Human embryonic stem cells: prospects for development

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Summary

It is widely anticipated that human embryonic stem (ES) cells will serve as an experimental model for studying early development in our species, and, conversely, that studies of development in model systems, the mouse in particular, will inform our efforts to manipulate human stem cells in vitro. A comparison of primate and mouse ES cells suggests that a common underlying blueprint for the pluripotent state has undergone significant species-specific modification. As we discuss here, technical advances in the propagation and manipulation of human ES cells have improved our understanding of their growth and differentiation, providing the potential to investigate early human development and to develop new clinical therapies.

Introduction

Human embryonic stem (hES) cells have been the subject of many review articles since the first report of their derivation over five years ago (Thomson et al., 1998), and the characteristics of the cells, their potential use in regenerative medicine, and the ethical issues surrounding their provenance, have been widely discussed in the scientific literature. However, few reviews have focused on hES cells from an embryological standpoint. We now have an experimental system that provides us with routine access to stages of the human life cycle that were previously out of reach to experimentation. How useful a tool will these cells be for studying these developmental stages? Conversely, will our understanding of the molecular regulation of mammalian development based on studies in the mouse provide us with a framework to control hES cells in vitro, enabling us to develop the many important clinical applications that are envisioned for these cells in the future?

This second question is particularly important because the answer profoundly affects how we devise strategies to manipulate ES cell differentiation. If differentiation in vitro is a reflection of events in the embryo, as exemplified by the conversion of mouse ES cells into motoneurons in vitro (Wichterle et al., 2002), then our efforts should be informed and strongly influenced by developmental studies in model systems. If hES cell differentiation does not closely resemble mouse embryonic development, a more empirical approach will be needed to identify the signaling pathways that control hES cell differentiation, as we discuss later.

In this article, we review aspects of primate embryology that are relevant to ES cell biology, survey the similarities and differences between mouse and primate ES cells, and then discuss recent advances in hES cell technology, and in understanding primate ES cell differentiation. We do not discuss here a very promising and relatively neglected alternative source of human pluripotent cells, the embryonic gonad (Shamblott et al., 1998); recent encouraging work confirms that pluripotent cell lines can be derived from this source, although there are substantial challenges in maintaining and manipulating these cells (Turnpenny et al., 2003).

Primate embryonic development

Our understanding of mammalian embryology, and of pluripotent stem cells, is based chiefly on studies in the mouse. However, there are significant differences between mouse and primate development (Box 1). We need to take these differences into account when we consider the embryological context in which the embryological counterparts of ES cells from the two species first appear (pre-implantation blastocyst) and then differentiate (early postimplantation period).

There is very little information about the molecular control of development in primates, and on gene expression patterns during postimplantation primate development, which makes comparison with the extensive data from the mouse very difficult. Comparison of the mouse and human genome shows that genes involved in reproduction are amongst those subject to the strongest evolutionary pressure, and that various types of non-coding regulatory elements are under strong selective pressure (Waterston et al., 2002). Thus, the comparison of coding sequences alone may overlook important species differences in gene expression control during development.

A recent study that compared gene expression patterns in rhesus monkey and mouse embryos found differences in the abundance of certain transcripts between the two species, in the oocyte, and at the cleavage, morula and blastocyst stages of development (Zheng et al., 2004). Although there is little information on gene expression in the immediate postimplantation period in human development, compared with mouse, some studies have found differences in the timing and expression patterns of certain developmental regulatory genes, such as *SOX9* and *SRY*, between the two species, at later stages of development (Fougerousse et al., 2000; Hanley et al., 2000). Conversely, gene expression surveys of placental tissue, which is readily available, have shown that the human and mouse trophoblast (the postimplantation derivatives of the outer epithelial layer of the blastocyst, which make up most of

Box 1. Differences between primate and mouse embryogenesis
• Different time scale for development (see main text).
• High levels of blastomere fragmentation and chromosomal abnormalities in human compared with mouse embryos (Almeida and Bolton, 1996; Almeida and Bolton, 1998; Katz-Jaffe et al., 2004).
• Later transition in human embryos from maternal to zygotic gene expression (this occurs at the 4- to 8-cell stage in humans and at the 2-cell stage in mice) (Braude et al., 1988).
• Differences in the temporal and spatial patterns of gene expression in the early embryo (see main text for more).
• The mouse blastocyst forms one cleavage stage later than the human (Mohr and Trounson, 1982).
• Human embryos have two phases of extraembryonic endoderm formation and limited reliance on yolk sac placentation. Mice have one phase of extraembryonic endoderm generation and more reliance on yolk sac placentation.
• The human epiblast is shaped into a disc rather than as a cup, as in the mouse.
• Interstitial implantation occurs in human embryogenesis but not in the mouse (see main text for more).
• Precocious formation of amnion and extraembryonic mesoderm in human embryos compared with in the mouse.

the fetal part of the placenta) do share certain regulatory pathways (Loregger et al., 2003). Overviews of the genetics of human developmental anomalies, for example of the heart (Ryan and Chin, 2003) or of the limb (Tickle, 2002), similarly suggest the conservation of the role of key genes. There is certainly a need for a more extensive analysis of the expression of developmental regulatory genes in the primate, particularly in the early postimplantation embryo. Until we have such data, the use of transcription factors or other genes as markers of progenitor cell populations in hES cell cultures, based on their expression in the mouse embryo, should be regarded as being presumptive.

The postimplantation period is when most key commitment and differentiation events take place, and is also the phase of primate development that is least accessible to study. Nevertheless, histological and ultrastructural analyses have provided some insights into the differences between primate and murine development (Fig. 1). It is important to remember that the primate embryo devotes its first two weeks almost exclusively to the formation of extraembryonic membranes (Enders and Schlafke, 1981; Enders et al., 1986; Luckett, 1978). Thus, in the time it takes the mouse embryo to develop past midgestation, the primate embryo has just begun germ layer formation.

