EMBRYONIC STEM CELLS: THE MOUSE SOURCE – VEHICLE FOR MAMMALIAN GENETICS AND BEYOND

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by

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In a developmental system there may be a complexity of environment, a progressive developmental time course and a multiplicity of cells and interacting components. Isolation of such systems into culture allows both simplification and experimental access. Tissue-culture of disaggregated cells, in particular, allows for isolation and purification of cell type by cloning and detailed manipulation of culture conditions. It also gives an entrée to a genetic analysis via somatic cell genetics. It was for such reasons that I sought an *in vitro* developmental system. Genetic knowledge for any culturable system from higher organisms was at that time sparse but best for chick and mouse.

I had been looking for messenger RNA changes during development mainly in early Xenopus embryos. By the end of the 60's it was becoming clear to me that for such molecular studies not only was a larger scale manipulable system needed it but also one with a better genetic potential. Excellent organ culture systems of the early and mid development of chick were available but these were difficult to deconstruct into the tissue-culture level. Some genetics was available but the chick karyotype was a problem for somatic cell genetic approaches. Mammalian embryos were, by contrast, extremely inaccessible but *in vitro* culture of the early preimplantation stages had been developed (for a comprehensive review see (Cockroft, 1997)). Mammalian long-term tissue culture was better established and mouse genetics was at least comparable to that of the chick. Somatic cell genetic approaches were more available.

Robin Weiss drew my attention to two important reviews of work with mouse teratocarcinomas published in 1967 which pointed the way to an opportunity to develop a tissue culture system for studies of cellular differentiation (Pierce, 1967; Stevens, 1967). Stevens reviewed his work in which he had established inbred strains of mice with a high incidence of spontaneous testicular teratocarcinomas, had shown that these tumours were transplantable and demonstrated their origin from primordial germ cells in the foetal testis. He also showed that they could be experimentally induced by ectopic

transplantation both of geminal ridges containing these primordial germ cells and of early embryos i.e. transplantation of sources of pluripotential cells. Prophetically Stevens and Little in their paper of 1954 (Stevens and Little, 1954) set the field by saying of their transplantable teratocarcinomas *"Pluripotential embryonic cells appear to give rise to both rapidly differentiating cells and others which like themselves, remain undifferentiated*"; this is the definition for an Embryonic Stem Cell.

Pierce and his colleagues provided a long series of experimental studies including demonstrating growth of cells from the tumours in tissue culture but the single most important demonstration was that of Kleinsmith and Pierce (1964) who showed that transplantation of a single cell *in vivo* could result in a fully differentiating teratocarcinoma; unequivocally establishing the presence of the pluripotential tumour stem cells. These cells were named following the human nomenclature as Embryonal Carcinoma cells.

In May 1969, Leroy Stevens very generously sent me stocks of 129 inbred mice some carrying transplantable teratocarcinomas (induced as described in (Stevens, 1970)). From tumours passaged from this stock I was able to establish clonal tissue cultures which retained their full pluripotency as demonstrated by their ability to differentiate as a tumour *in vivo* (Evans, 1972). One significant feature of this isolation and cloning was that irradiated chick embryo fibroblasts were used as a feeder layer. It was noted that when the use of this irradiated feeder layer was discontinued the cultures spontaneously generated differentiated cell types (E cells) as well as maintaining the stem cell line (C cells). Retrospectively we can see that these E cells do indeed arise by differentiation from the stem cells and that they provide a balanced, mixed population where the pluripotentcy of the stem cells is maintained by the feeder effect of the associated differentiated products. At the time, however, the processes of differentiation were unclear and it was not possible to see extensive differentiation *in vitro*. I published a detailed discussion of the situation in 1975 (Evans, 1975) which shows the difficulty of interpretation just as we were beginning to see differentiation *in vitro*. It is also of retrospective interest that it was here where I first proposed that pluripotential embryonic cells should be able to be cultured directly from normal embryos something which was not to be achieved for another six years; *" I should like to suggest that it may be quite feasible to obtain cultures of pluripotent cells directly from the embryo now that experience has been obtained handling such cells, and that the earlier results of Cole, Edwards & Paul (Cole et al., 1966) with cultures from rabbit blastocysts should not necessarily inhibit further efforts in this direction."*

During this time, moreover, studies started to indicate the very close relationship of these cultured Embryonal Carcinoma cells to their primordial germ cell and normal embryo counterparts.

