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Reversible programming of pluripotent cell differentiation

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SUMMARY

We have undertaken an in vitro differentiation analysis of two related, interconvertible, pluripotent cell populations, ES and early primitive ectoderm-like (EPL) cells, which are most similar in morphology, gene expression, cytokine responsiveness and differentiation potential in vivo to ICM and early primitive ectoderm, respectively. Pluripotent cells were differentiated in vitro as aggregates (embryoid bodies) and the appearance and abundance of cell lineages were assessed by morphology and gene expression. Differentiation in EPL cell embryoid bodies recapitulated normal developmental progression in vivo, but was advanced in comparison to ES cell embryoid bodies, with the rapid establishment of late primitive ectoderm specific gene expression, and subsequent loss of pluripotent cell markers. Nascent mesoderm was formed earlier and more extensively in EPL cell embryoid bodies, and resulted in the appearance of terminally differentiated mesodermal cell types prior to and at higher levels than in ES cell embryoid bodies. Nascent mesoderm in EPL cell embryoid bodies was not specified but could be programmed to alternative fates by the addition of exogenous factors.

EPL cells remained competent to form primitive endoderm even though this is not the normal fate of primitive ectoderm in vivo. The establishment of primitive ectoderm-like gene expression and inability to participate in embryogenesis following blastocyst injection is therefore not directly associated with restriction in the ability to form extra-embryonic lineages. However, the EPL cell embryoid body environment did not support differentiation of primitive endoderm to visceral endoderm, indicating the lack of an inductive signal for visceral endoderm formation deduced to originate from the pluripotent cells. Similarly, the inability of EPL cells to form neurons when differentiated as embryoid bodies was attributable to perturbation of the differentiation environment and loss of inductive signals rather than a restricted differentiation potential. Reversion of EPL cells to ES cells was accompanied by restoration of ES cell-like differentiation potential. These results demonstrate the ability of pluripotent cells to adopt developmentally distinct, stable cell states with altered differentiation potentials.

Key words: ES cell, EPL cell, Inner cell mass (ICM), Primitive ectoderm, Differentiation, Visceral endoderm, Embryoid body

INTRODUCTION

The inner cell mass (ICM) of the 3.5 days post coitum (d.p.c.) mouse blastocyst comprises a pool of pluripotent cells, which gives rise to all the differentiated cell types that make up the embryo and adult, and many of the extra-embryonic tissues (Gardner and Beddington, 1988). By 4.5 d.p.c., the pluripotent ICM cells that lined the blastocoelic cavity have differentiated to form primitive endoderm, an extra-embryonic cell lineage which gives rise to both visceral and parietal endoderm (Gardner, 1985). From 4.5 d.p.c. to 6.5 d.p.c. the remaining pluripotent cells, referred to collectively as ‘epiblast’, undergo extensive proliferation, selective apoptosis and migration to give rise to a columnar epithelial monolayer of pluripotent cells termed the primitive ectoderm (Snow, 1977; Coucouvanis and Martin, 1995). Remodelling of the pluripotent cell population during this time is accompanied by alterations in gene expression and differentiation potential (Beddington, 1983; Gardner and Beddington, 1988; Haub and Goldfarb, 1991; Rogers et al., 1991). The primitive ectoderm, through the process of gastrulation, gives rise to the mesoderm, ectoderm and endoderm of the embryo, germ cells and remaining extra-embryonic tissue (Gardner and Rossant, 1979). While this represents the normal course of embryogenesis, considerable developmental lability is evident from the observation that destruction of up to 85% of cells within the mouse embryo prior to organogenesis does not necessarily prevent formation of a normal embryo (Snow and Tam, 1979). This property is thought to reside in the capacity of pluripotent cells to reprogram their development in response to environmental cues (Smith, 1992).

A molecular understanding of early mammalian embryogenesis requires characterisation of the signals that regulate pluripotent cell biology. This has been complicated by experimental difficulties associated with direct experimentation on early mammalian embryos, although increasing evidence points to a role for visceral endoderm as a source of signals that regulate pluripotent cell maintenance,

survival, apoptosis and differentiation (Chen et al., 1994; Coucouvanis and Martin, 1995; Thomas and Beddington, 1996). Pluripotent cell lines (embryonic stem or ES cells) can be isolated from the pre-implantation mouse embryo and maintained in the undifferentiated state in vitro (Evans and Kaufman, 1981; Martin, 1981; Brook and Gardner, 1997). These cells exhibit gene expression profiles and differentiation potentials that are consistent with their embryonic origin (Beddington and Robertson, 1989; Rathjen et al., 1999), and can be used as a model system for the analysis of pluripotent cell biology. Factors contained within a conditioned medium, MEDII, cause specific and uniform differentiation of ES cells into a novel, morphologically distinct, cell population termed early primitive ectoderm-like or EPL cells (Rathjen et al., 1999). Gene expression, cytokine responsiveness and in vivo differentiation potential indicate that EPL cells are a pluripotent cell population distinct from ES cells and ICM, and most similar to embryonic primitive ectoderm following implantation of the embryo and cavitation of the epiblast, but prior to 6.0 d.p.c. The ES to EPL cell transition was shown to be reversible in terms of gene expression and formation of chimaeras in vivo. Pluripotent cells can therefore be maintained in vitro in two distinct but interconvertible states, which are most similar to alternative pluripotent cell populations of the pre- and post-implantation mouse embryo.

In this paper we describe a comparative analysis of ES and EPL cell differentiation. The inability of EPL cells to participate in development following blastocyst injection (Rathjen et al., 1999), while consistent with their relationship to early primitive ectoderm (Beddington, 1983), complicates the analysis of differentiation potential in vivo. For this reason, differentiation was analysed in embryoid bodies by culture of ES and EPL cell aggregates in suspension. Embryoid bodies formed from ES cells have been shown to undergo a differentiation program that is reminiscent of early mouse embryogenesis, with the ordered appearance of primitive endoderm, primitive ectoderm and their differentiated derivatives (Doetschman et al., 1985; Shen and Leder, 1992).

Analysis of EPL cell differentiation potential was carried out using EPL cells derived from ES cells cultured for 3 days in medium containing 50% MEDII. Typically, EPL cells formed in this way exhibit a 15-fold increase in *Fgf5* expression and a 7-fold decrease in *Rex1* expression (Rathjen et al., 1999) compared to ES cells. Uniform expression of *Oct4* and *Fgf5* within the EPL cell population indicates that these cells comprise a homogeneous pluripotent cell population. EPL cells were found to retain pluripotence as assessed by gene expression and the formation of differentiated embryonic and extra-embryonic cell types in vitro. EPL cell differentiation within embryoid bodies reflected the developmental progression of pluripotent cells in vivo. However, formation of an alternative differentiation environment within EPL cell embryoid bodies resulted in the production of an altered profile of differentiated cell types. Comparative analysis of ES and EPL cell differentiation revealed differences in the timing and extent of primitive ectoderm, visceral and parietal endoderm, mesoderm and neuron formation. ES cell differentiation potential was regained by EPL cells reverted to ES cells. Pluripotent cell populations can therefore be maintained in vitro in distinct, interconvertible developmental states with alternative differentiation potentials.