In primate embryos from natural matings, the extraembryonic endoderm is already significantly developed at the blastocyst stage. With further development, the extraembryonic endoderm forms a continuous sheet of squamous cells extending from below the epiblast outward to line the blastocoel cavity. Similar to in-vitro fertilised (IVF) human embryos at the blastocyst stage, as with earlier stages of development, there is abundant cell death, and the rate and extent of development of individual embryos is highly variable. At early implantation, visceral endoderm can be identified beneath the epiblast. Amnion formation begins when epiblast cells become polarised and radially arranged around the

presumptive amniotic cavity. Then, cavitation separates the epiblast and the amnion, which develop into columnar and cuboidal epithelia, respectively. The cytotrophoblast cell layer (the precursor cell of all other trophoblasts), where active cell division continues, is overlayed by the syncytiotrophoblast, which is the syncytial outer layer of the trophoblast, through which the embryo receives nutrients from the mother and which is formed by fusion of cytotrophoblast cells. Later, lacunae will form in the syncytiotrophoblast layer, which eventually fill with maternal blood. [For a comparison of placental morphology and development in mouse and humans, see Georgiades et al. (Georgiades et al., 2002).]

The origin of extraembryonic mesoderm in the primate embryo is unclear. Enders and King (Enders and King, 1988) obtained ultrastructural evidence that the extraembryonic mesoderm appears prior to overt formation of the primitive streak, which is observed at the caudal end of the embryonic disc. These authors in fact concluded that this tissue might arise from the hypoblast or extraembryonic endoderm (Bianchi et al., 1993; Enders and King, 1988). This extraembryonic mesoderm continues to expand upward and laterally to form part of the chorionic villi and to line the entire inner aspect of the trophectoderm, the outer epithelial layer of the blastocyst that gives rise to most of the embryonic part of the placenta. The formation of a secondary yolk sac between the embryonic disk and the primary yolk sac is a characteristic feature of primate embryos. The visceral endoderm shows asymmetry in the early embryo as it is thickened over the future cranial end of the epiblast. The secondary yolk sac undergoes considerable expansion, and only near the end of the third week of development is yolk sac hematopoeisis underway.

Structural studies of early postimplantation development in the baboon (Enders et al., 1990) and human (Hamilton and Mossman, 1972; Luckett, 1978) show that they closely resemble that in the rhesus macaque monkey. The human embryo undergoes interstitial implantation (it becomes entirely surrounded by endometrial tissue). Unlike in the rhesus, in the human and in some species of lemur, a mesh of endodermal cells forms within the blastocoel cavity, perhaps the equivalent of an ingrowth of parietal endoderm. The differentiation of the primitive streak is evident early in development in the form of loosely associated cells on the ventral surface of the epiblast. This earliest mesoderm is thought to be extraembryonic, and its subsequent development is similar to that of the rhesus embryo; in the mouse, extraembryonic mesoderm is not prominent at the equivalent stage. By around day 14 of development in human embryos, the embryonic disk with its three germ layers is evident.

Comparing pluripotent mouse and human stem cells

To a certain degree, the use of hES cells as a developmental model, and the success of applying embryological principles to manipulate their growth and differentiation, depends upon understanding their relationship to the pluripotent cells of the embryo. Mouse ES cells can be derived with highest efficiency from the epiblast of the peri-implantation embryo (Brook and Gardner, 1997). However, in many respects, including their developmental capacity, mouse ES cells seem to resemble the inner cell mass (ICM) (Pelton et al., 2002).

Human and other primate ES cells are also derived from the ICM of the pre-implantation embryo, and share with mouse ES
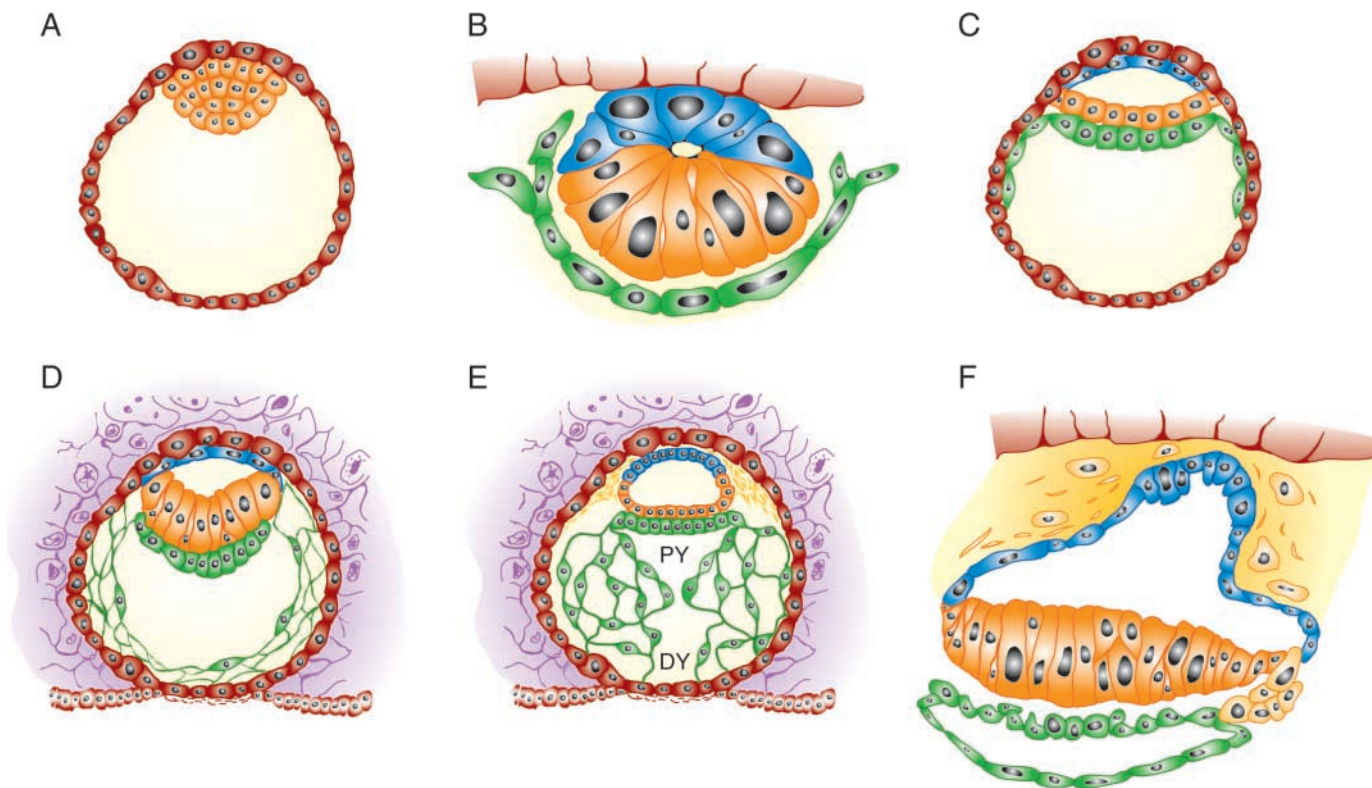
Fig. 1. Schematic of primate peri-implantation development. (A) A blastocyst-stage primate embryo showing the trophectoderm (red) and inner cell mass (ICM, orange). (B) A portion of a postimplantation day 10, rhesus macaque embryo, showing the development of the amnion (blue) through the cavitation of the ICM (orange), together with the trophectoderm (red) and extraembryonic endoderm (green). (C) A postimplantation day 9, human embryo, showing the extension of the extraembryonic endoderm (green) around the blastocoel cavity. (D) A postimplantation day 10.5, human embryo, showing the characteristic mesh of extraembryonic endoderm within the blastocoel cavity (green). Maternal tissue is shown in purple. (E) A day 11.5 human embryo, showing the extraembryonic mesoderm in yellow. (F) A portion of a day 13, rhesus macaque embryo, showing the formation of the primitive streak (light orange) from the caudal epiblast (dark orange), subsequent to the extensive development of the extraembryonic mesoderm (yellow). The secondary yolk sac is shown in green. Data from Enders et al., Hamilton and Luckett (Enders et al., 1986; Hamilton, 1972; Luckett, 1978).

