Genesis of embryonic stem cells

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Embryonic stem (ES) cells are permanent pluripotent stem cell lines established from pre-implantation mouse embryos. There is currently great interest in the potential therapeutic applications of analogous cells derived from human embryos. The isolation of ES cells is commonly presented as a straightforward transfer of cells in the early embryo into culture. In reality, however, continuous expansion of pluripotent cells does not occur *in vivo*, and *in vitro* is the exception rather than the norm. Both genetic and epigenetic factors influence the ability to derive ES cells. We have tracked the expression of a key marker and determinant of pluripotency, the transcription factor Oct-4, in primary cultures of mouse epiblasts and used this to assay the effect of experimental manipulations on the maintenance of a pluripotent cell compartment. We find that expression of Oct-4 is often lost prior to overt cytodifferentiation of the epiblast. The rate and extent of Oct-4 extinction varies with genetic background. We report that treatment with the MAP kinase/ERK kinase inhibitor PD98059, which suppresses activation of the mitogen-activated protein kinases Erk1 and Erk2, results in increased persistence of Oct-4-expressing cells. Oct-4 expression is also relatively sustained in cultures of diapause embryos and of isolated inner cell masses. Combination of all three conditions allowed the derivation of germline-competent ES cells from the normally refractory CBA mouse strain. These findings suggest that the genesis of an ES cell is a relatively complex process requiring epigenetic modulation of key gene expression over a brief time-window. Procedures that extend this time-window and/or directly regulate the critical genes should increase the efficiency of ES cell derivation.

Keywords: pluripotency; self-renewal; diapause; reprogramming; epigenetics; mitogen-activated protein kinase

1. INTRODUCTION

ES cells are pluripotent cell lines derived from the epiblast of the mouse embryo without any genetic transformation (Evans & Kaufman 1981; Martin 1981). They retain pluripotency in culture, can be genetically engineered and can be used to generate chimaeras with germline contribution (Bradley *et al.* 1992). Consequently, ES cells have found a number of important applications in basic research (A. Smith 2001). More recently, pluripotent stem cells similar to mouse ES cells have been isolated from non-human primate and human blastocysts (Thomson *et al.* 1995, 1998) opening the door to potential applications in drug discovery and cell replacement medicine (Smith 1998; Solter & Gearhart 1999). These aspirations for biotechnological and medical exploitation will require efficient and robust derivation of stable pluripotent stem cell lines (A. G. Smith 2001).

In this context, it is valuable to remember that ES cells are not readily derived from every mouse strain and no proven derivations have been described for related rodents such as rats, or for livestock species, despite considerable efforts over more than 20 years (Gardner & Brook 1997; Prelle *et al.* 1999). Stable mouse ES cell lines originate consistently only from the 129 or C57BL/6 strains, or

hybrids thereof. Specialized protocols have been applied in individual laboratories (McWhir *et al.* 1996; Brook & Gardner 1997) to establish lines from other strains, such as CBA, but the generality of these procedures is unclear.

ES cells, as well as the epiblast from which they are derived, express the POU transcription factor Oct-4, a specific marker of pluripotent cells *in vivo* and *in vitro* (Rosner *et al.* 1990; Schöler *et al.* 1990a). Oct-4 appears to be indispensable for pluripotent capacity both in the embryo and in ES cell lines (Nichols *et al.* 1998; Niwa *et al.* 2000). It is assumed, although not formally proven, that continual expression of Oct-4 in precursor cells is necessary throughout derivation for the establishment of ES cell lines.

However, when a blastocyst is cultured in standard ES cell derivation conditions, the number of Oct-4-expressing cells declines prior to overt cytodifferentiation of the epiblast (Buehr *et al.* 2002). This precipitate loss of Oct-4 may underlie the general inefficiency of ES cell derivation because if an outgrowth loses all Oct-4 expression during the first few days of culture, it is likely to have lost all ES cell precursors. By contrast, escape from initial extinction of Oct-4 may be a key event in the birth of an ES cell, since expression is stabilized in established ES cells. Therefore, any condition that helps maintain Oct-4 expression in epiblast cultures may improve the efficiency of ES cell derivation.

