Annu. Rev. Cell Dev. Biol. 2001. 17:435–62

EMBRYO-DERIVED STEM CELLS: Of Mice and Men

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Key Words self-renewal, pluripotency, differentiation, cell therapy, epiblast

■ **Abstract** Mouse embryonic stem cells are continuous cell lines derived directly from the fetal founder tissue of the preimplantation embryo. They can be expanded in culture while retaining the functional attributes of pluripotent early embryo cells. In particular, they can participate fully in fetal development when reintroduced into the embryo. The capacity for multilineage differentiation is reproduced in culture where embryonic stem cells can produce a wide range of well-defined cell types. This has stimulated interest in the isolation of analogous cells of human origin. Such human pluripotent stem cells could constitute a renewable source of more differentiated cells that could be employed to replace diseased or damaged tissue by cellular transplantation. In this review, the relationships between mouse embryonic stem cells, resident pluripotent cells in the embryo, and human embryo-derived cell lines are evaluated, and the prospects and challenges of embryo stem cell research are considered.

This review is dedicated to Rosa Beddington FRS, a great developmental biologist, a wonderful colleague, and an inspirational advocate of human stem cell research.

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1081-0706/01/1115-0435$14.00

ORIGINS AND PROPERTIES OF MOUSE EMBRYONIC STEM CELLS

Teratocarcinoma and Embryonal Carcinoma

In 1970, a striking observation was reported: Early mouse embryos grafted into adult mice produced teratocarcinomas (Solter et al. 1970, Stevens 1970). Teratocarcinomas are malignant multidifferentiated tumors containing a significant population of undifferentiated cells. Previously, studies of spontaneously occurring teratocarcinoma (reviewed by Stevens 1983) had established that the undifferentiated component, embryonal carcinoma (EC), could be propagated in culture. This also proved to be true for embryo-derived teratocarcinoma. The derivative EC cell lines can be expanded continuously but can also differentiate either in vitro or via teratocarcinoma formation. Most importantly, clonally isolated and expanded EC cells retain the capacity for differentiation and may produce derivatives of all three primary germ layers: ectoderm, mesoderm, and endoderm (Kleinsmith & Pierce 1964, Martin & Evans 1975). Therefore, individual EC cells are self-renewing, pluripotent stem cells. The presence and expansion of embryonal carcinoma in embryo-derived teratocarcinomas suggested that the epiblast founder cells of the embryo (Hogan et al. 1994), which are normally present only transiently, may in fact be predisposed for conversion to EC cells and thus for continuous self-renewal.

The epiblast origin of the EC cells was evidenced by the fact that teratocarcinomas can be produced only from embryos prior to gastrulation and from grafts containing epiblast but not from other regions of the early embryo (Diwan & Stevens 1976). Consistent with this origin, EC cells show a range of phenotypic features in common with epiblast cells (reviewed by Martin 1980). When this relationship was subjected to the ultimate test, reintroduction into the developing embryo (Brinster 1974), it was found that some EC cell lines could participate in embryogenesis and contribute derivatives to a variety of tissues in resultant chimaeric fetuses and liveborn mice. Thus EC cells can remain receptive to cues in the embryo microenvironment in an equivalent manner to resident epiblast cells. However, this proved to be the exception rather than the rule. Most EC cell lines show poor differentiation potential in vitro and in vivo and contribute poorly to chimeras and/or produce embryonic tumors. Moreover, EC cells are almost

always aneuploid, likely the result of uncontrolled selection pressures during tumor growth. Consequently, they are not capable of proceeding through meiosis to produce mature gametes.

The concept that stem cells for the mammalian embryo could be isolated and continuously cultured while retaining full developmental potency and identity therefore remained open to question for some years.

Derivation of Embryonic Stem Cells

Studies with EC cells did eventually pave the way for the establishment of "true" embryo stem cell cultures, however. A critical point was the appreciation that EC cell pluripotency was best sustained by co-culture. In primary teratocarcinoma explant cultures, EC cells tended to thrive in proximity to differentiated cell types but expanded poorly in isolation. This prompted investigation of the potential of established cell lines to support EC cell propagation. Co-culture with mitotically inactivated embryonic fibroblasts was found not only to allow the efficient establishment of EC cultures but also to result in stem cells with high differentiation capacity (Martin & Evans 1975, Martin et al. 1977). It was reasoned that the fibroblasts were providing some critical nutrient or trophic factor support, hence they were described as feeder cells.

These workers then investigated the culture of primary embryo cells in the presence of feeder. In 1981, the derivation of pluripotent cell lines directly from mouse blastocysts was reported (Evans & Kaufman 1981, Martin 1981). The protocols for embryonic stem (ES) cell derivation are relatively simple and remain unchanged to the present day (Robertson 1987). Embyros at the expanded blastocyst stage are plated, either intact or following immunosurgical isolation of the inner cell mass (ICM: forebear of the epiblast), onto a feeder layer. Conventional tissue culture medium is supplemented with 2-mercaptoethanol and 10–20% fetal calf serum. After several days of culture, the epiblast-derived cell mass is dissaggregated and replated onto fresh feeders. Various types of differentiated colonies arise along with colonies of a characteristic undifferentiated morphology (Robertson 1987). The latter are individually dissociated and replated. If secondary colonies of undifferentiated cells arise, these can generally be expanded further and continuous ES cell lines established. They proliferate rapidly in culture, and clonal populations can readily be initiated from single cells.

ES cells closely resemble EC cells in morphology, growth behavior, and marker expression. This relationship extends to the capacity to give rise to teratocarcinomas. ES cells produce tumors containing well-differentiated mesodermal, ectodermal, and endodermal tissue and cell types (Evans & Kaufman 1983). The ability to give rise to teratomas is a defining feature of pluripotent mouse embryo cells shared by ES and EC cells, and by germ cells (see below). The proportion of undifferentiated stem cells tends to be less in ES than in EC cell-generated tumors, most likely reflecting the EC cell history of selection for growth in teratocarcinomas and consequent transformation. An unresolved question is whether stem

cells re-isolated from a primary ES cell-derived teratocarcinoma would retain full developmental potential or would be compromised in the manner of EC cells.

Embryonic Stem Cell-Derived Mice

The most extraordinary attribute of ES cells is that, even after extended propagation and manipulation in vitro, they remain capable of re-entering embryogenesis (Figure 1). Several techniques can be used to introduce ES cells into the pre-implantation mouse embryo where they then colonize all fetal lineages plus yolk sac mesoderm, allantois, and amnion (Bradley et al. 1984). Consistent with their epiblast origin, ES cells contribute poorly to extraembryonic endoderm and rarely, if ever, to trophoblast (Beddington & Robertson 1989). In contrast to EC cells, ES cells behave relatively consistently in their ability to integrate into the embryo and produce viable chimeras. No systematic bias is detected in the colonization patterns of ES cells, and they produce functional differentiated progeny in all tissues and organs. Genetic coat color markers therefore provide a simple and reliable means of monitoring overall chimaeric contribution in liveborn offspring. Transgenic marker genes such as β-galactosidase or green fluorescent protein (eGFP) are used to visualize ES cell contributions to developing embryos (Figure 2).

ES cells maintain a diploid karyotype. This is crucial because a balanced chromosome complement is necessary for meoisis. Thus unlike EC cells, if ES cells colonize the germ cell lineage in a chimera, they are capable of generating functional gametes. The landmark of deriving mice from cultured stem cells was reported by the Evans laboratory in 1984 (Bradley et al. 1984). In the early days of ES cell culture, germ line transmission was often elusive. Retention of germ line competence depends absolutely on adherence to a rigorous tissue culture regime, with avoidance of any untoward selective pressures such as overgrowth or nutrient deprivation. Of course, random mutational events inevitably occur in the culture, and epigenetic modifications may also arise, for example, loss of imprinting (Dean et al. 1998). Therefore, it is advisable to use low-passage stocks and/or to isolate new sub-clones periodically. In addition, the extent of contribution of strain 129 ES cells to chimeras is strongly influenced by the genotype of the recipient embryo. In particular, microinjection into C57BL/6 blastocysts results in very high ES cell contributions and a greatly increased frequency of germ line transmission (Schwartzberg et al. 1989).

