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Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors

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SUMMARY

The primitive ectoderm of the mouse embryo arises from the inner cell mass between 4.75 and 5.25 days post coitum, around the time of implantation. Positioned at a pivotal time in development, just prior to formation of the three germ layers of the embryo proper, the primitive ectoderm responds directly to the signals generated during gastrulation. We have identified a conditioned medium, MEDII, which caused the homogeneous conversion of ES cells to a morphologically distinct cell population, termed early primitive ectoderm-like (EPL) cells. EPL cells expressed the pluripotent cell markers *Oct4*, SSEA1 and alkaline phosphatase. However, the formation of EPL cells was accompanied by alterations in *Fgf5*, *Gbx2* and *Rex1* expression, a loss in chimaera forming ability, changes in factor responsiveness and modified differentiation capabilities, all consistent with the identification of EPL cells as equivalent to the primitive ectoderm population of the 5.5 to 6.0 days post coitum embryo. EPL cell formation could be reversed in the presence of LIF and withdrawal of MEDII, which suggested that EPL cell formation was not a terminal differentiation event but reflected the ability of pluripotent cells to adopt distinct cell states in response to specific factors. Partial purification of MEDII revealed the presence of two separable biological activities, both of which were required for the induction and maintenance of EPL cells.

We show here the first demonstration of uniform differentiation of ES cells in response to biological factors. The formation of primitive ectoderm, both in vivo and in vitro, appears to be an obligatory step in the differentiation of the inner cell mass or ES cells into cell lineages of the embryonic germ layers. EPL cells potentially represent a model for the development of lineage specific differentiation protocols and analysis of gastrulation at a molecular level. An understanding of the active components of MEDII may provide a route for the identification of factors which induce primitive ectoderm formation in vivo.

Key words: Differentiation, Embryogenesis, ES cell, ICM, Pluripotent cell, Primitive ectoderm

INTRODUCTION

Early developmental events within the mammalian embryo entail the formation of extra-embryonic cell lineages and the construction of an embryonic environment in which later development can proceed. The resulting blastocyst contains two extraembryonic lineages, trophectoderm and primitive endoderm with the embryo itself comprising a pool of pluripotent cells located within the inner cell mass (ICM). As development proceeds, pluripotent cells of the ICM undergo rapid proliferation, selective apoptosis, differentiation and reorganisation as they form a second pluripotent cell population, the primitive ectoderm. In the mouse, pluripotent cells of the ICM begin to proliferate rapidly around the time of blastocyst implantation (4.75 days post coitum (d.p.c.)) and expand into the blastocoelic cavity (Snow, 1977). Between 5.0 and 5.5 d.p.c. the inner cells of the pluripotent cell mass are proposed to undergo apoptosis to form the proamniotic cavity (Coucouvanis and Martin, 1995). The outer, surviving cells, or primitive ectoderm, continue to proliferate and by 6.0-6.5 d.p.c. have formed a pseudo-stratified epithelial layer of pluripotent cells. The primitive ectoderm gives rise to the germ cells (Ginsburg et al., 1990) and, during gastrulation, acts as a substrate for the generation of the primary germ layers of the embryo proper and the extra-embryonic mesoderm (Gardner and Rossant, 1979).

The development of pluripotent cells from ICM to primitive ectoderm is poorly characterised, due to the small size, complexity and inaccessibility of the mouse embryo within the uterine environment. Developmental differences between the ICM and primitive ectoderm have been reported by Gardner and Rossant (1979) and Gardner (1985), who demonstrated that the pluripotent cells of the embryo progressively lose developmental potential such that primitive ectoderm (5.0 d.p.c.) can be distinguished from ICM by an inability to form primitive endoderm. Furthermore, pluripotent cells of the post-

implantation embryo, but not the pre-implantation embryo, are unable to contribute to development when introduced into host blastocysts (Gardner, 1971; Rossant, 1977; Beddington, 1983; Brook and Gardner, 1997). Compared to ICM, primitive ectoderm exhibits an up regulation of the fibroblast growth factor 5 gene (*Fgf5)* and down regulation of the zinc finger gene, *Rex1* (Haub and Goldfarb, 1991; Hébert et al., 1991; Rogers et al., 1991). The analysis of gene expression by wholemount in situ hybridisation, and the analysis of developmental potential by transplantation studies, suggest that pluripotent cells within primitive ectoderm remain homogeneous until just prior to gastrulation (Beddington 1983; Rosner et al., 1990; Haub and Goldberg, 1991; Hébert et al., 1991). The possibility that additional, temporally defined, pluripotent cell populations exist transiently between the ICM and primitive ectoderm has not been resolved. Little is known of the molecular signals involved in the development of pluripotent cells but expression mapping studies, gene knockouts and the analysis of in vitro assays suggest that signals emanating from the primitive and visceral endoderm are required for primitive ectoderm formation and differentiation (Chen et al., 1994; Spyropoulos and Capecchi, 1994; Coucouvanis and Martin, 1995; Thomas and Beddington, 1996).

Pluripotent cells can be isolated from the pre-implantation mouse embryo as embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981; Smith and Hooper, 1987; Nichols et al., 1990; Pease et al., 1990; Brook and Gardner, 1997). ES cells can be maintained indefinitely as a pluripotent cell population in vitro in the presence cytokines of the IL-6 family (Williams et al., 1988; Smith et al., 1988; Gearing and Bruce, 1992; Conovar et al., 1993; Pennica et al., 1995; Yoshida et al., 1994), and, when reintroduced into a host blastocyst, can contribute to all adult tissues of the mouse including the germ cells (Robertson et al., 1986; Thomas and Capecchi, 1987). ES cells, therefore, retain the ability to respond to all the signals that regulate normal mouse development, and potentially represent a powerful model system for the investigation of mechanisms underlying pluripotent cell biology and differentiation within the early embryo.

