Series

What history tells us
VII.
Twenty-five years ago: the production of mouse embryonic stem cells

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1. Introduction

Much research and lively debate focuses on the potential value of embryonic stem (ES) cells for regenerative medicine, the ethical issues raised by the use of human embryos to generate them, and the efforts presently made to circumvent this problem.

The preparation of human ES cells in 1998 (Thomson *et al* 1998), following on from that of ES cells from a rhesus monkey three years before (Thomson *et al* 1995), was the extension to primates of experiments done in rodents, in 1981. The origins of these scientific developments have recently been revisited by some of the main contributors to this field (Edwards 2001; Solter 2006). However, these historical descriptions leave some questions unanswered. Why were two decades necessary to adapt a technology developed in mice to humans? Were there ethical obstacles? If so, the comments that accompanied the production of human ES cells in 1998 make it obvious that they had not been solved to the satisfaction of all (Miller and Bloom 1998). Were there technical difficulties? It is hard to see how, when one considers that the tricks used in 1998 – addition of specific factors, need for feeder layers of cells – were the same as those used on mouse embryos. In any case, such a delay would not have been necessary if sufficient effort had been focused on overcoming the difficulties. The mere chronology shows that the production of human ES cells has not been a natural follow-up to the experiments done on mice.

This impression is strengthened after scrutinizing the two articles published in 1981 (Evans and Kaufman 1981; Martin 1981), and the comment on Evans and Kaufman's results by Brigid Hogan in *Nature* (Hogan 1981). No mention is made, either of any possible extension to humans, or of any medical use of these cells. The interest is seen at the fundamental level. These ES cells are a perfect *ex vivo* system to study differentiation in higher organisms, and in particular in mammals. Through the possibility of generating mosaic animals by injection of these cells into the cavity of the blastocyst, it will be possible to test the effect of mutations generated *ex vivo*. ES cells are an improved form of one category of cells that have been already used to study development – the embryonal carcinoma (EC) cells. As EC cells, they produce tumours when injected into recipient animals, and could therefore also help to disentangle the complex, antagonistic relations between differentiation and oncogenesis.

My aim here is to recall the long and tortuous path which led from observations on a specific – and rare – kind of tumour, a teratoma, to the development of human ES cells. The objective is not only to revisit a neglected chapter of the history of modern biology. It is also to explain why it took so long to recognize the value of ES cells for the future of medicine. I hope too that it will help to temper the enthusiasm surrounding present research, and show that some of the difficulties encountered today (Vogel 2000) were already familiar to biologists twenty years ago.

2. From the characterization of teratomas to the preparation of ES cells

Teratomas are spontaneous tumours of the testes and ovaries. They are characterized by the heterogeneity of the cells forming them. A distinction has later been made between different types of tumours: teratomas designate benign tumours, uniquely formed of variously differentiated cells and tissues; the more malignant teratocarcinomas contain both differentiated cells and small undifferentiated ones; and aggressive embryonal carcinomas only contain rapidly proliferating undifferentiated (EC) cells. As for all tumours, their occurrence was explained by Cohnheim in 1875 as the result of the anarchic development of early embryonic cells left behind during development. However, their localization in the genital organs supported an alternative hypothesis: they were the result of an anarchic multiplication of the

primordial germ cells. The observation by Leroy Stevens of the high frequency (1%) of teratomas in the 129 mouse strain permitted the development of work hitherto limited by the scarcity and diversity of tumours observed in humans (Stevens and Little 1954).

Further studies on the 129 mouse strain initially favoured the alternative hypothesis. The natural tumours were shown to have their origin within the seminiferous tubules (Stevens 1959, 1962; Pierce and Beals 1964); besides, it was possible to generate teratomas by grafting 12.5 to 15.5-day embryos (or only genital ridges) at ectopic positions in syngenic animals. Together, the findings suggested that tumours result from the anarchic development of germ cells at a specific phase of their development. An apparently decisive argument in favour of this hypothesis was the absence of tumours when the embryos used for the grafts bore a mutation preventing the formation of germ cells (Stevens 1967a).