Nowadays, production of germ line ES cell chimeras is a standard procedure in many laboratories. The capacity to introduce DNA into ES cells by a variety of infection or transfection protocols and the facility of clonogenic expansion allow precise genetic modifications to be created and then delivered into mice (Robertson 1986). Indeed, the principal interest of ES cells to date has been their utility as cellular vectors for engineering of the mouse genome. Such applications have been reviewed extensively elsewhere (Bradley et al. 1992, Joyner 1991, Zheng et al. 2000) and are not considered further here.

A surprising feature of mouse ES cell lines is that the great majority are 40XY. In XX ES cells, as in epiblast, both X chromosomes are active, a situation that appears to be unstable or else disadvantageous for ES cell propagation (Rastan & Robertson 1985). In any case, the XY genotype confers appreciable advantages for establishing germ line transmission. Not only can male chimeras produce more offspring than females, but XY cells can convert the indifferent genital ridge of an XX recipient embryo into testicular development. Because XX germ cells do not develop in a male gonad, this phenomenon of sex conversion results in chimeric males in which all the spermatocytes are of ES cell origin (Bradley et al. 1984).

ES cells are sometimes described as totipotent, referring to the fact that they can give rise to germ cells. However, mouse ES cells cannot produce all types of cells (Figure 1), in particular they do not produce trophectoderm. They cannot generate a blastocyst de novo and hence are not sufficient to produce an embryo. Therefore, in this author's opinion the term totipotent is best reserved for the fertilized egg and blastomeres of the cleavage stage embryo that actually do have total developmental potency, whereas ES cells are more accurately described as pluripotent.

The issue of whether ES cells are self-sufficient for generation of the fetal component of the conceptus has been addressed by Nagy and co-workers (Nagy et al. 1991, 1993) who introduced ES cells into tetraploid-recipient embryos. In tetraploid embryos, extraembryonic lineages are produced normally but fetal lineages develop poorly. Consequently, in chimeras between tetraploid and diploid embryos, the fetus becomes composed almost exclusively of diploid cells. ES cells show a similar propensity to dominate the tetraploid contribution, and such fetuses can develop to term with few if any persisting tetraploid cells. Thus it can be argued that ES cells alone are competent to generate the entire fetus. However, a resident tetraploid ICM compartment is present at the time the ES cells are introduced and is only subsequently diluted out. To date, fetal development has not been reported following microsurgical replacement of the ICM or epiblast with ES cells. Therefore, the requirement for a normalizing signal from the host ICM to induce ES cells to re-enter into an embryonic differentiation program should not be discounted.

Although liveborn offspring may be obtained from ES cell-tetraploid chimeras, many embryos die in utero, and those that do develop to term usually die at or shortly after birth. This does not happen with ICM/tetraploid chimeras. It is likely that cryptic epigenetic and/or mutational changes arise during derivation or propagation of the ES cells (Dean et al. 1998). Such changes can be masked in diploid chimeras, particularly if they are noncell autonomous. Consequently, ES cells that give good somatic and germ line colonization in diploid chimeras often perform poorly in the tetraploid setting (Nagy et al. 1993). Recent studies of nuclear transfer using ES cells as donors have also resulted in offspring that die at birth. These two phenomena may have common causality. It is intriguing that for both nuclear transfer and tetraploid chimeras, long-term survivors can be obtained if the ES cells are of a hybrid genetic background (Rideout et al. 2000).

Embryonic Germ Cells

Teratocarcinomas also occur spontaneously, apparently originating from germ cells. Testicular teratocarcinoma is particularly prevalent in strain 129 mice. In the absence of methods for propagating germ cells in vitro, the evidence that they can give rise to embryonal carcinoma remained something of a curiosity. Following molecular cloning of the *Steel* growth factor and the cytokine leukemia inhibitory factor (LIF) (see below), limited culture of germ cells became possible (Matsui et al. 1991). Building on this, Matsui et al. (1992) found that on further addition of basic fibroblast growth factor (FGF-2) to the cultures, mouse primordial germ cells (PGC) converted after several days into cells resembling ES cells that could then be maintained indefinitely. These cells are termed embryonic germ (EG) cells to denote their origin. In most respects they are indistinguishable from blastocyst-derived ES cells and, at least for some lines, can contribute efficiently to chimeras and give germ line transmission (Labosky et al. 1994, Stewart et al. 1994). However, imprinted gene status is progressively erased during germ cell development, and this compromises the developmental potential of EG cells derived from later stage PGCs (Tada et al. 1998). Interestingly, EG cells appear to retain the capacity of germ cells to erase imprints (Tada et al. 1997), a property that has not been shown in ES cells.

PROPAGATION OF EMBRYONIC STEM CELLS

Extrinsic Control of ES Cell Propagation

ES cells can routinely be expanded to give relatively homogenous, undifferentiated populations (Figure 3), as judged by morphology and expression of a range of markers. The reproducible colonization of chimeras from injection of a few cells and the efficient generation of equipotent subclones confirm that ES cells undergo symmetrical self-renewal. Their expansion can be continued indefinitely, and very large (10^9–10^{10}) populations of substantially pure stem cells can be rapidly generated. In fact, ES cells appear to be immortal and show no evidence of either crisis or senescence in contrast to other primary cultures.

However, maintenance of the undifferentiated stem cell phenotype is not cell-autonomous. Media containing all necessary metabolites and nutrients are not sufficient to support either derivation or maintenance of ES cells. Co-culture with a feeder layer was originally considered essential. Subsequently, it was discovered that the feeders could be substituted by conditioned media preparations (Smith & Hooper 1987). This suggested that the critical requirement is to provide trophic stimulation, without which the ES cells differentiate. This interpretation was confirmed by the subsequent finding that a single cytokine, LIF, could sustain ES cell self-renewal in the absence of feeders (Smith et al. 1988, Williams et al. 1988). LIF is produced by feeder cells, and this expression is stimulated by the presence of ES

