Cell

Capture of Authentic Embryonic Stem Cells from Rat Blastocysts

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SUMMARY

Embryonic stem (ES) cells have been available from inbred mice since 1981 but have not been validated for other rodents. Failure to establish ES cells from a range of mammals challenges the identity of cultivated stem cells and our understanding of the pluripotent state. Here we investigated derivation of ES cells from the rat. We applied molecularly defined conditions designed to shield the ground state of authentic pluripotency from inductive differentiation stimuli. Undifferentiated cell lines developed that exhibited diagnostic features of ES cells including colonization of multiple tissues in viable chimeras. Definitive ES cell status was established by transmission of the cell line genome to offspring. Derivation of germline-competent ES cells from the rat paves the way to targeted genetic manipulation in this valuable biomedical model species. Rat ES cells will also provide a refined test-bed for functional evaluation of pluripotent stem cell-derived tissue repair and regeneration.

INTRODUCTION

Authentic ES cells are defined by three cardinal properties: unlimited symmetrical self-renewal in vitro; comprehensive contribution to primary chimeras; and generation of functional gametes for genome transmission. ES cells are obtained from pluripotent epiblast cells of the mouse blastocyst extracted from the uterine environment and placed in the artificial context of laboratory cell culture (Buehr and Smith, 2003; Evans and Kaufman, 1981; Gardner and Brook, 1997; Martin, 1981). It is unclear whether ES cells themselves arise as a consequence of the synthetic culture milieu or represent a transient phase in ontogeny that is captured by arresting developmental progression (Buehr and Smith, 2003; Gardner and Brook, 1997; Niwa, 2007; Silva and Smith, 2008; Smith, 2001a).

Empirical evidence to date is that ES cells can reproducibly be derived from only a few inbred mouse strains. This is achieved using fibroblast feeders and/or the cytokine leukemia inhibitory factor (LIF) in combination with selected batches of fetal calf serum or the growth factor bone morphogenetic protein (Gardner and Brook, 1997; Ying et al., 2003a). The same conditions do not yield ES cells from most mouse strains and not at all from the rat (Brenin et al., 1997; Prelle et al., 1999). We (Buehr et al., 2003) and others (Fandrich et al., 2002; Vassilieva et al., 2000) have reported derivation of cell lines from preimplantation rat embryos that have superficial morphological resemblance to ES cells but do not express biologically relevant levels of the key transcription factor determinants of ES cell identity, Oct4 (Niwa et al., 2000) and Nanog (Chambers et al., 2003; Mitsui et al., 2003), and are not capable of germlayer differentiation in vitro, in tumors, or in chimeras. In our experience, such cells give rise only to extraembryonic trophoblast and hypoblast lineages, and we refer to them as extraembryonic stem (ExS) cells (Buehr et al., 2003).

Shortly after implantation the epiblast transforms into an epithelium and in rodents forms a cup-shaped structure termed the egg cylinder. Cell lines termed EpiSCs have recently been derived from the epithelialized epiblast of postimplantation mouse and rat egg cylinders (Brons et al., 2007; Tesar et al., 2007). These cells have fundamentally different growth factor requirements from ES cells. They are sustained by fibroblast growth factor (FGF) plus activin or nodal and not by LIF. In this respect they resemble primate embryo-derived stem cells (Thomson et al., 1998; Vallier et al., 2005; Xu et al., 2005). Like primate stem cells and unlike ES cells, EpiSCs are sensitive to single-cell dissociation and are generally passaged as clusters of cells rather than dispersed. They do exhibit capacity for multilineage differentiation and teratoma formation. Crucially, however, EpiSCs fail to incorporate properly into the inner cell mass (ICM) when injected into blastocysts and do not contribute significantly to chimeras (Tesar et al., 2007).

Although the tissue and stage of origin of ES cells and EpiSCs are defined, the identity between these cultured cell lines and resident cells in the embryo is uncertain (Rossant, 2008). The derivation of cell lines in culture may involve significant transcriptional and/or epigenetic reprogramming, particularly when potent stimuli such as FGF are involved. One example of this is the reprogramming of unipotent primordial germ cells to generate pluripotent EG cells (Durcova-Hills et al., 2006; Matsui et al., 1992; Resnick et al., 1992). Derivation of tripotent neural stem cells using FGF may be a similar instance (Gabay et al., 2003; Pollard et al., 2008). However, the key parameter determining derivation and propagation of mouse ES cells appears to be suppression or neutralization of extrinsic differentiation signals (Ying et al., 2003a, 2008), rather than provision of a self-renewal stimulus. Based on those findings we have postulated that ES cells represent a ground state in mammalian development and that this may be shared with preimplantation epiblast cells (Silva and Smith, 2008).

