

A germ cell origin of embryonic stem cells?

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Summary

Because embryonic stem (ES) cells are generally derived by the culture of inner cell mass (ICM) cells, they are often assumed to be the equivalent of ICM cells. However, various evidence indicates that ICM cells transition to a different cell type during ES-cell derivation. Historically, ES cells have been believed to most closely resemble pluripotent primitive ectoderm cells derived directly from

the ICM. However, differences between ES cells and primitive ectoderm cells have caused developmental biologists to question whether ES cells really have an in vivo equivalent, or whether their properties merely reflect their tissue culture environment. Here, we review recent evidence that the closest in vivo equivalent of an ES cell is an early germ cell.

Introduction

Embryonic stem (ES) cells are pluripotent (see Box 1) and can be expanded without limit in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). It is remarkable that permanent pluripotent stem cell lines can be derived from pre-implantation embryos at all, because, in vivo, pluripotent cells of the early mammalian embryo proliferate only briefly before becoming cells with a more restricted developmental potential. A few years after the initial derivation of mouse ES cells, it was suggested that they be called ‘embryo-derived stem cells’, a more precise term that would distinguish between these new pluripotent cell lines and cells within the embryo (Rossant and Papaioannou, 1984). However, this term was never adopted, and the extent to which these pluripotent stem cell lines represent any specific embryonic cell type or reflect their artificial tissue culture environment is still an open issue today – two decades later. Elucidating the origin of ES cells is of importance because it may help us to identify genes that are essential for the long-term maintenance of the pluripotent state. It could also assist with the derivation of ES cells from species whose ES cells have proved difficult to isolate. It will also help us to assess how accurately ES cell differentiation reflects events that normally occur in vivo. Here, we review the origin of ES cells, and explore recent evidence that ES cells are closely related to early germ cells.

The historical origins of ES cells: embryonal carcinoma cells

Historically, work with mouse teratocarcinomas paved the way for the derivation of ES cells. These germ cell tumors contain multiple differentiated tissues and undifferentiated stem cells, called embryonal carcinoma (EC) cells (Damjanov and Solter, 1974; Dixon and Moore, 1952; Kleinsmith and Pierce, 1964). Although teratocarcinomas had been known as medical curiosities for centuries (Wheeler, 1983), it was the discovery that male mice of strain 129 had a high incidence of testicular teratocarcinomas (Stevens and Little, 1954) that made these

tumors more routinely amenable to experimental analysis. Because their growth is sustained by the persistent EC cell component (Stevens and Little, 1954), teratocarcinomas can be serially transplanted between mice. Eventually, conditions were developed that allowed the culture of EC cells in vitro, establishing them as an in vitro model of mammalian development (Kahan and Ephrussi, 1970).

As pluripotent cells of the intact early embryo proliferate for only a limited period of time, it was not initially obvious whether pluripotent cell lines could be established without undergoing malignant transformation. However, the transplantation of genital ridges or of egg-cylinder-stage embryos into ectopic sites, such as under the kidney capsule of adult mice, gave rise to teratocarcinomas at a high frequency in strains that did not spontaneously produce these tumors (Solter et al., 1970; Stevens, 1970a; Stevens, 1970b). These teratocarcinomas could be serially transplanted between adult mice, depending on whether the EC cell component persisted or differentiated (Solter et al., 1981). If the EC compartment disappears, the resulting tumor develops as a benign teratoma. Indeed, the malignant phenotype of EC cells often depends on the strain of the host mouse, and not on the tumor strain. EC cells injected into mouse blastocysts can contribute to either the normal tissues of the resulting chimera (Brinster, 1974) or, in some cases, to tumors (Rossant and McBurney, 1982). Because the ectopic transplantation of normal peri-implantation embryos can give rise to pluripotent cell lines, the direct derivation of pluripotent cell lines in vitro was attempted without the teratocarcinoma step. The culture conditions that were established to support mouse EC cells, including the use of feeder cell layers, were essentially those used to isolate mouse, and eventually human, ES cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998).