Repeated innoculation of syngeneic mice with irradiated teratocarcinoma cell cultures results in antisera reacting with the cell surface of the EC cells (reviewed by Jacob, 1977). Unaltered teratomas are still, however, produced in these hyperimmunised mice by inoculation with live EC cells. The same specific cell surface antigens are present upon the cells of early mouse em-

bryos and germ cells (Artzt *et al.*, 1973). Although originally these antigens were thought to be cell surface proteins associated with the wild-type t-locus this became disproved by careful genetic studies (Erickson and Lewis, 1980) and it subsequently became apparent from studies with human monoclonal autoantibody sera that they were cell surface carbohydrate moieties. (Childs *et al.*, 1983; Gooi *et al.*, 1981; Kapadia *et al.*, 1981). The branching of these carbohydrate chains differs on the ES cells and their differentiated progeny as also seen in development of the early mouse embryo. Studies with these and the usefully discriminatory Forssman antigen demonstrated that the EC cells had a similar cell surface phenotype only to the pluripotential cells of the early pre and postimplantation mouse embryo (Evans *et al.*, 1979; Stinnakre *et al.*, 1981). In addition high resolution two dimensional electrophoretic analysis of nascent protein synthesis suggested that the EC cells were very similar to early embryo cell types but in particular matched 5 day ecto-derm (Lovell-Badge and Evans, 1980).

Perhaps the most dramatic indication of the similarity of EC cells to the early embryo was their ability to become reincorporated into a mouse blastocyst and develop into a healthy mouse with tissue contributions from the EC cells. The first indications of this were published by Brinster (Brinster, 1974). My experiments together with Richard Gardner and his colleagues (Papaioannou *et al.*, 1978; Papaioannou *et al.*, 1975) showed that very extensive chimaerism across most tissues of the mouse was achievable using tissue culture EC cells but that some of these animals showed later-origin tumours of differentiated cell types. (Some EC cell stocks (apparently those which through progression in cell culture differentiated more poorly) gave rise to animals bearing early-origin undifferentiated tumours.) None of these mice were able to transmit the teratoma-origin genome through their gametes, most probably because the cells used were aneuploid. In any case it became apparent that for effective germline transmission both euploidy and excellent and uncompromised cellular differentiation would be needed.

Progress in understanding the differentiation of the cells *in vitro* (which can be very extensive and equivalent to that seen in a teratoma) gave rise to one of the important conceptual breakthroughs – the realization that the differentiation of the EC cells was not abnormal, disorganized, random or stochastic but followed the normal pathways of early embryonic development. We noticed that in every situation where the EC cells were allowed to differentiate the first differentiated cells to appear were primary embryonic endoderm (Evans and Martin, 1975; Martin and Evans, 1975b). We had been investigating the relationship of the C-cells and E-cells in the culture and used very careful recloning of isolated single cells on feeder layers. Homogeneous cultures of the C-cells (the embryonal carcinoma cells) were able to be maintained by passage on feeder layers but when the feeders were removed the cells clumped up and some became detached as small colonies which formed embryoid bodies (Martin and Evans, 1975a). EC cell clumps in suspension formed simple embryoid bodies and when these were allowed to develop further they became more complex cystic bodies in suspension or if allowed to attach to the

