells as well as germ cells²⁴. In the embryo, the cell surface phenotype of EC cells matches ICM cells and early embryonic ectoderm.

ES cells. The homology of EC cells with early embryonic cells was becoming clear by 1975 and, in the following years, both

biochemical and, in particular, cell-surface antigenic markers continued to confirm this^{23,25–29}. By the early 1980s I was able to suggest that teratocarcinomas are formed from normal cells¹³ and also, in reviewing the situation, conclude that it was extremely likely that it would become possible to culture pluripotent cells directly from embryos, without an intervening tumour stage³⁰.

In 1981 we reported the establishment of such cells in culture. Matt Kaufman and I³¹ showed that it was possible to isolate pluripotent cell cultures directly from delayed blastocysts. These cultures proved to have all the properties previously established for EC cell cultures, as well as a completely normal karyotype. Later that year Gail Martin³² reported a technique that used conditioned medium and enabled her to recover teratocarcinoma-forming pluripotent cell cultures. She coined the term ES cells.

ES cells and genetic manipulation

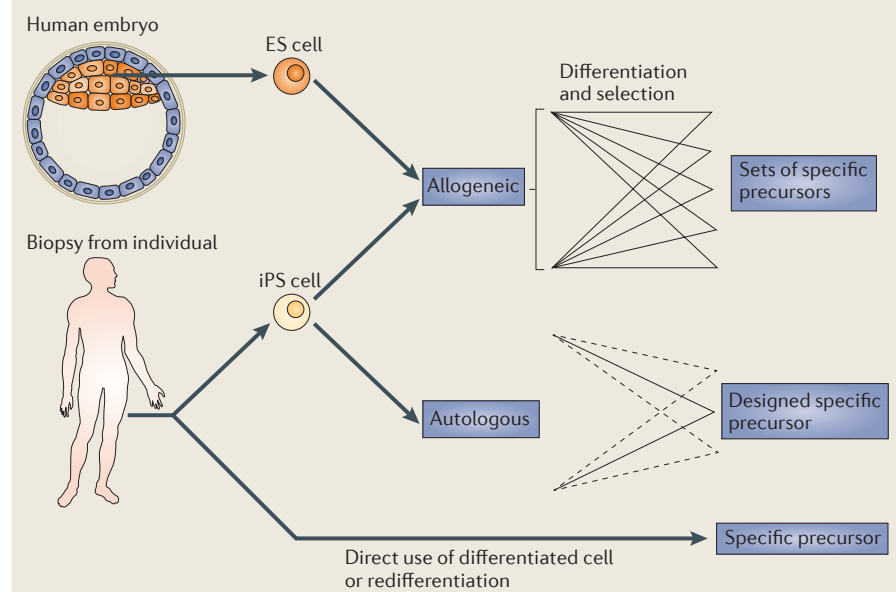
Because of all the antecedent work with the culture of mouse EC cells, the expected properties of directly isolated mouse

Box 2 | Fountain of eternal youth?

Unlike most differentiated cell types, embryonic stem (ES) cells under appropriate tissue culture conditions will proliferate indefinitely and remain normal with an uncompromized karyotype. There is no senescence or shortening of telomeres, and they maintain their pluripotency. They can also give rise to all cell lineages. Now, with the advent of human ES cells, this raises the possibility that such stem cells might be the perfect source for differentiation into specific progenitors for regenerative medicine tissue repair — if they were available for all patients and if appropriate protocols for their differentiation were available.

The problem of sourcing histocompatible cells for transplant had been approached by the idea of banks of embryo-derived cells, which would provide an acceptable degree of tissue match for use as allogeneic grafts for most patients, but the generation of induced pluripotent (iPS) stem cells changes this (see the figure; dashed lines indicate possible routes of differentiation; routes indicated by solid lines are taken). Human pluripotent cells may now be derived in tissue culture not only from the use of limited and ethically challenged human embryos, but from the virtually unlimited source of donated adult cells. The possibility of using large and well-chosen banks of cultures as an allogeneic resource remains. They could be pre-prepared, well characterized and validated. For most purposes, however, autologous treatment with cells derived from the individual patient would seem to be ultimately the best treatment. Is this technically and logistically feasible? It may not be feasible for immediate treatment of acute conditions when time is of the essence; however, many of the targets considered for regenerative medicine by cell transplantation are chronic conditions, in which speed is less important than long-term effectiveness and lack of side effects. In such cases, autologous therapy is the ideal.

