The origin and identity of embryonic stem cells

Jennifer Nichols1,2,* and Austin Smith1,3

Summary
Embryonic stem (ES) cells are used extensively in biomedical research and as a model with which to study early mammalian development, but their exact origin has been subject to much debate. They are routinely derived from pre-implantation embryos, but it has been suggested that the cells that give rise to ES cells might arise from epiblast cells that are already predisposed to a primordial germ cell (PGC) fate, which then progress to ES cell status via the PGC lineage. Based on recent findings, we propose that ES cells can be derived directly from early epiblast cells and that ES cells might arise via two different routes that are dictated by their culture conditions.

Key words: Cells, ES, Identity

Introduction
Pluripotency is first acquired in the mouse embryo as the epiblast forms in the inner cell mass (ICM). Under appropriate culture conditions, ICM cells can proliferate in vitro in the form of embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981). These cells retain the capacity to repopulate an embryo and to contribute descendants to all tissues of the adult, including the germline (Bradley et al., 1984), and are thus defined as being naïve pluripotent cells (Nichols and Smith, 2009). Pluripotent stem cell lines were originally derived from mouse testicular teratocarcinomas. That these teratocarcinomas arise from germ cells may be inferred from observations that teratocarcinomas are more common in the germ line than in any other tissue; that testicular tumours in mice usually arise soon after birth; and that nascent tumours have been detected specifically within the testicular tubules of foetuses (Stevens, 1962). Subsequently, it was shown that pluripotent cell lines that are almost indistinguishable from ES cells could be derived in vitro from primordial germ cells (PGCs) of the developing mouse embryo by epigenetic reprogramming (Matsui et al., 1992). Thus, a window of opportunity during which to capture naïve pluripotency seems to open twice during development: first in the early epiblast and later in the germ cell lineage.

The resemblance of embryonic germ (EG) cells to ES cells prompted the suggestion that ES cells might arise from epiblast cells that are already predisposed to a PGC fate (Gardner and Brook, 1997; Zwaka and Thomson, 2002). It was also speculated that PGCs might be induced in explants that are cultured from embryos at pre-implantation or post-implantation stages of development (Hayashi and Surani, 2009a; Tang et al., 2010). In this hypothesis article, we propose, based on recent findings, that ES cells can be derived in explant cultures via two routes: directly from the newly formed epiblast, as it transits the state of naïve pluripotency; or during the specification of PGCs in culture when cells undergo epigenetic reprogramming, resulting in their re-acquisition of pluripotency (Fig. 1).

Emergence of naïve pluripotency
Fertilisation in mammals produces a zygote that will give rise to the entire embryo and to the extra-embryonic lineages. Equipotent blastomeres are produced from the zygote during several rounds of cleavage divisions, but by the blastocyst stage, two distinct lineages have emerged that exhibit morphological and molecular differences. The trophectoderm, which is characterised by its epithelial nature and by the expression of the transcription factor Cdx2 (caudal type homeobox 2), among other specific markers (Beck et al., 1995), will contribute primarily to the placenta, whereas the ICM segregates into the epiblast (otherwise known as the primitive ectoderm) and the hypoblast, which is also commonly referred to as the primitive endoderm. The epiblast and hypoblast become completely distinct, at least in the mouse embryo, by the time of implantation. Each has its own molecular signature. Notably, the epiblast is characterised by the expression of the transcription factor Nanog, and the hypoblast by the expression of the transcription factors Gata6 and Gata4 (GATA-binding proteins 6 and 4) (Chambers et al., 2003; Chazaud et al., 2006; Plusa et al., 2008). The acquisition of epiblast identity coincides with the reactivation of the inactive X chromosome in female mouse embryos (Mak et al., 2004; Okamoto et al., 2004; Silva et al., 2009). X reactivation is also a hallmark of the successful formation of ES cells (Silva et al., 2008), and might be one of several epigenetic events that serve to alleviate chromatin inaccessibility to enable the establishment of the naïve pluripotent state in the epiblast. Hypoblast differentiation is not required for epiblast specification; embryos that carry mutations in genes encoding components of the fibroblast growth factor (Fgf)/Erk (Ephb2 – Mouse Genome Informatics) pathway, such as Grb2 (growth factor receptor-bound protein 2), lack hypoblasts but have apparently normal epiblasts (Chazaud et al., 2006; Cheng et al., 1998). Chemical inhibition of the Fgf/Erk pathway entirely suppresses hypoblast development and concomitantly expands the epiblast (Nichols et al., 2009b; Yamanaka et al., 2010). The epiblast cells from embryos treated in this way are not compromised in their developmental potential; they can contribute to normal embryogenesis and form functional germ cells when injected into host blastocysts (Nichols et al., 2009b).