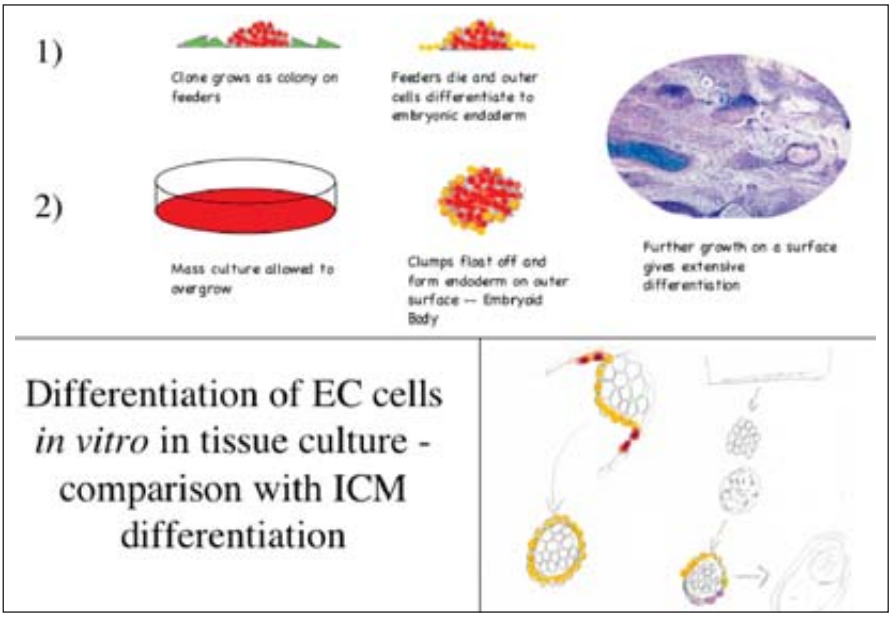

Figure 1.

tissue culture surface spread out and developed into a mixture of cells and tissues which on section proved equivalent in complexity and organisation to the wide diversity of tissues seen in a teratoma (Martin and Evans, 1975c). Similar extensive differentiation was seen if an individual colony arising from a clone on feeders was allowed to continue to grow after the feeders died out (Evans and Martin, 1975). It is clear that the initiation of this differentiation is the same process as that seen when cells on the blastocoelic surface of the inner cell mass (ICM) differentiate into primary endoderm; an isolated ICM becomes surrounded by a rind of endoderm (Rossant, 1975).

In about 1980, I had been trying again to isolate cells from ICM's and had generated numbers of endodermal cultures. I devoted some time to consider why it had not proved possible to isolate cells equivalent to EC cells directly from early mouse embryos and this is written up in a review published in 1981 (Evans, 1981a). The main points of note are:

1) EC cells from culture form teratocarcinomas upon transplantation *in vivo*.

2) Teratocarcinomas containing EC cells were able to be made by ectopic transplantation of embryos from the 2-cell stage through to the dissected embryonic ectoderm from embryos of 7.5 days of development.

3) The cell surface phenotype and the spectrum of protein synthesis suggested that the closest match to EC cells was later then the 3.5 day ICM and earlier than the 6.5 day ectoderm.

4) EC cells in culture enter into differentiation as though they are ICM cells.

5) EC cells could cooperate with ICM of a blastocyst in the development of a chimaeric mouse.

I considered that there might be three classes of reason why EC cells had not been grown directly from explanted embryos or dissected embryo tissues.

1) There might be only very small numbers of founder cells available and that therefore success *in vitro* would depend upon the highest efficiency of cloning. By that time I had been slowly improving the cloning efficiency of passaged EC cells (both mouse EC cells and Human teratocarcinoma derived cells) and using this as a test for optimising the media and conditions arrived at a mix known around the lab at the time as "Martin's Magic Medium" or MMM. The feeder layer used was also optimised by the same test. Retrospectively an optimised medium and procedure is entirely necessary but numerous variants are possible.

2) The timing might be more critical than *in vivo* where processes of onward development or even regression could take place more readily. Retrospectively, we now know that cultures of ES cells have been satisfactorily established from cleaving embryos through to late 4.5 day so this was not the main problem.

3) It was known that the amount of differentiation of teratocarcinomas tended to diminish with tumour passage. EC cell lines diminished in their readiness to differentiate *in vitro* with tissue culture passage. This raised the possibility that adaptation to tumour and to tissue culture growth involved selection of cell lines which were slower to trigger differentiation and that maybe native cells directly from the embryo would differentiate so readily that the stem cell line was immediately lost. Thus conditions most conducive to maintenance of the undifferentiated stem cell state would be needed. In addition to the media supporting the best cloning efficiency, this meant using optimised feeder cell layers and using repeated dissagregation and passage so as not to allow the cells to form local concentrations. I said "*embryonal carcinoma lines which differentiate in vitro are difficult to maintain in an undifferentiated state, even with the help of feeder layers. It is very likely that even these lines have already been highly selected for the ability to be maintained in tissue culture and concomitantly for less ready differentiation. Their genuine embryonic counterpart may differentiate and lose its pluripotency and rapid growth characteristics all too readily under culture conditions*" (Evans, 1981a). Retrospectively this was probably the most cogent reason. Freshly isolated ES cell lines can differentiate precipitately if not prevented.