Spontaneous differentiation of ES cells in culture, induced either by the withdrawal of LIF or by differentiation in response to chemical reagents, results in the formation of heterogeneous arrays of terminally differentiated cell types (Smith, 1991). The relevance of this differentiation to development in vivo is questionable. Differentiation as embryoid bodies, formed by cellular aggregation of ES cells, results in reproducible and ordered generation of derivatives of all three germ layers via a primitive ectoderm intermediate, and appears to reflect many of the normal processes of embryonic development (Shen and Leder, 1992; J.-A. Lake, J. Rathjen and P. D. Rathjen, unpublished). Although the analysis of embryoid body differentiation has shed some light on inductive signals involved in primitive ectoderm formation and differentiation to mesoderm (van den Eijnden-van Raaij et al., 1991; Coucouvanis and Martin, 1995; Johansson and Wiles, 1995), it involves the creation of a complex cellular environment in which the characterisation of defined differentiation events is difficult. Uniform differentiation of ES cells in response to factors of biological origin, has not been reported, possibly because ES cells, which are most like pluripotent cells of the pre-implantation embryo, require prior differentiation to a responsive intermediate, or primitive ectoderm-like cell (Shen and Leder, 1992).

In this paper we report the differentiation of ES cells to a homogeneous pluripotent cell population, termed early primitive ectoderm-like (EPL) cells, in response to two separable factors derived from medium conditioned by a human hepatocellular carcinoma cell line. EPL cells have been characterised by gene expression, chimera formation, factor requirements and differentiation potential and found to be most closely related to the primitive ectoderm of the pre-gastrulation embryo. The action of MEDII appears to parallel the formation of primitive ectoderm from the ICM, and EPL cells represent an in vitro equivalent of the post-implantation primitive ectoderm of the embryo, prior to 6.0 d.p.c. The transition of ES cells to EPL cells occurred in the absence of cellular aggregation and is the first report of factors which effect the specific differentiation of ES cells to a homogeneous cell lineage.

MATERIALS AND METHODS

Cell culture conditions

ES cells were cultured in the absence of feeders on tissue-culture grade plastic-ware (Falcon) pre-treated with 0.2% gelatin/PBS for a minimum of 30 minutes. Cells were cultured in Dulbecco's modified Eagles medium (DMEM; Gibco BRL #12800) supplemented with 10% foetal calf serum (FCS; Commonwealth Serum Laboratories), 40 mg/ml gentamycin, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (β-ME) and 1000 units of LIF under 10% CO_2 in a humidified incubator. EPL cells were formed and maintained in media containing 50% MEDII conditioned medium in DMEM supplemented with 10% FCS, 40 mg/ml gentamycin, 1 mM L-glutamine, 0.1 mM β-ME with or without the addition of 1000 units of LIF. Routine tissue culture was performed as described by Smith (1991). E14 ES cells (Hooper et al., 1987) were obtained from Anna Michelska (Murdoch Institute, Melbourne). CCE ES cells (Robertson et al., 1986) were obtained from Richard Harvey (Walter and Eliza Hall Institute, Melbourne). MBL5 (Pease et al., 1990) and D3 (Doetschman et al., 1985) ES cell lines were obtained from Lindsay Williams (Ludwig Institute, Melbourne). CGR8 and E14TG2a (Hooper et al., 1987) ES cells were provided by Austin Smith (Centre for Genome Research, Edinburgh). EPL formation was apparent with the addition of between 10% and 80% MEDII, however optimal culture conditions were observed at 50% (data not shown).

LIF was produced from COS-1 cells transfected with a mouse LIF expression plasmid, pDR10, as described by Smith (1991) with the following modifications. COS-1 cells were transfected by electroporation using a Bio-Rad Gene Pulsar at 270 V and a capacitance of 250 μD. Transfected cells were plated at 7×10^4 cells/cm^2 in DMEM, pH 7.4, containing high glucose and supplemented with 10% FCS, 40 mg/ml gentamycin and 1 mM L-glutamine. Medium was collected and assayed for LIF expression as described by Smith (1991).

Hep G2 cells (Knowles et al., 1980; ATCC HB-8065) were maintained in culture in DMEM supplemented with 10% FCS, 40 mg/ml gentamycin and 1 mM L-glutamine and passaged at confluence. To condition medium (MEDII) HepG2 cells were seeded into DMEM supplemented with 10% FCS, 40 mg/ml gentamycin and 1 mM L-glutamine at a density of 5×10^4 cells/cm^2. Medium was collected after 4-5 days, sterilised by filtration through a 0.22 μm membrane and supplemented with 0.1 mM β-ME before use. MEDII

was stored at 4°C for 1-2 weeks or at –20°C for up to 6 months without apparent loss of activity. To produce serum free MEDII (sfMEDII) Hep G2 cells were seeded as above and cultured for three days. Cells were washed twice with 1× PBS and once with serum free medium (DMEM containing high glucose but without Phenol Red, supplemented with 1 mM L-glutamine, 0.1 mM β-ME, 1× ITSS supplement (Boehringer Mannhiem), 10 mM Hepes, pH 7.4, and 110 mg/l sodium pyruvate) for 2 hours. Fresh serum free medium was added at a ratio of 0.23 ml/cm^2 and the cells cultured for a further 3-4 days. SfMEDII was collected, sterilised and stored as for MEDII.

Northern blot and RNA protection analyses

Cytoplasmic RNA was isolated from cultured ES and EPL cell layers using the method of Edwards et al. (1985). Northern blot analysis was performed as described by Thomas et al. (1995). DNA probes were prepared from DNA fragments using a Gigaprime labelling kit (Bresagen). DNA fragments were isolated form the following plasmids. An H*19* cDNA fragment was excised from LC10-8 (from Dr P. W. J. Rigby; Poirier et al., 1991) as a 778 bp fragment with *Pvu*II. A 462 bp *Stu*I cDNA fragment of *Oct4*, spanning positions 491-593, in bluescript was obtained from Dr H. Schöler (Schöler et al., 1990). A 484 bp fragment was released by *Xho*I/*Hin*dIII digestion and used for probe generation. A *Brachyury* specific probe was excised from pSK75 (obtained from Dr B. G. Herrmann) as a 1600 bp *Eco*RI fragment. F20A in pUC8, containing *uvomorulin*, was obtained from Dr R. Kremler (Ringwald et al., 1987). An *uvomorulin* probe was excised as a 620 bp *Eco*RI fragment. pAB11, containing *Evx-1* cDNA sequences, was obtained from Dr G. Martin (Dush and Martin, 1992). A 700 bp *Pst*I/*Bam*HI *Evx-1* cDNA fragment was used in northern analysis. *Fgf5* was obtained as a full length coding region clone in Bluescript from Dr J. Hébert (Hébert et al., 1990), from which an 800 bp *Eco*RI/*Bam*HI cDNA fragment was isolated and used as a probe. *AFP* was obtained as a 400 bp *Eco*RI fragment, encoding the first 350 bp of the mouse *AFP* cDNA in pBKSII+ from Dr R. Krumlauf. *Rex1* was obtained from Dr N. Clarke as a 848 bp PCR fragment cloned into pCR^{TM}II (Hosler et al., 1989). The 848 bp *Rex1* probe was excised from this plasmid by *Eco*RI digestion. A *mGAP* probe was synthesised by labelling a whole plasmid containing 300 bp of *mGAP* cDNA sequence (Rathjen et al., 1990).