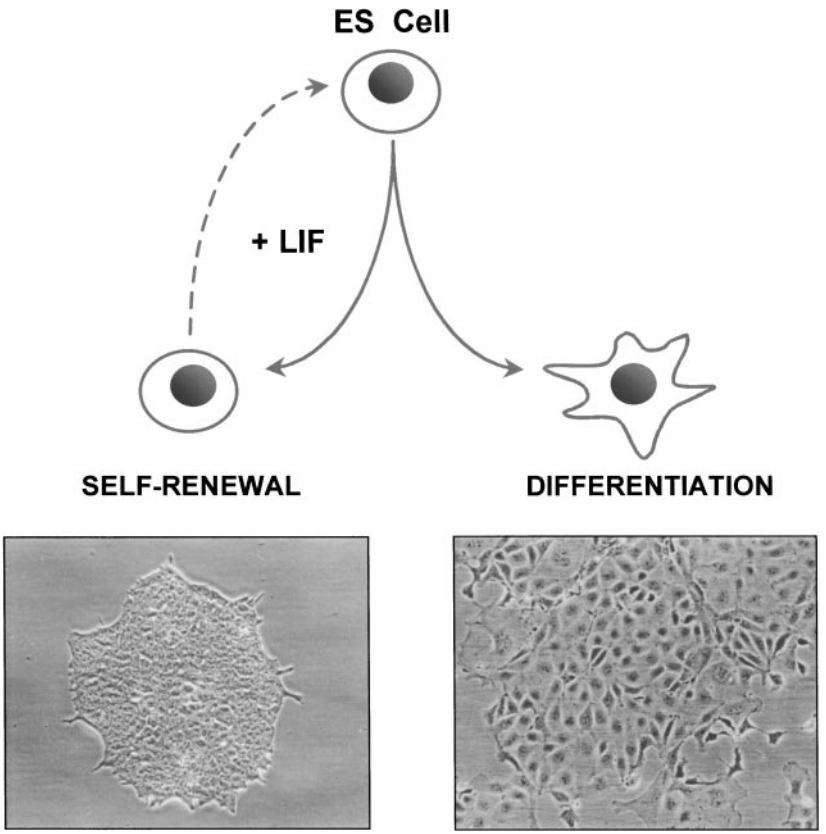

Figure 3 ES cell self-renewal and differentiation. *Upper panel*: diagram of alternative fates of a stem cell and the effect of LIF. *Lower panel*: photographs of self-renewing ES cell cultured in the presence of LIF (*left*) and differentiated derivatives 4 days after LIF withdrawal (*right*).

cells (Rathjen et al. 1990). Furthermore, feeders lacking a functional *Lif* gene do not support ES cell propagation effectively (Stewart et al. 1992). On withdrawal of LIF (or feeders), proliferation continues but differentiation is induced, and stem cells do not persist beyond a few days. The effect on ES cell self-renewal is exclusive to LIF and to a small group of related cytokines that all act via the gp130 receptor (Yoshida et al. 1994).

The signaling processes downstream of gp130 that suppress differentiation have now been delineated (Niwa et al. 1998) and are reviewed in detail elsewhere (Burdon et al. 1999a). In summary, cytokine engagement of gp130 results in JAK kinase-mediated recruitment and activation of the latent transcription factor STAT3. By using a directly activatable variant of STAT3, Matsuda et al. (1999) have

provided evidence that activation of this transcription factor alone is sufficient to support ES cell self-renewal at high density in serum-supplemented medium.

Neither the LIF family of cytokines nor STAT3 signaling are dedicated to stem cell regulation. In fact, they have diverse effects on a variety of cell types (Kishimoto et al. 1994). Interestingly, most of these actions are to promote differentiation, for example of myeloid cells or astrocyte precursors, or to induce expression of differentiated functions, such as acute phase protein synthesis by hepatocytes. Therefore, ES cell self-renewal is stimulated by a conventional signal transduction pathway, but the output of this signal, inhibition of differentiation, is peculiar to the stem cell context.

Why should ES cells be responsive to gp130 signaling? LIF and LIF receptor/gp130 are expressed in the early embryo (Nichols et al. 1996), but gene inactivation studies indicate that they are not required for development prior to gastrulation (Stewart et al. 1992, Ware et al. 1995, Yoshida et al. 1996). Further investigation, however, has uncovered a key adaptive function for cytokine signaling in the mouse epiblast (Nichols et al. 2001). This cryptic role is revealed during diapause, a state of arrested development occurring in mated female mice that are lactating or have been experimentally ovariectomized. In this situation, embryos develop to the hatched blastocyst stage but do not implant until maternal estrogen is restored. The physiological significance of diapause is to delay the arrival of a second litter until the preceding pups are weaned. Normal embryos can resume development even after 3 to 4 weeks of implantation delay. In contrast, embryos lacking gp130 fail to recover after only a few days in diapause, owing specifically to an inability to maintain the epiblast. Therefore, signaling through gp130 is essential for prolongation of epiblast lifespan. It is noteworthy that ES cells were first derived from blastocysts in diapause, and this generally appears to enhance the efficiency of ES cell generation (Brook & Gardner 1997, Gardner & Brook 1997). Implantation delay may preconfigure epiblast cells optimally for continued self-renewal by interrupting normal developmental progression and activating dependence on gp130 signaling.

The knockout studies in the gp130 pathway demonstrate that the normal transient phase of epiblast expansion in vivo does not rely on gp130 signaling. Evidence has been presented for a gp130-independent mechanism of supporting at least limited expansion of mouse ES cells (Dani et al. 1998). Moreover, as discussed below, human embryo-derived cells may utilize an alternative pathway to sustain self-renewal. The identities of the factors and signals involved in any of these cases are currently unknown, however.

Self-Renewal Versus Differentiation: Signaling Homoeostasis

Somewhat surprisingly, signals other than STAT3, and in particular activation of the ERK mitogen-activated protein kinase (MAPK) pathway, seem not to be required for ES cell propagation (Burdon et al. 1999b, Cheng et al. 1998). ERK

activation is normally a key signal for cell cycle progression through the cyclin D/CDK checkpoint in G1 (Roovers & Assoian 2000). ES cells, however, have an unorthodox cell cycle, another feature in common with epiblast cells, in which a G1 checkpoint does not appear to be operative (Savatier et al. 1995). The possible significance of an autonomous cell cycle for maintenance of pluripotency will be considered elsewhere (T. Burdon, A.G. Smith & P. Savatier, in preparation). In fact, inhibition of the ERK-activating enzyme MEK actually enhances self-renewal, implying that there is a prodifferentiative effect of ERK activation. This may relate to growth factors and other inductive stimuli that signal through the Ras-Raf-MEK-ERK cascade and also to integrin-mediated ERK activation (discussed in Burdon et al. 1999a).

Although ES cells utilize classical signal transduction mechanisms, the potential contribution of stem cell–specific signaling adaptors should not be overlooked. For example, ERK activation in response to various stimuli appears attenuated in ES cells relative to other cell types, despite the presence of comparable levels of ERK proteins. This may result in part from the specific expression in ES cells of an altered form of the Gab1 scaffold protein that suppresses linkage of certain receptors to the Ras-ERK cascade (T. Burdon & A.G. Smith, unpublished data). It will be important to determine whether such redirection of primary signal transduction to minimize prodifferentiative outputs is a significant component of homeostatic signaling in pluripotent cells.

Overall, the self-renewal of ES cells appears to depend on the balance between conflicting intracellular signals. The dual requirement to achieve and maintain a high level of STAT3 activation and low level of ERK activity may underlie some of the difficulties experienced in ES cell derivation where the alternative outcome of differentiation is generally favored. In this context, application of the MEK inhibitor PD05809 does appear to increase the efficiency of ES cell establishment by promoting expansion of primary stem cell colonies (M. Buehr, J. Nichols & A.G. Smith, in preparation). The signaling network is likely to be far more complex than outlined above, however. Even in established ES cell cultures, some level of spontaneous differentiation is usually evident. Indeed, in the author's experience this is the hallmark of a good, that is, fully pluripotent, ES cell line.

Intrinsic Determination of Pluripotent Identity

Oct-3/4 is a POU family transcriptional regulator restricted to early embryos, germ line cells, and undifferentiated EC, EG, and ES cells (Pesce et al. 1998). In vivo, zygotic expression of Oct-3/4 is essential for the initial development of pluripotential capacity in the ICM (Nichols et al. 1998). In ES cells, continuous function of Oct-3/4 is necessary to maintain pluripotency (Niwa et al. 2000). If Oct-3/4 expression is acutely eliminated in ES cells, self-renewal ceases and an unusual transdifferentiation process is triggered. Instead of forming the normal ES cell derivatives in monolayer culture, endoderm and mesoderm, the cells differentiate

into trophoblast. In the presence of FGF-4 and feeders, it is even possible to isolate trophoblast stem (TS) cells. The interest of this observation is that the differentiation of trophectoderm and ICM in the mouse blastocyst is associated with a segregation of developmental capacity such that the former can generate only trophoblast lineages and the latter only yolk sac and fetal tissues. Consistent with this, ES cells do not normally form trophoblast in vitro, in teratomas, or in chimeras. It appears that this developmental restriction may be necessary for manifestation of pluripotency and is imposed directly by Oct-3/4. In other words, Oct-3/4 acts in part as a lock that prevents differentiation into trophoblast (Niwa et al. 2000).