Collaboration with Matt Kaufman brought, critically, expertise and experience with early mouse embryo manipulation. He had been exploring the developmental potential of parthenogenetic embryos in particular haploid embryos and had discovered that such haploid embryos could be persuaded to develop to an early postimplantation stage (Kaufman, 1978). These em-

bryos tend to have a reduced cell number at the blastocyst stage and in order to allow a compensatory increase before their implantation Kaufman had utilised implantational delay. We therefore sought to use such delayed blastocysts as a source of haploid cells in culture. In the first place we used diploid delayed blastocysts from strain 129 mice and upon explantation I was able to see outgrowth of instantly recognisable EC-like cells. These were able to be picked and maintained in passage tissue culture and had all the expected properties of the sought after primarily isolated pluripotent cells (Evans and Kaufman, 1981). Most importantly they were euploid XY cells and with careful culture maintained a stable karyotype. Interestingly the XX cells from female embryos were also isolated but had a less stable karyotype presumably because of the long term chromosomal imbalance without X-inactivation (Robertson *et al.*, 1983).

We viewed these cells as normal derivatives from the embryo and confidently expected that they would prove useful vectors to the mouse germ line. Martin later in the year, using a different method, reported the establishment of similar cultures directly from embryos but these did not retain a normal karyotype (Martin, 1981). She provided the important nomenclature of Embryonic Stem Cells.

Together with Liz Robertson and Allan Bradley we were soon able to show that progeny of the ES cells were able to form functional germ cells (both sperm and ova) in chimaeric mice. Interestingly male ES cells were often able to transform the sexual differentiation of a female host blastocyst and result in a male chimaeric mouse where, as only the ES derived cells carring a Y chromosome were able to make sperm, 100% of the germline transmission was from the tissue culture derived cells (Bradley *et al.*, 1984).

Transgenesis and mutagenesis was clearly the next step and I chose to use retroviral vectors which have the advantage of cleanly integrated transgenesis and that any mutation caused by this integration is clearly marked by the foreign DNA. It should be remembered that at this time the genetic maps were rudimentary, there was little gene and virtually no genomic sequence data. Thus clean transgene integration associated with mutation was a route to gene discovery.

Using this technique we were able to demonstrate the transmission of sequences introduced by retroviral vectors *in vitro* into the mouse germline (Evans *et al.*, 1985; Robertson *et al.*, 1986) and used several methods to recover newly induced mutation of endogenous loci.

The way was now clear to an experimental genetics for mice. Transgenes could be introduced in culture and the structure verified before introduction into the germline. New mutations could be tested both *in vitro* and *in vivo*. It was around this time that the possibilities of using homologous recombination gene targeting that had been developed by both Oliver Smithies and Mario Capecchi to specifically alter endogenous loci became available and subsequently this has been the most important method for the experimental genetics. These techniques depend upon the availability of cloned sequence for the target gene and the advances in knowledge of mammalian and in par-

ticular mouse genomic sequences has been pivotal. Possibly about one quarter of available loci have already been targeted, and indeed complete coverage of specifically induced mutation in the mouse is now planned (Austin *et al.*, 2004). This is all dependent upon the technology of using mouse ES cells as a vector to the mouse germline. We have described studies on numbers of induced and specifically targeted mutations. I shall here, however, mention only some examples of our experiments using retroviral vectors and one example of gene targeting using homologous recombination.

In the first place it would be useful to be able to select a specific mutation in culture. The most feasible candidate was Hprt which being X-linked is present as only a single copy in XY cells and in which mutation is selectable because in its absence the cells are resistant to otherwise lethal incorporation of 6-thoguanine. Two independent mutations were recovered from ES cell cultures superinfected with retroviral vectors and transferred to the mouse germline (Kuehn *et al.*, 1987). Retrospectively one of the two turned out to be not the expected clean proviral insertion but example of retroposition of an endogenous processed message. This is an interesting observation of an unusual event; such elements are commonly found in genomic sequences and may well be the products of retroviral reverse transcriptase. Our proof that the alpha tubulin processed pseudogene was indeed the cause of this Hprt mutation is an interesting example of the of the use of homologous recombination in ES cells. It is particularly clear because it is without complications of associated vector or selection elements (Carlton *et al.*, 1995).