MATERIALS AND METHODS

Cell culture and in vitro differentiation assays

All cultures were incubated in 10% CO_2 (in air) at 37°C on tissue-culture grade plasticware (Falcon) pretreated with 0.2% gelatin/PBS. Routine maintenance of pluripotent cells was performed as outlined in Rathjen et al. (1999). Feeder independent D3 (Doetschman et al., 1985), MBL5 (Pease et al., 1990; obtained from Lindsay Williams, Ludwig Institute, Melbourne, Australia) and E14 (Hooper et al., 1987; obtained from Anna Michaelska, Murdoch Institute, Melbourne, Australia) ES cell lines were routinely cultured in ES DMEM (DMEM high glucose; Gibco BRL) supplemented with 40 mg/ml gentamycin, 10% foetal calf serum (FCS), 0.1 mM β-mercaptoethanol (β-ME) and 1 mM L-glutamine, to which COS-1 cell-conditioned medium containing LIF, equivalent to 10^5 U/ml LIF (ESGRO; Amrad), was added at 1% (v/v) (Smith, 1991). The KSF-4 ES cell line, which constitutively expresses nuclear localised *LacZ* (Berger et al., 1995; obtained from Patrick Tam, CMRI, New South Wales, Australia), was maintained on inactivated primary mouse embryonic fibroblast feeder layers (Abbondanzo et al., 1993) in DMEM (high glucose) supplemented with 40 mg/ml gentamycin, 20% FCS, 0.1 mM β-ME, 1 mM L-glutamine and 1% LIF as before. Prior to the formation of EPL cells, KSF-4 cells were cultured on gelatin-treated plasticware, in the absence of a feeder layer, for two passages. EPL cells were formed and reverted as described by Rathjen et al. (1999). Briefly, EPL cells were formed by the culture of ES cells in ES DMEM containing 50% MEDII (medium conditioned by human hepatocellularcarcinoma cell line HepG2; ATCC HB-8065; Rathjen et al., 1999) and without LIF for 3 days before use in differentiation experiments. Reverted EPL cells were formed by seeding EPL cells, formed as above, into ES DMEM supplemented with LIF. Reverted EPL cells were cultured for 6 days prior to use in differentiation experiments.

For the majority of experiments detailed here, embryoid bodies were formed in ES DMEM using the partial trypsinisation method (Robertson, 1987). Alternatively, embryoid bodies were formed from a single cell suspension plated at 1×10^5 cells/ml in bacteriological dishes in ES DMEM, or suspended in a hanging drop of medium (50 μl) at 1.6×10^4 cells/ml on the inside surface of a Petri dish lid. Cell aggregates formed in suspended drops were transferred to bacteriological dishes for further culture after 48 hours. Embryoid bodies were maintained with regular replenishing of medium. Mixed cell embryoid bodies were formed from a single cell suspension as previously described, with the two cell populations mixed at the specified ratio.

Beating muscle formation was assessed in individual embryoid bodies plated onto pre-equilibrated agarose plugs (1% agarose in DMEM equilibrated against ES DMEM for 3 hours at 37°C, 10% CO_2 in air in 24-well plates) in ES DMEM. The presence or absence of beating muscle was assessed at days 4, 6, 8, 10 and 12 by microscopic examination. Alternatively, the presence of beating muscle and neurons was assessed in individual embryoid bodies seeded into 24-well trays at day 6 of development and scored microscopically at days 7, 8, 9, 10 and 12. Neuron identity was confirmed by positive staining with the neurofilament 200 antibody N-4142 (Sigma; data not shown). Macrophage formation was assessed in aggregates formed from ES and EPL cells in ES DMEM. After 48 hours, aggregates were transferred into 1.25 ml MC medium (0.9% methyl cellulose in IMDM, 15% FCS, 50 μg/ml ascorbic acid, 4.5×10^{-4} M α-monothioglycerol) supplemented with 400 U/ml recombinant mouse IL-3 (courtesy of Dr T. Gonda, Institute of Medical and Veterinary Science, Adelaide, Australia) and 10 ng/ml recombinant human M-CSF (R&D Systems). Colonies were scored for the presence or absence of macrophages by microscopic examination on days 12, 15 and 18. Macrophage identity was confirmed by morphology when stained with May-Grünwald-Giemsa stain and by positive staining

with the macrophage-specific antibody F4/80 (Austyn and Gordon, 1981; data not shown).

Retinoic acid (RA) differentiation was carried out by plating cells in bacteriological dishes, at a density of 1×10^5 cells/ml, in ES DMEM supplemented with 1 μM RA. After 48 hours, aggregates were transferred to ES DMEM and maintained for 2 days before being seeded individually or collectively into tissue culture dishes. The presence of neurons was assessed by microscopic examination 2 days after seeding. Neuron identity was confirmed by positive staining with the neurofilament 200 antibody N-4142 (Sigma).

Results consistent with those presented in this manuscript were obtained using EPL cells derived from a variety of ES cell lines including D3 (Doetschman et al., 1985), MBL5 (Pease et al., 1990), KSF-4 (Berger et al., 1995), E14 (Hooper et al., 1987) and E14TG2a (Hooper et al., 1987), and using alternative methods for embryoid body formation, including single cell suspension, hanging drop and partial trypsinisation. Furthermore, the differentiation potential reported for EPL cells was specifically associated with these cells as it could not be recapitulated by ES cells that had been spontaneously differentiated by culture in the absence of LIF, nor by ES cells that had been cultured in the presence of alternative fractions of MEDII for equivalent time periods (data not shown).

Gene expression analysis

RNA was isolated from ES and EPL cells using the method of Edwards et al. (1985). RNA was isolated from embryoid bodies and RA-treated aggregates by the method of Chomczynski and Sacchi (1987). Northern blot hybridisation analysis was performed as described by Thomas et al. (1995). ^{32}P-labelled DNA probes were synthesised using either the Gigaprime kit (Bresatec) or Megaprime kit (Amersham). Riboprobes were synthesised as described by Kreig and Melton (1987). Whole-mount in situ hybridisation on embryoid bodies was carried out as described by Rathjen et al. (1999). Probe fragments used for northern blot analysis and/or in situ hybridisations were: *AFP*, linearisation of a plasmid containing a 400 bp *Eco*RI fragment encoding the first 350 bp of mouse *AFP* cDNA in pBluescript II KS+ with *Hin*dIII, followed by transcription with T7 polymerase; *Brachyury*, linearisation of pSK75 (Herrmann, 1991) with *Bam*HI and transcription with T7 polymerase; *Fgf5*, an 800 bp *Bam*HI/*Eco*RI fragment derived from a plasmid containing the full-length *Fgf5* cDNA (Hébert et al., 1991); *Goosecoid*, linearisation of a plasmid containing 909 bp *Goosecoid* genomic DNA (Blum et al., 1992) with *Hin*dIII and transcription with T3 polymerase; *mGAP*, whole plasmid containing 300 bp of *mGAP* cDNA sequence (Rathjen et al., 1990); *Nkx2.5*, linearisation of plasmid containing 1.6 kb *Nkx*2.5 cDNA (Lints et al., 1993) with *Hin*dIII and transcription with T3 polymerase; *Oct4*, a 462 bp *Xho*I/*Hin*dIII fragment derived from a plasmid containing *Oct4* cDNA (Schöler et al., 1990). Antisense *Oct4* riboprobe was obtained by digestion of the foregoing with *Hin*dIII and transcription with T7 polymerase; *SPARC*, 570 bp *Eco*RI fragment derived from pG43 (Mason et al., 1986); *Rex1*, 848 bp *Eco*RI fragment derived from *Rex1*-containing plasmid (kindly provided by Dr Neil Clarke, Department of Genetics, University of Cambridge).

RNase protection assays were performed as described in Chapman et al. (1997). A riboprobe for the detection of *Sox1* was transcribed from pR1X*Sox1*, a derivative of plasmid #1022 comprising 1100 bp of the *Sox1* cDNA in Bluescript (obtained from Dr R. Lovell-Badge, National Institute for Medical Research, Mill Hill, London), from which an approx. 360 bp *Eco*RI/*Xho*I *Sox1* fragment was subcloned into Bluescript KS+. pR1X*Sox1* was digested with *Bam*HI and transcribed with T3 RNA polymerase.