One indication that these early EC cell lines may be derived from germ cells (Solter et al., 1970; Stevens, 1967; Stevens, 1970a) came from mouse genital ridge-transplantation experiments. These experiments showed that genital ridges

Box 1. Glossary**Inner cell mass (ICM)**

The second lineage of the early embryo that is located inside the blastocyst. It gives rise to all embryonic tissues.

Pluripotency

Refers to the unique ability of cells within the early embryo to differentiate into all cell types.

Primitive ectoderm

The remaining ICM tissue formed during the second differentiation event of embryonic development (also known as epiblast or embryonic ectoderm)

Primitive endoderm

An epithelial layer derived from cells that are in contact with the blastocyst cavity.

Trophectoderm

During the first differentiation event in mammalian development, morula cells segregate into two cell lineages: the first, the trophoctoderm, forms the outer layer of the blastocyst. It eventually becomes part of the placenta.

effectively give rise to teratocarcinomas only in a narrow time window (E12.0-12.5). It is around this time that migratory germ cells start arriving in the genital ridge. In the egg cylinder-transplantation experiments, however, the origin of the EC cells was less clear. Primitive ectoderm seemed the most likely candidate for several reasons: because the potential to form teratocarcinomas is lost at the time when primitive ectoderm disappears at E8.5 (Damjanov et al., 1971); because EC cells have phenotypic similarities to primitive ectoderm cells *in vivo* (Diwan and Stevens, 1976); and because EC cells, when reintroduced into blastocysts, contribute to the same tissues as primitive ectoderm (Brinster, 1974). In addition, when analyzing the earliest stages of teratocarcinoma formation in 129/Sv mouse fetal gonads, Stevens observed clusters of polarized epithelial cells surrounding a central cavity that morphologically resembled primitive ectoderm cells (Stevens, 1983). He also observed that the formation of teratocarcinomas in ovaries included parthenogenic activation of the oocyte, the formation of blastocyst-like structures and the subsequent formation of structures that resembled early egg cylinders, which eventually became disorganized. Isolated transplanted primitive ectoderm itself gives rise to teratocarcinomas (Diwan and Stevens, 1976), but because early germ cells are just appearing at this stage, a germ cell origin cannot be completely ruled out by these experiments.

Are ES cells a tissue culture artifact?

ES cells clearly exhibit some properties that are not normally shown by cells of the intact embryo. For example, although ES cells retain properties of early embryonic cells *in vitro*, no pluripotent cell demonstrates long-term self-renewal *in vivo*. Embryonic cells, once brought into tissue culture, are exposed to numerous extrinsic signals to which they never would be exposed to *in vivo*. ES cells certainly adapt to selective tissue culture conditions and acquire novel functions that allow them to proliferate in an undifferentiated state indefinitely, and,

because of this, ES cells are in some sense tissue culture artifacts (Buehr and Smith, 2003; Rossant, 2001; Smith, 2001).

As these changes are inevitable, the issue is not whether ES cells exhibit some properties that merely reflect their tissue culture environment, but rather whether they are most closely related to a specific *in vivo* cell type in the embryo, or if the influence of the culture environment is so dominant that it is impossible to relate ES cells to a single, *in vivo* cell type. We will certainly not completely resolve this issue here, but will re-explore the relationship of ES cells to specific early embryonic cell types.

Are ES cells most closely related to primitive ectoderm?

Although ES cell lines are generally derived from the culture of the ICM, some experiments suggest that ES cells more closely resemble cells from the primitive ectoderm. For example, isolated primitive ectoderm from the mouse gives rise to ES cell lines at a higher frequency than does isolated ICM. Moreover, the culture of primitive ectoderm allows the isolation of ES cell lines from mouse strains that have been previously refractory to ES cell isolation (Brook and Gardner, 1997). Indeed, ES cell lines can be derived from single, isolated, mouse primitive ectoderm cells, which is not possible with ICM cells (Gardner and Brook, 1997). Although these experiments suggest that ES cells are more closely related to primitive ectoderm than to ICM, they do not reveal whether ES cells more closely resemble primitive ectoderm or a cell derived from it *in vitro*.