The pleiotropic activity of Oct-3/4 is evidenced by the finding that marginally increased expression in ES cells also provokes differentiation but in this case into endoderm and mesoderm (Niwa et al. 2000). This has been attributed to perturbation of the combinatorial interactions between Oct-3/4 and a set of co-activators and co-repressors. Competition and co-ordination between various transcriptional complexes involving Oct-3/4 may even guide cell determination in vivo. Cell commitment during gastrulation could conceivably involve differential regulation by inductive signals either of Oct-3/4 itself or of Oct-3/4 partners and consequent selective effects on subsets of target genes.

According to this model, Oct-3/4 actively participates in lineage choice. This is feasible because, although Oct-3/4 is invariably downregulated during pluripotent cell differentiation, the timing suggests that this is a consequence rather than a cause of cell commitment. Indeed, Oct-3/4 levels transiently increase in nascent hypoblast in the embryo (Palmieri et al. 1994), suggesting that the quantitative upregulation of Oct-3/4 could direct hypoblast commitment. Furthermore, downregulation of Oct-3/4 is not required for differentiation. This is clear because ES cells in which Oct-3/4 is produced constitutively from a transgene remain dependent on LIF and differentiate just as do parental ES cells when LIF is withdrawn (Niwa et al. 2000). A key conclusion from this last finding is that maintenance of Oct-3/4 expression is not in itself sufficient to sustain the pluripotent phenotype. Propagation of the pluripotent ES cell phenotype thus requires both the intrinsic activity of Oct-3/4 and the cytokine-induced action of STAT3.

PLURIPOTENCY OF EMBRYONIC STEM CELLS

Multilineage Differentiation Capacity

A major aspiration at the outset of EC and ES cell research was to elucidate the decision-making processes in lineage commitment and cell type differentiation of pluripotent cells. This issue is now re-emerging with increasing interest in the application of ES cell systems for efficient in vitro analysis of gene function and pharmacological screening and for the potential development of cell therapy (see below). From their differentiation in teratomas and chimeras, ES cells clearly have the capacity to produce every type of fetal and adult cell. Understanding and controlling cell fate determination remains a major challenge, however.

ES cells differentiate readily in monolayer culture when deprived of LIF or feeder support (Figure 3). Various differentiated morphologies emerge, and markers of mesoderm and endoderm become expressed. However, the identities of the major cell types produced under such conditions have not been carefully defined. It is possible that many of the cells do not represent bona fide embryonic or fetal phenotypes but rather are aberrant products arising from misregulated or scrambled differentiation program.

The principal method used to trigger differentiation of ES cells into defined cell types is cell aggregation in suspension culture. This technique, originally developed with EC cells (Martin & Evans 1975, Martin et al. 1977), leads to formation of multidifferentiated structures called embryoid bodies. In these structures, the developmental program of ICM/epiblast cells is reactivated in the ES cells. Cellular differentiation proceeds on a schedule similar to that in the embryo but in the absence of proper axial organization or elaboration of a body plan (Doetschman et al. 1985). Each embryoid body develops multiple cell types, and further differentiation is elaborated on subsequent attachment and outgrowth. A range of differentiated products can readily be obtained as indicated in the following list (Table 1). It is possible to bias the differentiation for or against certain cell types by addition of retinoic acid (Rohwedel et al. 1999). However, the final cultures are invariably a heterogeneous mixture.

Gut endoderm marker gene expression has been reported (Abe et al. 1996, Levinson-Dushnik & Benvenisty 1997), but production of defined endodermal cell types such as hepatocytes or pancreas cells remains inefficient. Evidence for in vitro differentiation of mouse ES cells into hepatic and pancreatic cells has now been reported (Hamazaki et al. 2001, Lumelsky et al. 2001). Neural crest derivatives have also not been demonstrated convincingly. In such cases there are two likely contributory factors to current failures: Either appropriate conditions for primary induction of the particular lineage have not been established, and/or the culture environment is not adequate for sustaining the lineage precursors. A further consideration is the limited availability of reliable markers of early lineage precursors.

Controlling Embryonic Stem Cell Differentiation: Instruction and Selection

Our abilities to direct pluripotent cells into specific pathways and then to support the viability and maturation of individual differentiated phenotypes in vitro are currently limited and the approaches rather unsophisticated. There is not yet a single case where differentiation of ES cells is in any meaningful sense directed. The range and proportion of differentiation products is influenced by different protocols for embryoid body formation and treatment with retinoic acid, but these determinations are purely empirical; the result remains a mixture of cell types. In the absence of understanding how to instruct ES cells uniformly to enter a lineage of choice, the alternative is to isolate cells of interest from the mixed cell populations

TABLE 1 Differentiated cell types produced with moderate efficiency and reproducibility from mouse ES cells in vitro

Cell type	Reference
Yolk sac endoderm	Doetschman et al. 1985
Yolk sac mesoderm	Doetschman et al. 1985
Primitive hematopoietic	Doetschman et al. 1985, Nakano et al. 1996
Definitive hematopoietic	Nakano et al. 1996, Nishikawa et al. 1998, Wiles & Keller 1991
Lymphoid precursor	Potocnik et al. 1994
Mast cell	Tsai et al. 2000
Dendritic cell	Fairchild et al. 2000
Endothelial cell	Risau et al. 1988, Yamashita et al. 2000
Cardiomyocyte	Doetschman et al. 1985, Maltsev et al. 1993
Striated muscle	Rohwedel 1994
Smooth muscle	Yamashita et al. 2000
Adipocyte	Dani et al. 1997
Osteoblast	Buttery et al. 2001
Chondrocyte	Kramer et al. 2000
Keratinocyte	Bagutti et al. 1996, Yamashita et al. 2000
Neuron	Bain 1995, Strubing et al. 1995
Astrocyte	Fraichard et al. 1995
Oligodendrocyte	Brustle et al. 1999, Liu et al. 2000

(Figure 4). In some cases this can be achieved through selective culture conditions. Hematopoietic cells can readily be enriched and expanded by culture in semi-solid media in the presence of hematopoietic growth factors (Wiles & Keller 1991). A second strategy is to purify lineage-committed precursors or specific differentiated phenotypes based on restricted expression of a marker gene(s). Immunopurification can be applied where suitable cell surface markers and antibodies are available. In an elegant study, Nishikawa showed that distinct mesodermal subsets are produced during monolayer differentiation on a collagen substratum, out of which clonogenic endothelial and hematopoietic progenitors can be isolated by fluorescence-activated cell sorting (FACS) (Nishikawa et al. 1998). This is a significant result because it establishes that the initiation of true differentiation can be uncoupled from complex morphogenetic and multicellular interactions that occur in an embryoid body (or embryo).

Transgenes conferring drug resistance and/or cell sorting capacity present another way of isolating a particular subset of cells. In this approach, a selection marker/reporter is placed under transcriptional control of a promoter that is

expressed only in the cell type of interest, either by introduction of a transgene or by knockin to the endogenous locus. This strategy can be applied to either a terminally differentiated cell type or a lineage-restricted precursor (Klug et al. 1996, Li et al. 1998). An advantage of the selection route in isolation of somatic stem cells/precursors is that the drug can be maintained to eliminate differentiating progeny and facilitate continued amplification of the lineage precursors (Li et al. 1998).