Histological analysis

Embryoid bodies were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight before embedding in paraffin wax and sectioning as described in Hogan et al. (1994). Sections were stained with Toluidine Blue for 10 seconds. Embryoid bodies that had been subjected to whole-mount staining were fixed in 4% PFA overnight, washed several times with PBS, 0.1% Tween-20, treated with 100% methanol for 5 minutes and then isopropanol for 10 minutes. Bodies were then treated and embedded as described above.

To detect β-galactosidase activity embryoid bodies were fixed in 0.2% glutaraldehyde in PBS for 15 minutes on ice, washed 3×15 minutes with detergent rinse (0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40) and stained in detergent rinse containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal for 2 hours at 37°C. Bodies were then washed 3× with detergent rinse and transferred to 70% ethanol. For sectioning embryoid bodies were dehydrated to 100% ethanol and embedded in paraffin wax as described above.

Immunological detection

Alpha fetoprotein (AFP) expression in sectioned embryoid bodies was carried out essentially as described by Dziadek and Adamson (1978) with the following modifications. Prior to antibody incubations, endogenous peroxidase activity was blocked by incubation of the section in 3% H_2O_2 in methanol for 30 minutes. Sections were then incubated with a 1/200 dilution of rabbit anti-AFP (ICN) for 2 hours, then washed several times with PBS before incubation with 1/200 dilution of HRP-conjugated anti rabbit IgG (Silenus).

RESULTS

Advanced pluripotent cell differentiation in EPL cell embryoid bodies

A clear morphological difference between ES and EPL cell embryoid bodies was evident within 4 days of embryoid body formation. ES cell embryoid bodies comprised a homogeneous population of round, relatively smooth and ordered aggregates (Fig. 1A). EPL cell embryoid bodies also formed a homogeneous population; however, the bodies appeared irregular and disorganised (Fig. 1B). Sectioning and staining indicated that while ES cell embryoid bodies exhibited a regular internal morphology characterised by compacted round cells (Fig. 1C), reminiscent of undifferentiated pluripotent cells (Martin et al., 1977), internal cells in EPL embryoid bodies were loosely packed and heterogeneous, possibly reflecting differentiation (Fig. 1D; Martin et al., 1977).

The progression from ICM to primitive ectoderm to germ layer formation can be monitored via alterations in gene expression both in vivo and during embryoid body differentiation in vitro (Shen and Leder, 1992). *Rex1* is expressed by ICM and ES cells, and is downregulated in EPL cells, the primitive ectoderm at 6.0 d.p.c. and during ES cell differentiation in vitro (Hosler et al., 1989; Rogers et al., 1991; Rathjen et al., 1999). *Fgf5* is not expressed by ICM or ES cells, but is expressed in EPL cells and the primitive ectoderm prior to and during gastrulation, and upregulated transiently during ES cell differentiation in vitro (Hébert et al., 1990; Haub and Goldfarb, 1991; Hébert et al., 1991). *Oct4* is expressed by all pluripotent cell populations of the embryo, EPL cells and ES cells, and is downregulated upon differentiation of these cells in vivo and in vitro (Schöler et al., 1990; Schöler, 1991; Rathjen et al., 1999).

Although the expression of *Rex1* was found to be downregulated in both ES and EPL cell embryoid bodies, the kinetics of *Rex1* regulation differed between the two cell populations. Downregulation to a barely detectable, basal level

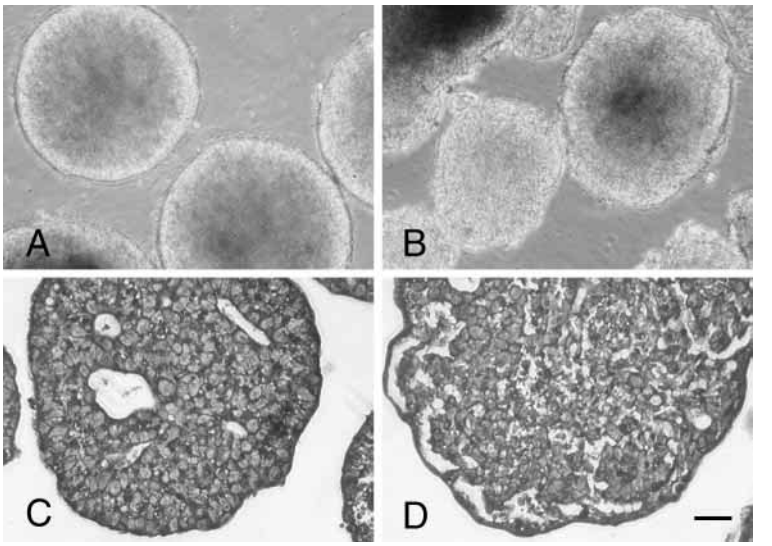

Fig. 1. Morphology of ES and EPL cell embryoid bodies at day 4 of differentiation. (A,C) Day-4 ES cell embryoid bodies, (B,D) day-4 EPL cell embryoid bodies. Embryoid bodies in C and D were sectioned and stained with Toluidine Blue. Bars: 25 μm (A,B); 15 μm (C,D).

was observed by day 1 in EPL cell embryoid bodies (Fig. 2). In contrast, a similar level of *Rex1* expression was not observed in ES cell embryoid bodies until day 2, with intermediate levels of expression observed on day 1 (Fig. 2). Similarly, the kinetics of *Fgf5* regulation differed between embryoid bodies derived from ES or EPL cells. *Fgf5*, already expressed in EPL cells, was upregulated rapidly from day 1 in EPL cell embryoid bodies to a peak on day 2/3, followed by a marked decrease in expression on day 4. Upregulation of *Fgf5* expression in ES cell embryoid bodies was not observed until day 3. While expression levels in these bodies increased further at day 4, they remained below *Fgf5* levels in EPL cell bodies at days 2 and 3. *Oct4* expression decreased with time in both ES and EPL cell embryoid bodies but was downregulated more rapidly in EPL cell embryoid bodies, with a fourfold decrease seen between days 3 and 4 (Fig. 2). This decrease in *Oct4* expression followed the highest levels of *Fgf5* expression and presumably reflected differentiation of pluripotent cells within the bodies.

The changes in *Rex1*, *Fgf5* and *Oct4* expression observed in both ES and EPL embryoid body development suggested that differentiation reflected the events of normal embryogenesis and proceeded through the formation of late stage primitive ectoderm. This was consistent with our previous alignment of ES and EPL cells with pluripotent cell populations occurring prior to 6.0 d.p.c. Further, the rapid increase in *Fgf5* expression and the earlier onset of differentiation, as detected by the loss of *Oct4* and *Fgf5* expression, indicated that pluripotent cell differentiation within EPL cell embryoid bodies was advanced compared to ES cell embryoid bodies.

The EPL cell embryoid body environment is non-permissive for visceral endoderm formation

By 4.5 d.p.c. pluripotent cells exposed to the blastocoelic cavity have differentiated to form primitive endoderm. The primitive endoderm gives rise to two distinct endodermal cell populations, visceral endoderm, which remains in contact with the epiblast, and parietal endoderm, which migrates away from the pluripotent cells to form a layer of endoderm adjacent to the trophectoderm (Gardner, 1985). While others have analysed extra-embryonic endoderm formation at relatively late stages (day 9-12) of embryoid body development (Soudais et al., 1995; Duncan et al., 1997), formation of an outer layer of endoderm, containing both visceral and parietal endoderm cells, can be observed in embryoid bodies by day 4 or 5 of development (Doetschman et al., 1985; Robertson, 1987). This timing is coincident with formation of primitive ectoderm and creation of an inner cavity, events that accompany endoderm specification in the embryo (Coucouvanis and Martin, 1995). Analysis of endoderm formation at this stage is therefore more likely to reflect normal embryonic events.

