

COMMENTARY

Human embryonic stem cells

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SUMMARY

Embryonic stem (ES) cells are cells derived from the early embryo that can be propagated indefinitely in the primitive undifferentiated state while remaining pluripotent; they share these properties with embryonic germ (EG) cells. Candidate ES and EG cell lines from the human blastocyst and embryonic gonad can differentiate into multiple types of somatic cell. The phenotype of the blastocyst-derived cell lines is very similar to that of monkey ES cells and pluripotent human embryonal carcinoma cells, but differs

from that of mouse ES cells or the human germ-cell-derived stem cells. Although our understanding of the control of growth and differentiation of human ES cells is quite limited, it is clear that the development of these cell lines will have a widespread impact on biomedical research.

Key words: Human embryonic stem cell, Human embryo, Blastocyst, Primordial germ cell, Embryonal carcinoma, Mouse ES cell, Mouse EG cell, Marker, Growth regulation, Gene expression

INTRODUCTION

In November of last year, groups in the United States led by James Thomson and John Gearhart published data describing the derivation of candidate human pluripotent embryonic stem (ES) and embryonic germ (EG) cell lines from blastocysts or primordial germ cells, respectively (Thomson et al., 1998; Shambloott et al., 1998). Readers will probably agree that few if any previous scientific papers reporting the characterisation of cultured cell lines would have attracted a similar degree of public attention. The interest stems in part from the ethical controversy surrounding the origins of the cells but chiefly from the widespread conviction that their availability will profoundly alter our approaches to many problems in human biology and medicine. Several features define ES cells (below), but the two key properties that make these cells so remarkable are these: ES cells can be grown in vitro and expanded in number indefinitely in the primitive undifferentiated state characteristic of the embryonic cells from which they are derived, and throughout long periods of cultivation in vitro they retain a key property of those embryonic cells – pluripotency, or the ability to develop into any cell type in the adult body (Fig. 1). The scope of even the more obvious applications envisioned for human cells with these properties is breathtaking: new approaches to the study of human embryonic development and disorders thereof, such as birth defects and embryonal tumours; access to hitherto-unexplored territories of human embryonic gene expression for modern genomics data mining; new tools for the discovery of polypeptide growth and differentiation factors that might find application in tissue

regeneration and repair; new means to creating human disease models in vitro for basic research, drug discovery and toxicology; a potential answer to the issue of the chronic shortage of tissue for transplantation in the treatment of degenerative diseases, and an end to the use of immunosuppressive therapy in transplantation, if cloning techniques can be used to derive stem cells from a patient's own tissue; new delivery systems for gene therapy.

Given the potential applications of these cells, and the ethical controversy regarding the use of in vitro fertilised embryos or tissue from aborted foetuses to derive them, the widespread public discussion of these issues is understandable, warranted, and welcome. However, since the sheer volume of commentary on human ES cell ethics, scientific applications and commercial potential now threatens to overwhelm the peer-reviewed scientific literature on the subject, we will focus here on human ES cells themselves: the background to their discovery, their known properties, and what we need to learn about them before we begin to use them to address the futuristic agenda outlined above.

PLURIPOTENT STEM CELLS IN MAMMALS

A brief historical account

The development of mouse ES cells in 1981 (Evans and Kaufman, 1981; Martin, 1981) provided the paradigm and as we will see below, much of the technology, for the development of human ES cells, but the concept of a pluripotent embryonic cell is far older than that. Development of ES cells evolved out