We have assessed the impact on Oct-4 expression in cultured epiblast and, subsequently, on ES cell derivation of three manipulations: (i) exposure to PD98059, a well-

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One contribution of 14 to a Discussion Meeting Issue 'Epigenesis versus preformation during mammalian development'.

Table 1. Expression of Oct-4 in cultures of 129 and CBA blastocysts in the absence or presence of PD98059 (PD). (Abbreviation: n.d., not determined.)

days in culture	embryo outgrowths with positive cells (%)			
	CBA		129	
	without PD	50 μ M PD	without PD	50 μ M PD
4	96 ($n = 26$)	96 ($n = 23$)	n.d.	n.d.
5	20 ($n = 40$)	68 ($n = 22$)	75 ($n = 80$)	88 ($n = 34$)
6	8 ($n = 135$)	48 ($n = 112$)	41 ($n = 22$)	20 ($n = 54$)

Figure 1. Oct-4 *in situ* hybridization on CBA blastocyst cultures in the absence or presence of PD98059. (a) Control blastocyst outgrowths in the absence of PD98059. After 6 days Oct-4 expression is absent from most outgrowths and present in a small cluster of cells in one outgrowth. (b) Outgrowths cultured for 6 days in the presence of PD98059. Prominent patches of Oct-4-positive cells are present in the central masses of cells. Scale bars, 100 μ M.

characterized specific inhibitor of the Erk activating enzyme MEK-1 (Dudley *et al.* 1995) previously shown to enhance self-renewal of established ES cells (Burdon *et al.* 1999); (ii) prior induction of diapause; and (iii) immunosurgical isolation of the ICM.

2. MATERIAL AND METHODS

(a) Animals

Mice used were of the 129/Ola and CBA/CA strains. Diapause was induced by the method of MacLean Hunter & Evans (1999): animals were injected intraperitoneally with 10 μ g of tamoxifen (Sigma) and 1 mg of Depo-Provera (Upjohn) at 2.5 dpc.

(b) Culture conditions

Blastocysts and ICMs were collected at 3.5 dpc; diapause embryos were collected 5 days after treatment with tamoxifen. All material was cultured on feeder cells expressing the matrix-localized form of LIF (DIA-M cells) (Buehr *et al.* 2002) in Glasgow modification of minimal essential medium (Sigma) supplemented with 15% foetal calf serum, penicillin-streptomycin

Table 2. Oct-4-positive colonies after disaggregation and 10 days culture in the absence or presence of PD98059 (PD).

cell strain	percentage of embryos giving rise to Oct-4-positive colonies	
	without PD	50 μ M PD
CBA	0 ($n = 85$)	0 ($n = 56$)
129	11.7 ($n = 51$)	11.3 ($n = 54$)

and 2-mercaptoethanol. Human LIF at a concentration of 200 U ml⁻¹ (Smith 1991) was added to all cultures at all stages.

The MEK-1 inhibitor PD98059 (New England Biolabs) was prepared as a stock solution of 50 mM in DMSO, and where required added to the medium at a final concentration of 50 μ M. PD98059-supplemented medium was renewed every 2 days. Two control conditions were used: (i) 1 μ l ml⁻¹ DMSO was added to the medium as a vehicle control; and (ii) no DMSO was added. The results from both groups were indistinguishable and were subsequently pooled.

(c) Culture of primary undifferentiated cell colonies and ES cell derivation

To monitor the frequency with which primary ES cell colonies were obtained, outgrowths were individually disaggregated after brief exposure to trypsin (0.025%) and replated in the same conditions. Blastocyst cultures were disaggregated after 5 days and isolated ICMs after 6 days. The disaggregated cultures were maintained for 10 days, examined for the presence of undifferentiated cell colonies of ES morphology and then fixed and processed for Oct-4 *in situ* hybridization.