A maximum of three individual cultured primitive ectoderm cells per embryo have been shown to give rise to ES cell colonies (Gardner and Brook, 1997). This low frequency could have been due to some variability in the potential of primitive ectoderm cells, to some variability in the environment in which they were placed or to damage caused by the dissociation of the primitive ectoderm into individual cells. However, by tracking the expression of the octamer-binding transcription factor 4 (*Oct4*) gene, a marker of pluripotency, in intact cultured ICM/epiblast cells, it was shown that *Oct4* expression was maintained in only a small proportion of outgrowing cells (Buehr et al., 2003), which also suggests that only a minority of primitive ectoderm cells can transit to a new stable, proliferative pluripotent state, and, subsequently, be expanded as ES cells. These results could be due to a requirement for a relatively rare intrinsic or extrinsic stochastic event, or to an inherent heterogeneity of the primitive ectodermal cell population. Recent data indicate that even the earliest ICM is heterogeneous and consists of a mixture of cells that express either *Oct4* or *Gata6* (Rossant et al., 2003), and a similar later heterogeneity could account for the fact that only a minority of primitive ectoderm cells generally give rise to ES cells in culture.

Established mouse ES cell lines express some specific markers of primitive ectoderm at a very low level, if at all (Table 1), such as fibroblast growth factor 5 (*Fgf5*) (Haub and Goldfarb, 1991; Hebert et al., 1991; Rathjen et al., 1999). Culture conditions have been established that convert mouse ES cells into early primitive ectoderm-like cells that express both *Fgf5* and *Oct4* (Rathjen et al., 1999), but these cells fail to form chimeras when injected into mouse blastocysts. Taken together, these results suggest that ES cells are most closely

Table 1. Marker genes expressed in embryonic stem cell (ES), early germ (EGC) and later germ cells (LGC), in the inner cell mass (ICM) and in the primitive ectoderm (PE)*

Gene	Species	ES	EGC	LGC	ICM	PE
<i>Pou5f1</i> (Pesce and Scholer, 2001)	M	+	+	+	+	+
<i>Nanog</i> (Chambers et al., 2003)	M	+	+	+	+	+
<i>Dppa3</i> (Saitou et al., 2002)	M	+	+	+	+	+
<i>Ifitm3</i> (Saitou et al., 2002)	M	+	+	+	+	+
<i>Kit</i> (Horie et al., 1991)	M	+	+	+	–	N/D
<i>DAZL</i> (Clark et al., 2004)	H	+	+	+	–	N/D
<i>Ddx4</i> (Toyooka et al., 2003)	M	–	–	+	–	–
<i>Akp2</i> (Chiquoine, 1954)	M	+	+	+	+	+
<i>Zfp42</i> (Rogers et al., 1991)	M	+	N/D	N/D	+	–
<i>Fgf5</i> (Haub and Goldfarb, 1991; Hebert et al., 1991)	M	–	N/D	N/D	–	+
<i>Gbx1</i> (Chapman et al., 1997)	M	+	N/D	N/D	+	–

*Data are based on mouse (M) and human (H) studies, some are preliminary.

+ denotes expression at that developmental stage, – denotes the gene is not expressed
N/D, not done.

related to a subpopulation of primitive ectoderm cells, or to a close derivative of primitive ectoderm cells.

One of the curious species-specific differences between human and mouse ES cells is that human ES cells give rise to trophoblast cells at a high efficiency (Xu et al., 2002), but mouse ES cells do not (Beddington and Robertson, 1989). In the intact mouse embryo, the last cells capable of giving rise to trophoblast cells are early ICM cells, so the failure of mouse ES cells to differentiate into trophoblast is good evidence that they are not the equivalent of early ICM cells (Brook and Gardner, 1997). The differentiation of human ES cells to trophoblast could be explained if they are related to an earlier cell type than mouse ES cells, or if the specification of the trophoblast lineage occurs differently in human embryos. However, a third possibility is that ES cells represent a different cell type altogether. It is therefore worthwhile examining the relationship between ES cells and germ cells.