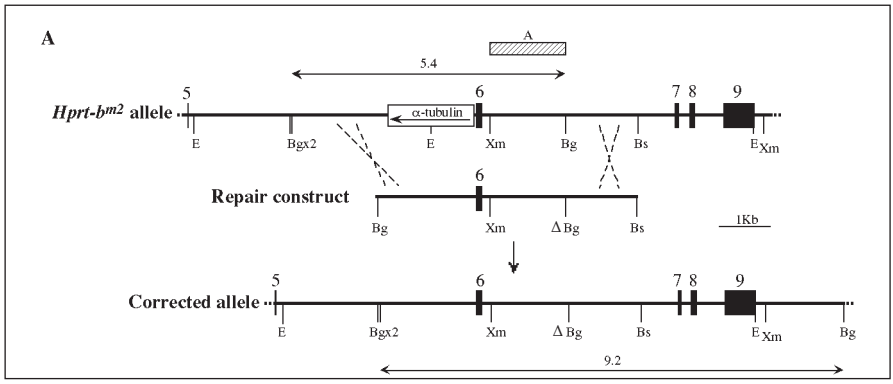

Figure 2. Adapted from (Carlton *et al.*, 1995). We found that the change in the Hprt-b^{m2} allele appeared to be not the expected retroviral vector insertion but an insertion of an α-tubulin processed pseudogene in inverse orientation close to but not disrupting the coding sequence of exon 6. In order to prove that this genomic change was responsible for the mutation we used homologous recombination with a repair construct which was a purified DNA fragment identical with the normal gene sequence across this interval but with a single change to remove a Bgl II restriction site for diagnostic purposes. This construct efficiently targeted the mutant allele and restored function. Southern blot analysis confirmed that only change was removal of the α-tubulin insert. This is a clear example of homologous recombination gene targeting without any complications of selectable markers or associated vectors.

A retroviral vector insertion transmitted through the germline may be screened for phenotypic effect. The absence of homozygous offspring from a heterozygote intercross is indicative of an embryonic lethality. One such example is the insertion 413d which identified a homozygous lethal locus (subsequently renamed nodal). Robertson and her colleagues (Conlon *et al.*, 1991; Robertson *et al.*, 1992) demonstrated that death occurred in the homozygous embryos at an early postimplantation stage but was not a cell autonomous lethality as ES cells homozygous for the insertion could be isolated from blastocysts. Kuehn (Zhou *et al.*, 1993) cloned the locus and showed that it was expressed as a secreted factor controlling axis formation in gastrulation. It is interesting to note that nodal expression may be a key controller of differentiation of ES cells (Takenaga *et al.*, 2007).

Direct physical phenotype may also be observed, for instance (Carlton *et al.*, 1998) described a dominant mutation resulting from the proviral integration which caused a craniofacial dysmorphology resulting from constitutive upregulation of Fg F3 and Fg F4 in the developing skull.

Another very useful technique has been that of gene trapping (Joyner *et al.*, 1992; Skarnes *et al.*, 1992) (reviewed by Evans *et al.*, 1997) where a reporter gene is used to find retroviral vector insertion which falls within a functional locus. Numerous interesting mutations have been recovered in this way and the complex developmental and behavioural consequences of partial inactivation of the histone H3.3a may be quoted as an example (Couldrey *et al.*, 1999).

These types of approaches allow gene function discovery by phenotype but nowadays gene targeting technology allows any designer mutation to be introduced into the mouse germline as a direct experimental approach. In addition to simple mutation, methods have been developed which allow both spatial and temporal control of gene deletion or of function (e.g. see review by Clarke, 2000). All these studies are dependant upon 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 by tissue culture of embryonic stem cells.