The findings that lineage commitment can be induced in a monolayer and that specific precursor cell populations can be isolated have implications for future efforts to direct differentiation. Dispersed cell culture is much more amenable than aggregate culture for observation, dissection, and interventions, such as administration of soluble factors. The development of some lineages is actually suppressed by cell-cell interactions and is therefore enhanced by isolating the precursors from the rest of the cell society (Nishikawa et al. 1998). Inductive signaling molecules such as hedgehog and bone morphogenetic proteins are likely to have more profound and interpretable consequences if applied to homogeneous monolayer cultures than to embryoid bodies or mixed cell populations. Further effort is needed to obtain differentiation of a range of cell lineages from ES cells in monolayer monoculture. Another approach is to co-culture ES cells with stromal cell lines. Different stroma have been employed to promote hematopoietic commitment (Nakano et al. 1996) and to produce dopaminergic neurons (Kawasaki et al. 2000). In the latter case, the inducing activity is at least in part soluble, which gives hope that eventually it may be possible to generate individual lineages instructively. Ideally, this would be achieved entirely by extrinsic stimulation, using defined media supplemented with specific inductive factors. It is likely, however, that genetic manipulation to force expression of master transcription factors may be required for maximal efficiency. Definition of all the steps required to induce any desired cell, tissue, or organ from ES cells in culture could be proposed as the ultimate challenge for developmental biologists.

Fidelity and Functionality of In Vitro Generated Cell Types

Can cells generated in vitro really acquire the full specialization of differentiated cell types in vivo? Remarkably, the available data indicate that ES cell derivatives can become specialized to a high degree in culture. ES cell progeny can express appropriate markers and functional attributes not just of generic differentiation but of specific sub-types of neuron or cardiomyocyte, for example (Lee et al. 2000, Maltsev et al. 1993, Strubing et al. 1995). The accumulating evidence from transplantation studies indicates that the specification of at least some ES cell derivatives is sufficient to allow their integration into adult tissue (see below).

With the exception of trophoblast, it seems that there is no intrinsic limitation to the ability of ES cells to differentiate in vitro. The restriction is in the availability of suitable culture systems that will support development and expression of differentiated functions. A dramatic illustration of the potential of in vitro differentiation

systems is the generation in isolation of entire vascular structures (Yamashita et al. 2000). The opportunity to observe and then to manipulate such complex differentiation processes opens unheralded opportunities for mammalian developmental biology and cell engineering.

PLURIPOTENT STEM CELLS FROM OTHER SPECIES

Is the ES Cell Phenotype Unique to Mice?

In considering this question, it is first necessary to agree on a definition of an ES cell. Although pluripotency is an essential feature of an ES cell, it does not follow that all pluripotent stem cells are equivalent to ES cells. Below is a list of the defining properties that are functionally important and/or unique to mouse ES (and EG) cells.

- origin from the ICM/epiblast (or from primordial germ cells for EG cells);
- derivation without transformation or immortalization;
- stable diploid karyotype;
- clonogenic;
- unlimited self-renewal capacity;
- high amplification capacity;
- pluripotent, can generate all fetal and adult cell types in vitro and in teratoma;
- extrinsic suppression of differentiation by gp130 cytokine or other stimuli;
- Oct-4-mediated transcriptional orchestration;
- absence of G1 cell cycle checkpoint;
- absence of X inactivation (in XX lines);
- incorporation into embryonic development and contribution to all germ layers in chimera;
- germ line colonization and transmission.

In the case of human pluripotent stem cells, the last two properties cannot be determined for obvious ethical reasons.

As discussed above, mouse ES cells originate from the epiblast (Evans & Kaufman 1983, Gardner & Brook 1997), which might be considered a conditional stem cell population. In the embryo epiblast, cells persist only transiently before inevitably undergoing differentiation or cell death. Establishing an ES cell culture therefore involves capture of this transient phase of pluripotency (Figure 1). Disruption of and liberation from inductive signals in the embryo microenvironment is essential. The subsequent derivation of ES cells is not a result of a conventional transformation event because they remain fully responsive to regulatory signals if returned to the embryo microenvironment. Yet the process by which a

state of continuous self-renewal is arrived at is not simply a default in the absence of gastrulation. ES cell lines can be derived reproducibly from embryos of inbred 129 strains and somewhat less efficiently from C57BL/6 strains. However, usually only a minority of embryos give rise to ES cells, suggesting that some epigenetic event is rate limiting. Separation of epiblast cells from extraembryonic tissue is reported to enhance the efficiency of ES cell derivation and has allowed lines to be established from the CBA strain (Brook & Gardner 1997). Nonetheless, the isolation of ES cell lines from other strains of mice has generally proven problematic. Thus there is also a strong genetic component to ES cell derivation. Interestingly, this is not reflected in the propensity of embryos to give rise to teratocarcinomas, which does not exhibit such dramatic strain dependency (Damjanov et al. 1983). Aside from mice, although various primary cultures and cell lines derived from early embryos have been described, evidence of pluripotency is rare, and in no case has the criterion of germ line colonization after long-term culture been satisfied (Gardner & Brook 1997).

An ES cell retains the essential features and identity of an epiblast cell. Nonetheless, it is altered in certain respects, such as dependence on cytokines and fidelity of imprinting, from an embryonic cell in situ. In reality, ES cells should be considered a cell culture phenomenon or even an artifact. From this perspective it is not surprising that there are significant differences between species, reflecting not the intrinsic status of the epiblast but its capacity to adapt to an arbitrary set of artificial conditions. Therefore, the phenotypes that may survive or emerge in culture will not necessarily be invariant between different genotypes. Even in mouse there may be differences in developmental staging between ES cells of different genetic backgrounds (Yagi et al. 1993). In the rat, cell lines that show some characteristics of ES cells can be established at high efficiency, but these cells are not, in fact, pluripotent (Vassilieva et al. 2000; M. Buehr & A.G. Smith, in preparation). In stark contrast to the situation with various rodents and ungulates, however, pluripotent cell lines have been established from primates. The relationship of these cells to mouse ES cells is considered below.

Human Pluripotent Stem Cells (hPSCs)

Teratocarcinomas occur in humans as in mice, and several human EC cell lines have been isolated. The dual capacities of mouse ES cells for unlimited expansion and for multilineage differentiation have subsequently provoked interest in establishing similar embryo-derived cell lines of human origin. The motivations for this are essentially fourfold:

a. human embryology—to recapitulate in vitro otherwise inaccessible aspects of early differentiation of the human embryo;

b. functional genomics—to investigate and manipulate specific gene functions in diploid human cells;

c. pharmaceutical development—to provide large numbers of phenotypically

defined human cell types for compound screening and toxicological testing;

d. regenerative medicine—to create a renewable supply of cells for clinical use in cell replacement, tissue repair, and delivery of gene therapy.

The path-finding studies of Thomson and co-workers first demonstrated that pluripotent cells with some of the expected features of embryonic stem cells could be isolated from nonhuman primates (Thomson et al. 1995, Thomson & Marshall 1998). Then in 1998, this was extended to derivation of cell lines from human blastocysts (Thomson et al. 1998). In parallel, Gearhart and colleagues described the isolation of candidate human EG cells (Shamblott et al. 1998). These courageous studies have aroused considerable controversy but are now increasingly seen as ushering in a new age of opportunities in human medicine (see Robertson 2001 for review of ethical and legislative issues). Against a background of intense political, commercial, media, and public interest, however, it is vital to maintain scientific objectivity and in particular to appreciate that we are confronted by major challenges to current knowledge and understanding.