Germ cells and the primitive ectoderm

In elegant, clonal-fate mapping studies in the mouse (Lawson and Hage, 1994), germ cells were shown to arise from a founder population in the E6.0-6.5 proximal epiblast adjacent to the extra-embryonic ectoderm. These founder cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive germ cells is present at the base of the allantois (Fig. 1) (Ginsburg et al., 1990). The E6.5 distal epiblast, which would not normally contribute to germ cells, will contribute to germ cells if transplanted to a proximal location (Tam and Zhou, 1996), which demonstrates that location and inductive signals, rather than germ plasm determinants, are responsible for the specification of germ cells in mice (Extavour and Akam, 2003). This flexibility suggests that cultured primitive ectoderm cells could spontaneously give rise to early germ cells in culture.

Bone morphogenetic protein 4 (Bmp4) (Lawson et al., 1999) and Bmp8b (Ying et al., 2000) are required for the formation of the proximal posterior extra-embryonic region that gives rise to primordial germ cells (PGCs) and to cells of the allantois in the mouse. The addition of Bmp4 and Bmp8b to distal mouse epiblast cultures increases the formation of cells strongly positive for Tnap (Ying et al., 2001), a marker shared by early

germ cells and ES cells. These Tnap-positive cells were interpreted as being germ cells in this study. Recently, BMP signaling has been shown to be important for the self-renewal of mouse ES cells (Ying et al., 2003), and although BMPs are involved in many differentiation decisions in the early embryo, these results do further hint at a relationship between ES cells and early germ cells.

Similarities between germ cells and ES cells

In mice, PGCs migrate and proliferate until ~25,000 are present in the genital ridge at E13.0 (Tam and Snow, 1981). Pluripotent cell lines from pre- and post-migratory (Resnick et al., 1992; Matsui et al., 1992; Shambloott et al., 1998), as well as from migratory (Durcova-Hills et al., 2001), germ cells have been isolated, and these cell lines are termed embryonic germ (EG) cells to distinguish their origin. Mouse EG cell lines are remarkably similar to mouse ES cell lines (Donovan and de Miguel, 2003). During germ cell migration and maturation, however, the somatic status of imprinted genes is progressively erased (Yamazaki et al., 2003), and EG cells isolated at various stages of migration retain some of these differences, such as the reduced methylation of many imprinted genes, including *H19* and *Snrpn* (Hajkova et al., 2002). The analysis of mouse PGCs at E10.5 suggests that methylation erasure has already begun by this time, as supported by studies of the expression of imprinted genes (Yamazaki et al., 2003). This study showed that imprinted genes, such as *H19* and *Snrpn*, exhibit imprinted (somatic) expression patterns in E9.5 PGCs, but by E10.5 have switched to a bi-allelic mode of expression (Yamazaki et al., 2003). Because the genes expressed in ES cells exhibit somatic imprinting patterns (Geijsen et al., 2004), their change in imprinting status suggests that if ES cells are derived from germ cells, this derivation must occur before E9.5.

There is a paucity of known molecular markers that distinguish early germ cells from other pluripotent cells of the early embryo. One marker, Tnap, is strongly expressed by early germ cells and by ES cells, but is weakly expressed by the epiblast and other surrounding embryonic cells (Chiquoine, 1954; Ginsburg et al., 1990). Two new markers for early germ cells, fragilis (Ifitm3 – Mouse Genome Informatics) and *Dppa3* (also known as stella or PGC7), have recently been identified that allow the better separation of early germ cell precursors from their differentiated neighboring cells (Saitou et al., 2002).