To confirm the ES cell status of these primary colonies, four undifferentiated colonies from PD98059 cultures (2 $\times$ 129, 2 $\times$ CBA), identified by morphology after 10 days of culture, were picked and replated for expansion into ES cell lines. After picking, the PD98059 concentration was reduced in a stepwise manner over several days to 37.5, 25 and 12.5 μ M. Three lines (the 129 lines and CBA line 1) were then removed completely from PD98059, while the other line (CBA line 2) was maintained in 12.5 μ M inhibitor.

(d) In situ hybridization

Whole-mount *in situ* hybridization of cells was performed as previously described (Buehr *et al.* 2002). The template for the Oct-4 riboprobe is a *Stu1* fragment corresponding to nucleotides 951–489 of GenBank accession no. X52437 (Schöler *et al.* 1990b).

Figure 2. The effect of PD98059 on formation of Oct-4-positive colonies from strain 129 blastocyst outgrowths after disaggregation and replating. (a) Two small colonies of Oct-4-positive cells typically seen 10 days after replating in the absence of PD98059. (b) A typical large Oct-4-positive colony formed 10 days after disaggregation and replating with culture throughout in the presence of PD98059. (c) Phase-contrast view of a large primary colony of undifferentiated cells 10 days after disaggregation and replating with culture throughout in the presence of PD98059. Scale bars, 100 μ M.

(e) *Immunosurgery*

Immunosurgery was performed according to Solter & Knowles (1975). The antibody was anti-mouse whole serum (Sigma) and the source of complement was whole rat serum.

3. RESULTS

(a) *Effect of PD98059 on retention of Oct-4 expression in blastocyst outgrowths*

We have previously shown (Buehr *et al.* 2002) that when blastocysts are placed in culture, both the proportion of Oct-4-expressing cells per outgrowth and the number of outgrowths retaining Oct-4-expressing cells decline even though the mass of morphologically undifferentiated ICM-derived tissue increases. This downregulation of Oct-4 takes place over several days (table 1). After 6 days in culture, less than 10% of CBA outgrowths retain any Oct-4-expressing cells. Loss of Oct-4 expression also occurs in 129 outgrowths, but to a notably lesser degree: nearly half of 6-day 129 outgrowths retain some Oct-4-positive cells. This strain difference is striking given the well-established differences in the permissiveness of these two strains for ES cell generation (Brook & Gardner 1997).

Blastocysts of both strains were then cultured in the presence of PD98059 at 50 μ M, a concentration previously shown to suppress Erk activation in ES cells and to enhance self-renewal (Burdon *et al.* 1999). The addition of PD98059 slightly diminished the expansion in cell mass compared with untreated controls over the 6-day period but had no overt effect on differentiation. Outgrowths were examined for the presence of Oct-4-expressing cells (table 1). The proportion of 129 outgrowths containing Oct-4-positive cells was not significantly altered. By contrast, PD98059 increased the proportion of CBA outgrowths with Oct-4 expression to a level comparable with strain 129, that is, nearly 50% retaining positive cells after 6 days. Figure 1a shows representative CBA outgrowths with patches of strong Oct-4 mRNA hybridization after 6 days' culture in PD98059,

while in the absence of PD (figure 1b) the Oct-4 signal is minimal.

(b) *Effect of PD98059 on the generation of undifferentiated primary colonies from non-manipulated blastocysts*

As PD98059 appeared to enhance the persistence of Oct-4 expression in CBA outgrowths, we then disaggregated outgrowths cultured with or without PD98059 and replated them in the same conditions. After 10 days, cultures were inspected for the presence of undifferentiated cell colonies and then fixed and analysed for Oct-4 expression. We observed complete correspondence between Oct-4 expression and undifferentiated morphology.