Following implantation, the rodent epiblast grows from a ball of cells into a cup-shaped epithelium. This morphological change is accompanied by the reduced expression of transcription factors such as Nanog and Rex1 (zinc-finger protein 42; Zfp42 – Mouse Genome Informatics) and by the upregulation of Fgfs and brachyury (Chambers et al., 2003; Pelton et al., 2002; Thomas and Beddington, 1996). Additionally, in female embryos, random X inactivation occurs throughout the epiblast by 5.5 days post coitum (dpc) (Rastan, 1982). Over the next few days, specification of the
germ cell lineage commences in the proximal epiblast, which has initiated expression of Fgf5 and brachyury. Germ cell precursors are specified by signalling molecules, such as the bone morphogenetic proteins (BMPs) Bmp4 and Bmp8b, which are secreted by the extra-embryonic ectoderm and visceral endoderm, and are subsequently marked by expression of PGC markers such as Stella (developmental pluripotency associated 3; Dppa3 – Mouse Genome Informatics), Blimp1 (PR domain containing 1, with ZNF domain; Prdm1 – Mouse Genome Informatics) and Prdm14. Expression of somatic genes such as homeobox A1 (Hoxa1), Hoxb1, Lim1 (LIM homeobox protein 1; Lhx1 – Mouse Genome Informatics) and Evx1 (even skipped homeotic gene 1 homolog) is suppressed in the Stella-positive cells, but brachyury and Fgf8 remain expressed in these cells, indicating that they have been diverted from the naïve pluripotent state of the epiblast and have embarked upon the acquisition of a somatic fate (Saitou et al., 2002). PGCs subsequently multiply and migrate along the developing hindgut to arrive at the genital ridges at around midgestation, maintaining expression of the POU domain transcription factor Oct4 (Pou5f1 – Mouse Genome Informatics) during this time. Although Oct4 is considered to be a marker of pluripotency, PGCs are actually unipotent in vivo; however, they can be induced to acquire pluripotency when explanted or by transformation into teratocarcinoma cells.

**Derivation of ES cell lines: a brief history**

The first ES cells were successfully derived directly from blastocysts in 1981 (Evans and Kaufman, 1981; Martin, 1981). The initiative to derive pluripotent cell lines from early embryos was inspired by studies of teratocarcinoma cells. In addition to their spontaneous appearance in the testes of mice, these tumours can be generated by grafting embryos at the egg cylinder stage into an adult mouse (Damjanov et al., 1971). Undifferentiated embryonal carcinoma (EC) cell lines can be propagated from explanted tumours and can contribute to multiple tissues when injected into host blastocysts to form chimaeras (Brinster, 1974). The culture regime for retaining pluripotency included a ‘feeder’ layer of mitotically inactivated mouse fibroblasts and foetal calf serum that was batch-selected to promote proliferation and retain an undifferentiated phenotype. ES cells were derived in these conditions by allowing the blastocysts (or isolated ICMs) to develop intact for a few days prior to their disaggregation. A satisfactory outcome of ES cell derivation is confirmed by the appearance of ES cell colonies after several days of further culture (see Fig. 2B). For many years, ES cell derivation remained inconsistent and poorly understood. Interestingly, it became apparent that the facility for ES cell derivation was dependent upon the genetic background of the embryo; the 129 strain of mouse was found to be generally permissive, whereas the derivation of ES cells from embryos of the CBA strain was not possible without modification of the original protocol (Batlle-Morera et al., 2008; Brook and Gardner, 1997; Buehr and Smith, 2003).