RNA protection assays were performed as described by Chapman et al. (1997). A riboprobe for the detection of *Oct4* was transcribed from the *Oct4* containing plasmid described above which had been linearised with *Nci*I.

Wholemount in situ hybridisation

Wholemount in situ hybridisation on cell layers was performed using the method of Rosen and Beddington (1993) with the following modification. Cell layers were pre-hybridised, hybridised and washed at 65°C. Cell layers were blocked with 10% heat inactivated FCS and antibodies were added in TSBT/1% FCS. The antibodies were not pre-adsorbed. Anti-sense *Oct4* probes were synthesised by T3 RNA polymerase as run-off transcripts from bluescript containing a 462 bp *Stu*I *Oct4* cDNA fragment (Schöler et al., 1990), linearised with *Hin*dIII. Sense transcripts, used as controls, were obtained from the same plasmid linearised with *Xho*I and transcribed by T7 RNA polymerase. Sense and anti-sense *Fgf5* transcripts were generated from a plasmid clone containing the full-length *Fgf5* coding sequence which had been linearised with either *Eco*RI or *Bam*HI and transcribed with T7 or T3 RNA polymerase respectively.

Alkaline phosphatase staining

Alkaline phosphatase was visualised using diagnostic kit 86-R (Sigma). The kit was used according to the manufacturer's specifications with the following modification: cell layers were fixed in 4.5 mM citric acid, 2.25 mM sodium citrate, 3 mM sodium chloride, 65% methanol and 4% para-formaldehyde prior to washing and staining.

Blastocyst injection

ES and EPL cells were introduced to into CBA/C57 F2 blastocysts using standard blastocyst injection technology, as described by Stewart (1993). E14TG2a (Hooper et al., 1987) cells for injection were grown for 2 and 4 days in either medium containing LIF or MEDII before trypsinisation to a single cell suspension in preparation for injection.

GPI analysis

GPI analysis of blood and tissues was described by Bradley (1987). Blood samples were collected from the tail. The tissue samples analysed were taken from the brain, eye, femur, heart, intestines, kidneys, liver, lung, muscle, stomach, skin, spleen, tongue, thymus and the ovary or testes. Tissues samples were prepared by freeze/thawing and by homogenisation in water before analysis.

Column chromatography

Anion exchange column chromatography was performed using a FPLC system fitted with a 1 ml Resource Q column (Pharmacia). 2 mg of protein (retained fraction) was bound in 50 mM Tris-Cl, pH 8.5, and eluted with an increasing concentration of 1 M NaCl in 50 mM Tris-Cl, pH 8.5, at a flow rate of 1 ml/minute over 40 minutes. Proteins were detected at 280 nm. 1 ml fractions were collected across the gradient, desalted and concentrated using Centricon-10 units (Amicon) before being assayed for activity. Activity was assessed by seeding 500 D3 ES cells into gelatin coated 1 ml tissue culture wells containing 0.5 ml ES DMEM, 1000 units mLIF, 0.5 ml eluted fraction (obtained by ultrafiltration of sfMEDII on a Centricon-10 unit; Amicon) reconstituted to 10% FCS and the column fractions. After 5 days cells were stained for alkaline phosphatase and assessed for EPL cell formation morphologically.

Normal phase chromatography was performed using a Waters 510 HPLC machine fitted with a 10 mm Waters radial pak normal phase silica column. Eluted material was detected with a Waters 490E programmable multi-wavelength detector set at 215 nm. The eluted fraction, prepared by ultrafiltration of sfMEDII over a Centricon-10 unit (Amicon), was pre-treated on a Sephadex G10 column, lyophilised and resuspended in 30:70 methanol:acetonitrile before application to the column. Material was eluted using increasing concentrations of water at a rate of 0.5 ml/minute. 1 ml fractions were collected, lyophilised, resuspended in 50 μl DMEM and assayed for activity. D3 ES cells were seeded as before into 1 ml ESDMEM, 1000 units LIF, 50 μg of retained fraction and the column fractions. Activity was assessed as before.

RESULTS

MEDII effects the transition of ES to EPL cells

When grown in medium supplemented with recombinant mouse LIF (mLIF) and in the absence of a feeder cell layer ES cells grow as a homogeneous population with greater than 95% of the colonies displaying a distinctive domed colony morphology (Fig. 1A.; Pease et al., 1990; Conovar et al., 1993). To assay for factors capable of inducing ES cell differentiation, ES cells were seeded at a density of 250 cells/cm^2 and cultured in the presence of mLIF and conditioned medium derived from mammalian cell lines. After 5 days the cells were assessed for divergence from the ES cell colony morphology.

When cultured in the presence of 10-80% medium (MEDII) conditioned by Hep G2 cells (Knowles et al., 1980; ATCC HB-8065) ES cells gave rise to a morphologically distinct population of cells, which we have termed early primitive ectoderm-like, or EPL, cells. The formation of EPL cells was