Data in table 2 indicate that despite its influence on Oct-4 expression in CBA blastocyst cultures, PD98059 treatment does not result in propagation of undifferentiated stem cells after disaggregation. Furthermore, it has no appreciable effect on the frequency with which strain 129 blastocysts give rise to Oct-4-positive colonies. However, the MEK-1 inhibitor does affect the size of colonies from 129 outgrowths. Figure 2 shows primary colonies of 129 ES cells 10 days after outgrowth disaggregation, cultured in the absence (figure 2a) or presence (figure 2b) of PD98059 and stained for Oct-4 expression. Treatment with PD98059 has clearly enhanced the size of the primary colony. This was a consistent observation. A phase-contrast photograph of a similar large colony is presented in figure 2c, showing characteristic ES cell morphology.

During previous studies, it has been observed that once undifferentiated primary colonies are obtained they can almost invariably be propagated as continuous ES cell lines (J. Nichols, personal communication). To confirm that this remained the case after PD98059 treatment, two primary strain 129 colonies were picked and expanded. Both yielded cultures which were indistinguishable from regular ES cells and continued to proliferate upon complete withdrawal of PD98059. One of these lines was

Table 3. Expression of Oct-4 in 6 day cultures of 129 and CBA blastocysts and isolated ICMs in the absence or presence of PD98059 (PD).

(Abbreviation: n.d., not determined.)

	outgrowths with Oct-4 positive cells (%)	
	without PD	50 μ M PD
CBA		
3.5 dpc blastocyst	8 (<i>n</i> = 135)	48 (<i>n</i> = 112)
diapause blastocyst	21 (<i>n</i> = 29)	54 (<i>n</i> = 50)
3.5 dpc ICM	33 (<i>n</i> = 60)	52 (<i>n</i> = 40)
diapause ICM	56 (<i>n</i> = 34)	50 (<i>n</i> = 18)
129		
3.5 dpc blastocyst	41 (<i>n</i> = 22)	20 (<i>n</i> = 54)
diapause blastocyst	76 (<i>n</i> = 30)	100 (<i>n</i> = 24)
3.5 dpc ICM	36 (<i>n</i> = 11)	100 (<i>n</i> = 3)
diapause ICM	100 (<i>n</i> = 17)	n.d.

Table 4. Oct-4-positive colonies from disaggregated CBA embryos cultured in the absence or presence of PD98059 (PD).

	percentage of embryos giving rise to Oct-4-positive colonies	
	without PD	50 μ M PD
3.5 dpc blastocyst	0 (<i>n</i> = 85)	0 (<i>n</i> = 56)
diapause blastocyst	0 (<i>n</i> = 16)	0 (<i>n</i> = 20)
3.5 dpc ICM	0 (<i>n</i> = 28)	0 (<i>n</i> = 24)
diapause ICM	0 (<i>n</i> = 41)	26 (<i>n</i> = 43)

Table 5. Chimaeras from ES cells derived in PD98059.

strain	blastocysts injected	mice born	chimaeras
129	32	12	3
CBA line 1	25	8	2
CBA line 2	24	14	2

3 males: 2 germline chimaeras, 1 did not breed
1 male: not a germline chimaera. 1 female: did not breed
2 males: both germline chimaeras

injected into blastocysts and produced chimaeras that gave germline offspring.

(c) Effect of diapause, immunosurgical isolation of the ICM, and PD98059 on the retention of Oct-4-expressing cells

ES cell lines are more efficiently derived from blastocysts in diapause than from intact 3.5 dpc blastocysts (Kawase *et al.* 1994; Brook & Gardner 1997). They may also be derived from immunosurgically isolated ICMs (Martin 1981). We therefore investigated the effects of diapause and ICM isolation on retention of Oct-4-expressing cells and the impact of PD98059 on such cultures (table 3).