Formation of parietal and visceral endoderm during EPL and ES embryoid body development was analysed by *SPARC* and *AFP* expression respectively (Holland et al., 1987; Dziadek and Adamson, 1978). *SPARC* expression followed similar kinetics in ES and EPL cell embryoid bodies, with an approximately twofold increase over the first 4 days (Fig. 3A). Whole-mount in situ hybridisation and sectioning of ES and EPL cell embryoid bodies indicated that *SPARC* expression was confined to an outer endodermal cell layer in both ES and EPL cell embryoid bodies at day 4 (Fig. 3B,C), indicative of parietal endoderm formation. *AFP* levels were too low to be detected during these stages by northern analysis or RNase protection (Shen and Leder, 1992), so whole-mount in situ hybridisation of embryoid bodies was used to detect *AFP* expression. At day 3, 1% of ES cell embryoid bodies exhibited discrete patches of *AFP* expressing cells on their surface. This level rose to 52.9±9.5% of ES cell embryoid bodies by day 4 (Fig. 3D). Sectioning confirmed that *AFP* expression was confined to outer cells and was therefore representative of visceral endoderm (Fig. 3E). *AFP* expression could not be detected on surface or interior cells of EPL cell embryoid bodies at day 3 or day 4 of embryoid body development (Fig. 3F).

Cell mixing experiments were carried out to determine

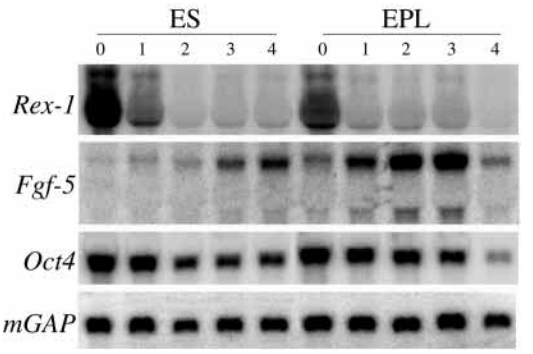

Fig. 2. Expression of pluripotent cell markers, *FGF5*, *Rex1* and *Oct4*, in differentiating ES and EPL cell embryoid bodies. Northern blot analysis was carried out on 20 μg total RNA derived from ES and EPL cell embryoid bodies at the indicated days of differentiation. Expression of *mGAP* was used as a loading control (Rathjen et al., 1990).

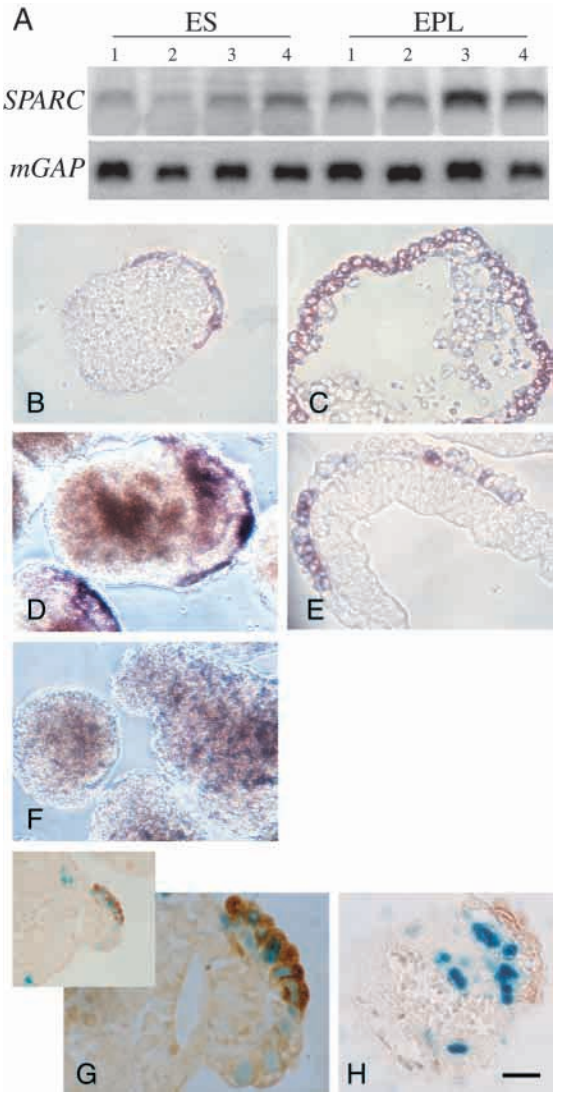

Fig. 3. EPL cells form parietal endoderm but not visceral endoderm when differentiated as embryoid bodies. (A) *SPARC* expression in embryoid bodies derived from ES and EPL cells at days 1-4 of differentiation as determined by northern blot analysis. (B,C) Sections of day-4 ES (B) and EPL (C) cell embryoid bodies subjected to whole-mount in situ hybridisation with antisense DIG-labelled *SPARC* specific riboprobes. (D,E,F) Whole-mount in situ hybridisation of day-4 ES (D) and EPL (F) cell embryoid bodies with antisense DIG-labelled *AFP* specific riboprobes, and (E) cross-section of an ES cell embryoid body stained for AFP expression. (G,H) Day-4 chimeric embryoid bodies formed by mixing 1:1 ES (*LacZ*–) and EPL (*LacZ*+) cells stained for β-galactosidase activity (blue, nuclear localised), sectioned and stained for AFP expression by immunoperoxidase antibody staining (brown). Inset shows a lower magnification of the embryoid body shown in G. Bars: 12.5 μm (B,C,E); 36 μm (D,F); 8.5 μm (G,H).

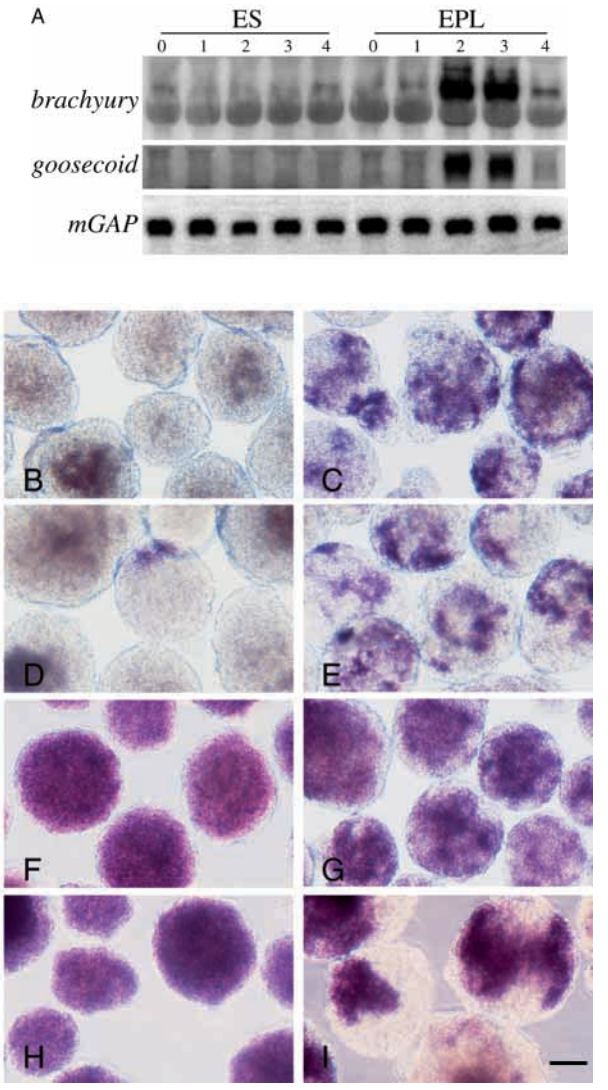

Fig. 4. Mesoderm induction occurs in EPL cell embryoid bodies earlier and at higher levels than in ES cell embryoid bodies. (A) The expression of *Brachyury* and *goosecoid* in ES and EPL cell embryoid bodies at days 0-4 of differentiation as determined by northern blot analysis of 20 μg of total RNA. Whole-mount in situ hybridisation of ES (B,D,F,H) and EPL (C,E,G,I) cell embryoid bodies with antisense DIG-labelled riboprobes specific for *Brachyury* (B-E) and *Oct4* (F-I) at day 3 (B,C,F,G) and day 4 (D,E,H,I). Bar, 90 μm.