The first question is whether the human cell lines qualify for the designation of ES or EG cells. Current aspirations and intentions are largely predicated on the assumption that human embryo-derived cells are in essence identical to mouse ES cells. This should not be taken for granted. As discussed above, ES cell status does not equate to some immutable cell state in the early embryo but represents an epigenetic adaptation to the ex vivo environment. The nature of cells that can be propagated from early embryos varies greatly both between and within species. In animal species, there is general acceptance of an absolute definition of an ES cell. This is the capacity, after clonal propagation, to contribute to somatic and germ line chimerism. In humans, this criterion is not acceptable, and in any case is irrelevant for the desired applications. In the latter regard, there is convincing evidence from differentiation in teratomas that human embryo-derived cells are pluripotent, and evidence is amassing that they can differentiate into germ layer derivatives in vitro (Amit et al. 2000, Itskovitz-Eldor et al. 2000, Reubinoff et al. 2000, Schuldiner et al. 2000). The outstanding question concerns the interplay between extrinsic stimuli and the intrinsic signaling and transcription network that determines stem cell fate. Does this operate according to the same general principles in human pluripotent stem cells (hPSCs) as in mouse ES cells? Current evidence on this point is fragmentary and does not allow any conclusions. For example, evidence has been presented that human cells do not require or respond to gp130 stimulation (Reubinoff et al. 2000, Thomson et al. 1998), suggesting a rather fundamental distinction, but until downstream signals are examined, notably STAT and ERK activation, the picture is incomplete. On the other hand, human cells have been shown to express Oct-4, but in this case the level of expression, which appears to be so important in mouse cells (Niwa et al. 2000), has not been accurately determined or manipulated. Undoubtedly, these issues will become clear in the near future as hPSCs become more widely available. The answers will dictate whether it will be relatively straightforward to translate our

increasing knowledge of how to manipulate mouse ES cell differentiation over to human cells or whether it will be necessary to unravel a new set of biological specifics.

A second important issue to be borne in mind with human cells is the likely impact of genetic heterogeneity. Mouse ES cells are predominantly derived from completely inbred embryos of strain 129, although some lines have been established from 129 hybrids and from a small number of other inbred strains. It is manifestly clear that the genetic background has a major influence both on the initial frequency of establishing ES cells and on their subsequent stability in culture. There is currently nothing known of the genes involved, but given the fine balance of processes required to sustain self-renewal, it is conceivable that the expression, qualitative or quantitative, of many genes could mitigate against self-renewal. Therefore, one can anticipate that hPSCs may differ greatly in their ease of propagation and expansion.

A third concern is the genetic and epigenetic integrity of hPSCs. If mouse ES cells are not maintained with scrupulous care, they rapidly lose developmental potency. This is often subtle, however, and only revealed by poor or biased contribution to chimeras and/or absence of germ line colonization. In the context of hPSCs, which are reported to grow more slowly and require more fastidious handling than ES cells, the risk of incurring an untoward genetic or epigenetic change that gives a growth advantage in vitro may be even greater. If such an event is not accompanied by an overt phenotypic or karyotypic transformation, it may go undetected. It may therefore be necessary to establish assays for routine monitoring of hPSC populations. These would include assessment of genetic integrity and gene expression profile, along with measurement of key functional parameters, namely growth rate and self-renewal and differentiation efficiencies.

Finally, reproducing early human differentiation processes in vitro may prove more problematic than for mouse ES cells. The formative processes of gastrulation, primary tissue specification, and organogenesis take place over approximately 4 days in the mouse embryo and are well described. The corresponding window of human embryogenesis, between 2 and 5 weeks after fertilization, is inaccessible. The lack of characterization of key events and, probably more importantly, the longer-time course of human development may present significant hurdles in attempts to produce differentiated cell types efficiently from hPSCs.

CURRENT AND FUTURE DIRECTIONS

The Molecular Basis of Pluripotency and "Stemness"

Although the definition of a stem cell, that is as a cell that can both self-renew and undergo differentiation, is entirely functional, the underlying processes by which a stem cell preserves, chooses, and effects alternative fates are, of course, molecular in nature. Elucidation of these molecular pathways remains one of the outstanding challenges in cell and developmental biology. Compared with other

stem cell types, the ability to intervene genetically in ES cells and to expand relatively pure populations of undifferentiated cells offers major advantages for molecular and biochemical interrogation. ES cell investigations seem likely to yield valuable insights into stem cell biology in general and in particular if there are common features and principles to regulation of different stem cell types.

Crucial requirements have been identified for transcriptional regulation by Oct-4 and STAT3 in mouse ES cells. An immediate issue, therefore, is the characterization of respective target genes. However, identifying genes regulated by specific transcription factors is not a trivial task, particularly for repressed targets, which could be the most important in the context of suppressing differentiation. Neither is a catalogue of targets, in itself, likely to be overly informative. It is already known that Oct-4 regulates multiple genes (Niwa et al. 2000, Saijoh et al. 1996), at least some of which, for example *Opn* (osteopontin), do not appear to have any function in the ES cell state (Botquin et al. 1998). A similar caveat applies to the current enthusiasm for global expression profiling using microarrays or SAGE (serial analysis of gene expression). ES cells seem able to express many otherwise tissue-specific genes without functional consequence. Indeed, this is a major aspect of their utility for gene trapping (Friedrich & Soriano 1991, Gossler et al. 1989, Leighton et al. 2001, Skarnes et al. 1995). Thus one can anticipate a high degree of noise in studies of gene expression during ES cell differentiation. The experiments will need to be carefully designed and the data rigorously filtered. One approach to consider might be comparative analyses of single ES cells. For example, genes expressed in a mosaic fashion in the population typical of many developmentally regulated mRNAs could be eliminated, allowing closer approximation to the core stem cell gene expression program. Ultimately, however, any models or hypotheses based on expression data will have to be tested by loss- and gain-of-function experimentation. This will necessitate implementation of methods for regulatable transgene expression for rapid gene ablation by homologous recombination or possibly via antisense or RNAi (see below).

The protein partners of Oct-3/4 and STAT3 are a further obvious line of enquiry. It is known that Oct-3/4 interacts with the Sry-related HMG box transcription factor Sox2 (Ambrosetti et al. 1997) and with one or more unidentified proteins that constitute the so-called E1A-like activity of pluripotent cells (Scholer et al. 1991). Partners of STAT3 in ES cells have not been described, although interestingly STAT3 has been shown to interact with CBP/p300 in neuroepithelial cells (Nakashima et al. 1999), and a recent report suggests a possible interaction with neurogenic bHLH proteins (Sun et al. 2001). The characterization of synergistic and antagonistic transcription factor combinations in ES cells will help to inform and explain observed patterns of gene expression. Investigation of signaling proteins and complexes in ES cells is also still in its infancy. This area would seem particularly likely to yield insights into how the self-renewal cycle can be broken and differentiation triggered. It is possible that a proteomic approach to ES cell characterization may prove more incisive than transcriptional profiling, although it too will require validation by genetic manipulation.

Another critical but currently underinvestigated area is that of chromatin organization and epigenetic control in ES cells. A singular feature of ES cells in this respect is that DNA methylation apparently plays little or no role in heritability of gene expression. Thus ES cells lacking maintenance methyltransferase activity are unaffected by the resultant global demethylation, whereas this cannot be tolerated by somatic cells (Lei et al. 1996). This goes to the heart of self-renewal: If pluripotency is a state of general transcriptional permissiveness (Smith 2001), what is the mechanism(s) that ensures this condition is stably inherited? A search for ES cell-specific chromatin components and/or modifications may be illuminating.

Technologies for Rapid and Versatile Genetic Manipulation

Analytical dissection of ES cell self-renewal, commitment, and differentiation requires tools for refined genetic intervention. Two features of ES cells can confound reliable phenotype observation and interpretation after genetic manipulation. First, transgenes introduced into ES cells tend to be progressively silenced, resulting in mosaic expression and heterogeneous phenotypes. Second, either deletion of a gene required for self-renewal or forced expression of a transgene that drives differentiation will result in inability to form proliferative colonies and consequent recovery only of nonexpressing or deviant cells. Thus gain-of-function experimentation by conventional stable transfection is fraught with potential artifact and requires painstaking characterization. A method that provided efficient transfection and robust expression in ES cells would be of great utility.