cells the key biological properties of pluripotentiality and immortality (Reubinoff et al., 2000; Thomson et al., 1998). Unfortunately, to date, no studies have examined the ability of rhesus (Thomson et al., 1995), marmoset (Thomson et al., 1996) or cynomolgus (Suemori et al., 2001) monkey ES cells to participate in chimera formation following blastocyst injection, and so the assessment of the developmental capacity of primate ES cells is limited to data from teratoma formation in xenografts in immunodeprived mice. These studies show that such grafts contain a wide variey of cell types, often with a considerable degree of histotypic differentiation. However, the finding of multilineage differentiation in teratomas provides no information about the functional status of the differentiated cells and, of course, gives no indication of the ability of the ES cells to participate in normal embryogenesis.

Human and mouse ES cells also appear to express a set of genes that are found in pluripotent cell populations in the mouse embryo, including some that are known to be important in establishing or maintaining the pluripotent lineage. Many studies of the hES cell transcriptome (Bhattacharya et al., 2004; Richards et al., 2004; Sato et al., 2003; Sperger et al., 2003) have identified genes (see Table 1) that are expressed at a higher level in hES cells relative to differentiated ES cultures

or to other human cell types. Although the results of these studies vary appreciably (depending on the technology platform used, the cell populations to which the ES cells were compared, and the ES cell lines that were used), there are consistent findings. The overall pattern indicates that there may be a molecular blueprint for the pluripotent state, which is conserved across species (Pera et al., 2000). The most extensive conservation of gene expression between mouse and human is most likely to be amongst genes encoding transcriptional regulators or DNA-modifying enzymes. By contrast, a direct comparison of human and mouse ES cells identified many differences in the expression of cytokines between the two species (Ginis et al., 2004). It is noteworthy that the various studies did not all identify these genes as stem cell markers. Also, some molecules are notable for their absence; CD9, which is expressed by mouse and hES cells (Oka et al., 2002; Carpenter, 2004), was not reported as a prominently expressed gene in any of these assays. The embryonic markers defined by antibodies SSEA-1, -3 and -4 against cell-surface glycolipids are expressed differently in mouse and hES cells. The cell-surface proteoglycan recognised by several monoclonal antibodies reactive with hES cells, including TRA-1-60, TRA-1-81 and GCTM-2, is not detected

Table 1. A selective list of genes that are highly expressed in human ES cells relative to other cells types

Class of molecule	Name	Nature/function	ES cell specific*	Expressed in mouse ES cells
Transcription factor				
	POU5F1	Pluripotency	+	+
	NANOG	Pluripotency	+	+
	SOX2	Pluripotency	–	+
	FOXD3	Pluripotency	+	+
	UTF1	Transcriptional co-regulator	+	+
	REX1	?	+	+
	SRY	Sex determination	–	–
DNA modifying				
	TERF1	Telomerase	–	+
	CHK2	Cell cycle checkpoint	–	+
	DNMT3	DNA methyltransferase	–	+
Surface marker				
	GJA1	Gap junction protein	–	+
	CD24	Red cell, B cell development	–	?
	GCM1	CD34-related glycoprotein/podocyte development	–	?
Growth/differentiation factor				
	LEFTB	Axis determination	–	+
	CER1	BMP antagonist	–	?
	GDF3	TGF superfamily member, unknown function	+	+
	FGF2	Mitogen	–	–
	GAL	Neuropeptide	–	+
Receptor				
	TDGF1	Co-receptor for nodal	+	+
	ACVR2B	Activin/BMP receptor	–	+
	CRABP1	Retinoic acid binding	–	
	FZD5	WNT receptor	–	?
	FZD7	WNT receptor	–	?
	FGFR1	FGF receptor	–	–
	FGFR2	FGF receptor	–	–
	BMPR1A	BMP receptor	–	+
Other				
	STELLA	Maternal lethal	+	+
	FLJ10713	?	+	+

*A gene that is expressed in early embryos or primordial germ cells, ES cells, and a few other cell types.