whether the failure of EPL cells to form visceral endoderm reflected an inherent restriction in the developmental potential of these cells or an alteration in the embryoid body environment. The KSF-4 ES cell line constitutively expresses a *LacZ* gene modified to target β-galactosidase protein to the nucleus (Berger et al., 1995). Analysis of embryoid bodies formed from KSF-4 ES cells demonstrated formation of visceral endoderm at levels comparable with D3 ES cells (data not shown). After passaging in the absence of mouse embryonic fibroblast feeder cells, KSF-4 ES cells were cultured for 2 days in MEDII to form KSF-4 EPL cells (EPL (*LacZ*+)). Embryoid bodies generated by co-aggregation of a 1:1 ratio of D3 ES cells and EPL (*LacZ*+) cells were assessed for visceral endoderm formation by *AFP* expression at day 4. ES cell embryoid bodies and EPL (*LacZ*+) cell embryoid bodies gave rise to visceral endoderm at levels consistent with those previously described (65% and 0% of bodies respectively). Embryoid bodies formed from the mixed cell population gave rise to visceral endoderm at levels comparable to ES embryoid bodies (51%). Double staining for β-

galactosidase activity and *AFP* expression revealed the presence of *LacZ*+ and *LacZ*– cells within both the internal cell populations and the visceral endoderm (Fig. 3G,H). Visceral endoderm, of either ES or EPL cell origin, was located adjacent to internal cells of ES (*LacZ*–) or EPL (*LacZ*+) cell origin. For example, visceral endoderm of ES cell origin could be found adjacent to *LacZ*+ EPL-derived cells, and vice versa. These results indicate that EPL cell descendants were competent to form both embryonic and extra-embryonic cell types, and suggest that the inability of EPL embryoid bodies to form visceral endoderm resulted from a deficiency in the embryoid body environment rather than an inherent developmental restriction of EPL cells.

EPL cell embryoid bodies are predisposed to the formation of mesodermal progenitors, which can be programmed to alternative cell fates

Formation of early mesoderm was monitored by analysing expression of the early mesodermal markers, *brachyury* and *goosecoid* (Herrmann, 1991; Blum et al., 1992; Fig. 4A). Consistent with previous reports (Keller et al., 1993; Johansson and Wiles, 1995), *brachyury* expression was barely detectable on days 0-3 of development but was upregulated on day 4 in ES cell embryoid bodies (Fig. 4A). *Goosecoid* expression could not be detected in ES cell embryoid bodies during the course of this experiment. In contrast, *brachyury* and *goosecoid* expression were upregulated 30- and 6-fold, respectively, on days 2 and 3 of EPL cell embryoid body development, followed by 9- (*brachyury*) and 6.5- (*goosecoid*) fold decreases in expression on day 4 (Fig. 4A). In both ES and EPL cell embryoid bodies the expression of mesodermal markers immediately preceded decreases in the expression levels of primitive ectoderm markers *Fgf5* and *Oct4* (Fig. 2). *Brachyury* expression in ES cell embryoid bodies did not reach the levels seen in EPL cell bodies at day 3/4 even after extended culture.

Whole-mount in situ hybridisation was used to detect the extent of *brachyury* expression within the embryoid body populations. *Brachyury* expression was detected in 1% of ES cell embryoid bodies at day 3 and 16% of bodies at day 4 (Fig. 4B,D). By contrast, 98% and 92% of EPL cell embryoid bodies exhibited *brachyury* expression on days 3 and 4, respectively (Fig. 4C,E). A similar expression pattern was observed with *goosecoid* (data not shown). *Oct4* expression within ES and EPL cell embryoid bodies exhibited the expected inverse correlation with the onset and extent of *brachyury* expression. *Oct4* expression was relatively uniform throughout ES cell embryoid bodies on days 3 and 4 (Fig. 4F,H), but patchy within EPL cell embryoid bodies where mesoderm differentiation had commenced (Fig. 4G,I). The comparison of markers specific for nascent mesoderm therefore suggested that EPL cell embryoid bodies undergo a differentiation program which results in the earlier appearance and more extensive formation of mesoderm when compared to ES cell embryoid bodies.

Terminal differentiation of nascent mesoderm was monitored by the appearance of beating cardiocytes (Fig. 5A, Doetschman et al., 1985). These were first detected in ES cell embryoid bodies at day 8 of development (8%) and increased with time, reaching 36% of bodies at day 12. In EPL cell embryoid bodies, beating muscle was observed in 14% of embryoid bodies by day 6, 2 days prior to its appearance in ES cell embryoid bodies, and increased steadily to 60% by days 10 and 12 of embryoid body development. The proportion of EPL cell embryoid bodies containing beating muscle was higher than ES cell embryoid bodies at all time points. Consistent with this profile, expression of *Nkx2.5* (Lints et al., 1993) was induced earlier in EPL cell embryoid bodies, by day 6 compared to day 8 in ES cell embryoid bodies (Fig. 5B). Furthermore, while *Nkx2.5* expression levels increased in both ES and EPL cell embryoid bodies to day 12, levels in ES cell embryoid bodies were approximately threefold below those observed in EPL cell embryoid bodies throughout this period. The enhanced ability of EPL cells to form cardiac muscle when differentiated as embryoid bodies presumably reflects the increased formation of nascent mesoderm during EPL cell embryoid body differentiation.

Formation of macrophages in response to exogenous factors (Wiles and Keller, 1991) was assessed to determine if the elevated levels of nascent mesoderm in EPL cell embryoid bodies were developmentally restricted to myogenic lineages or could be programmed to alternative developmental fates. ES and EPL cell embryoid bodies were differentiated in MC medium supplemented with mIL-3 and hM-CSF, and scored for the presence of macrophages on days 12, 15 and 18 (Fig.