How will the appropriate therapeutic tissue-specific precursors be isolated? *In vitro* cell differentiation may be enhanced or channelled by specific growth conditions and the addition of induction factors to a culture from which the desired population may be purified. Alternatively, direct artificial manipulation of cell fate may be possible by, for instance, exogenously introduced transcription factors. This is a technology in its infancy.



pluripotent stem cells were well understood and characterized. These were all rapidly verified, including, importantly, the ability of ES cells to make chimeras in the context of a carrier mouse embryo. Liz Robertson, Allan Bradley and colleagues^{33–35} showed that chimeras made with karyotypically normal ES cell lines chimerized not only the soma but also the germline of the resulting mice, hence giving a route to genetic manipulation from culture to creature.

During normal early development there may be no more than a couple of dozen ES cells, and they are constrained in the normal time course of development and present for only a short time. By contrast, many millions of ES cells may be maintained in tissue culture indefinitely, with all of them retaining their pluripotency. This means that clones bearing a rare genetic change may be identified and used to pass that change into the mouse germline, and hence the mouse genome. These

cells are therefore a vector to a form of experimental mammalian genetics³⁶.

There are essentially two approaches for obtaining specific mutations in ES cells: random mutagenesis and selection or screening; and targeted mutagenesis.

Random mutagenesis and selection or screening. ES cells in culture are amenable to most forms of mutagenesis, so the screen is pivotal. In the late 1980s, when we were first able to use ES cells to transfer a mutation from culture to the mouse germline, sequence information for the mouse genome was very limited, and many loci were unidentified. When a mutation is being induced randomly into as-yet-unidentified loci, it is useful to ensure that these are marked, and for this reason methods of insertional mutagenesis have tended to be favoured. Retroviral vectors were our vehicle of choice because of their efficient transfection and clean integration, leading to a readily identifiable mutation³⁷.

With the notable exception of *Hprt*^{34,38}, an interesting mutation was not readily selected *in vitro*, and so screening of the resultant progeny after intercrossing was required. This was a lengthy process that did, however, result in the identification of some interesting loci, for example *Nodal*^{39,40}. The random insertional mutagenesis approach was greatly strengthened by the introduction of gene trapping, achieved by transfection of ES cells using vectors with reporters that would be transcribed only when integrated into a suitable site in the genome (reviewed in REF. 41). This allowed a screen not only of mutagenic effects (most usually in homozygote offspring), but also of developmental and tissue-specific expression *in vitro*, in the immediate chimera and the heterozygote offspring. Integration using a retroviral vector has the apparent disadvantage that the trapping construct is surrounded by long terminal repeats. However, the internal promoter and enhancer may be removed, and the interference of the retroviral splice acceptor may be obviated by using a splice acceptor as the trapping element that is placed in reverse orientation⁴². This brought the advantages of retroviral transfection to insertional mutation gene trapping.

Gene targeting by homologous recombination. The alternative approach of generating specific mutations in ES cells is directed mutagenesis, a method that was pioneered by both Oliver Smithies⁴³

and Mario Capecchi⁴⁴. They showed that a DNA construct containing substantial homology with a chromosomal sequence could recombine into the endogenous sequence at relatively high efficiency when introduced into cells in tissue culture. With the advent of full knowledge of the mouse genome, this method of gene targeting by homologous recombination has become the choice for gene inactivation. Even when a specific point mutation or random point mutagenesis limited to a particular locus is desired, it is more effective to recurrently target a marked locus with mutagenized vector than hitting the whole genome⁴⁵.

Homologous recombination works well with ES cells and it is limited only by the need to culture the cells in conditions in which they retain their full pluripotency and ability to form mice with chimaeric germ lines. The design of the targeting vector and the screening method to find correctly targeted clones are the main considerations. In addition to simple mutations, methods have been developed that allow both spatial and temporal control of gene deletion or function (reviewed in REF. 46). All these studies depend on the combination of *in vitro* cell genetic manipulation and selection, coupled with true *in vivo* observation of the physiological consequences in the context of the whole animal. This has been made possible through the culture of ES cells. It is important to note that virtually any desired designer change may be made by using the technique of homologous recombination gene targeting, ranging from single point mutations to large chromosomal alterations. All of these techniques are applied to mice, and this has provided the experimental genetic approach to mammalian genetics, illuminating our understanding of the human genome data.

Conclusion

Pluripotent cells have established at least two platforms for research: an *in vitro* system of cell differentiation that is equivalent to that in the early embryo, and a vector-based system to experimental mammalian genetics *in vivo*. These depend on the ability of pluripotent cells to differentiate as in an embryo to all types of stably differentiated cell. The ability to experimentally manipulate the differentiative state of cells, and revert a terminally differentiated cell to pluripotency with germline potential⁴², now closes the developmental loop (BOX 2).

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Competing interests statement

The author declares [competing financial interests](#): see web version for details.

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