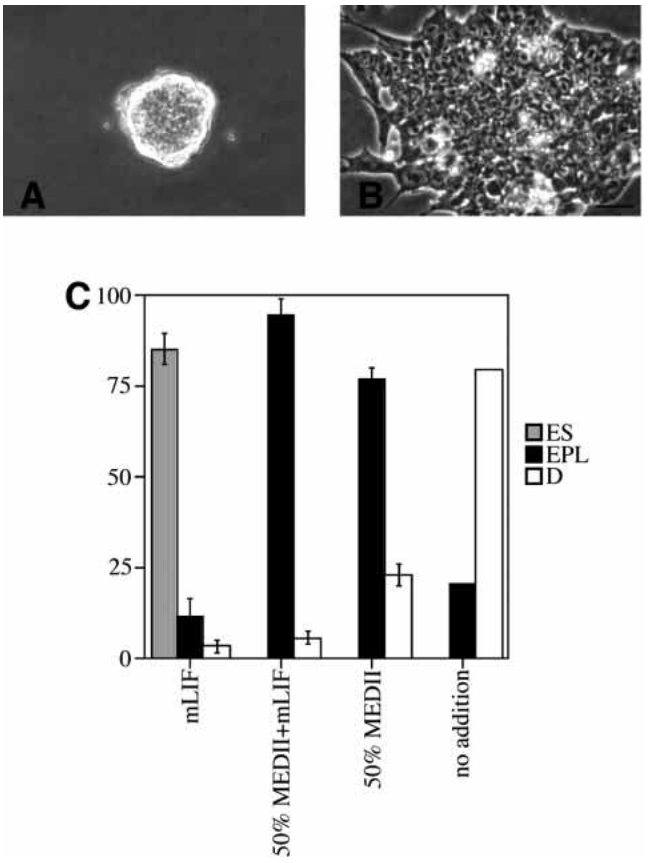

Fig. 1. MEDII effects the transition of ES cells to EPL cells. ES cells grown in medium containing mLIF (A) or 50% MEDII+mLIF (B) after 4 days in culture. ES cells cultured in the presence of MEDII show a characteristic morphology associated with the formation of EPL cells. Bar, 50 μm. (C) ES cells were seeded at a density of 250 cells/cm^2 in medium containing mLIF, 50% MEDII+mLIF, 50% MEDII and without addition. After 5 days the cultures were stained for alkaline phosphatase and haematoxylin, and the percentage of ES, EPL and differentiated colonies determined. Alkaline phosphatase positive colonies were subdivided into ES and EPL cell colonies on the basis of morphology, differentiated colonies (D) were determined as alkaline phosphatase negative. Plating efficiencies (%) for each condition were 42.8±8.7 (mLIF); 47.26±2.6 (50% MEDII+mLIF); 40.5±5.9 (50% MEDII); 41.4±6.6 (no addition).

specific for MEDII and was not seen in response to any of thirteen other conditioned media assayed. The EPL cell morphology was observed both on gelatin coated plastic and in co-culture with inactivated feeders (data not shown).

In contrast to the characteristic ES cell colony morphology, EPL cells grew as monolayer colonies in which individual cells, containing nuclei with one or more prominent nucleoli, were easily discernible (Fig. 1B). Morphologically, EPL cells resembled P19 embryonal carcinoma (EC) cells (McBurney and Rogers, 1982; Rudnicki and McBurney, 1987), which are considered similar to cells of post implantation primitive ectoderm (van den Eijnden-van Raaij, 1991; Rogers et al., 1991). The culture of ES cells in the presence of 50% MEDII+mLIF resulted in a relatively homogeneous cell population in which greater than 95% of colonies were of the EPL cell colony morphology and in which no residual ES cell colonies could be detected (Fig. 1C). In the absence of added LIF 77% of the colonies were morphologically EPL cells with the remaining 23% of colonies comprising overtly differentiated cell types (Fig. 1C). This instability was not observed when EPL cells were formed or maintained at high densities in MEDII without added mLIF (data not shown). The formation of a relatively uniform cell population from ES cells contrasted with the differentiated cell types produced when ES cells differentiate spontaneously in response to LIF withdrawal (Smith, 1991; Fig. 1C). However, within spontaneously differentiated ES cell cultures a small proportion of EPL-like colonies were seen (Fig. 1C), suggesting that EPL cells are a normal derivative of ES cells. EPL cell morphology could be maintained with extended culture of greater than 40 passages, or 100 days (data not shown) and was dependent on the continued presence of MEDII in the culture medium. Withdrawal of MEDII and mLIF resulted in the generation of an array of differentiated cell types (data not shown) similar to those arising from spontaneous ES cell differentiation (Smith, 1991).

The transition of ES cells to EPL cells, in response to MEDII, was demonstrated for a number of independently derived ES cell lines including MBL5, D3, CCE, E14 and CGR8 (data not shown). The appearance of EPL cells generated from each ES cell line was comparable.

EPL cells express pluripotent cell markers

The pluripotent cells of the early mouse embryo and germ line, and ES cells in culture, are characterised by expression of the homeobox gene *Oct4* (Rosner et al., 1990; Schöler et al., 1990; Yeom et al., 1991), the cell surface antigen SSEA1 (Solter and Knowles, 1978; Smith, 1992) and by alkaline phosphatase activity (Hahnel et al., 1990; Pease et al., 1990). EPL cells were formed by culturing ES cells in the presence of MEDII or MEDII+mLIF, for 2, 4, 6 and 16 days, with passaging every 2 days. Northern analysis of RNA from these populations showed expression of *Oct4* at levels equivalent to the levels seen in ES cells (Fig. 2A). *Oct4* expression persisted, at levels analogous to those seen in ES cells, for at least 16 days in the presence of MEDII or MEDII+mLIF (Fig. 2A), suggesting that the formation of EPL cells is not equivalent to the process of spontaneous differentiation (Fig. 2A). In situ hybridisation of ES cells and EPL cell monolayers with an *Oct4* specific anti-sense RNA probe showed *Oct4* expression uniformly distributed across the EPL cell colonies, and not restricted to sub-populations of cells within the culture (Fig. 2B,C). Sporadic differentiated cells within the population did not express *Oct4* (data not shown). Analysis of alkaline phosphatase activity and SSEA1 distribution also showed uniform expression by EPL cells (Fig. 2D,E; data not shown), with down regulation in the differentiated cells (data not shown). The uniform distribution of these pluripotent cell markers in EPL cell cultures demonstrated the homogeneity of the EPL cell population.

EPL cell formation is accompanied by establishment of primitive ectoderm-like gene expression

The expression of pluripotent cell marker genes by EPL cells suggested that these cells could be equivalent to a pluripotent cell population of the early embryo (Schöler et al., 1990; Hahnel et al., 1990), or possibly a differentiated cell lineage in