Several studies have been performed to try to correlate the changes that occur during explant outgrowth with the successful establishment of ES cells. For example, the expression levels of Oct4, a POU domain transcription factor and marker of pluripotency, have been shown to decrease more dramatically in outgrowths of embryos explanted from mouse strains that are resistant to ES cell
derivation compared with Oct4 levels in explanted embryos from the permissive 129 strain (Buehr et al., 2003). Even in 129 embryo explants, the domain of Oct4 expression has been found to decrease to a few cells during the outgrowth period, suggesting that successful ES cell derivation does not simply depend upon the expansion of the Oct4-positive population of cells, and might actually entail epigenetic and transcriptional resetting of the expression of key genes, which possibly creates an artificial, culture-induced state (Buehr and Smith, 2003). As we discuss below, in order to dissect the process of ES cell derivation and to understand the origin of ES cells, it has been necessary to refine the crude culture regime for murine ES cell derivation.

The essential self-renewal function of feeder cells in ES cell culture protocols can be reproduced by supplementing the culture medium with the cytokine leukaemia inhibitory factor (LIF), at least for ES cells derived from embryos of the 129 strain of mice (Smith et al., 1988; Williams et al., 1988). More recently, serum has been replaced by the addition of Bmp4 or of related growth factors to the culture medium, allowing germline-competent ES cells to be propagated in defined culture conditions by supplementation with the two cytokines LIF and Bmp4 (Ying et al., 2003). Unfortunately, even under these defined conditions, it has not been possible to derive germline-competent ES cells efficiently from embryos of non-129 strains of mice. This marked variation in ability to derive ES cells from a range of different mouse strains might be related to strain-specific variation in Erk signalling (Batlle-Morera et al., 2008; Wray et al., 2010). The Erk pathway is activated independently by Fgf4 and LIF (Burdon et al., 1999; Kunath et al., 2007; Wang et al., 1994), both of which are produced in early mouse embryos (Nichols et al., 1996; Rappolee et al., 1994). Erk activity has been associated with differentiation of ES cells; inhibiting this pathway has been shown to promote ES cell self-renewal (Burdon et al., 1999). The inhibition of Erk activity has also improved the efficiency of ES cell derivation from embryos of non-129 strains of mice (Batlle-Morera et al., 2008; Buehr and Smith, 2003).

The strain specificity that has hampered ES cell derivation has now been eliminated by a new approach. Instead of activating pathways to block differentiation, small molecules are employed to inhibit specific kinases. This new ES cell culture regime, known as ‘2i’ (for two inhibitors), operates by inhibiting both Erk signalling and glycogen synthase kinase 3 (Gsk3) (Ying et al., 2008). In essence, the inhibition of Erk signalling in the developing embryo abrogates the pro-differentiation activities that are promoted during normal development (Nichols et al., 2009b). Expansion of ES cell cultures is inefficient unless GSK3 is inhibited or the Stat3 pathway is stimulated, but the mechanism for this rescue is not entirely clear (Ying et al., 2008). It is possible that Gsk3 inhibition promotes translation in the context of ES cell culture in 2i (Wray et al., 2010). By using 2i culture conditions, ES cells can be derived efficiently from all mouse strains tested to date, including the non-obese diabetic (NOD) mouse, previously considered to be the strain most recalcitrant for ES cell derivation (Hanna et al., 2009; Kiyonari et al., 2010; Nichols et al., 2009a; Ying et al., 2008). This important finding demonstrates that a particular inbred genetic predisposition is not required for ES cell derivation, but that ES cells arise owing to the generic features of early mouse development.