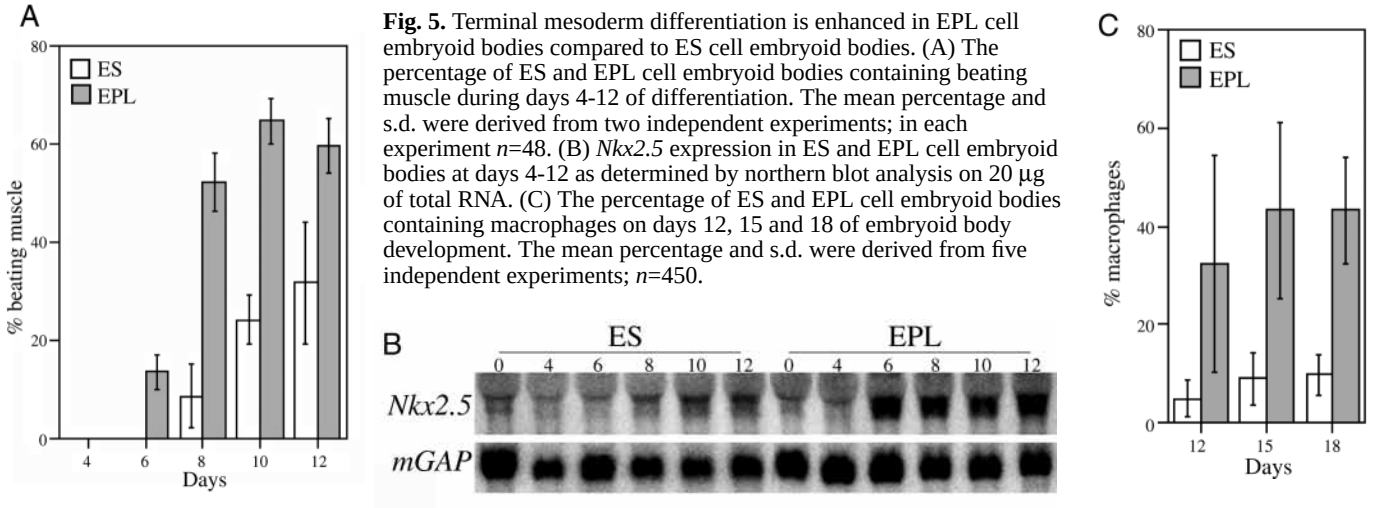

Fig. 5. Terminal mesoderm differentiation is enhanced in EPL cell embryoid bodies compared to ES cell embryoid bodies. (A) The percentage of ES and EPL cell embryoid bodies containing beating muscle during days 4-12 of differentiation. The mean percentage and s.d. were derived from two independent experiments; in each experiment n=48. (B) *Nkx2.5* expression in ES and EPL cell embryoid bodies at days 4-12 as determined by northern blot analysis on 20 μg of total RNA. (C) The percentage of ES and EPL cell embryoid bodies containing macrophages on days 12, 15 and 18 of embryoid body development. The mean percentage and s.d. were derived from five independent experiments; n=450.

5C). On day 12, 32.4% of EPL cell embryoid bodies were observed to contain macrophages, compared to 4.8% of ES cell embryoid bodies. On days 15 and 18 the proportion of bodies containing macrophages had increased to 43% of EPL cell embryoid bodies and 9-10% of ES cell embryoid bodies. Consistent with the earlier formation of mesoderm in EPL cell embryoid bodies, formation of macrophages initiated approximately 2 days earlier in EPL cell embryoid bodies compared to ES cell embryoid bodies (data not shown). Enhanced formation of multiple mesodermal lineages in EPL cell embryoid bodies suggests that the elevated nascent mesoderm in these bodies contains a multipotent mesodermal progenitor which can be programmed to alternative fates in response to specific environmental cues.

EPL cell embryoid bodies fail to form neural and ectodermal lineages

Differentiation of ES and EPL cells into ectoderm-derived lineages was assessed by the presence of neurons within individual embryoid bodies (Fig. 6A). Neurons were not detected in either embryoid body population before day 10. On day 10, 20% of ES cell embryoid bodies contained obvious neural networks. This rose to 42% by day 16. Neurons were not detected in embryoid bodies derived from EPL cells on any of the days assessed.

ES cells and P19 embryonal carcinoma (EC) cells form neurons when differentiated as aggregates in the presence of retinoic acid (RA) (Jones-Villeneuve et al., 1982; Bain et al., 1995). To examine whether the inability of EPL cells to form neurons when differentiated as embryoid bodies resulted from an inherent restriction in neuron differentiation capacity, the ability of EPL cells to differentiate into neurons after aggregation in the presence of RA was assessed. Individual RA-treated ES and EPL cell aggregates were scored for the presence of neurons (Fig. 6B) and found to exhibit similar frequencies of neuron formation at 63% and 68%, respectively. This indicated that the lack of neurons within EPL cell embryoid bodies did not reflect an inherent restriction in the capacity of EPL cells to form neurons, but an altered embryoid body environment, which resulted in reduced neural specification.

Sox1 is expressed in ectodermal cells committed to neural fate and expression persists in undifferentiated neural progenitors throughout embryonic development (Pevny et al., 1998). To investigate the development of neurectoderm within embryoid bodies, RNA isolated from ES and EPL cell embryoid bodies on days 5 to 10 of development was assessed for the presence of *Sox1* by RNase protection. *Sox1* expression could be detected in ES cell embryoid bodies during this period of development, consistent with later differentiation into neurons. By contrast, no *Sox1* expression was observed in EPL cell embryoid bodies, indicating that these failed to form neurectoderm or neural progenitors (Fig. 6C).

Reverted EPL cells regain ES cell differentiation capability

EPL cells revert to ES cells when cultured in the absence of MEDII but in the presence of LIF, as assessed by morphology, gene expression, cytokine responsiveness and ability to contribute to chimaeras following blastocyst injection (Rathjen et al., 1999). Reversion to ES cells was demonstrated to occur

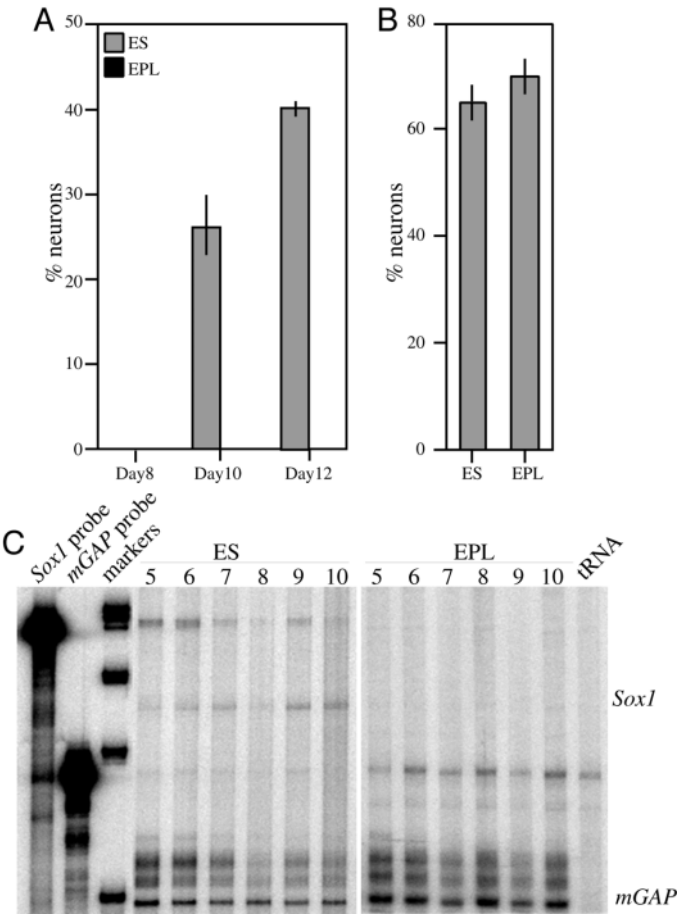

Fig. 6. EPL cell embryoid bodies fail to form neurons. (A) The percentage of ES and EPL cell embryoid bodies forming neurons from days 8-16 of differentiation. The mean percentage and s.d. were derived from two independent experiments; in each experiment n=48. (B) The percentage of ES and EPL cell RA-treated aggregates containing neurons. The mean percentage and s.d. were derived from four independent experiments; in each experiment n=32. (C) RNase protection assay of 20 μg RNA from ES and EPL cell embryoid bodies on days 5 to 10 of development with radiolabelled anti-sense probes specific for *Sox1* and *mGAP*. Marker lane contains radiolabelled pUC19/*Hpa*II fragments.

in clonal EPL cell populations, suggesting that the ES cells arise from reversion of EPL cells, not as a consequence of residual ES cells with an EPL cell population (Rathjen et al., 1999).