An empirically determined culture construction that is effective for derivation of ES cells only in specific inbred laboratory mouse strains may actually be counterproductive for maintaining ground state pluripotency more broadly. Serum and serum substitutes contain a variety of inductive stimuli that may activate commitment and differentiation programs. ES cells will continue to proliferate in the absence of serum. However, simple withdrawal of serum or other exogenous stimuli is not sufficient to prevent differentiation because of the autoinductive action of fibroblast growth factor 4 (FGF4) (Kunath et al., 2007; Stavridis et al., 2007; Ying et al., 2003b). FGF4 signaling through the MEK/ERK pathway drives ES cells into commitment. Genetic impairment or selective chemical blockade of this pathway can sustain self-renewal of mouse ES cells, even in the absence of LIF signaling (Chen et al., 2006; Ying et al., 2008). However, to suppress differentiation entirely and maintain high viability and growth rate when FGF/ERK signaling is reduced it is necessary either to provide LIF or to restrict activity of glycogen synthase kinase 3 (GSK3). GSK3 is a central node for negative modulation of a range of anabolic processes and generally acts to suppress cellular biosynthetic capacity (Frame and Cohen, 2001). GSK3 is inhibited by phosphorylation downstream of growth factors that activate phosphatidyl inositol 3 kinase and Akt. GSK3 is also a key component of the β-catenin destruction complex and pharmacological inhibition of GSK3 increases cytoplasmic and nuclear β-catenin, mimicking canonical Wnt signaling (Ding et al., 2000). The small molecule CHIR99021 selectively inhibits both GSK3α and GSK3β (Murray et al., 2004). The combination of three inhibitors (3i) that target FGF receptor, MEK, and GSK3 enables efficient derivation and propagation of germline-competent ES cells from a range of mouse strains (Ying et al., 2008) (J. Nichols and A.S., unpublished data). These findings suggest that the key to deriving and maintaining ES cells may be to shield the native epiblast ground state from activation of the ERK pathway by either exogenous or autocrine inductive stimuli that will normally drive developmental progression. Complementary inhibition of GSK3 stabilizes the ground state, likely via a combination of β-catenin-dependent action and β-catenin-independent anabolic effects (Silva and Smith, 2008) (J. Wray and A.S., unpublished data). To test the generality of this concept we have investigated the possibility of deriving true ES cells from the rat.

RESULTS

Sustained Expression of Pluripotent Markers in Rat Inner Cell Masses Explanted in 3i

We first examined the effect of the neutralizing 3i culture regime on ICMs explanted from rat blastocysts. In addition to 3i we provided LIF because although this is dispensible for mouse ES cell culture in 3i, we find that clonogenicity and ES cell derivation are invariably enhanced by addition of LIF (Ying et al., 2008) (J. Wray, J. Nichols, and A.S., unpublished data). As a source of LIF we used DIA-M feeders that have been genetically engineered to express the matrix-associated form of LIF (Buehr et al., 2003; Rathjen et al., 1990b). The feeders also support attachment of the ICMs.

Retained expression of the transcriptional determinant Oct4 can be used as a surrogate assay for presence of pluripotent cells (Buehr et al., 2003). In ICM explants in conventional culture Oct4 is rapidly extinguished (Buehr and Smith, 2003). ICMs were isolated from embryonic day (E) 4.5 rat blastocysts by immunosurgery and plated on DIA-M feeders. Explants were fixed at different time points and analyzed by immunostaining (Figure 1A). In standard culture with or without serum we observed loss of Oct4 by 3 days as previously described (Buehr et al., 2003). Concurrent with downregulation of Oct4 we noted ectopic appearance of Cdx2, the trophectoderm determinant and antagonist of Oct4 (Niwa et al., 2005). Expression of Oct4 and Cdx2 were generally mutually exclusive. In contrast, in the presence of 3i we found that Oct4 protein was maintained in the majority of cells after 3 days and expression of Cdx2 was suppressed. By 4 days the majority of cells in the core of the explants were Oct4 positive (Figure 1B). A second critical marker of pluripotent status, Nanog (Chambers et al., 2003; Mitsui et al., 2003), which is downregulated in similar fashion to Oct4 in conventional culture, was similarly maintained in serum-free medium supplemented with 3i, although with a more heterogeneous expression than Oct4. The effect of 3i is unlikely to be due to cell selection because the total number of ICM cells was actually higher in the 3i-treated cultures (Table S1 available online).

We then dissociated ICMs cultured in 3i for 3 days into small clumps and replated them in the same conditions. Cells remained viable and formed colonies of morphologically undifferentiated cells. These colonies continued to express Oct4 and Nanog (data not shown). In many previous experiments under various conditions (Buehr et al., 2003; Buehr and Smith, 2003), we have very rarely detected expression of Oct4 after replating and only confined to small clusters of cells, never throughout the outgrowths as seen in 3i. These observations suggest that 3i may suppress loss of pluripotency and differentiation of cultured rat epiblast.

Derivation of Continuous Cell Lines

We therefore investigated longer-term effects. ICMs from two different inbred strains, Dark Agouti (DA) and Fischer 344, were plated on DIA-M feeders in serum-free 3i. After 3–4 days, around one-third of the ICMs had attached and proliferated such that