A question mark indicates that expression is not known.

Additional references indicating gene function or specific expression in pluripotent cells: POU5F1 (Nichols et al., 1998); NANOG (Chambers et al., 2003); SOX2 (Avilion et al., 2003); FOXD3 (Hanna et al., 2002); UTF1 (Nishimoto et al., 1999); DNMT3 (Chen et al., 2003); GDF3 (Caricasole et al., 1998); TDGF1 (Baldassarre et al., 1997; Ding et al., 1998); STELLA (Goto et al., 2002; Payer et al., 2003).

on mouse ES cells by these reagents, but it is unclear whether these monoclonal antibodies show any cross-reactivity with mouse tissue.

There are also phenotypic differences between mouse and hES cells (Pera et al., 2000). Mouse cells grow in rounded colonies with a glassy appearance and indistinct cell borders, whereas hES colonies are flatter and often display more distinct cell borders. ES cells from the two species also show different growth regulation. Whereas both mouse and hES cells require feeder cell support, leukemia inhibitory factor (LIF) cannot substitute for a feeder cell layer in maintaining hES cells (Reubinoff et al., 2000; Thomson et al., 1998) or embryonal carcinoma cells (Pera et al., 1989). The basis of this lack of response to LIF is unclear; some researchers have attributed it to the poor activation of the STAT3 pathway in human cells following receptor engagement (Sato et al., 2004), while others suggest that it is because of the absence, or relatively low level of expression, of components of the LIF pathway (Ginis et al., 2004; Richards et al., 2004). There is a specific physiological requirement for LIF for pluripotent stem cell survival during embryonic diapause in the mouse (Nichols et al., 2001). Diapause, a resting state induced in the blastocyst to enable the mother to complete lactation of a previous litter prior to further development of the new conceptus, has no equivalent in the human, and because LIF is an example of a gene that is involved in both reproduction and regulation of the immune response, its function might have been subject to evolutionary pressures. It is possible that mouse ES cells derived or grown under different conditions, in the absence of LIF, might resemble their human counterparts more closely.

There are likely to be other differences in the extrinsic control of stem cell maintenance between the species. The MEK kinase pathway promotes differentiation in mouse ES cells (Nichols et al., 2001), but in the human, fibroblast growth factor 2 (FGF2) (Amit et al., 2000), which activates this pathway, can maintain hES cells in the undifferentiated state. Bone morphogenetic protein (BMP) 2 or BMP4, under serum-free culture conditions and in cooperation with an active gp130 signaling pathway, will inhibit neural differentiation and thereby assist in the maintenance of pluripotentiality in mouse

ES cells (Ying et al., 2003). In the human, BMPs will induce differentiation into extraembryonic lineages, either in the presence (Pera et al., 2004) or absence (Xu et al., 2002b) of serum.

Are the differences between mouse and hES cells related to genuine species differences in pluripotent cell phenotype, or do they reflect that the ES cells correspond to a different stage of embryonic development in the two species? Some data suggest that the same pattern of antigen expression is seen in the ICM of the human blastocyst and in hES cells. SSEA-3, SSEA-4 and TRA-1-60 were all found to be expressed in the ICM in a limited series of human pre-implantation blastocysts cultured in vitro (Henderson et al., 2002). As these markers are not expressed in the mouse ICM, this finding suggests that there are actual species-specific differences in the expression of these markers in pluripotent cells. Moreover, a general argument in favor of the existence of species-specific differences in pluripotent stem cell phenotype is that pluripotent cells in mice and humans show these differences irrespective of whether they originate directly from embryos or from primordial germ cells through the protracted process of teratocarcinogenesis (Pera et al., 2000). Thus, human embryonal carcinoma stem cells resemble hES cells, mouse embryonal carcinoma stem cells resemble mouse ES cells, and the two cell types show the same interspecies differences.

Human ES cell technology

Although our understanding of the regulation of human pluripotent stem cells is still limited, substantial recent advances in ES cell technology will facilitate their use in studying the cellular and molecular control of human development.

Deriving and maintaining human ES cells

Human ES cells were first successfully derived using mouse embryonic fibroblast feeder cells and serum-containing medium (Reubinoff et al., 2000; Thomson et al., 1998), in a culture method that has since been widely used (Cowan et al., 2004; Hovatta et al., 2003; Mitalipova et al., 2003; Park et al., 2003; Reubinoff et al., 2000; Richards et al., 2002). Recently, one group described the derivation of a hES cell line from a blastocyst developed through somatic cell nuclear transfer (Hwang et al., 2004), a technique that might prove useful for generating histocompatible ES cell lines, or ES cell lines from individuals with known genetic predisposition to disease. Interestingly, in contrast to mouse ES cell work, hES cells with a diploid XX genotype can be readily established and maintained. A lack of X-inactivation and X-chromosome dosage compensation could account for the difficulty in maintaining XX mouse ES cells, but the status of X-inactivation in hES cells is unknown.

Technical advances have partially overcome some of the limitations of the original systems for culturing hES cells, such as the spontaneous differentiation of the cells and the need to mechanically dissect ES colonies for subculture. A serum-free system based on combining a proprietary serum substitute and FGF2 enables the propagation of cultures with a higher proportion of stem cells. This system removes the need to mechanically isolate stem cells, which can instead be dissociated enzymatically (Amit et al., 2000). This technique has been widely adapted for the routine growth of hES cells.