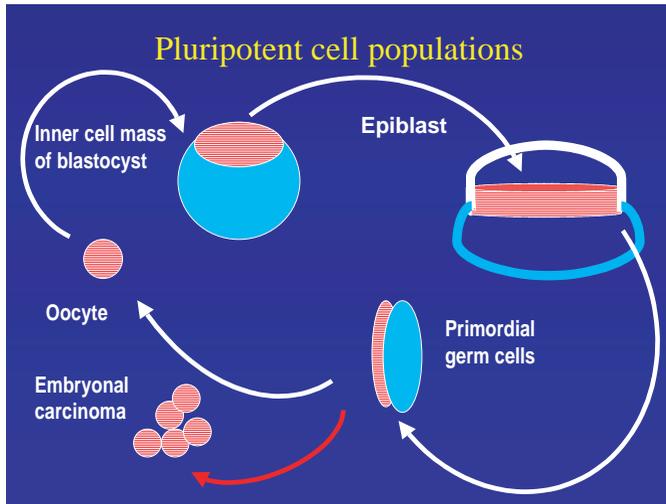


Fig. 1. Pluripotent stem cells. The oocyte, the cells of the early embryo up to compaction, the epiblast, primordial germ cells and the stem cells of teratocarcinomas all are pluripotent. Epiblast cells, primordial germ cells, and embryonal carcinoma cells can all give rise to cultured pluripotent stem cell lines.

of work on mouse teratocarcinomas, tumours that arise in the gonads of a few inbred strains, and consist of a remarkable array of somatic tissues juxtaposed together in a disorganised fashion. Classical work on teratocarcinomas established their origins from germ cells in mice and provided the concept of a stem cell (the embryonal carcinoma or EC cell) that can give rise to the multiple types of tissue found in the tumours (Kleinsmith and Pierce, 1964; review, Stevens, 1983). The field of teratocarcinoma research (review, Martin, 1980) expanded considerably in the 70's: the remarkable developmental capacity of the EC stem cell became apparent following the generation of chimaeric mice by blastocyst injection of EC cells, and investigators began to realise the potential value of cultured cell lines from the tumours as models for mammalian development. EC cells however had limitations: they often contained chromosomal abnormalities, and their ability to differentiate into multiple tissue types was often limited.

Since teratocarcinomas can also be induced by grafting blastocysts to ectopic sites, it was reasoned that it might be possible to derive pluripotent cell lines directly from blastocysts rather than from tumours, and that is what was done in 1981 by Gail Martin and Martin Evans independently. The result was a stable diploid cell line that could generate every tissue of the adult body, including germ cells. Teratocarcinomas also develop spontaneously from primordial germ cells in some mouse strains, or following transplantation of primordial germ cells to ectopic sites. In 1992 Brigid Hogan and her colleagues reported the direct derivation of EG cells from mouse primordial germ cells (Matsui et al., 1992). These EG cells have a developmental capacity very similar to that of ES cells, though they differ in their expression of some imprinted genes.

Testicular teratocarcinomas occur spontaneously in humans, and pluripotent cell lines were also developed from these (review, Andrews, 1988). Two groups reported the derivation of cloned cell lines from human teratocarcinoma that can differentiate *in vitro* into neurons and other cell types (Andrews

et al., 1984; Thompson et al., 1984). Subsequently, cell lines were developed that can differentiate into tissues representative of all three embryonic germ layers (Pera et al., 1989). As analysis of the properties of human EC cells proceeded, it became clear that they are always aneuploid, usually (though not always) have a limited capacity for spontaneous differentiation into somatic tissue, and differ in phenotype from mouse ES or EC cells.

In 1995, James Thomson's team derived primate ES cells from rhesus monkey blastocysts and later from those of the marmoset (Thomson et al., 1995, 1996). These primate cell lines are diploid, and give rise to an extensive variety of tissues representative of all three embryonic germ layers, but otherwise closely resembled their nearest counterpart, the human EC cell. The implication of the monkey work and the work on human EC cells was that a pluripotent stem cell, which would be rather different in phenotype from a mouse ES cell, could probably be derived from a human blastocyst.

A generic functional definition of an ES cell

In considering the properties of ES or EG cells, there are certain generic features that any ES cell might be expected to possess, and other properties which may be peculiar to bona fide pluripotent cells isolated from different species or different tissues, or representative of a different stage of embryonic development. The mouse ES cell provides a benchmark for definition of the generic requirements for ES cells. Its key features are these: it is derived from a pluripotent cell population; it is stably diploid and karyotypically normal *in vitro*; it can be propagated indefinitely in the primitive embryonic state; it can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers, both in teratomas after grafting or *in vitro* under appropriate conditions; and it can give rise to any cell type in the body, including germ cells, when allowed to colonise a host blastocyst. The criteria for pluripotency usually include derivation of the stem cell line from a single cloned cell. This experiment eliminates the possibility that several distinct committed multipotential cell types are present in the culture that together account for the variety of differentiated derivatives produced.)