Generating pluripotent cell lines from post-implantation embryos

The generation of EC cells from teratocarcinomas that had arisen spontaneously in male gonads (Stevens, 1962) prompted speculation that it may be possible to derive pluripotent stem cell lines directly from PGCs. This was achieved by culturing the posterior region of 8.5 dpc mouse embryos on feeder cells in the presence of LIF, steel factor and Fgf4 (Matsui et al., 1992). These cell lines are called embryonic germ (EG) cells and they closely resemble ES cells. It is also possible to derive EG cells from explanted genital ridges at 11.5 and 12.5 dpc, but by this stage the developing germ cells have undergone imprinting erasure, which renders the derivative EG cells less able to integrate into chimaeras, and particularly into the germline (Labosky et al., 1994; Tada et al., 1998). The derivation of EG cells involves transient supplementation with Fgf at explantation, and it has been suggested that this addition triggered an epigenetic reprogramming process (Durcova-Hills et al., 2006). However, Fgf appears not to be necessary if PGCs are cultured directly in 2i medium with LIF (Leitch et al., 2010). It appears that under these conditions the epigenetic barrier from PGC to EG cell status may be more easily traversed. This may be attributable to the capacity of Erk and Gsk3 inhibition to prevent the PGCs from progressing along their normal differentiation pathway.

The post-implantation mouse epiblast has been shown to give rise to multi-lineage-competent teratocarcinomas (Diwan and Stevens, 1976; Solter et al., 1970). However, it has not been possible to derive ES cells from post-implantation epiblasts directly. Recently, pluripotent cell lines were generated from post-implantation mouse embryos using the culture conditions employed for the derivation of stem cell lines from human blastocysts; specifically, explanting on to a substrate of feeder cells or fibroblastin and supplementation of the medium with activin and Fgf2 (Brons et al., 2007; Tesar et al., 2007; Thomson et al., 1998). These ‘EpiSCs’ cannot be propagated efficiently from single cells and do not readily contribute to chimaeras. They share many characteristics with the pluripotent lines derived from human embryos, suggesting that human ‘ES’ cells may actually represent a more advanced stage of development than the blastocyst (Fig. 3) (Brons et al., 2007; Nichols and Smith, 2009; Rossant, 2008; Tesar et al., 2007). Fgf2 is crucial for the derivation and subsequent expansion of both human ‘ES’ cells and EpiSCs. EpiSCs are
transcriptionally and epigenetically distinct from ES cells; EpiSCs are characterised by expression of Fgf5 and brachyury, whereas a hallmark of ES cells is the expression of Rex1 and Klf4 (Kruppel-like factor 4), and the absence of X chromosome inactivation (Brons et al., 2007; Guo et al., 2009; Tesar et al., 2007). EpiSCs can be reprogrammed to assume the defining characteristics of ES cells, including germ line competence, by transfecting them with single pluripotency factors, such as Klf4 (Guo et al., 2009; Hall et al., 2009). Extended culture in the presence of feeders and LIF can also reprogramme EpiSCs at low efficiency. This may be attributable to their reversion to an early epiblast state (Bao et al., 2009) or to their progression to PGCs, which then convert to EG cells (Hayashi and Surani, 2009b). Interestingly, naïve pluripotent cells can be derived from single cells isolated from the mouse post-implantation epiblast and explanted into culture on feeders in medium supplemented with serum and LIF, but this process requires a culture period of 2-5 weeks (Bao et al., 2009).

How do ES cells arise?