Diapause blastocysts showed greater retention of Oct-4 after 6 days, which was further enhanced by PD98059. This was observed for both CBA and 129 embryos. Purified ICMs showed even greater persistence of Oct-4-expressing cells in the CBA strain (data for 129 were inconclusive), which was again enhanced by PD98059. Finally, CBA ICMs isolated from diapause blastocysts retained Oct-4-expressing cells in 50% of outgrowths.

At this level, there was no additional effect of the MEK inhibitor, even though a higher proportion of expressing outgrowths can readily be obtained from the 129 strain.

(d) A combination of manipulations allows derivation of CBA ES cells

Based on the preceding observations we disaggregated CBA outgrowths of normal and diapause blastocysts, and normal and diapause ICMs, cultured in the absence or presence of PD98059, and monitored the replated cultures for primary undifferentiated cell colonies.

Data summarized in table 4 show that the individual effects of diapause and ICM isolation, with or without PD98059, are not sufficient to engender undifferentiated stem cell colonies from CBA embryos. By contrast, when we combined the three experimental treatments and cultured ICMs isolated from diapause blastocysts in 50 μ M of PD98059, undifferentiated Oct-4-positive colonies were obtained from 25% of starting embryos.

To test whether these primary colonies were true ES cells, two colonies were picked and passaged. They each

gave rise to mass cultures identical in appearance to regular ES cells. Line 1 was withdrawn entirely from PD98059, while line 2 was maintained in 12.5 μ M of PD98059. Both lines were frozen and thawed. At passage 4–5, cells from each line were injected into recipient C57BL/6 blastocysts, and chimaeras were obtained and tested for germline transmission (table 5). Although the number of animals is small, it is clear that germline-competent ES cells can be derived from pure inbred CBA/Ca mice using this protocol.

4. DISCUSSION

We have previously suggested that a critical factor limiting ES cell derivation may be the unexpectedly rapid and progressive loss of Oct-4 expression that occurs during the initial phase of epiblast culture (Buehr *et al.* 2002). The present results indicate that while maintenance of Oct-4 expression through the outgrowth stage is probably a prerequisite for ES cell derivation, this is not in itself sufficient. The implication is that Oct-4 expression alone is a fairly crude readout of developmental identity and that other genes may provide a more accurate gauge of cell potency. A further implication from this study is that the three factors, diapause, isolation of the ICM and culture in PD98059, which in combination allow the derivation of CBA ES cells, presumably affect different aspects of the derivation process. Interestingly, although PD98059 had little or no additive effect on Oct-4 expression in outgrowths from diapause CBA ICMs, it nonetheless played an essential role in the derivation of primary ES cell colonies from this material (table 4). This may indicate that the critical contribution of PD98059 is to sustain self-renewal after disaggregation and replating.

ES cell lines derived in the presence of PD98059 can contribute normally to chimaeras and populate the germline (table 5). A more extensive study is required to establish whether all ES cell lines established using the MEK inhibitor have full developmental potential, but the present data provide no contraindication. PD98059 should therefore be a useful tool in the establishment of ES cell lines. This applies both to permissive strains, in which the increased size of primary undifferentiated colonies facilitates their identification and picking (figure 2), and, when used in conjunction with diapause and ICM isolation, to at least some mouse strains in which derivation has previously proven difficult. It must be added, however, that this is not a generic solution. When the same combined protocol is applied to rat embryos, the persistence of Oct-4-expressing cells is similarly increased during the initial outgrowth phase and even for several days after disaggregation, but this does not extend through to production of ES cells (Buehr *et al.* 2002; unpublished data). In the case of human blastocyst-derived stem cells, diapause cannot be examined but current derivation protocols do involve immunosurgical isolation of the ICM (Thomson *et al.* 1998; Reubinoff *et al.* 2000). The effect of MEK inhibitor on human pluripotent stem cells has not been described.