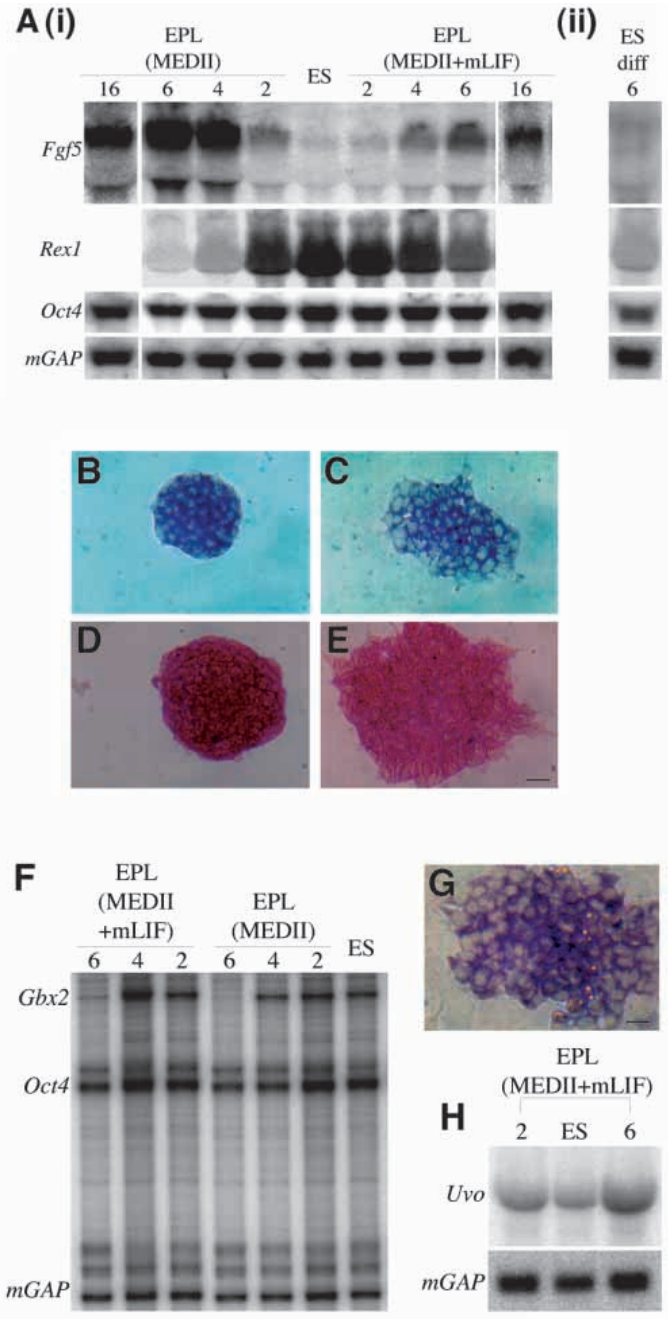

Fig. 2. (A) Northern blot analysis of ES and EPL cell RNA. 20 μg of total RNA, isolated from E14 ES cells, EPL cell derivatives cultured in MEDII or MEDII+mLIF for 2, 4, 6, and 16 days (i), and spontaneously differentiated ES cells cultured for 6 days in the absence of exogenous factors (ii), was analysed for the expression of *Fgf5*, *Rex1*, *Oct4*, and *mGAP*. *Fgf5* transcripts were 2.7 and 1.8 kb (Hébert et al., 1990), *Rex1* 1.9 kb (Hosler et al., 1989), *Oct4* 1.55 kb (Rosner et al., 1990) and *mGAP* 1.5 kb. (B,C) In situ analysis of ES (B) and EPL (C) cell layers for the expression of *Oct4*. (D,E) ES (D) and EPL (E) cells were stained for the presence of alkaline phosphatase. (F) 10 μg RNA from ES cells and EPL cells cultured for 2, 4 and 6 days in MEDII+mLIF and MEDII was analysed for the expression of *Gbx2*, *Oct4* and *mGAP* by Rnase protection. (G) In situ analysis of EPL cells for the expression of *Fgf5*. (H) 20 μg of total RNA from ES cells and EPL cells maintained in MEDII+mLIF for 2 and 6 days was analysed by northern for the expression of *uvomorulin* (*Uvo*). *mGAP* expression was used to normalise RNA levels. Bars: 32 μm (B-E); 25 μm (F).

which *Oct4* expression was not fully down-regulated (Pruitt, 1994). The pluripotent cell populations of the embryo can be discriminated from differentiated cell lineages by morphological and developmental criteria (Snow, 1977; Beddington, 1983; Gardner, 1985) and by the temporal and spatial expression of marker genes (Haub and Goldfarb, 1991; Hébert et al., 1991; Rogers et al., 1991). The embryonic equivalent of EPL cells was investigated by analysis of the expression of marker genes that identify the pluripotent cell populations of the embryo, and the differentiated cells of the extra-embryonic lineages and gastrulating embryo.

Three genes, *Fgf5*, *Rex1* and *Gbx2*, have been reported to be differentially transcribed between cells of the ICM and primitive ectoderm (summarised in Table 1). *Fgf5* expression is up regulated on the formation of primitive ectoderm from the ICM (Haub and Goldfarb, 1991; Hébert et al., 1991), whereas both *Rex1* and *Gbx2* expression can be detected in the ICM but not in the primitive ectoderm by 6.5 d.p.c. (Rogers et al., 1991; Bulfone et al., 1993; Chapman et al., 1997). The expression of *Fgf5* and *Rex1* in ES and EPL cells was assessed by northern blot (Fig. 2A; Table 1). *Gbx2* expression was analysed by RNase protection assay (Fig. 2F; Table 1). *Fgf5* expression, which was barely detectable in ES cells, was elevated 50-fold in EPL cells grown for 2 days in MEDII. *Fgf5* expression increased in EPL cells with time in culture such that maximal expression, representing a 340 fold induction of *Fgf5*, was reached by day 6 (Fig. 2A) and persisted for at least 16 days in culture (Fig. 2A). *Rex1* was expressed at high levels by ES cells, but this expression was down regulated 50% within 2 days of EPL cell formation (Fig. 2A; Table 1). *Rex1* expression was reduced further in EPL cells cultured for longer periods of time such that EPL cells grown in MEDII for 6 days showed a 5-fold reduction in *Rex1* expression compared to ES cells. *Gbx2* expression was high in ES cells and maintained

Table 1. Summary of the gene expression patterns of ES cells and EPL cells compared to gene expression in the ICM and primitive ectoderm of the embryo

	ES cells	ICM	EPL cells	Primitive ectoderm
Oct4	+	+	+	+
Alkaline phosphatase	+	+	+	+
Uvomorulin	+	+	+	+
Fgf5	–	–	+	+
Rex1	high	+	low	–
Gbx2	+	+	–	–
Alphafetoprotein (AFP)	–	–	–	–
H19	–	–	–	–
Evx1	–	–	–	–
Brachyury	–	–	–	–