Leroy Stevens therefore considered that, during their oncogenic development, these germ cells only mimicked what happened during normal development in the mouse (Stevens 1967b). But additional experiments progressively showed that the formation of these tumours had more in common with the development of the embryo as such, thereby confirming early intuitions (Peyron 1939). When transplanted into peritonea, teratocarcinoma cells generate floating structures called embryoid bodies in which the organization of endodermic and ectodermic cells is identical to that observed in the embryos. Most of all, teratocarcinomas utterly indistinguishable from those previously described could be directly produced by transfer of embryos at the late blastocyst stage, i.e. day 5.5 of development, to extra-uterine sites in syngenic mice (Stevens 1970).

The value of the teratocarcinoma system for studying the early steps of embryogenesis was progressively demonstrated by embryologists like Boris Ephrussi (Kahan and Ephrussi 1970), and molecular biologists who had turned to embryology, for example François Jacob (Jacob 1977). As argued by Jacob and Monod in 1963 (Jacob and Monod 1963), the study of gene regulation in microorganisms had been made possible by the conjunction of genetic and biochemical studies on *Escherichia coli*. The study of the mechanisms of regulation occurring during early development in mammals had hitherto been impossible due to the scarcity of material, and the impossibility of experimental access to the early post-implantation embryo. What was required was the production of embryonic cell lines exhibiting the characteristics of cells at different stages of their differentiation; the possibility of observing the differentiation of multipotential embryonic cells *ex vivo;* the capacity to alter the genetic characteristics of these primitive cells *ex vivo*; and the ability to re-inject them usefully into normal embryos. Teratocarcinomas provided exactly that. A clear distinction between the undifferentiated EC cells and

the differentiated derivatives was established. EC cells were shown to be multipotential (Kleinsmith and Pierce 1964). They could be maintained in an undifferentiated state by culture *ex vivo*, or by transplantation into the animal. Despite the number of passages, most of them conserved their multipotentiality and remained able to generate derivatives of the three primordial germ layers (Stevens 1958). Their karyotype apparently remained unaltered during these passages. But they could also be differentiated *ex vivo*. More and more studies were done, and the morphological, immunological and biochemical resemblance between these EC cells and the cells forming the inner cell mass of late blastocysts became increasingly obvious (Jacob 1977; Martin *et al* 1978). It led to the discovery that the same protein controlled the crucial interactions taking place between cells of the early embryo at the morula-blastocyst stage, and cell-cell contacts in EC cell cultures (Hyafil *et al* 1980). The developments prompted Ralph Brinster (Brinster 1974), Beatrice Mintz and Karl Illmensee (Mintz and Illmensee 1975; Illmensee and Mintz 1976) and Papaioannou, McBurney, Gardner and Evans (Papaioannou *et al* 1975) to inject these cells into blastocysts. This technique had previously been used to produce chimaeric animals by injecting cells from a blastocyst into another one (Gardner 1968). After the reimplantation of these blastocysts into the uterus of surrogate mothers, mosaic animals were obtained, with different tissues derived from the EC cells.

The experiments of 1981 – consisting in the direct generation of embryonic stem cells from blastocysts *ex vivo* – were therefore a further confirmation of the similarity between EC cells and early embryonic stem cells, and, considering the previous unsuccessful attempts, an achievement (Sherman 1975; Edwards 2001). The limited degree of mosaicism reached with EC cells, and the difficulty of obtaining transmission through the germinal line, were considered as limits probably resulting from modifications of the EC cells during the long time they had spent out of the organism. These obstacles were overcome only in 1981 by Beatrice Mintz with a teratocarcinoma cell line recently established in her laboratory (Stewart and Mintz 1981). One could hope to overcome these difficulties with the newly obtained ES cells. In addition to their embryonic character, these ES cells were also oncogenic. The capacity, when injected into adult animals, to form teratocarcinomas indistinguishable from the teratocarcinomas obtained by injection of EC cells was considered, by those who produced them, as one of the important criteria of the embryonic nature of ES cells.