Generic criteria for pluripotent ES or EG cells

- Originate from a pluripotent cell population
- Maintain normal karyotype
- Immortal and can be propagated indefinitely in the embryonic state
- Clonally derived cultures capable of spontaneous differentiation into extraembryonic tissue and somatic cells representative of all three embryonic germ layers in teratomas or *in vitro*

Thus far, only mouse EG or ES cells meet these generic criteria. Primate ES cells meet the first three four criteria, but not the last. Numerous other candidate mammalian ES cells have been described over the years in domestic and laboratory species, but only in the mouse have all criteria been met rigorously. Some workers restrict the designation of ES cell to those cells capable of colonising all tissues including the germ line. Such a test can never be applied to human ES cells for

ethical reasons. One can therefore refer to human cells as ES cells if they meet all other generic criteria, but should note that it may be very difficult to prove from *in vitro* data and teratoma formation *in vivo* that an ES cell really can give rise to every type of tissue cell, given that some tissue types are seen rarely, if ever, in teratomas or *in vitro*. Here, we will use the following terminology: a pluripotent stem cell is a stem cell derived from an embryo, primordial germ cells or a teratocarcinoma that is capable of indefinite self renewal and differentiation into extraembryonic tissue and tissue representative of all three embryonic germ layers; an ES cell is a preimplantation embryo-derived cell that meets the first four criteria listed above; and EG cell is a cell derived from the embryonic gonad that meets the first four criteria listed above.

THE HUMAN CELLS

Derivation and evidence for pluripotency

James Thomson and co-workers (1998) derived ES cells from surplus blastocysts donated by couples undergoing treatment for infertility. It is remarkable that their methodology was not very different from that used 17 years earlier to derive mouse ES stem cells: the trophectoderm, thought to be inhibitory to ES cell establishment, was removed by immunosurgery, the inner cell mass was plated on to a mouse embryonic fibroblast feeder cell layer, and following a brief period of attachment and expansion, the resulting outgrowth was disaggregated and replated onto another feeder cell layer. There were no significant departures from mouse ES protocols in the media or other aspects of the culture system and a relatively high success rate was achieved. Given that others have attempted to grow human ES cells, what factors were critical for success? The experience of Thomson's group with monkey ES cells was undoubtedly helpful. Primate pluripotent stem cells are different in many respects to mouse ES cells, particularly in their morphology and their ability to withstand dissociation into single cells, so it is important to recognise the correct cell type and handle it appropriately during subculture. But another factor that must be highlighted is the improvement in human embryo culture procedures, including the development of two stage culture systems that employ different media for appropriate developmental stages; this has allowed a high rate of production of good quality blastocysts (review, Gardner, 1998).

Shamblott and colleagues (1998) isolated pluripotent cells from embryonic and foetal gonads at 5-9 weeks post-fertilisation. Although we know little about the details of primordial germ cell maturation in man, we do know that this period encompasses those developmental stages in which primordial germ cells arrive in the gonads and proliferate, and overt sexual differentiation of the gonads occurs. Cells expressing markers characteristic of primordial germ cells are found in the human embryonic and foetal gonad throughout this period (Jorgensen et al., 1995). The culture system used by this group incorporated factors known to support mouse primordial germ cell survival and mitogenesis *in vitro*: STO fibroblast feeder cell layers, basic fibroblast growth factor, Leukemia Inhibitory Factor (LIF), and forskolin (review, Donovan, 1994).