Genes differentially transcribed between these two cell populations have been highlighted. RNA was isolated from ES cells and EPL cells grown in the presence of MEDII for 2 days and analysed by northern blot for the expression of *Oct4*, *uvomorulin*, *Fgf5*, *Rex1*, alphafetoprotein (AFP), *H19*, *Evx1* and *Brachyury*. *Gbx2* expression was detected using RNA protection assays. Alkaline phosphatase activity was detected using an enzymatic stain on ES and EPL cell layers. Embryonic expression patterns were determined by Rosner et al., 1990; Schöler et al., 1990; Yeom et al., 1991 (*Oct4*); Hahnel et al., 1990 (alkaline phosphatase); Sefton et al., 1992 (*uvomorulin*); Haub and Goldfarb, 1991 (*Fgf5*); Rogers et al., 1991 (*Rex1*); Bulfone et al., 1993; Chapman et al., 1997 (*Gbx2*); Dziadek and Adamson, 1978 (*AFP*); Poirier et al., 1991 (*H19*); Bastian and Gruss, 1990; Dush and Martin, 1992 (*Evx1*); and Herrmann, 1991 (*Brachyury*).

with 2 days of culture in MEDII, but was reduced with further culture in MEDII to undetectable levels by day 6. The changes in expression of *Fgf5*, *Rex1* and *Gbx2* were delayed when the transition of ES to EPL cells was carried out in MEDII+mLIF when compared to EPL cells formed in MEDII (Fig. 2A), suggesting that LIF retarded the ES to EPL cell transition. The persistence of EPL cell morphology and high levels of *Fgf5* and *Oct4* expression (Fig. 2A) demonstrated the ability of MEDII to maintain EPL cells as a stable cell population and was clearly distinct from the gene expression changes observed during spontaneous differentiation of ES cells (Fig. 2A).

EPL cell monolayers, cultured for 4 days in MEDII, were probed for *Fgf5* expression using DIG-labelled anti-sense *Fgf5* transcripts. Equivalent levels of *Fgf5* specific staining were seen in all EPL cells within the culture (Fig. 2G) confirming both the uniformity of the ES to EPL cell transition and the homogeneity of EPL cell populations.

The expression of *uvomorulin*, a cadherin expressed by the pluripotent cells of the ICM and the primitive ectoderm but down-regulated in cells of the primordial germ cell lineage (Sefton et al., 1992), was assessed in ES and EPL cell cultures by northern blot analysis. *Uvomorulin* expression was maintained in EPL cells at levels equivalent to or greater than those observed in ES cells (Fig. 2H) suggesting that these cells did not represent an in vitro equivalent of primordial germ cells.

Consistent with the expression pattern of pluripotent cell markers EPL cells did not express detectable levels of marker genes specific for the extra-embryonic lineages of the early embryo (H*19*; Poirier et al., 1991; *AFP*; Dziadek and Adamson, 1978;), the primitive streak (*Evx1*; Bastian and Gruss, 1990; Dush and Martin, 1992) or nascent mesoderm (*Brachyury*; Wilkinson et al., 1990; Herrmann, 1991) (Table 1; data not shown).

The morphological transition of ES to EPL cells was found to be accompanied by differential regulation of marker genes that discriminate pluripotent cell populations in vivo. Although gene expression in ES cells was equivalent to their origin from the pluripotent cells of the pre-implantation embryo, EPL cells expressed a repertoire of marker genes which is shared by only one cell population of the early embryo, the primitive ectoderm (Table 1).

ES and EPL cells represent distinct, but interchangeable, cell states

EPL cells seeded and cultured in medium containing mLIF, but not MEDII, were observed to adopt an ES cell-like colony morphology, a process we termed reversion. Further, the withdrawal of MEDII, in the presence of mLIF, from established EPL cell colonies resulted in the formation of a three-dimensional colony structure suggestive of the ES cell phenotype (data not shown). Northern blot analysis was used to examine the expression of *Oct4*, *Fgf5* and *Rex1* in ES, EPL and reverted EPL cells. EPL cells cultured and passaged in MEDII and MEDII+mLIF for 2, 4 and 6 days were reverted by passaging the cells into medium containing mLIF alone for 6 days. Reverted EPL cells exhibited low *Fgf5* expression and high *Rex1* expression, comparable to the gene expression profile observed in ES cells and distinct from the expression of high levels of *Fgf5* and low levels of *Rex1* in the parental EPL cells (Fig. 3A). These data indicated that the phenotypic reversion of EPL cells was accompanied by the establishment of an ES cell gene expression profile. These data also demonstrated a requirement for MEDII for both the establishment and maintenance of EPL cell characteristics.

Clonal EPL cell lines were generated and reverted to ensure that reversion was not a consequence of residual ES cells within EPL cell populations. EPL cells, grown for 4 days in medium containing MEDII but without added mLIF, were seeded at limiting dilution in MEDII+mLIF to produce clonal EPL cell colonies. Two clones were expanded in MEDII+mLIF over 3 weeks before seeding into medium containing mLIF alone or MEDII+mLIF. The resulting cultures were assessed for the presence of ES cells, EPL cells and differentiated colonies (Fig. 3B). A high proportion of cells from both lines formed alkaline phosphatase positive colonies with ES cell morphology in cultures seeded and maintained in mLIF alone which were not seen in the cultures maintained in MEDII+mLIF, indicating efficient reversion of the clonal lines.

Reverted EPL cells, but not EPL cells, were able to contribute to chimeric mice following blastocyst injection

Pluripotent cells from the pre- and post-implantation embryo differ by the ability of the former but not the latter to contribute to embryo development following blastocyst injection (Gardner, 1971; Rossant, 1977; Beddington, 1983; Brook and Gardner, 1997). ES cells retain the ability to contribute to all embryonic and adult tissues (Robertson, 1986; Thomas and Capecchi, 1987). E14TG2a ES cells (Hooper et al., 1987) and their EPL cell derivatives were tested for their ability to contribute to chimeric mice when injected into CBA/C57 F2 blastocysts. The contribution of ES cell and EPL cell derivatives to mouse offspring was assessed by coat colour contribution and GPI analysis of blood and tissues. E14TG2a ES cells contributed to the development of 44% of injected blastocysts (Table 2). In contrast, E14TG2a derived EPL cells, grown in MEDII without added mLIF for 2 and 4 days such that EPL cell gene expression was established, did not contribute to chimera formation as assessed by coat colour of the 33 and 50 live born pups, respectively (Table 2). GPI analysis of blood samples taken from 20 of these mice also failed to detect any contribution from the EPL cells. Analysis of 15 tissue samples taken from two mice also failed to detect any EPL cell contribution. This could not be explained by adverse effects of EPL cells on the viability of injected blastocysts as the percentage of live born pups was