Unfortunately, transduction with retroviral expression vectors has not proven particularly effective to date in ES cells because of promoter silencing. A method that does show promise is extrachromosomal propagation of plasmid vectors exploiting the replication system of polyoma virus (Gassmann et al. 1995). ES cells that stably express the polyoma large T protein from an introduced transgene can efficiently support the episomal maintenance of subsequently introduced plasmids containing the polyoma origin of replication. This allows stable transfectants to be established at a frequency of 1–5% of total cells compared with less than 0.1% for stable integration. The reliable generation of large numbers of expressing transfectants provides a reliable primary screen, even in cases where the phenotype is induction of differentiation (Niwa et al. 1998, 2000). Importantly, expression of polyoma large T protein at levels sufficient to support episomal replication appears to have no effect on ES cell self-renewal or pluripotency.

Site-specific recombination provides a route for efficient activation of a previously introduced but silent genetic modification. This may be deletion of an endogenous gene sequence or activation of a transgene via excision of a blocking element. Cre or Flp recombinase can be introduced into significant fraction of ES cells by transient transfection, but a preferable option is stable introduction of a regulatable recombinase, such as a steroid hormone fusion (Vallier et al. 2001), that can then be activated in all cells. The recombinase deletion approach can also be used to delete a transgene and determine whether a gain-of-function phenotype is

reversible. This is an important consideration because ES cells are mutable, and the possibility always exists that a phenotype observed in relatively rare transfectant clones is either unrelated to the transgene or involves a second event.

Due to the intricacy of the biological networks at play, control over the level and timing of transgene expression is particularly desirable. The ideal system would allow inducible expression of a transgene over a broad linear range in ES cells and differentiating progeny and would also be fully reversible. The tet regulatory system devised by Bujard and colleagues can fulfil these requirements (Baron & Bujard 2000). This system has been demonstrated to function effectively in ES cells, allowing tight on/off regulation of transgene expression (Niwa et al. 1998, 2000). Implementation is demanding because of the need to integrate two separate transgenes, both of which may be subject to position effect and silencing. Nonetheless, inducible transgene expression will be essential to dissect and ultimately to direct ES cell differentiation with any precision.

Derivation of Pluripotent Cells from Somatic Tissues?

Recent reports indicate that stem cells in certain adult tissues may be able to give rise to cell types of other tissues or organs. The clearest case is that cells isolated from mouse bone marrow can participate in liver regeneration and form hepatocytes (Lagasse et al. 2000). There is also good evidence that bone marrow–derived cells can contribute to muscle regeneration (Ferrari et al. 1998). A more dramatic claim is that cells derived from the brain can reconstitute adult hematopoiesis (Bjornson et al. 1999) and can contribute to a diversity of developing fetal tissues (Clarke et al. 2000). There are various possible explanations for these results that include the presence of reservoirs of tissue-specific stem cells in favorable ectopic locations such as the bone marrow, persistence of rare pluripotent stem cells in privileged sites, and interconversion of stem cells in response to some external insult or stimulation (Weissman 2000). If either of the two latter explanations is correct, it follows that it ought to be possible to either generate or directly isolate pluripotent cells from tissues such as brain and bone marrow. In such an event, it will be of great interest to examine the relationship of soma-derived stem cells to ES and epiblast cells. The production of EG cells from primordial germ cells could be considered a precedent for reversion of a more differentiated cell to pluripotent stem cell status. However, PGCs may be uniquely permissive for reacquisition of pluripotency precisely because they have not been subjected to somatic specification or restriction. In the case of somatic cells, one might anticipate a requirement for a significantly greater degree of epigenetic reprogramming. It remains to be determined whether this might occur and if so under what constraints.

Prospects for Cell Therapy

Of course the great dream for hPSCs (and adult stem cells) is to provide regenerative therapies (Figure 5). Cell transplantation to restore tissue function after disease or injury is in theory applicable to a huge variety of human ailments,

spanning neurodegeneration (Svendsen & Smith 1999), diabetes (Soria et al. 2000), and myocardial infarction (Klug et al. 1996). Recent studies with mouse ES cells offer encouragement in this regard, provided (as discussed above) that mouse cells are a valid model for human hPSCs. Thus ES cell-derived neural cells have been demonstrated to survive and to exhibit at least some aspects of appropriate region-specific neuronal differentiation when introduced into the developing mouse brain (Brustle et al. 1997). In adults, transplantation of glial derivatives of ES cells results in a degree of remyelination in a rat model of multiple sclerosis (Brustle et al. 1999) and, most dramatically, to a partial functional recovery in a spinal cord injury model (Liu et al. 2000). One problem that has arisen in such studies is the development of teratomas from undifferentiated ES cells present in the grafted population. For any clinical application, it would be essential to ensure that such cells were eliminated by rigorous purification and/or genetic selection. Encouragingly, genetically purified ES cell-derived cardiomyocytes have been shown to form intracardiac grafts in adult dystrophic mice without development of teratomas over a 7-week period (Klug et al. 1996). A second point still to be established unequivocally is whether the ES cell derivatives are fully functional after transplantation. In addition, the long-term stability of grafted cells has not been demonstrated. These questions can be addressed and resolved in coming years. There may be requirements to develop new or better transplantation models with more precise and quantitative determination of functional restoration. In the context of hPSCs, although preliminary studies can often be carried out in rodent recipients, the use of primate models would ultimately seem unavoidable.

An important variable to consider in cell transplantation is the developmental stage of the donor cells. For therapies aimed at long-term reconstitution of a continuously renewing tissue such as the hematopoietic system, only stem cell transplantation will suffice. This is not the case for treatment of tissues or organs in which there is little or no cell turnover. For example, in replacement of dopaminergic neurons in Parkinson's disease, it is generally considered to be important to transplant cells that are committed to mesencephalic dopaminergic differentiation (Dunnett & Bjorklund 1999). However, it is possible that the plasticity of naïve neural precursor cells may be advantageous for integration, provided the adult environment retains the appropriate cues to direct them efficiently into dopaminergic fates (Svendsen & Smith 1999). How far cells will have to be pushed along desired differentiation pathways in vitro and how much may be guided by the recipient environment are open questions at this stage. It is likely that the answer will vary for different tissues/organs and for different types of damage.

A limitation to hPSC-based cell therapy is the nonidentity between introduced cells and recipient, which will provoke immunological rejection. This problem can be reduced by having a panel of hPSC lines available that would allow matching of major histocompatibility determinants, as practiced in kidney transplantation for example. Nonetheless, life-long immunosuppressive treatment would be required. Approaches to circumvent this include induction of tolerance by prior creation of a chimeric lymphoid system via inoculation with hPSC-derived

lymphohematopoietic stem cells, or customization of the hPSCs for the recipient by genomic substitution of histocompatibility loci. The idea that has aroused the greatest excitement and controversy, however, is the possibility of deriving hPSCs de novo from the recipient via somatic cell nuclear transfer. This so-called therapeutic cloning procedure would involve the transfer of a nucleus from a somatic cell of the patient into an enucleated oocyte, development of the reconstituted embryo to the blastocyst stage, and then isolation and expansion of hPSCs (Figure 5). The feasibility of this scenario has now been demonstrated in mice by derivation of pluripotent ES cells from nuclear transfer embryos (Munsie et al. 2000). Bearing in mind the high levels of fetal abnormalities and perinatal mortality after nuclear transfer, it will be important to show that any ES cells derived in this way are not compromised. However, it is also worth noting that the requirements for creation in entirety of a viable fetus are likely to be much greater than those for generation of specific cell types. Therefore, a lower threshold of reprogramming fidelity may be acceptable to derive useful ES cells and hPSCs.