To what extent do the cells described by both groups meet

the generic criteria for ES or EG cells? Both types of culture are derived from pluripotent cell populations, and both retain a normal karyotype during extensive cultivation *in vitro*. The cells described by Thomson et al. have been grown for extensive periods and possess telomerase activity, both findings which indicate that they are immortal. The EG cells have not been grown for such long periods, but there is no indication that their lifespan is finite. The blastocyst-derived cells form teratomas containing derivatives of all three germ layers, and in some cases the tissues show a high degree of histotypic organisation (formation of ganglia, for example). Evidence for *in vitro* differentiation, however, is limited to expression of markers characteristic of trophoblast and endoderm formation (production of human chorionic gonadotrophin and alphafoetoprotein); whether the cells found producing alphafoetoprotein represent extraembryonic (yolk sac) endoderm or definitive (embryonic) endoderm is not clear. In the case of the germ-cell-derived cultures, no evidence was presented regarding formation of teratomas *in vivo*, but the authors did observe *in vitro* differentiation within embryoid bodies. Embryoid bodies are structures formed by pluripotent stem cells grown in three-dimensional culture under conditions non-permissive for stem cell growth. In the mouse, embryoid bodies consist of two layers, one of extraembryonic endoderm and one of ectoderm, and interactions between these two cell types probably drive the differentiation of ectoderm into multiple cell lineages, which mimics the situation in the early postimplantation embryo *in vivo* (review, Martin, 1981). Shamblott et al. sectioned embryoid bodies that formed spontaneously in culture and, using immunocytochemistry, demonstrated expression of single markers in different cell types consistent with the representation of mesodermal, ectodermal and endodermal lineages. It may be premature to refer to these structures as embryoid bodies, given that as yet no convincing evidence based on marker or gene expression shows that they resemble any structure found in the human peri-implantation embryo. However, they clearly contain a heterogeneous mixture of cell types. Neither group was able to clonally derive cell lines, although this might be only a matter of persistence. Our own studies on ES cell lines independently derived from blastocysts confirm the findings of J. A. Thomson et al. (B. E. Reubinoff, M. F. Pera, C. Y. Fong, A. O. Trounson and A. Bongso, unpublished).

In summary the evidence that ES cells can be derived from human blastocysts is quite convincing, although stronger data regarding somatic differentiation *in vitro* and clonal derivation would prove this conclusively. The evidence for pluripotency of the EG cells is also strongly suggestive, although additional data on teratoma formation, the characterisation of the differentiated cells found *in vitro*, and clonal derivation would make the case more convincing.

Morphology, marker expression and growth requirements

How do the phenotypes of the cells – morphology, antigen expression, growth requirements – compare with one another and with those of other types of pluripotent cells, such as EC cells or mouse ES cells? Human EC cells and monkey and human ES cells have phenotypes which are very similar and readily distinguished from those of their counterparts in the mouse and from those of human EG cells. The primate cells

Table 1. Marker expression and growth properties of mouse and primate pluripotent cells

	Mouse EC, ES, EG cells	Human EC cells	Monkey ES cells	Human ES cells	Human EG cells
SSEA-1	+	-	-	-	+
SSEA-3	-	+	+	+	+
SSEA-4	-	+	+	+	+
TRA-1-60	-*	+	+	+	+
TRA-1-81	-*	+	+	+	+
GCTM-2	-*	+	‡	+	?
Alkaline phosphatase	+	+	+	+	+
Oct-4	+	+	‡	?	?
Genesis	+(ES)	+	?	?	?
Germ cell nuclear factor	+(ES, EC)	+	?	?	?
GDF-3	+(ES, EC)	+	?	?	?
Cripto (TDGF-1)	+(inner cell mass, ectoderm)	+	?	?	?
Feeder-cell dependent	ES, EG, some EC	Some; few show high cloning efficiency	Yes	Yes	Yes
Factors known to aid stem cell renewal	LIF and other factors acting through gp130	?	?	?	LIF, bFGF

*Antibodies do not react with mouse cells. It is unknown whether this is due to lack of expression or species specificity of the antibody.