3.   EC cells and cancer

On this long journey from teratocarcinoma to ES cells, the inverse relation between the differentiated character

of a tumour and its malignancy was more and more firmly established: EC cells were tumorigenic, whereas the differentiated cells derived from them were not. Some considered that the undifferentiated embryonic cell was not oncogenic *per se*, and that an epigenetic modification was necessary for the transition to the transformed phenotype. Even if one adopts this point of view, it was obvious that this transition was much easier for this embryonic cell than for any differentiated one. One should remember that the "oncogenic paradigm" – the hypothesis that cancer results from somatic mutations in a limited number of genes, oncogenes and tumour-suppressor genes, only emerged at the beginning of the 1980s. Before that, although the hypothesis of somatic mutations in cancer had already been around for decades, the dominant model was that cancer was a problem of deregulation, of de-differentiation of cells. This hypothesis was in full agreement with the observation that cancerous cells frequently exhibit characteristics normally absent from the adult, but present in the embryo. This antagonistic relation between cancer and differentiation was evident for those involved in the study of teratocarcinomas. For François Jacob (Jacob 1978) and Gail Martin (Martin 1975, 1980), it provided an additional interest in the system; for researchers like Barry Pierce, it was its main interest, the possibility of firmly grounding a "model for a developmental concept of cancer" (Pierce 1967), with major potential therapeutic applications, since this hypothesis suggested that cancer could be 'reversed' (Braun 1965).

4.   From mouse embryonic stem cells to human embryonic stem cells

The scientific context in which they were developed explains why ES cells were extensively used in the exploration of early mammalian development. Of two possibilities – a biochemical study of the differentiation process *ex vivo*, and the introduction of *ex vivo* generated mutations into animals, it was the second that was favoured. ES cells were first seen as another way to generate transgenic animals. But a new possibility rapidly emerged when the first developmental genes of mammals – the homeobox-containing genes – were isolated by analogy with *Drosophila* genes, three years after the production of mouse ES cells. In the absence of natural mutations, the function of these genes in mammalian development had to be demonstrated. The path to be followed became obvious: introduce non-functional copies of these genes in ES cells, select the rare ES cells in which the process of homologous recombination had been successful, create mosaic animals by injection of these modified ES cells into blastocysts, and at the next generations obtain heterozygous and homozygous animals derived from the manipulated ES cells. Decisive progress was made by Mario

Capecchi to select the rare recombinant ES cells (Mansour *et al* 1988; Capecchi 1989). The production of knock-out, and then knock-in, animals became the major application of ES cells.

The value of obtaining human ES cells was therefore initially seen as opening the door to germinal gene therapy through homologous recombination: the possibility of replacing a non-functional copy of a gene by a functional one. The possibility of using these cells to regenerate diseased tissues came some years later, in a very different context. The rapid development of transplantations – thanks to the use of efficient immunosuppressive drugs like cyclosporin – was rapidly limited by the number of donors. Simultaneously, the spectacular attempts to replace the traditional pharmacological treatment of Parkinson's disease by the grafting of cells extracted from human aborted embryos led to the idea that any human disease in which the symptoms come from a massive cell death could be 'cured' in the same way. The 1997 demonstration (Wilmut *et al* 1997) that the nucleus of the fertilized egg could be replaced by the nucleus of a somatic cell, which is called cloning, opened the way to the generation of ES cells with the same MHC characteristics as those of any adult organism (thereby avoiding any form of immunological rejection) (Solter 1998). The rapid investment of biotech companies in such work, triggered by the difficulties and disillusions of the first gene therapies, explains the abrupt shift of interest toward the therapeutic use of ES cells at the end of the 1990s. The major part of the work was done at Geron, a biotechnological firm already famous for its 1998 preparation of immortal human cells by expression of the telomerase gene (Bodnar *et al* 1998). Human ES cells were obtained from primordial germ cells nearly simultaneously by another group, confirming the observations made more than thirty years before by Stevens (Shamblott *et al* 1998).

5.   ES cell therapy and cancer

A new generation of scientists was responsible for preparing human ES cells. This generation had at its disposal a wealth of information – on controlling genes and signaling pathways – that the previous generation lacked. This is not a sufficient excuse for the partial oblivion of the numerous observations made by their predecessors on EC and ES cells, showing how difficult the manipulation and control of these cells was. In particular, the close relation between the undifferentiated characteristics of these cells and their propensity to generate tumours was forgotten. What encouraged this amnesia was the development of the oncogene paradigm. Since the development of cancer is considered to be the result of somatic mutations, combined with germinal mutations in the case of familial forms, the previous observations

on the oncogenic potential of EC cells were immediately interpreted as resulting from the accumulation of mutations in these cells during the *in vitro* cultivation process, parallel to the aneuploidy of many EC cell lines.