CONCLUDING NOTE

The faculty for propagating pluripotent stem cells from mouse and human embryos is essentially fortuitous in biological origin. This gift from nature has provided unparalleled research tools for investigating mammalian development, genetics, and physiology. Now these cells offer the foundations for an entirely new form of human medicine.

ACKNOWLEDGMENTS

This review also could not have been produced without the efforts of all past and present members of my laboratory. Our research is supported by the Biotechnology and Biological Sciences Research Council and the Medical Research Council of the UK and by the International Human Frontier Science Programme Organisation. I thank Val Wilson and Jenny Nichols for comments on the manuscript and Andras Nagy for Figure 2. I am forever indebted to Chris Graham for introducing a disillusioned undergraduate to “the cell that can make everything.”

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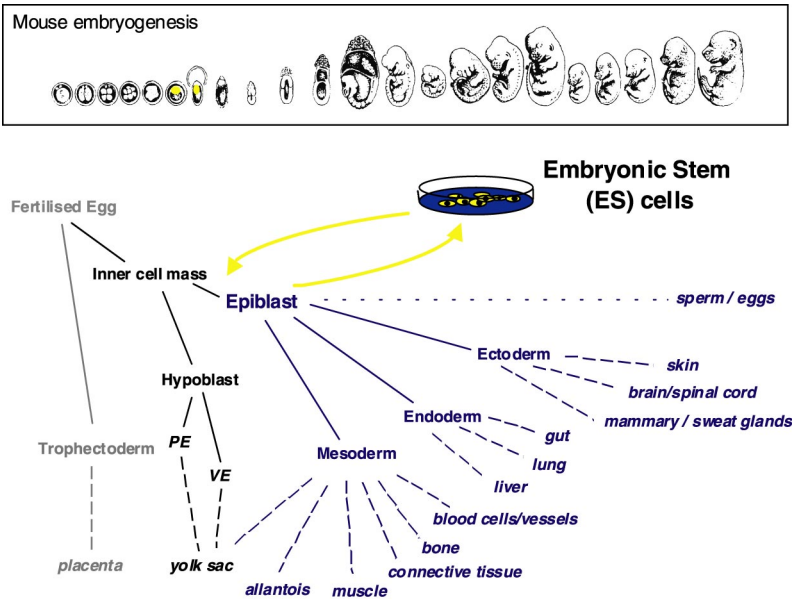

Figure 1 Diagram depicting ES cells in context of mouse development. *Upper panel* shows development of mouse embryo with the inner cell mass from which ES cells can be derived highlighted in yellow (adapted from Cold Spring Harbor Laboratory Manual: *Manipulating the Mouse Embryo*). *Lower panel* shows lineage diagram of mouse development with lineages colonized by ES cells highlighted in blue. ES cells can produce hypoblast derivatives in vitro but rarely do so in vivo.

Figure 2 Chimeric embryo produced by blastocyst injection of ES cells. ES cell descendants visualized by β-galactosidase staining are present throughout the fetus and in yolk sac mesoderm but not in yolk sac endoderm or placenta. Image provided courtesy of A. Nagy.

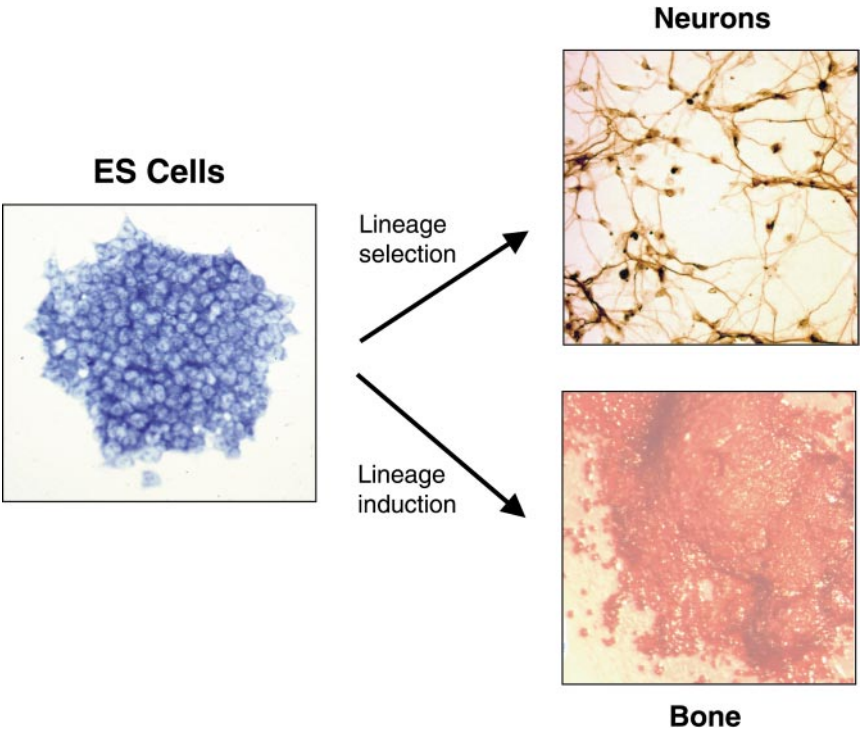

Figure 4 ES cell differentiation potential. ES cells differentiate into different germ layer derivatives via embryoid body formation. Particular cell types can subsequently be enriched for by selection and/or induction. In the examples shown, substantially pure populations of neurons are obtained following isolation of neural precursors by selection for expression of the *Sox2* gene (Li et al. 1998), and osteoblast differentiation is induced by treatment of embryoid body outgrowths with bone morphogenetic protein-4 (J. Kawaguchi & A.G. Smith, unpublished data).

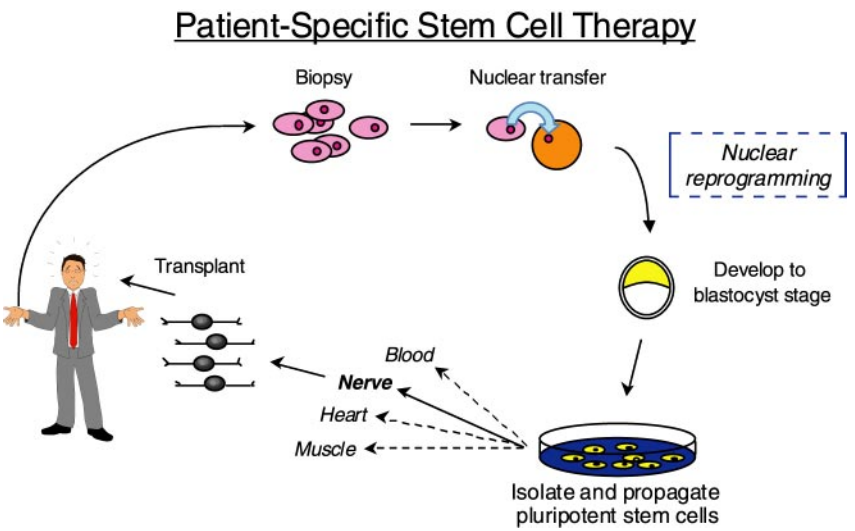

Figure 5 Stem cell therapy. Outline of the isolation of human pluripotent stem cells, expansion in culture, differentiation into the cell type of relevance, and transplantation. Stem cells may be derived from surplus preimplantation embryos generated in the course of IVF treatment and donated for research, or possibly in future from embryonic entities produced by nuclear transfer. The second route would avoid any requirement for immunosuppression after transplantation.