Because ES cells have been produced from explanted ICMs, historically they have been equated to ICM cells from 3.5 dpc mouse blastocysts. However, it is also possible to derive ES cells from blastomeres isolated from mouse embryos at the eight-cell stage (Eistetter, 1989). During the process of ES cell derivation from isolated blastomeres, cell division results in the formation of a ‘mini-blastocyst’ environment, from which ES cells may subsequently emerge. The derivation of ES cells directly from individual ICM cells isolated before segregation of the epiblast and hypoblast has not been reported. In a study involving the microdissection of peri-implantation mouse embryos at 4.5 dpc, Brook and Gardner demonstrated conclusively that the origin of ES cells is the early epiblast, following its overt segregation from the hypoblast (Brook and Gardner, 1997). They also showed that ES cells can be derived directly from single epiblast cells isolated from freshly retrieved 4.5 dpc embryos, but achieved a maximum of only three ES cell clones per embryo. This led to speculation that the process of ES cell derivation may capture a subpopulation of the epiblast that contains specified germ cell precursors (Gardner and Brook, 1997; Zwaka and Thomson, 2005). However, by using the more permissive culture conditions of 2i medium supplemented with LIF, the number of ES cell clones that can be obtained from a single embryo can be increased (Nichols et al., 2009b). This suggests that all epiblast cells may transiently have the capacity to become ES cells, although the realisation of this potential depends upon the timing of their isolation and their culture environment.

Recently published studies have provided evidence that ICM cells undergo significant changes during the outgrowth phase of ES cell derivation. A single-cell gene expression analysis, performed using cells from whole-mouse embryos plated in conventional ES cell culture conditions, has shown that the molecular profile of a subgroup of cells changes dramatically as they progress from ICM to ES cell status (Tang et al., 2010). These changes include the upregulation of genes that are associated with self-renewal, such as Lin28a, Tcf3 (transcription factor 3) and Nr0b1 (nuclear receptor subfamily 0, group B, member 1), and the downregulation of genes associated with developmental differentiation, such as Gata6, Cdx2 and Hoxd8, coincident with specific alterations in the levels of certain epigenetic regulators, such as an increase in Dnmt3a (DNA methyltransferase 3A), Hdac5 (histone deacetylase 5) and Cbx1 (chromobox homologue 1). Interestingly, a proportion of genes involved in the specification of PGCs, such as fragilis (interferon induced transmembrane protein 3; Ifitm3 – Mouse Genome Informatics), Blimp1 and Prdm14, are slightly upregulated during outgrowth and in the resulting ES cells (Tang et al., 2010). This observation raises the possibility that in whole-epiblast culture under serum-containing conditions, it may be possible for induction of germ cell precursors to occur. These might potentially be converted to EG cells by further culture, resulting in the generation of pluripotent cell lines. Thus, we speculate that there may be two alternative routes to the derivation of naïve pluripotent cell lines during the derivation process. The first captures the state of naïve pluripotency as it arises in the newly formed epiblast; the second depends upon induction of the germ cell lineage during outgrowth culture (Fig. 1). It would be interesting to perform single-cell gene
expression analysis on embryos and their derivative ES cell lines during the process of ES cell derivation in 2i medium. It might be anticipated that PGC-associated genes, such as fragilis and Blimp1, would not be detected if ES cells can be derived directly from the early epiblast.

**The role of embryonic diapause in ES cell derivation**

An interesting property of rodents is their potential for embryonic diapause, which may be significant for the facility of ES cell derivation. Diapause is a phenomenon that can occur in mice and rats when embryos are produced in a suckling mother. In diapause, the embryos advance to the blastocyst stage, hatch from the zona pellucida and segregate the epiblast and hypoblast, but then remain in an unimplanted, non-progressive state until oestrus is restored. The epiblast retains expression of Oct4 and Nanog, and both X chromosomes are active in female embryos (Silva et al., 2009). LIF signalling is specifically required in the epiblast in diapause, consistent with there being a close relationship between the early epiblast and ES cells (Nichols et al., 2001). The implementation of diapause has been shown to improve the efficiency of ES cell derivation (Brook and Gardner, 1997; Kawase et al., 1994). This may be because development is paused when the epiblast is in the state of naïve pluripotency, thus increasing the likelihood of capturing this state in culture. From mammals that do not have diapause, true ES cells have not yet been derived, and little is known about the details of epiblast development (such as the relative timing of commitment, the expression of markers and the reactivation/inactivation status of the X chromosome) that would facilitate culture optimisation. Does this failure to produce true ES cells reflect an evolutionary divergence of development in which the state of naïve pluripotency is rapidly transited or bypassed in non-murine mammals, or are the current culture conditions unsuitable to capture this state in vitro? The means to harness naïve pluripotent cells from non-rodent mammals may require the development of culture conditions that will promote the progression of an explanted epiblast cell through development to PGC specification, and from PGC specification to reprogramming to an EG cell.