‡B. E. Reubinoff and M. F. Pera (unpublished).

grow in flat colonies with distinct cell borders in monolayer culture whereas mouse ES cells grow in more rounded clumps with indistinct cell borders. A series of surface antigens characterise primate pluripotent stem cells. The stage-specific embryonic antigens 1, 3 and 4 are globoseries glycolipids recognised by monoclonal antibodies originally raised to distinguish early stages of mouse development. Primate pluripotent cells express SSEA-3 and SSEA-4 (the epitope recognised by the latter is more readily detected than that seen by the former), and express SSEA-1 only upon differentiation (Andrews et al., 1996; Thomson and Marshall, 1998; Thomson et al., 1998). Essentially the reverse is true of mouse ES cells. Also characteristic of human EC cells is the expression of a set of antigens associated with a pericellular matrix proteoglycan found on the surface of these cells that is also secreted or shed into the culture medium by them (Cooper et al., 1992; Badcock et al., in press). The TRA-1-60 epitope is a sialidase-sensitive epitope associated with this proteoglycan; the antibody GCTM-2 reacts with its core protein, and antibodies TRA-1-80 and K21 react with other unknown epitopes on the same molecule. Human ES cells, as well as monkey ES cells, react with TRA1-60, TRA1-80 and GCTM-2. Although GCTM-2 and TRA1-60 do not label mouse ES or EC cells, it is not clear whether the mouse cells lack the surface proteoglycan or whether the antibodies are species specific. Peter Andrews and co-workers (Badcock et al., 1999) have pointed out that mouse ES and EC cells, and their human counterparts, all express some form of polylysosamine glycoconjugate on their surface, whether it be keratan sulphate or a non-sulphated form. All primate pluripotent stem cells, like mouse EC and ES cells, express alkaline phosphatase activity. In humans, there are four different isozymes of alkaline phosphatase. EC cells express the tissue non-specific form and a form of the enzyme that can be detected by antibodies that react with the germ cell or placental form (available immunological reagents do not distinguish between these closely related isoforms). It is not clear which form of alkaline phosphatase the human ES cells express.

It must be noted that none of these surface markers is completely specific and all can be detected in other tissue

types. The markers analysed thus far in the primate or human ES or EG cells are only immunochemically defined epitopes or enzymatic activities; there is as yet little information on gene expression in human ES cells or primate ES cells.

Like mouse ES and EG cells, primate pluripotent cells, including some human EC cells, require a mouse embryonic fibroblast feeder-cell layer for support. (The terminology used for these feeder cells is misleading. They are better described as primitive mesenchymal cells rather than connective tissue fibroblasts, and they are derived from midgestation foetuses, not embryos.) In the case of mouse ES and EG cells, this requirement can be replaced by LIF or related members of this cytokine family, but pluripotent human EC cells, rhesus monkey ES cells, and human ES cells will not respond to LIF in such a fashion (Pera et al., 1989; Roach et al., 1993; Thomson and Marshall, 1998; Thomson et al., 1998). Even on a feeder cell layer, all primate pluripotent cells grow very poorly when dissociated to single cells, whereas mouse ES cell lines can be cloned at a relatively high efficiency in the presence of LIF under these conditions.

The morphology, marker expression and growth requirements of pluripotent cells derived from the gonad differ in some ways from those of other primate pluripotent stem cells. The cells grow in more rounded clumps that lack distinct cell borders and are very difficult to dissociate. They express SSEA-1 in addition to SSEA-3, SSEA-4, and TRA 1-60, and they contain alkaline phosphatase activity. The EG cells appear to exhibit some degree of dependence on LIF and basic fibroblast growth factor, although this has not yet been systematically investigated. It must be noted that the markers expressed by these germ cell derived cultures are consistent with the identification of these cells as primordial germ cells. As in the mouse, the process of conversion from a primordial germ cell to a cell that can be continuously cultured and that is pluripotent is poorly defined. Shablott et al. noted that only a small fraction of the cells give rise to embryoid bodies containing multiple types of differentiated cell. This process of conversion to pluripotency may be slower in humans, since the time frame of germ cell maturation is different to that in the mouse.

Generic molecular markers of pluripotent stem cells?