However, it has only modestly improved the cloning efficiency of hES cells, and because the proprietary serum replacement used has an undefined protein component, it is possible that it may modulate the effects of added differentiation inducers in an unknown fashion. In one modification of this technique, the feeder cell component is replaced with Matrigel, an extracellular matrix (ECM) preparation, and conditioned medium from the feeder cell layer (Xu et al., 2001). This system enables the long-term maintenance of the stem cell phenotype, with strong suppression of the spontaneous differentiation observed at high passage levels (Carpenter et al., 2004). Amit and co-workers (Amit et al., 2004) have reported that the combination of FGF2, TGFβ, LIF and a proprietary serum replacer can achieve serum-free, feeder-free maintenance of hES cells on a fibronectin ECM. A recent report also suggests that Wnt signaling modulation can support the short-term maintenance of some stem cell markers in hES cell cultures in the absence of a feeder cell layer (Sato et al., 2004). However, new hES cell culture methods must undergo testing to ensure that the key properties of pluripotent stem cells are maintained and that the system does not select for karyotypically altered cells (Draper et al., 2004). No culture method to date enables high-efficiency clonal propagation of hES cells.

Manipulating human ES cells

Developing improved technology for the genetic manipulation of hES cells will also be crucial for their effective application in research. Although hES cells can be modified by transgenesis and gene targeting, there are still questions over the efficiency of the techniques in different cell lines. The generation of stable transformants of hES cells has been achieved using conventional DNA delivery systems (Eiges et al., 2001), or through the use of lentiviral (Gropp et al., 2003; Ma et al., 2003) or adenoviral (Smith-Arica et al., 2003) vectors. One group (Zwaka and Thomson, 2003) used gene targeting via electroporation to obtain homologous recombination in hES cells at frequencies similar to those observed in mouse ES cells. The use of short interfering (si)RNA to knockdown gene expression is another methodology that holds promise for use in ES cell research, and several recent reports have shown that this technique can be used to knockdown gene expression in ES cells (Hay et al., 2004; Vallier et al., 2004).

ES cell differentiation: a model for embryonic cell commitment

It seems unlikely that hES cells, at least as we understand them currently, will yield information that is of direct relevance to the mechanisms of patterning, axis formation or segmentation in the primate embryo. There is no evidence to date that differentiating ES cells can reproducibly generate the spatial organisation of embryonic and extraembryonic tissue that is seen in the embryo in vivo.

However, hES cells might provide important new information about the cellular and molecular basis of commitment and differentiation events during human development. To enable mechanistic studies of the events controlling the formation of a specific cell lineage, the differentiation system should meet specific criteria, as outlined in Box 2. Well-defined differentiation methodology,

the ability to manipulate gene expression through siRNA, transgenesis or targeted genetic modification, and the use of engineered lineage-specific reporters, will enable the study of gene expression and function in a human developmental context.

Spontaneous differentiation

Most early reports of hES cell differentiation studied spontaneous differentiation in vitro, either during long-term maturation of adherent cell layers in situ (Reubinoff et al., 2000), or after the formation of embryoid bodies (Itskovitz-Eldor et al., 2000) (see Fig. 2). The formation of embryoid bodies, three-dimensional multicellular structures formed by non-adherent cultures of differentiating ES cells (see Fig. 2B), is thought to mimic the environment of the peri-implantation embryo, where interactions between various cell types facilitate inductive events. The spontaneous differentiation of adherent ES cell cultures in situ has not been widely studied, but it is likely that the interactions between the various cells is similar to that which occurs in embryoid bodies.

The mouse ES cell embryoid body first forms a bilayered structure with extraembryonic endoderm on the outside and primitive ectoderm on the inside (Doetschman et al., 1985). Although there is little evidence for continued spatial regulation of cell differentiation beyond this rudimentary relationship, detailed studies of gene expression during the differentiation of mouse ES cells into hematopoietic cells, or neural differentiation in embryoid bodies, strongly suggest that the temporal sequence of events is very similar to that in postimplantation mouse embryos cultured in vitro (Loebel, 2003). It is not clear that embryoid bodies formed by hES cells display any consistent structural organisation of the extraembryonic endoderm and primitive ectoderm, as mouse embryoid bodies do.

Investigators have used various means to influence the outcome of differentiation in embryoid bodies, such as treating them with growth factors or with differentiation inducers, such as retinoic acid (Schuldiner et al., 2000). Although these treatments can influence the outcome of differentiation, they still generally result in a mixed population of cells that is enriched only to a limited degree for the cell of interest. Nonetheless, when combined with the selection for certain cell types, based on their expression of surface markers (Levenberg et al., 2002), by using lineage-specific promoters to drive selectable marker genes, or by using selective culture methodology (Reubinoff et al., 2001), spontaneous differentiation does enable the isolation and analysis of lineage-committed human progenitor cells from ES cultures.

> **Box 2. Desirable features of an in vitro differentiation system**
> • Commitment or differentiation events can be induced reproducibly in a controlled, stepwise fashion.
> • Most of the cell population responds to an inducer.
> • Factors that control the induction are well defined.
> • It is possible to identify, propagate and expand progenitor cells at various stages along the lineage.
> • These progenitors yield differentiated end cells with expected patterns of marker and gene expression and functional capacity.

Directed differentiation

Both co-culturing ES cells with inducing cells and treating cultures with growth factors have been used to direct the differentiation of hES cells. The effect of these two treatments can be based on one or both of two mechanisms: inducing differentiation along a lineage of interest, or the enhanced growth or survival of a spontaneously differentiating cell population. It is not always easy to delineate these effects in the experiments described to date, and, although in principle it should be possible to identify the key factor(s) produced by the inducing cell lines in co-culture experiments, the effects of co-culture are complex and often involve multiple factors.