But this explanation is clearly insufficient. The karyotype of EC cells is no more abnormal than the karyotype of many other cell lines, which do not have the same oncogenic potential. Quite the opposite; the first description of EC cell lines insisted on the stability of their karyotype! And the first mouse ES cells were shown to be no more or less tumorigenic than the EC cell lines. The oncogenic character of these cells was progressively rediscovered in the numerous studies that were initiated after the production of human ES cells (Björklund *et al* 2002). From a practical point of view, this has progressively emerged as one – if not the major – obstacle which will have to be overcome if these cells are to be used for human therapies. Since the oncogenic potential is linked with the undifferentiated state, a solution consists in the elimination of these undifferentiated cells from the cells which will be injected to the patients – for instance by the use of specific antibodies. The design of a safe protocol will surely require a lot of additional works and careful controls.

6. Conclusion

Historical studies are frequently viewed as a luxury reserved for well-established scientists when they reach the end of a brilliant career. I consider that they can have two additional very useful functions. The first is to help to discern the permanent transformation in science of 'objects' and objectives. Human ES cells are not the human equivalent of mouse ES cells if one considers the motivations and goals attendant upon their creation. The road from mouse EC cells to human ES cells is far from straight – which explains the long delay needed to go from mouse ES cells to human ES cells. A scientific discovery has no value *per se*. It acquires this value and interest in a particular scientific and general context. Even if each discovery can be considered as a stone in the construction of the huge building of scientific knowledge, the form and characteristics of these stones permanently evolve. The second function of the history of science is, eventually, to learn lessons and retrieve information from the past. The difficulty of manipulating mouse EC and ES cells and the high capacity of these cells to generate tumours, were observations familiar to the small community of researchers working on EC and ES cells in the 1970s and at the beginning of the 1980s. Maybe reminiscences about these studies can be of some help in moderating sensational and premature announcements, which allow funds to be raised for research, but which also generate difficulties and dramatically alter the lay public's perception of scientific research.

References

Björklund L M, Sanchez-Pernaute R, Chung S *et al* 2002 Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model; *Proc. Natl. Acad. Sci. USA* **99** 2344–2349

Bodnar A G, Ouelette M, Frolkis M *et al* 1998 Extension of life-span by introduction of telomerase into normal human cells; *Science* **279** 349–352

Braun A C 1965 The reversal of tumor growth; *Sci. Am.* **213** 75–83

Brinster R L 1974 The effect of cells transferred into the mouse blastocyst on subsequent development; *J. Exp. Med.* **140** 1049–1056

Capecchi M R 1989 Altering the genome by homologous recombination; *Science* **244** 1288–1292

Edwards R G 2001 IVF and the history of stem cells; *Nature* (*London*) **413** 349–351

Evans M J and Kaufman M H 1981 Establishment in culture of pluripotential cells from mouse embryos; *Nature* (*London*) **292** 154–156

Gardner R L 1968 Mouse chimaeras obtained by the injection of cells into the blastocyst; *Nature* (*London*) **220** 596–597

Hogan B 1981 From embryo to teratocarcinoma in tissue culture; *Nature* (*London*) **292** 111–112

Hyafil F, Morello D, Babinet C and Jacob F 1980 A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos; *Cell* **21** 927–934

Illmensee K and Mintz B 1976 Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts; *Proc. Natl. Acad. Sci. USA* **73** 549–553

Jacob F 1977 Mouse teratocarcinoma and embryonic antigens; *Immunol. Rev.* **33** 3–32

Jacob 1978 The Leeuwenhoek lecture 1977: Mouse teratocarcinoma and mouse embryo; *Proc. R. Soc. London B* **201** 249–270

Jacob F and Monod J 1963 Genetic repression, allosteric inhibition and cellular differentiation; in *Cytodifferential and macromolecular synthesis* (New York: Academic Press)