I have been interested in the relationship between embryonal carcinoma cells, normal embryo cells and embryonic stem cells for many years (Evans, 1981b). It was the close relationship between EC cells and early embryo pluripotential cells as shown by both their cell surface phenotype and by the extensive match of nascent protein synthesis patterns that helped to lead the way to the isolation of Embryonic Stem Cells. Together with Susan Hunter we have been utilising an analysis of global transcriptional patterns to compare Embryonic Stem Cells in culture with normal early mouse embryo pluripotential tissues. These studies show considerable differences between ICM from blastocysts of either 3.5 or 4.5 days of development and ES cells but a remarkable match with ectoderm from 5.5 days of development (Figure 3). This match is all the more remarkable as we are comparing cells isolated directly from the normal, unmanipulated, *in vivo* embryo with ES cells from an

established cell line growing in an artificial serum-containing tissue culture medium on a plastic surface. It was always possible that mouse ES cells are effectively an artefact of culture and only become "normalised" by re-incorporation into an embryo and re-entrained into normal development by virtue of the influence from the environment of the host embryo. Alternatively they might represent a normal stem cell population. These present studies suggest that any culture adaptations away from a normal are minimal.

Embryonic Stem Cells have, therefore, delivered a major platform technology for experimental genetic manipulation which is delivering most important theoretical understanding and practical medical benefit. They are also proving greatly instrumental in delivering a second platform technology of

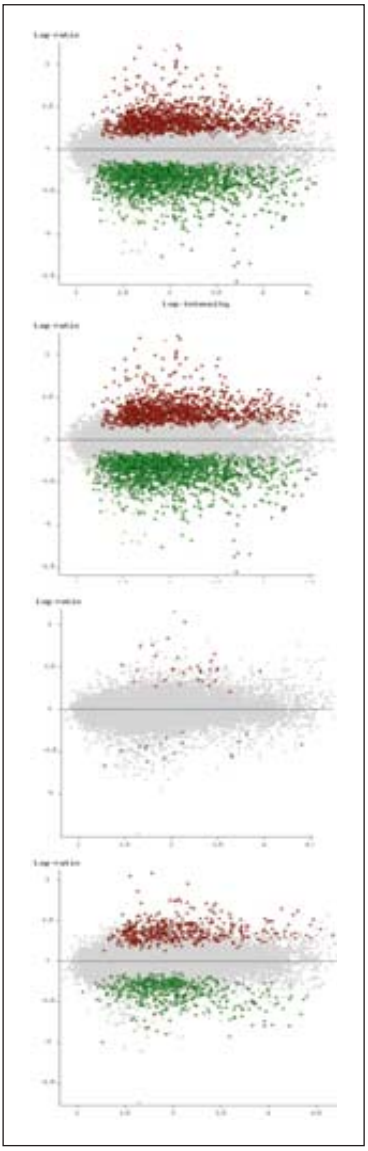

Figure 3. ES cells in culture were compared with tissues isolated from early mouse embryos by microarray transcriptomics. c-DNA was isolated from ICM's or dissected epiblast and compared with c-DNA isolated from ES cells, amplified by two rounds T7 transcription and hybridised to slides printed with the 15k NIA c-DNA probes. Unpublished results Susan Hunter and Martin Evans.

Charts of log ratio vs log intensity

A 3.5 day immunosurgically isolated ICM vs ES cells
B 4.5 day immunosurgically isolated ICM vs ES cells
C 5.5 day dissected epiblast vs ES cells
D 6.5 day dissected primary ectoderm vs ES cells

Global anova analysis; red spots significantly overexpressed by embryo samples, green spots significantly underexpressed by embryo samples. False discovery rate set to <0.01.

Note the exceptional match between ES cells and 5.5 day epiblast.

stem cell based regenerative medicine. One of the original aims of the tissue culture of EC cells was to provide a tractable system for the study of cellular determination and differentiation in vitro. This was achieved but with the mouse cells has not yet been fully exploited. With the advent of human ES cells and the possibilities of using them as a renewable source of tissue-specific precursors for tissue transplant therapies and regenerative medicine (see review by Lerou and Daley, 2005) the importance of understanding and controlling ES cell determination and differentiation in vitro has been highlighted. It is clear that the utility of isolation, maintenance and use of pluripotential stem cells has a long and important future.

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