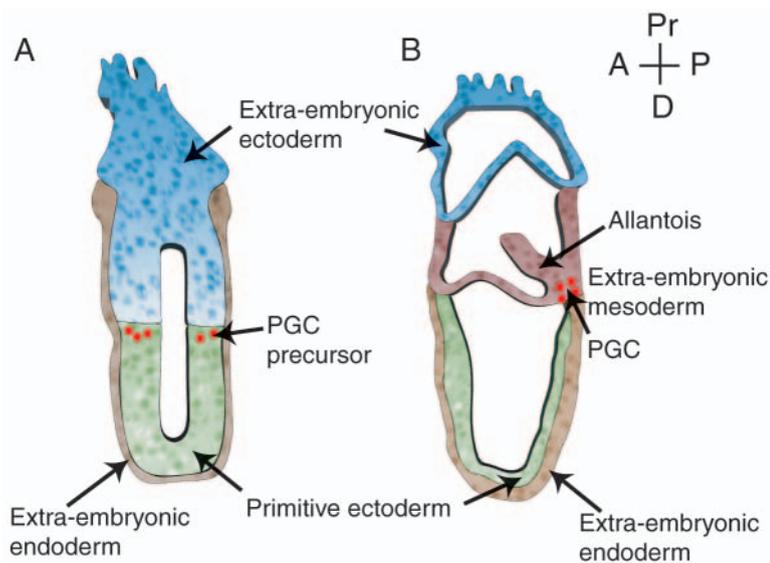


Fig. 1. Early development of the mouse embryo. (A) Six days after fertilization (E6.25), the mouse embryo consists of three layers. The inner cell mass (ICM) cells that are in contact with the blastocyst cavity differentiate into an epithelial layer called the extra-embryonic (primitive) endoderm. The rest of the ICM becomes the epiblast (primitive ectoderm). Primordial germ cells (PGCs, red dots) arise from a cell population in the proximal epiblast adjacent to the extra-embryonic ectoderm. These cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. (B) By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive PGCs is present at the base of the allantois within the extra-embryonic mesoderm (red dots). Once these PGCs are specified, they begin to migrate to the future gonadal anlagen. A, anterior; P, posterior; Pr, proximal; D, distal.

Dppa3 is expressed in pre-implantation embryos and in germ cells (Sato et al., 2002) and has recently been reported to have a role as a maternal transcript in preimplantation embryonic development (Bortvin et al., 2004). Dppa3-positive cells show increased expression of fragilis and remain positive for *Tnap* (*Akp2* – Mouse Genome Informatics) and *Oct4* (Saitou et al., 2002). Once Dppa3-positive PGCs start to migrate, they begin to express additional markers, such as steel factor receptor, followed by markers of more mature germ cells, such as murine vasa homolog (MVH; *Ddx4* – Mouse Genome Informatics) (Saitou et al., 2002).

Several recent reports describing the differentiation of mouse ES cells into cells that express markers of mature male and female germ cells (Geijsen et al., 2004; Hubner et al., 2003; Toyooka et al., 2003) are important for our understanding of the origin of ES cells. In each of these reports, germ cell markers were expressed by ES cells themselves, including those, such as Dppa3, that help distinguish germ cells from primitive ectoderm (Table 1). Only the expression of more mature germ cell markers (such as MVH) enabled in vitro-derived germ cells to be distinguished from ES cells themselves. In one study that examined the differentiation of human ES cells into germ cells (Clark et al., 2004), the expression of each of eight genes that are characteristic of early germ cells was detected in human ES cells, but the expression of each of six genes that are characteristic of later germ cells was not detected, strongly suggesting that the expression of the early germ cell-genes was not merely a result of the broadly 'leaky' transcription that is often attributed to ES cells. Using immunocytochemistry, it was also shown that most individual human ES cells in a population express the early germ cell markers stella related (STELLAR) and deleted in azoospermia-like (DAZL), indicating that a minor subset of randomly differentiating cells in a mixed population is not responsible for the expression of germ cell markers in ES cell cultures. Importantly, it was also shown that at least one germ cell-specific gene, *DAZL*, was expressed by human ES cells but not by human ICM. The existing gene expression data, then, are

consistent with the idea that the closest in vivo equivalent to ES cells is not the ICM or primitive ectoderm, but an early germ cell.