Human ES cells have now been shown to differentiate into the following cell types in vitro: neural progenitors and cells differentiated thereof (Carpenter et al., 2001; Reubinoff et al., 2001; Schuldiner et al., 2001; Schulz et al., 2003), blood cell precursors (Chadwick et al., 2003; Kaufman et al., 2001), endothelial cells (Gerecht-Nir et al., 2003; Levenberg et al., 2002), osteogenic cells (Sottile et al., 2003), cardiomyocytes (Kehat et al., 2001; Mummery et al., 2003; Xu et al., 2002a), insulin producing cells (Assady et al., 2001), hepatocytes (Rambhatla et al., 2003), keratinocytes (Green et al., 2003) and trophoblast cells (Xu et al., 2002b). The different ways in which these cell types have been induced, and the various inducing factors that have been identified and used in these experiments, are discussed below.

Differentiating lineages from ES cells in vitro

Extraembryonic lineages

Two studies have shown that hES cells can be used to study the formation of extraembryonic tissues from pluripotent cells, thus modelling the first commitment events in mammalian development.

In the first study, hES cells grown under serum-free conditions with BMP4 were induced to form flat epithelial cells that express many of the genes associated with trophoblast or placental development (Xu et al., 2002b). In this study, early trophoblast-lineage-specific genes, such as *MSX2*, were switched on very rapidly and expression remained elevated. With prolonged BMP4 treatment, markers of fully differentiated trophoblast cells, such as human chorionic gonadotrophin, were activated, and human chorionic gonadotrophin, estradiol and progesterone were secreted into the culture medium. Some cells underwent fusion to form syncytial giant cells that expressed human chorionic gonadotrophin. The time-lapse studies carried out by Xu et al. suggested that BMP4 directly effects differentiation, rather than cell survival (Xu et al., 2002b).

Although there is no evidence that BMPs are involved in trophoblast differentiation in the mammalian embryo, this culture system could be used to analyse the early stages of commitment to the trophoblast lineage.

By contrast, in the second study, hES cells treated with BMP2 or related molecules, in the presence of serum and a feeder cell layer, differentiate into flattened epithelial cells that express genes characteristic of the extraembryonic endoderm (Pera et al., 2004), as reported previously in human embryonal carcinoma cells treated with BMP2 (Pera and Herszfeld, 1998).

Thus, the differentiation of two main extraembryonic lineages in the human embryo, trophoblast and extraembryonic endoderm, can be analysed using hES cell cultures. The

controlled differentiation of ES cells into these two lineages will provide important information on the molecular regulation of these crucial events in peri-implantation development (Hay et al., 2004).

Ectodermal lineages

The neural differentiation of hES cells has been studied by several groups (Carpenter et al., 2001; Reubinoff et al., 2001; Schuldiner et al., 2001; Schulz et al., 2003; Zhang et al., 2001). The first three studies (Carpenter et al., 2001; Reubinoff et al., 2001; Schuldiner et al., 2001) used the spontaneous differentiation of ES cells as a starting point for the isolation and culture of highly purified populations of neural progenitors using selective serum-free culture conditions. These progenitors could be cultivated for ~25 population doublings (Reubinoff et al., 2001) as neurospheres in suspension culture, and they expressed markers of the early neuroectoderm, such as nestin, polysialylated N-CAM, musashi and Pax6. The neural progenitor cells could differentiate into neurons and astrocytes, and, to a minor degree, into cells expressing oligodendrocyte markers. However, rigorous proof that the progenitor cells were actually stem cells (such as studies of the differentiation of progenitor clones into neural lineages) was not obtained. Following engraftment into newborn mouse brains, the precursor cells survived, migrated out from the injection site, and underwent regionally appropriate differentiation (Reubinoff et al., 2001; Zhang et al., 2001). Electrophysiological studies showed that the ES-derived neurons could also respond to neurotransmitters in vitro (Carpenter et al., 2001). The use of retinoic acid and nerve growth factor (Schuldiner et al., 2001), or conditioned medium previously shown to induce ectoderm differentiation of mouse ES cells (Schulz et al., 2003), enhanced the yield of neuronal cells from hES cell embryoid bodies.

As noted above, we have recently reported a paracrine loop involving BMP-driven differentiation of hES cells into the extraembryonic endoderm (Pera et al., 2004). This study showed that a blockade of BMP signaling by noggin, a BMP antagonist, caused hES cells to differentiate into an intermediate cell type that lacked neural markers, but that could be easily converted into neural progenitor cells upon transfer into suspension culture in basal medium supplemented with FGF2. This effect of noggin is consistent with its role in neurogenesis in the embyro.