ES cells were differentiated to EPL cells by culture in MEDII for 2 days, before passaging into ES DMEM containing LIF to form reverted EPL cells (EPL^R). Embryoid bodies were formed from each cell population and their gene expression and differentiation compared. Representative results are shown in Fig. 7. Embryoid bodies derived from EPL^R cells were identical in morphology to ES cell embryoid bodies and could be distinguished easily from EPL cell embryoid bodies at day 4 (Fig. 1; data not shown). *Brachyury* expression in EPL^R cell embryoid bodies was similar to that observed for ES cell embryoid bodies, with expression detected at low levels on day 4 of development and not at high levels on day 2 and 3 as described for EPL cells. Compared to EPL embryoid bodies,

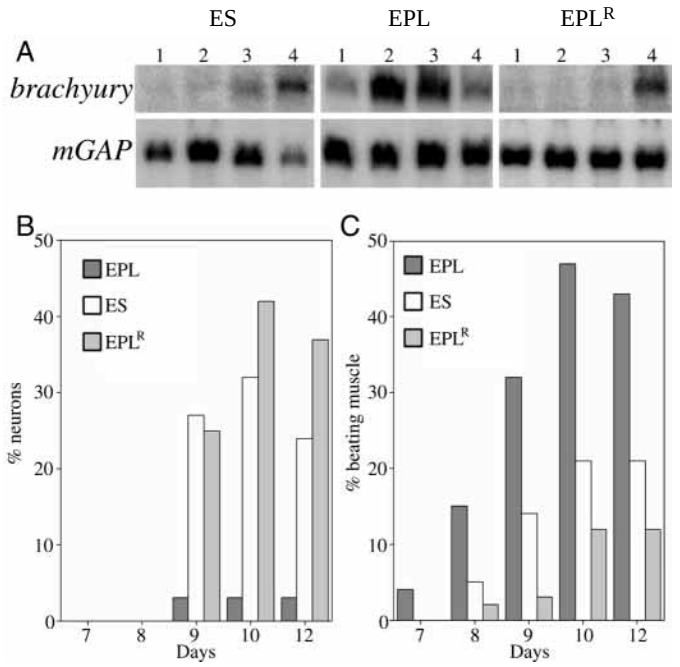

Fig. 7. Reverted EPL cells have a differentiation potential similar to ES cells when differentiated as embryoid bodies. (A) Northern analysis of *Brachyury* expression in embryoid bodies derived from ES, EPL and EPL^R cells at days 1-4 of differentiation. (B) The percentage of ES, EPL and EPL^R cell embryoid bodies exhibiting neurons during days 7-12 of differentiation; n=36. (C) The percentage of ES, EPL and EPL^R cell embryoid bodies forming beating muscle from days 7-12 of differentiation; n=36.

which failed to give rise to visceral endoderm, EPL^R embryoid bodies gave rise to visceral endoderm at levels comparable to ES embryoid bodies (31% and 46%, respectively), as shown by whole-mount in situ hybridisation. Differentiation of ES and EPL cell embryoid bodies into beating cardiocytes and neurons was consistent with the profiles previously described. Analysis of EPL^R embryoid bodies indicated that reversion of EPL to EPL^R cells was accompanied by restoration of high levels of neuron formation (Fig. 7B) and reduced levels and later appearance of beating cardiocytes (Fig. 7C). Reverted EPL cells therefore regain ES cell-like differentiation capacity in vitro.

DISCUSSION

The use of ES cells as an in vitro model system for the analysis of development has largely been exploited by differentiation of ES cells as embryoid bodies. Embryoid body differentiation recapitulates many aspects of early embryogenesis and circumvents some difficulties associated with experimental manipulation of the early embryo. Exploitation of this system would benefit from the ability to carry out comparative analyses of alternative differentiation via controlled perturbation of embryoid body differentiation. Some progress has been made towards this end through the addition of exogenous growth factors (Johansson and Wiles, 1995; Keller et al., 1993; Shen and Leder, 1992) and analysis of genetically altered ES cells (Lints et al., 1993; Soudais et al., 1995; Robb et al., 1996) during embryoid body differentiation. An alternative approach is the comparison of related pluripotent cell types which show different differentiation potentials in vitro. Such a system potentially allows the origin, target and identity of inductive signals controlling developmental events to be determined, as demonstrated by the investigation of embryonic cavitation using comparative ES and EC cell differentiation (Coucouvanis and Martin, 1995).

Alternative differentiation potentials reveal distinct pluripotent cell states

We demonstrate here that differentiation within embryoid bodies can be reversibly altered by prior differentiation of ES cells to an alternative pluripotent cell population, EPL cells. Like ES cells, EPL cells exhibited pluripotent differentiation capabilities in vitro and differentiated within embryoid bodies in a manner consistent with differentiation in the early embryo, with an ordered appearance of primitive endoderm, primitive ectoderm, germ layers and terminal cell differentiation. However, EPL cell differentiation within embryoid bodies was advanced in comparison with ES cell embryoid bodies, as indicated by the earlier formation and differentiation of late-stage primitive ectoderm and the earlier appearance of terminally differentiated mesoderm derivatives. This difference is consistent with our suggestion that, compared to ES cells, EPL cells represent an alternative pluripotent cell state that succeeds the ICM in embryogenesis. As predicted for an alternative pluripotent cell population (Rossant, 1993), reversion of EPL cells to an ES cell equivalent was accompanied by establishment of ES cell-like differentiation potential in vitro. This is consistent with our previous demonstration (Rathjen et al., 1999) that EPL cell reversion, which can occur in clonal EPL cell lines, is accompanied by establishment of ES cell gene expression, and restoration of the ability to contribute to chimaera formation in vivo. These data establish the fact that pluripotent cells can exist in at least two developmentally distinct states, which can be interconverted in response to environmental signals.

Over 85% of the pre-organogenesis gastrulating egg cylinder can be destroyed without preventing formation of a normal foetus (Snow and Tam, 1979). This developmental plasticity is thought to reside in pluripotent cells of the epiblast that are capable of reprogramming to overcome restrictions in developmental potential (Smith, 1992). The reversibility of the ES to EPL cell transition is the first demonstration that cells with properties equivalent to epiblast or early primitive ectoderm cells can revert, or dedifferentiate, to a more primitive pluripotent cell type in response to environmental cues, and may provide a cellular basis for the developmental lability of the early mammalian embryo.

The ability of EPL cells to convert to an ‘ES’ phenotype appears to be shared by other cells, including primordial germ cells in culture (Matsui et al., 1992; Resnick et al., 1992). Reversion of pluripotent cells to a more primitive state may also underlie the ability of EC cell lines obtained from grafts of 6.5 d.p.c. embryos to contribute to embryonic development when reintroduced into host blastocysts (Papaionnou et al., 1978; Rossant and McBurney, 1982). This reversion may reflect a more general ability for cell respecification since neural stem cells have been shown to contribute to the

haematopoietic system of sub-lethally irradiated mice (Bjornson et al., 1999).

Formation of primitive endoderm derivatives from pluripotent cells

In vivo, the extra-embryonic endodermal lineage arises from ICM of the 4.0 d.p.c. blastocyst with the differentiation of the pluripotent cells exposed to the blastocoelic cavity into primitive endoderm. The capacity of pluripotent cells after this differentiation event to form extra-embryonic endodermal lineages has not been resolved. Developmental restriction prior to primitive ectoderm formation is implied by the inability of microsurgically isolated primitive ectoderm from day 5.0 embryos to regenerate primitive endoderm and derivatives in vitro (Gardner, 1985). These data are supported by the observation that day 5.0 primitive ectoderm cells are able to contribute to foetal soma but unable to contribute to primitive endoderm/derivatives when reinjected into host blastocysts (Gardner and Rossant, 1979). However, using alternative experimental approaches primitive endoderm formation from later pluripotent cell populations has been observed in vitro and in vivo. ICM cores isolated from aggregated blastocysts were capable of regenerating primitive endoderm (Pedersen et al., 1977), and primitive endoderm has been observed in teratocarcinomas produced by 6/7 d.p.c. primitive ectoderm (Damjanov et al., 1987) and in differentiated EC cells derived from these teratocarcinomas (Martin and Evans, 1975). P19 EC cells, which exhibit morphology and gene expression consistent with primitive ectoderm (Rogers et al., 1991; Mummery et al., 1993), retain the capacity to form primitive endoderm cell types in vitro (Smith et al., 1987). Furthermore, primitive ectoderm cells of the 6.5 d.p.c. embryo can be recruited to the extra-embryonic endoderm cell layer by direct delamination (Tam and Beddington, 1992).