Some of the properties of ES cells, however, suggest that they are not merely the equivalent of early germ cells. For example, the earliest PGCs do not self-renew for prolonged periods of time, but instead begin a series of maturation steps, beginning with germ cell migration and ending in the highly specialized development of sperm or egg (Wylie, 1999). Although ES cells can differentiate into more mature germ cells in vitro, they do so relatively inefficiently. Indeed, the ability to colonize the germline of chimeras is one of the most easily lost properties of ES cells. If ES cells most closely represent early germ cells, it is unclear why they are not better at giving rise to more mature germ cells. In addition, isolated PGCs have never been demonstrated to contribute to chimeras when injected into blastocysts, so an exact equivalence to ES cells is unlikely.

Because a comprehensive and comparative analysis of the transcriptomes of isolated ICM, primitive ectoderm and early germ cells has not yet been reported, it is not yet clear how much the particular repertoire of genes expressed by ES cells represents an early germ cell, another specific in vivo cell type, a response to the tissue culture environment, or a combination of all three. If the ICM and primitive ectoderm are inherently heterogeneous, transcriptome analysis may need to be carried out at the single-cell level to ultimately understand these relationships. However, at the moment, the greatest concordance of known markers appears to be between ES cells and early germ cells.

Conclusions

We hypothesize that ES, EC and EG cells represent a family of related pluripotent cell lines, whose common properties reflect a common origin from germ cells (Fig. 2). Although a more detailed transcriptional analysis could ultimately refute the proposed relationship between ES cells and early germ cells, we hope this idea will at least help to stimulate a healthy