**Pluripotent cell lines from human embryos**

The overlap in properties between human ‘ES’ cells and EpiSCs suggests that during the derivation process in humans, the epiblast may advance to the equivalent of the post-implantation state. Although these pluripotent human cell lines are widely used for academic and pharmaceutical research, they tend to be somewhat restricted in their differentiation capacity and are generally more difficult to manipulate than are mouse ES cells. Will it be possible to derive naïve pluripotent human ES cells that more closely resemble mouse ES cells than mouse EpiSCs? Optimism for the attainment of this goal is provided by a recent study in which the key pluripotency factors Oct4, Klf4 and Klf2 were ectopically induced in human ‘ES’ cells in 2i medium supplemented with LIF and forskolin. These conditions led to the conversion of these cells into a cell type that possesses the characteristics of mouse ES cells, including the possession of two active X chromosomes and a responsiveness to LIF signalling (Hanna et al., 2010). It remains to be determined whether ES cells from human embryos could be captured by devising a strategy to maintain the early epiblast cells in a state of naïve pluripotency. This may require the isolation of epiblast cells from trophoderm and extra-embryonic endoderm or the deployment of additional kinase inhibitors to prevent explanted cells from progressing to the state of the post-implantation epiblast. Alternatively, if such attempts are unfruitful, it may be worth further investigating the possibility of capturing the PGC-EG transition by inductive explant culture.

**Conclusions**

We have presented here the possibility that the state of naïve pluripotency of mouse ES cells may be reached in vitro by two main alternative routes (captured directly from the nascent epiblast or via induction of PGCs), which depend upon the culture conditions used and the cellular environment. This reflects the complex intertwining of pluripotency and the germline in mammalian embryos. We propose experimental approaches to validate this hypothesis that include single-cell molecular profiling and time-lapse imaging of reporter expression in explanted embryos or ICMs during ES cell derivation, using alternative culture regimes and at various time points. The key information to be gained from these experiments will be whether or not genes associated with germ cell specification, such as fragilis and Blimp1, are turned on during ES cell derivation in 2i medium, as was previously reported for ES cell derivation using feeders and serum (Tang et al., 2010). If not, it may be concluded that the progression from ICM to ES cell need not be achieved via PGCs. However, that these PGC-associated genes have been detected during ES cell derivation on feeders with serum implies an alternative, inductive route to attaining naïve pluripotency. To obtain naïve pluripotent cells from non-murine mammals, monitoring the expression of these genes may provide a means to optimise protocols for ES cell derivation that maximise the induction of PGCs in explant cultures. It may then be possible to develop protocols that convert PGCs to EG cells. The recent report describing the efficient production of EG cells from rat embryos using 2i medium may shed light on how to achieve this goal (Leitch et al., 2010).

**Acknowledgements**

We thank all members, past and present, of the Smith and Nichols laboratories, especially Graziano Martello for critical discussion of the manuscript and Deborah Goode for help with the figures. A.S. is funded by The University of Cambridge, UK.

**Competing interests statement**

The authors declare no competing financial interests.

**References**


Labosky, P. A., Barlow, D. P. and Hogan, B. L. (1994). Mouse embryonic germ (EG) cell lines: transformation through the germ and epiblast stages in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development 120, 3197-3204.