In several interesting studies of cynomolgus monkey ES cells (Kawasaki et al., 2002; Mizuseki et al., 2003), neural differentiation has been induced by co-culturing ES cells with a PA6 cell line that produces an undefined, cell-associated differentiation inducer known as SDIA (stromal cell derived inducing activity). The co-culture of cynomolgus monkey ES cells with PA6 cells results in 35% of the ES cells forming tyrosine hydroxylase-positive cells, which also express the transcription factors NURR1 and LMX1b, thus confirming their identity as midbrain neuronal cells. SDIA induces neural differentiation of mouse ES cells in about the same time scale as that in the embryo, whereas the time that it takes to induce monkey ES cells to form midbrain neurons is longer than in cultures of mouse ES cells, but far more rapid than during monkey development and neurogenesis in vivo. Further experiments showed that these neural precursors could be committed to regionally specific fates. The authors suggested
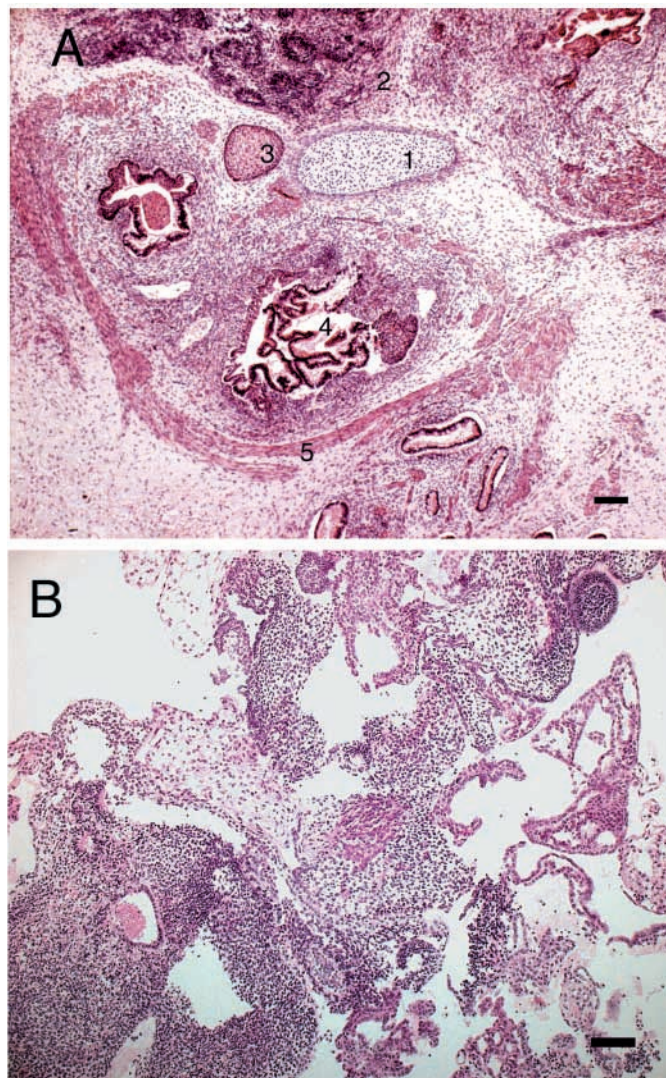

Fig. 2. Spontaneous differentiation of human ES cells. (A) Micrograph of a Hematoxylin and Eosin-stained section of a typical teratoma following the grafting of human ES cells into the testis capsule of immunodeficient mice, showing the presence of cartilage (1), primitive neural cells (2), stratified squamous epithelium (3), glandular epithelium (4), muscle (5) and other cell types. (B) Histological section of a human ES cell embryoid body. Scale bars: A, 800 µM; B, 100 µM.

that SDIA induced the ES cells to form ectoderm, which spontaneously gave rise to rostral CNS precursors; further treatment with appropriate inducers, such as sonic hedgehog (SHH), can yield cells that express markers characteristic of the full range of dorsal and ventral neural fates.

In studies of the neural differentiation of primate stem cells, it has therefore been possible to identify factors (such as SDIA and noggin) that predispose ES cells to undergo transition to neuroectoderm, to isolate early neural precursors, to convert them into neurons or astrocytes, and to direct regionally specific patterns of CNS differentiation. These model systems thus provide a means to study the cellular and molecular basis of the early stages of neurogenesis in the human embryo, and enable these events to be directly compared with those in the mouse.

Recently, Green and co-workers (Green et al., 2003) described the stepwise differentiation of keratinocytes, representing another ectodermal lineage, from hES cell-derived embryoid bodies that were replated onto monolayers following suspension culture. The transcription factor p63, required in the mouse for the development of the epidermis and its appendages, was first expressed, followed by markers of more mature stages of the keratinocyte lineage, including cytokeratin 14 and basonuclin.

Mesodermal lineages

Hematopoietic precursors have been obtained from hES cells by either co-culturing or by inducing them with growth factors. In the first study to isolate blood cell progenitors from hES cells, Kaufman et al. (Kaufman et al., 2001) cultured ES cells on marrow stromal or yolk sac-derived cell lines, and monitored the culture for the expression of blood cell lineage markers, such as CD34. The appearance of CD34$^+$ cells peaked at 17 days, at a level of 1-2% of the total cells, and declined thereafter; these cells were also CD45$^-$ (a general marker for hematopoetic cells), but many were CD31$^+$ (a marker of the endothelial lineage). These progenitors could form both erythroid and myeloid colonies in agar. The expression of adult and fetal hemoglobin, but not embryonic globin, was observed during erythroid differentiation.

Chadwick et al. (Chadwick et al., 2003) used a combination of embryoid body formation and treatment with various hematopoietic cytokines, plus BMP4, to induce the formation of hematopoietic progenitors from hES cultures. The combination of BMP4 and the cytokines SCF, FLT3 ligand, IL3, IL6 and granulocyte colony stimulating factor (GCSF) enhanced the yield of CD34$^+$CD45$^+$ cells from embryoid bodies cultures by almost sixfold. The CD34$^+$CD45$^+$ phenotype is similar to that of hematopoietic precursors identified from the dorsal aorta of humans. The precursors could form erythroid and myeloid colonies in agar, and the yield of colony-forming units, which showed some capacity for self renewal, was greatly enhanced by BMP4. The results were consistent with the direct induction of hematopoietic differentiation by the cytokines, or with an action of the factors on the multiplication and survival of an early precursor cell (hemangioblast) in the embryoid body.

Human ES cells have also been shown to differentiate into endothelial cells in embryoid bodies (Levenberg et al., 2002). At 13-15 days following embryoid body formation, transcripts of CD31 (P-selectin) and VE-cadherin were detected. Expression of CD34 and GATA2 was also elevated in the differentiating cultures, as expected during the early phases of endothelial cell differentiation. Curiously, unlike mouse ES cells, hES cells themselves expressed VEGF-R2, as well as the receptor TIE2 and AC133; the expression of these genes is characteristic of differentiating endothelial cells. CD31 expression was used to isolate the putative endothelial cell precursors by flow cytometry, with positive cells comprising about 2% of the population. The further culturing of sorted cells produced cells that expressed markers of the mature endothelium, such as von Willebrand factor; they could also uptake low-density lipoprotein. Furthermore, they formed tube-like structures in vitro, and functional microvessels when grafted into immunodeprived mice on artificial matrices in vivo.