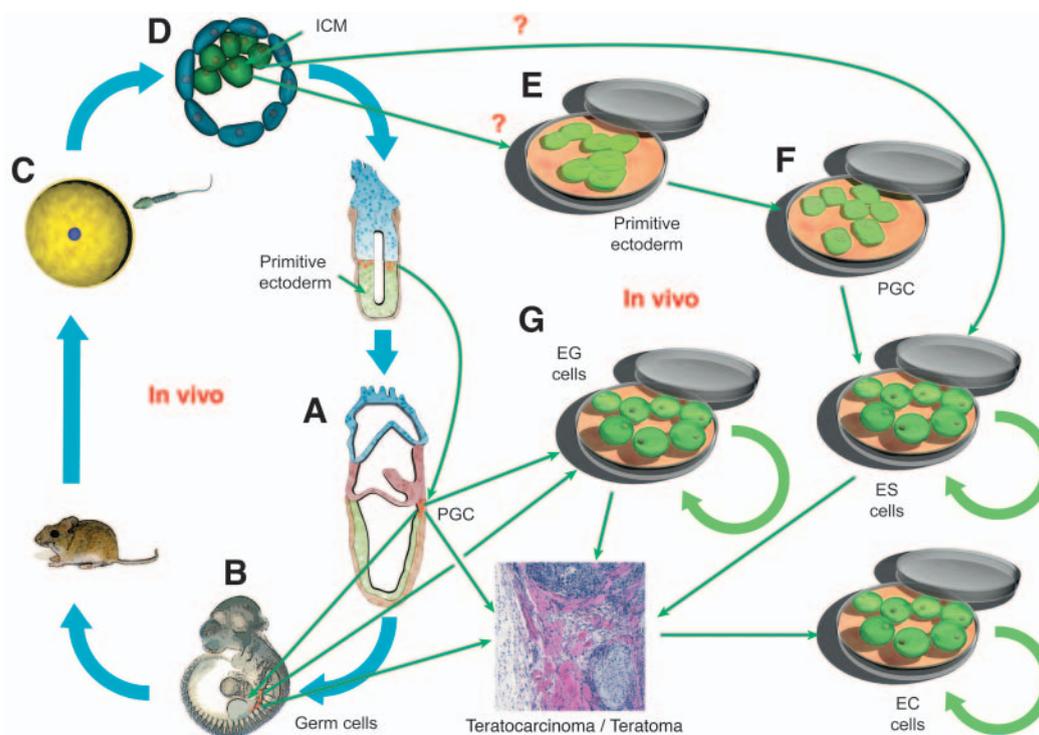


Fig. 2. Germ cell propagation in vitro and in vivo. (A-C) The germ cell cycle in the mouse. (A) Primordial germ cells (PGCs) appear at E7.25 as a small group of cells (red) in the extra-embryonic mesoderm. (B) After E8.5, PGCs start to migrate to the gonadal anlagen and contribute (C) during puberty to oocytes or sperm. (D) Embryonic stem (ES) cells are in vitro derivatives of inner cell mass (ICM) cells. (E,F) The formation of ES cells occurs either directly from the ICM/primitive ectoderm (E) or according to our hypothesis, through in vitro differentiation of ICM outgrowth into primitive ectoderm, then into extra-embryonic mesoderm and finally into PGCs (F). (G) PGCs that form in vivo (A-C) give rise to embryonic germ (EG) cells in vitro. Germ cells, PGCs, ES and EG cells are all capable of forming teratomas and teratocarcinomas. ES and EG cells can reintegrate into the normal embryo after injection into the blastocyst (not shown). Circular green arrows denote unlimited self-renewal.

re-evaluation of what is actually being studied when ES cells differentiate in vitro.

What is the relevance of a putative close relationship between ES cells and early germ cells? One prediction of this hypothesis is that at least some of the germ cell-specific genes expressed by ES cells, and not by primitive ectoderm cells, are essential for the long-term maintenance of the pluripotent state. If true, then it should be possible to generate knockout mice to identify genes that are essential for the specification or maintenance of PGCs, which are also essential for the derivation of ES cell. A related prediction of the hypothesis is that genes that are responsible for increasing susceptibility to spontaneous germ cell tumors should increase the efficiency of ES cell derivation. It is interesting, for example, that in species where teratocarcinomas occur at a clinically significant frequency, such as in mouse and human, ES cells have been successfully derived, whereas in species where teratocarcinomas are exceedingly rare, such as the rat, ES cells have proven difficult to derive. Understanding basic species differences in the specification or maintenance of early germ cells could allow the derivation of ES cells from species that have been hitherto resistant to the isolation of ES cells, such as the rat (Buehr et al., 2003).

Another implication of our hypothesis is that when looking for evolutionary clues to understand the pluripotent state, the comparative germ cell literature will be the most instructive.

In a species such as the zebrafish, which has a germ plasm that strictly separates germ cells from somatic cells, it makes sense that pluripotent cell lines that can contribute to the germline in chimeras (Ma et al., 2001) would have to be derived from germ line-lineage cells.

Another prediction arising from the hypothesis that ES cells most closely represent early germ cells is that the very earliest events of ES cell differentiation into somatic and extra-embryonic lineages will not accurately reflect events that normally occur in vivo. The idea that ES cells represent an in vitro equivalent to the ICM, however, is firmly entrenched and continues to strongly influence our thinking about these cells. When examining the differentiation of ES cells in vitro, the pervasive mental image is of a forward progression that recapitulates normal embryonic events. For example, one thinks of ICM cells progressing to primitive ectoderm cells, then to neural ectoderm cells, and finally to more specialized neural cell types. If ES cells most closely represent early germ cells, this mental image needs revision, as the earliest transition would appear to be more 'lateral' or even 'backward' than 'forward'. It will be illuminating to define each of the distinct transitions that ES cells can make in a single step and to determine how much these initial transitions resemble in vivo or artificial differentiation. If ES cells really represent early germ cells, the initial events in differentiation would be expected to be transitions that do not normally occur in intact

embryos, except, perhaps, when the transition is to more mature germ cells.

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