Table 2. ES cells and reverted EPL cells, but not EPL cells, contribute to the development of chimeric mice when injected into CBA/C57 black host blastocysts

	Blastocysts injected	Pups born	% Liveborn	% Chimera born
ES; E14TG2a	163	65	40	58
EPL; 2	65	33	50	0
EPL; 4	77	50	65	0
EPL; 2^R	78	22	28	36
EPL; 4^R	74	31	42	58

Summary of results from the injection of E14TG2a ES cells, their EPL cell derivatives grown in 50% MEDII for 2 and 4 days (EPL;2 and EPL;4 respectively) and reverted EPL cells, formed by the culture of EPL cells in medium containing mLIF but not MEDII for 6 days (EPL;2^R and EPL;4^R).

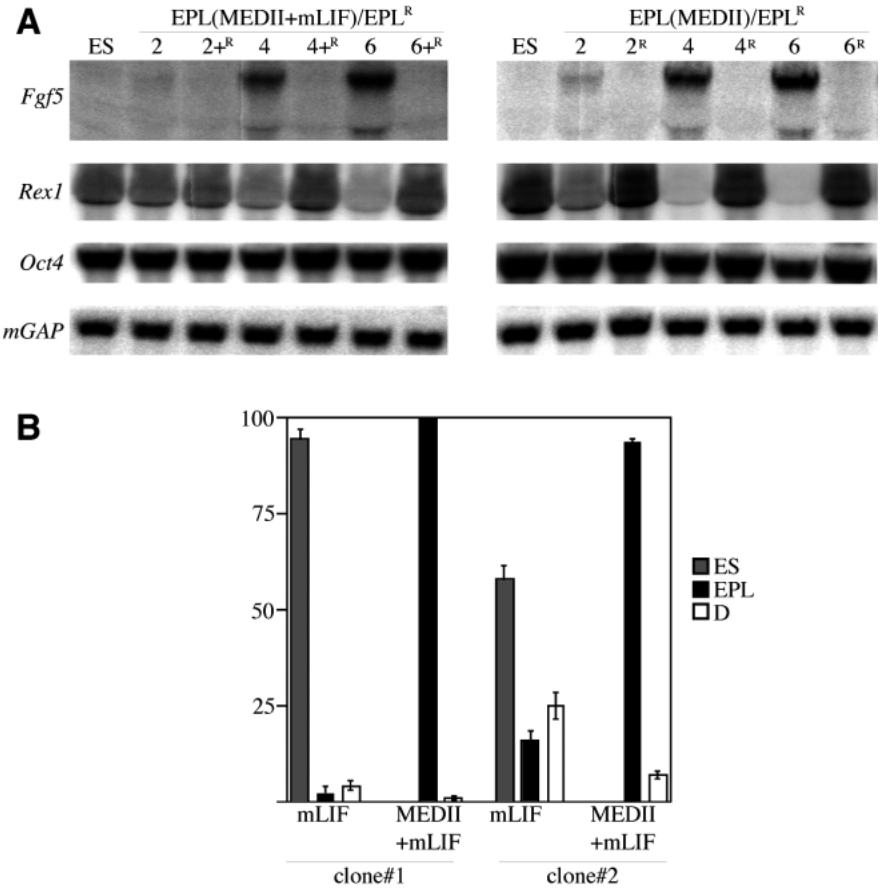

Fig. 3.(A) Northern blot analysis of EPL cells and reverted EPL cells. 20 µg of total RNA from EPL cells, cultured in the presence of MEDII or MEDII+mLIF for 2, 4 and 6 days, and their reverted derivatives, reverted in medium containing mLIF alone (2^R, 4^R and 6^R), was analysed by northern blot for the expression of *Fgf5*, *Rex1*, *Oct4* and *mGAP*. (B) Clonal EPL cell lines #1 and #2 were seeded at a density of 250 cells/cm^2 and 500 cells/cm^2, respectively, into medium containing mLIF and 50% MEDII + mLIF. After 5 days the cultures were stained for alkaline phosphatase and the percentage of ES, EPL and differentiated colonies determined. Alkaline phosphatase positive colonies were subdivided into ES and EPL cell colonies on the basis of morphology, differentiated colonies (D) were determined as alkaline phosphatase negative. Plating efficiencies (%) were 18.4±2.62 (clone #1, mLIF); 34.8±4.56 (clone #1, 50% MEDII + mLIF); 20.4±1.71 (clone #2, mLIF); 31.16±2.93 (clone #2, 50% MEDII + mLIF).

comparable from blastocysts injected with ES cells and EPL cells (Table 2).

The EPL cells used in the preceding blastocyst injection experiments were reverted by culture in media containing mLIF, but not MEDII, for 6 days (2^R and 4^R). These cells contributed to embryonic development in 36% (2^R) and 63% (4^R) of blastocysts injected (Table 1). GPI analysis of blood and tissue samples taken from 10 and 2, respectively, of the chimeric mice generated using reverted EPL cells established that these cells were able to contribute to mesodermal, endodermal and ectodermal derived cell lineages of the adult mouse (data not shown). These data indicated that the ES to EPL cell transition resulted in cells of differing developmental capabilities, reflected in the ability of ES cells, but not EPL cells, to contribute to development when introduced into a host blastocyst. The ability of reverted EPL cells to contribute to chimera development suggests that this loss in chimera forming ability by EPL cells can not be attributed to a loss of pluripotence on EPL cell formation.

Soluble biological factors within MEDII are responsible for the ES to EPL cell transition

Serum free medium conditioned by Hep G2 cells for 3-4 days (sfMEDII) was shown to cause the ES to EPL cell transition in a manner analogous to MEDII (data not shown). SfMEDII was separated into two fractions by ultrafiltration through a 3×10^3 M_r cut-off membrane (Centricon-3 unit; Amicon). Both fractions were assayed for EPL cell forming activity, defined as the ability to cause the complete conversion of D3 ES cells to alkaline phosphatase positive EPL cells in the presence of mLIF. ES cells seeded into the retained fraction (>3×10^3 M_r), at concentrations equivalent to 50% MEDII, did not form EPL cells (Fig. 4A). ES cells seeded into the eluted fraction (<3×10^3 M_r), at concentrations equivalent to 50% MEDII, gave rise to an array of colony morphologies including ES, EPL and differentiated. The presence of ES cells was uncharacteristic of the ES to EPL cell transition and demonstrated that the eluted material alone was unable to induce EPL cell formation (Fig. 4A). Seeding of ES cells into medium containing both the retained and eluted fractions resulted in uniform EPL cell formation (Fig. 4A), equivalent to that seen for sfMEDII. These data indicated that two separable biological factors were required for the conversion of ES to EPL cells.