Although there are apparent differences in the phenotype of murine and primate pluripotent cells, a universal set of molecular markers might be common to all types of stem cell. Such markers might not be absolutely specific to pluripotent cells but would probably have a very restricted expression in other cell types. The data on primate ES and EG cells are very limited at present, but a comparison of mouse ES and EC cells and human EC cells enables a preliminary sketch of the universal pluripotent mammalian stem cell to be drawn. Potential molecular markers include high molecular mass cell surface polylectosamine glycoconjugates, expression of some form of alkaline phosphatase, expression of the growth factors GDF-3 (Caricasole et al., 1998) and Cripto or Teratocarcinoma Derived Growth Factor 1 (Baldassare et al., 1997; Xu et al., 1998), and expression of the transcription factors OCT-4 (review, Brehm et al., 1998), germ cell nuclear factor (Lei et al., 1997) and Genesis (Sutton et al., 1996). The biological roles of most of these molecules in the context of ES cell growth remain to be defined, with the exception of Oct-4, which is required for the establishment of the pluripotent cell lineage in the mouse (Nichols et al., 1998).

CONCLUSIONS: HUMAN ES CELL PROSPECTIVES

Most of the applications of human ES cells will require cells to be grown and manipulated as a relatively pure stem cell population on a large scale, and the availability of methods for producing and isolating specific types of differentiated cell from them. At present, no one has reported large scale growth, efficient cloning or genetic manipulation of human ES or EG cells. It will be important to identify factors that facilitate growth and inhibit differentiation of human ES cells. Liberation from a feeder cell requirement may be essential for certain types of experiments, as well as for production of cells for transplantation. Despite many years of use of mouse embryonic fibroblast feeder cell layers for support of mouse ES cells and other cell types, it is not yet clear at the molecular level exactly what these cells provide for their clients. The feeder cell effect on primate pluripotent cells is not reproduced by either supernatants from the cells or by the extracellular matrix which they secrete onto the monolayer (Pera et al., 1989; Thomson and Marshall, 1998), which suggests either that secreted factors and matrix function synergistically, or that the important factors are either membrane bound, are passed through gap junctions, or are highly unstable. In the case of primate pluripotent cells, even in the presence of the mouse feeder cell layer, both their cloning efficiency and their tolerance for dissociation to single cells is very low. Therefore it seems likely that juxtacrine factors, again possibly membrane bound ligands binding to receptors on neighbouring autologous cells, are also critical to human ES cell growth or survival. There are several candidate positive regulators of ES cell growth, none of which has yet been purified to homogeneity or cloned; these factors appear unrelated to LIF and might eventually be found to play an important role in the maintenance of human ES cells (Roach et al., 1993; Dani et al., 1998; Rathjen et al., 1999).

In the case of mouse ES cells, and probably in the case of human ES cells, certain types of differentiated cell inhibit continuous stem cell growth. A methodology called stem cell selection, in which a selectable marker under the control of a stem cell specific promoter such as Oct-4 is introduced into stem cells, enables selection against differentiated cells during routine subcultivation, facilitating removal of inhibitors and enhanced stem cell growth (McWhir et al., 1996). Modulation of the action of whatever inhibitors these differentiated cells produce should also promote stem cell proliferation.

Directed differentiation of human ES cells into specific lineages has not yet been achieved. Spontaneous differentiation of mouse ES, human EC, and human EG cells has been observed both in embryoid bodies and in high density cultures. In mouse ES and human EC systems, retinoic acid or polar solvents, or growth factors, induce differentiation into cell populations that are enriched for particular cell types. The range of cells observed in teratomas formed in xenografts of human ES or human EC cells usually exceeds that seen during routine cultivation in vitro. Therefore, either the induction and proliferation of committed progenitor cells depend upon environmental factors that are absent in vitro, or factors present in vitro block differentiation into specific lineages. Alternatively, a combination of these two possibilities might act to limit the variety of cell types seen in culture. It is possible that simpler culture systems that eliminate the effects of feeder cells and components of serum will be necessary to enable a given purified factor or combination of factors to drive stem cells into commitment to a particular lineage (Wiles and Johansson, 1999). Alternatively, selective culture conditions or the use of lineage specific stem cell selection might allow isolation of pure populations of precursor cells appearing in a mixed background of cell types after spontaneous differentiation (Li et al., 1998).

There are clearly many challenges for cell biologists in this arena, and the opportunities are vast. The rapid dissemination of this technology, full support of this research from governmental and philanthropic as well as private sector sources, and cooperation and collaboration amongst workers in the area, will ensure that the potential benefits to research and medicine are realised soon.

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