Cynomolgus monkey ES cells have also been shown to differentiate into endothelial precursors by culturing them on a feeder cell layer of OP9 cells (Sone et al., 2003). A VEGF-R2$^+$ VE-cadherin$^-$ cell population was isolated by flow cytometry from these cultures, and under different conditions differentiated into either CD31$^+$VE-cadherin$^+$ endothelial cells, or into vascular mural cells (vascular smooth muscle cells or pericytes).

Cardiac muscle has also been derived from either spontaneously differentiating hES cells or from co-culture systems. Kehat and colleagues (Kehat et al., 2001) isolated beating cardiomyocyte foci from spontaneously differentiating human embryoid bodies, and showed that the cells had properties of fetal or neonatal cardiocytes, as evidenced by their subcellular distribution of gap junctions, their myofibrillar organisation, and their electrical activity. Xu et al. (Xu et al., 2002a) also exploited the spontaneous generation of beating foci in embryoid bodies to study cardiomyocyte differentiation. Beating foci were observed in up to 70% of embryoid bodies in this study. The cells expressed specific myocardial markers, including cardiac troponin 1 and alpha myosin heavy chain, as well as the transcription factors Nkx2.5, GATA4 and MEF2. The expression of atrial natriuretic factor characterised the ventricular myocytes as developing cells, and the study showed that the expression of adrenergic receptors underwent maturation patterns in culture that were similar to those seen in the developing heart in vivo. Recent studies have closely analysed the phenotype of the immature cardiac cells formed in human embryoid bodies electrophysiologically (Amit et al., 2003), and have documented the maturation of these cells by the ultrastructural analysis of sarcomere development (Snir et al., 2003).

By contrast, the hES cell lines studied by Mummery et al. (Mummery et al., 2003) showed no tendency to spontaneously differentiate into cardiac muscle. This group co-cultured ES cells with the END-2 murine cell line, which is thought to resemble visceral endoderm and has been shown previously to induce cardiac differentiation of mouse ES and EC cells. The END-2 line caused hES cells to differentiate into two lineages, one exemplified by cysts that express genes characteristic of the visceral endoderm, and the other consisting of foci of beating muscle. Sarcomeric organisation, the expression of atrial natriuretic factor and the L-type calcium channel, and an action potential similar to fetal ventricular cells indicated that the cells were immature cardiomyocytes.

Thus, several mesodermally derived cell types can be isolated from hES cells and their maturation studied in vitro. The cells involved in the earliest stages of ES commitment to mesoderm formation are unknown, and the factors that induce mesodermal progenitors and control their subsequent development remain largely uncharacterised. However, with further study, it should prove possible to isolate cells at early stages of mesoderm differentiation and to identify factors that drive their commitment to particular mesodermal lineages.

Endodermal lineages

The differentiation of hES cells into embryonic endodermal lineages has been more difficult to achieve. As in the mouse, studies are hampered by an incomplete understanding of the commitment and differentiation of embryonic endoderm in the embryo proper, by a lack of specific markers for the early

progenitors of this lineage, and by an overlap of gene expression patterns between extraembryonic and embryonic cell lineages. One study (Assady et al., 2001) has demonstrated that genes characteristic of the pancreatic lineage are switched on during human embryoid body formation, and cells stained by anti-insulin antibodies have been found in human embryoid bodies. However, little is known about what controls the appearance of these cells or their precursors. Another group (Rambhatla et al., 2003) have shown that hES cells grown as embryoid bodies and treated with sodium butyrate, or adherent hES cultures treated with dimethyl sulphoxide followed by sodium butyrate, differentiate into cells that express various hepatocyte markers.

Future prospects

The first five years of hES cell research have achieved some significant objectives. The original observations on the derivation and properties of hES cells have been repeatedly confirmed, and the technology has been successfully disseminated to a number of research groups. The ethical debate over the use of human embryos in research is unlikely ever to be fully resolved, as it is driven by religious and philosophical considerations of the nature of human existence, in addition to scientific and practical considerations. However, in most jurisdictions, extensive discussion, debate, and, ultimately, compromise have led to the formulation of laws and regulations that enable the work to progress on an ethical basis. It is most important for the future that researchers can generate and gain access to new hES cell lines derived using improved technologies.

Several key questions remain to be answered in the coming years. For example, what are the functions of the genes that maintain the pluripotentiality of hES cells? And what do the early differentiated progeny of hES cells represent in relation to the primate peri-implantation embryo, and why are there species-specific differences in the biology of cultured pluripotent cells? Our ability to grow and manipulate the cells has improved markedly, but there are several technical challenges that have still not been met, such as the large-scale propagation of pure ES cell cultures in defined media in the absence of feeder cells, their clonal growth, and easy and efficient ways to genetically manipulate them.

We also need to better understand the events that control ES cell commitment and differentiation. The appearance of a low proportion of differentiated cells in a complex mixture complicates the interpretation of the currently available data, and obscures whatever cell lineage relationships and cell interactions may have led to the final outcome. However, it has been possible to isolate and identify progenitors, to study their gene expression, and to assess the effect of exogenous factors on their generation, proliferation and differentiation. As in the mouse (Loebel et al., 2003), there is some evidence that the behaviour of hES cells can be predicted from what we know of mammalian embryogenesis, but the data on hES cell differentiation are very limited as yet. In the future, it is likely that high throughput screening approaches (Ding et al., 2003) will complement embryological studies in the effort to discover new means of manipulating ES cells. This approach could identify synthetic or natural small molecules that could serve as lead compounds for pharmaceutical development in the field of regenerative medicine.

It is important to recognise that ES cell differentiation events and the extrinsic factors that control them may prove to be extremely context dependent. ES cells may have the ability to respond to normal developmental cues, but the cells are in a totally different environment to the embryo. These considerations notwithstanding, progress during the first five years of hES cell research strongly suggests that these versatile cells will provide an important resource for understanding human development, alongside their anticipated roles in regenerative medicine.

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