It has been noted elsewhere that there is 'persisting ignorance about the genetic basis of permissivity [for ES cell derivation]' (Brook & Gardner 1997). One can go further and argue that the entire process of ES cell deri-

vation is obscure (Gardner & Brook 1997). Indeed, there is no obvious physiological rationale for epiblast cells to have a potential for sustained self-renewal, since their role and existence *in vivo* are only transient (discussed in A. Smith 2001). ES cell generation may therefore be an 'accidental' feature. This could explain why they have not been derived from a variety of mammals. This would also mean that those stem cells that can be derived from different strains or species might not represent identical cellular entities. In this regard, it may be significant that several differences have been described between mouse ES cells and human blastocyst-derived stem cells (Thomson *et al.* 1998; Reubinoff *et al.* 2000). Alternatively, capacity for self-renewal may be an intrinsic attribute of epiblast cells associated with their 'unprogrammed' status as embryo founder cells (Burdon *et al.* 2002). According to this scenario, the embryonic environment actively suppresses self-renewal and masks stem cell potential. Failures to derive ES cells would therefore reflect inadequate culture conditions that extinguish self-renewal. The truth may lie somewhere between these two extremes. There are probably 'hard-wired' constraints on epiblast self-renewal, but these could be subject to epigenetic modification or 'rewiring' in culture. Relative degrees of permissiveness for stem cell generation would then be determined by the balance between limiting genotypic factors and the facility for epigenetic reprogramming.

The authors thank Frances Stenhouse for *in situ* hybridization, Sharon White for chimaera production, and Carol Manson and staff for excellent animal husbandry. They are grateful to Tom Burdon and Jenny Nichols for advice and discussion. This research is supported by the MRC, BBSRC and NIH.

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Discussion

A. Camus (*Laboratoire de Développement des Vertèbres, Institut Jacques-Monod, CNRS, Universités Paris 6 et 7, Paris Cedex 05, France*). Are there any regional markers expressed by the Sox1-GFP cells? Do they have anterior identity?

A. Smith. We are examining this now. One of the reasons we developed this monolayer differentiation protocol was to avoid use of retinoic acid, which acts as a caudalizing agent. We anticipate therefore that the cells may have an anterior neural specification. The observation that we can readily generate tyrosine hydroxylase-positive putative mesencephalic dopaminergic neurons would suggest that at least a proportion of the cells should have a midbrain identity.

C. Merry (*Department of Medical Oncology, Cancer Research UK, Christie Research Centre, Christie Hospital, Manchester, UK*). Can you suggest possible reasons for the asynchronicity of Sox1 expression across the population on withdrawal of LIF? Do FGF receptor levels or isoforms play a role?

A. Smith. The heterogeneous behaviour of the ES cells may indicate that there is a stochastic component to initiation of differentiation, but what this means in molecular terms is unclear. Variable responsiveness to FGF4 could be involved.

P. M. Kumar (*Biological Sciences Department, Manchester Metropolitan University, Manchester, UK*). What is the role of extracellular matrix in your differentiation protocol?

A. Smith. Efficient neural differentiation in our serum-free system requires plating the ES cells on gelatin-coated plastic. Plating on fibronectin or in particular laminin suppresses neural differentiation and induces alternative cell fates. This might indicate that integrin signalling is anti-neural, though this requires further investigation. Once neural precursors are generated, the choice of substrate depends on the desired outcome. For good neuronal differentiation it is best to replate the cells on poly-D-lysine/laminin-coated dishes. To maintain neural precursors the cells can be kept on gelatin.

GLOSSARY

- dpc: days *post coitum*
 DMSO: dimethyl sulphoxide
 Erk: extracellular signal-regulated protein kinase
 ES: embryonic stem
 FGF: fibroblast growth factor
 ICM: inner cell mass
 LIF: leukaemia inhibitory factor
 MEK: mitogen-activated protein kinase/extracellular signal-regulated kinase