Efficient formation of parietal endoderm, a derivative of primitive endoderm, within EPL cell embryoid bodies, with kinetics similar to ES cell embryoid bodies, indicated that these cells retained the ability to form primitive endoderm. Formation of primitive ectoderm therefore appears to be a complex process in which alterations in gene expression and ability to form chimaeras following blastocyst injection are not necessarily accompanied by restriction in the ability to form primitive endoderm. The ability of EPL cells to form extra-embryonic lineages is therefore consistent with persistence of developmental plasticity in pluripotent cells beyond the acquisition of primitive ectoderm characteristics. Variable results obtained in earlier experiments may reflect the use of culture conditions that were not capable of maintaining primitive ectoderm in vitro, or environments in vivo and in vitro that were non-permissive or not instructive for primitive endoderm formation.

An inductive signal for visceral endoderm differentiation

Although parietal endoderm was formed efficiently in EPL cell embryoid bodies, they displayed a clear restriction in visceral endoderm formation. This is intriguing given that experimental evidence strongly suggests that visceral and parietal endoderm arise from a common precursor, the primitive endoderm (Gardner, 1982, 1983, 1985). EPL cells within an ES cell embryoid body environment were able to form visceral endoderm, with the appearance of both EPL- and ES-cell-derived visceral endoderm coincident within the bodies. This indicates that the failure to form visceral endoderm did not represent an inherent restriction in EPL differentiation potential but rather the absence of an appropriate environment or inductive signal in the EPL embryoid bodies, which is present in the ES cell embryoid bodies. This signal is unlikely to originate from parietal endoderm, which is present in both ES and EPL embryoid bodies, suggesting that induction of visceral endoderm requires an inductive signal from the pluripotent cells. A similar conclusion was reached by Gardner (1983), who proposed that primitive endoderm cells required contact with pluripotent cells of the epiblast/primitive ectoderm for visceral endoderm instruction. Interestingly, visceral endoderm formation was observed in extra-embryonic cells overlying pluripotent cells of both ES and EPL cell origin in mixed embryoid bodies. The pluripotent cell-derived signal must therefore either be restricted to ES-derived cells and diffusible in nature, or expressed by both ES-derived cells and EPL-derived cells present in an ES cell embryoid body environment. The failure of EPL-derived cells to support visceral endoderm in EPL cell embryoid bodies may be explained by reprogramming of EPL cells in mixed embryoid bodies to an ES cell state, as demonstrated here and previously (Rathjen et al., 1999).

The formation of late-stage primitive ectoderm and onset of mesodermal differentiation was advanced in EPL cell embryoid bodies compared to ES cell embryoid bodies, perturbing the normal temporal relationship between primitive endoderm and epiblast. As a result, visceral endoderm-competent primitive endoderm cells and pluripotent cells expressing the presumed inductive signal may not coincide temporally. A requirement for close coupling between pluripotent cells and primitive endoderm for visceral endoderm formation may also explain the observation that visceral endoderm formation is reduced in LIF-treated embryoid bodies, where primitive ectoderm formation is inhibited (Fisher et al., 1989; Shen and Leder, 1992). Default differentiation of primitive endoderm into parietal endoderm, in the absence of inductive signals and cell contacts required for visceral endoderm formation, may underlie the observation that isolated primitive endoderm cells predominantly form parietal endoderm when introduced into host blastocysts (Gardner, 1982, 1983). An understanding of the origin of visceral endoderm is important in view of increasing evidence pointing to the instructive ability of this cell population during early embryogenesis (Chen et al., 1994; Spyropolus and Capecchi, 1994; Coucouvanis and Martin, 1995; Thomas and Beddington, 1996). The use of pluripotent cells with alternative capacities to form visceral endoderm potentially allows mechanistic investigation of this event.

EPL cell embryoid bodies exhibit early defects in the formation of neural lineages

Embryoid bodies derived from EPL cells display a reduced or absent capacity for neuron formation when compared to ES cell embryoid bodies. However, RA differentiation of aggregates demonstrated that ES and EPL cells were equally competent to form neurons, consistent with the postulated pluripotence of EPL cells. This suggests that the defect in neuron formation in EPL embryoid bodies reflects an alteration

in the embryoid body environment rather than a cell-autonomous restriction in EPL differentiation ability.

The earliest defect that we identified in EPL cell embryoid bodies was a failure to form visceral endoderm. This suggested that the failure of EPL cell embryoid bodies to form neurons might be explained by perturbation of early differentiation events. Accordingly, we analysed formation of early neural progenitors by expression of *Sox1*, and demonstrated that EPL cell embryoid bodies fail to form these cells. Embryonic *Sox1* expression is first detected in ectodermal cells destined to become neurectoderm at 7.5 d.p.c. (Pevny et al., 1998) and persists in undifferentiated neural cells. A role for visceral endoderm in ectoderm/neurectoderm formation has not been demonstrated directly but is consistent with results reported by others. For example, the visceral endodermal cell line END-2 secretes a factor which can induce differentiation of P19 EC cells into muscle and neurons in vitro (Mummery et al., 1991; van den Eijnden-van Raaij et al., 1991). Furthermore, the anterior visceral endoderm has been postulated to be required for correct molecular patterning of the rostral neurectoderm (Thomas and Beddington, 1996; reviewed in Beddington and Robertson, 1998). We are currently investigating the formation of ectoderm and neurectoderm in ES and EPL cell embryoid bodies, and the possible role of visceral endoderm in these processes.

EPL cells embryoid bodies exhibit increased formation of nascent mesoderm, which can be programmed into alternative cell fates

Mesoderm formation within ES embryoid bodies described in this work was relatively consistent with that described by others, despite differences in the ES cell lines and culture conditions employed. *Brachyury* expression, detected by RT-PCR, was also shown to initiate in ES cell embryoid bodies on days 4-5 (Keller et al., 1993; Johansson and Wiles, 1995), and cardiac muscle formation is generally detected in ES cell embryoid bodies at day 8, with 40-60% of embryoid bodies beating by day 12 (Doetschman et al., 1985; Sanchez et al., 1991; Wang et al., 1992; Lints et al., 1993).

In comparison to ES cell embryoid bodies, the nascent mesoderm-specific marker *brachyury* was expressed earlier and in a greater proportion of EPL cell embryoid bodies, consistent with the earlier onset of differentiation in these bodies, observed both morphologically and as a reduction in *Oct4* expression. Further differentiation of EPL bodies confirmed the enhanced mesodermal differentiation with the appearance of terminally differentiated cells occurring earlier and at higher levels than in ES cell embryoid bodies. The apparent alteration in germ layer formation, in which EPL cells showed elevated formation of mesoderm at the expense of neurons/ectoderm, could reflect alterations in cell/cell interactions and/or cell/extracellular matrix interactions resulting from a lack of visceral endoderm. The importance of visceral endoderm in cellular specification in vivo has been repeatedly demonstrated (Beddington and Robertson, 1998).

The developmental fate of nascent mesoderm induced in EPL cell embryoid bodies was not determined but could be influenced by the addition of exogenous cytokines, as shown by the enhanced formation of macrophages in embryoid bodies cultured in the presence of IL-3 and M-CSF. This demonstrated that the effect of MEDII on ES cells did not predispose them to muscle formation, but increased the frequency of differentiation into multipotent mesodermal progenitors, which could be differentiated into alternative lineages by manipulation of the differentiation environment. The ability to compare differentiation within ES and EPL cell embryoid bodies provides a system for the identification and characterisation of signals that regulate mesodermal cell specification. The potential to produce cell populations enriched in mesoderm progenitor bodies also provides an improved system for the production of mesodermal cell types for applications such as cell-based therapy (Rathjen et al., 1998).

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