Kahan B W and Eprussi B 1970 Developmental potentialities of clonal *in vitro* cultures of mouse testicular teratoma; *J. Natl. Cancer Inst.* **44** 1015–1036

Kleinsmith L J and Pierce G B Jr 1964 Multipotentiality of single embryonal carcinoma cells; *Cancer Res.* **24** 1544–1551

Mansour S L, Thomas K R and Capecchi M R 1988 Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes; *Nature* (*London*) **336** 348–352

Martin G R 1975 Teratocarcinomas as a model system for the study of embryogenesis and neoplasia; *Cell* **5** 229–243

Martin G R 1980 Teratocarcinomas and mammalian embryogenesis; *Science* **209** 768–776

Martin G R 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells; *Proc. Natl. Acad. Sci. USA* **78** 7634–7638

Martin G R, Smith S and Epstein C J 1978 Protein synthetic patterns in teratocarcinoma stem cells and mouse embryos at early stages of development; *Dev. Biol.* **66** 8–16

Miller L J and Bloom F E 1998 Publishing controversial research; *Science* **282** 1045

Mintz B and Illmensee K 1975 Normal genetically mosaic mice produced from malignant teratocarcinoma cells; *Proc. Natl. Acad. Sci. USA* **72** 3585–3589

Papaioannou V E, McBurney M W, Gardner R L and Evans M J 1975 Fate of teratocarcinoma cells injected into early mouse embryos; *Nature* (*London*) **258** 70–73

Peyron A 1939 Faits nouveaux relatifs à l'origine et à l'histogénèse des embryomes; *Bull. Cancer* **28** 658–681

Pierce G B 1967 Teratocarcinoma: model for a developmental concept of cancer; *Curr. Topics Dev. Biol.* 223–246

Pierce G B Jr and Beals T F 1964 The ultrastructure of primordial germinal cells of the fetal testes and of embryonal carcinoma cells of mice; *Cancer Res.* **24** 1553–1567

Shamblott M J, Axelman J, Wang S *et al.* 1998 Derivation of pluripotent stem cells from cultured human primordial germ cells; *Proc. Natl. Acad. Sci. USA* **95** 13726–13731

Sherman M I 1975 The culture of cells derived from mouse blastocysts; *Cell* **5** 343–349

Solter D 1998 Dolly *is* a clone – and no longer alone; *Nature* (*London*) **394** 315–316

Solter D 2006 From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research; *Nature Reviews/genetics* **7** 319–327

Stevens L C 1958 Studies on transplantable testicular teratomas of strain 129 mice; *J. Natl. Cancer Inst.* **20** 1257–1275

Stevens L C 1959 Embryology of testicular teratomas in strain 129 mice; *J. Natl. Cancer Inst.* **23** 1249–1295

Stevens L C 1962 Testicular teratomas in fetal mice; *J. Natl. Cancer Inst.* **28** 247–267

Stevens L C 1967a Origin of testicular teratomas from primordial germ cells in mice; *J. Natl. Cancer Inst.* **38** 549–552

Stevens L C 1967b The biology of teratomas; *Adv. Morphogen.* **6** 1–31

Stevens L C 1970 The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos; *Dev. Biol.* **21** 364–382

Stevens L C Jr and Little C C 1954 Spontaneous testicular teratomas in an inbred strain of mice; *Proc. Natl. Acad. Sci. USA* **40** 1080–1087

Stewart T A and Mintz B 1981 Successive generations of mice produced from an established culture line of euploid tetratocarcinoma cells; *Proc. Natl. Acad. Sci. USA* **78** 6314–6318

Thomson J A, Kalishman J, Golos T G *et al* 1995 Isolation of a primate embryonic stem cell line; *Proc. Natl. Acad. Sci. USA* **92** 7844–7848

Thomson J A, Itskovitz-Eldor J, Shapiro S S *et al* 1998 Embryonic stem cell lines derived from human blastocysts; *Science* **282** 1145–1147

Vogel G 2000 Stem cells: new excitement, persistent questions; *Science* **290** 1672–1674

Wilmut I, Schnieke A E, McWhir J *et al* 1997 Viable offspring derived from fetal and adult mammalian cells; *Nature* (*London*) **385** 810–813