The retained and eluted fractions of sfMEDII were partially purified by column chromatography. The retained fraction was bound to a Resource Q anion exchange column and eluted with an increasing salt gradient. Individual fractions were assessed for EPL cell forming activity on ES cells in the presence of mLIF and the presence and absence of the eluted fraction. In the absence of the eluted fraction the ES to EPL cell transition could not be detected in any fraction. EPL cell forming activity was shown to elute from the column between 250 and 350 mM NaCl when assayed in the presence of the eluted fraction (Fig. 4B). Partial purification of the smaller activity was achieved by normal phase chromatography on a Waters radial-pak phase chromatography cartridge. Column fractions were assayed for EPL cell forming activity on ES cells in the presence and absence of the retained fraction. In the presence of the retained

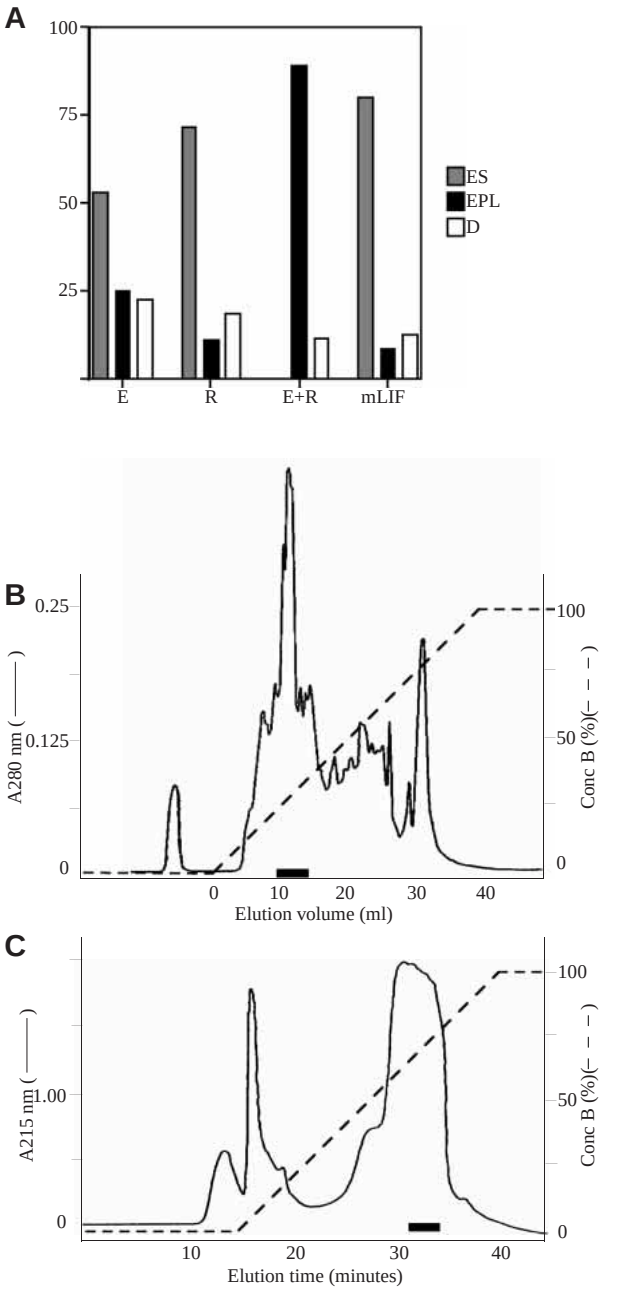

Fig. 4. Two active components are required for the formation of EPL cells. (A) sfMEDII was separated via ultrafiltration on a centricon-3 unit into two fractions; the retained fraction, containing all medium components of $>3\times10^3$ M_r and the eluted fraction, containing smaller, $<3\times10^3$ M_r, components. ES cells were seeded at a density of 250 cell/cm^2 into medium containing the eluted fraction, the retained fraction or the retained and eluted fractions together and compared to mLIF. After 5 days the cultures were stained for alkaline phosphatase and the percentage of ES, EPL (alkaline phosphatase positive) and differentiated colonies (alkaline phosphatase negative; D) determined. Plating efficiencies (%) were 48.4 (eluted fraction); 41.6 (retained fraction); 49.2 (eluted and retained fractions); 32.8 (mLIF). (B,C) Anion exchange chromatography of the retained fraction (B) and Normal phase chromatography of the eluted fraction (C). EPL cell forming activity is indicated in each case by the black bar. Buffer B was 1 M NaCl in 50 mM Tris-Cl, pH 8.5, for anion exchange chromatography (B) and water for the normal phase chromatography (C).

fraction, EPL cell forming activity was detected eluting from the column at 70% water:30% methanol:acetonitrile (Fig. 4C). In the absence of the retained fraction the ES to EPL cell transition was not observed. The ability to retain EPL cell forming activity on chromatographic columns indicates that at least two particulate activities within MEDII are responsible for the transition of ES to EPL cells.

A role for gp130 signalling in EPL cell maintenance

The increased level of differentiated cell colonies seen in EPL cell cultures without added mLIF when compared to cultures with added mLIF (Figs 1C, 5), suggested a possible role for LIF, or other cytokines which signal through gp130, in the maintenance of EPL cells. Antibodies to mouse gp130 (RX-19), which neutralises the activity of mLIF, hOSM, hIL-6 and hIL-11, but not hLIF, on myeloid leukemic M1 cells and ES cells (Koshimizu et al., 1996; Lake, 1996), and to hLIF (R&D Systems), which neutralises hLIF activity (Lake, 1996), were used to assess the role of gp130 signalling in EPL cell maintenance.

While EPL cell formation was observed in the presence of anti-gp130 (10 μg/ml) or anti-hLIF (10 μg/ml) antibodies, the addition of anti-hLIF antibodies resulted in marked destabilisation of EPL cells compared to cells cultured in MEDII alone (Fig. 5). This suggested that the presence of hLIF

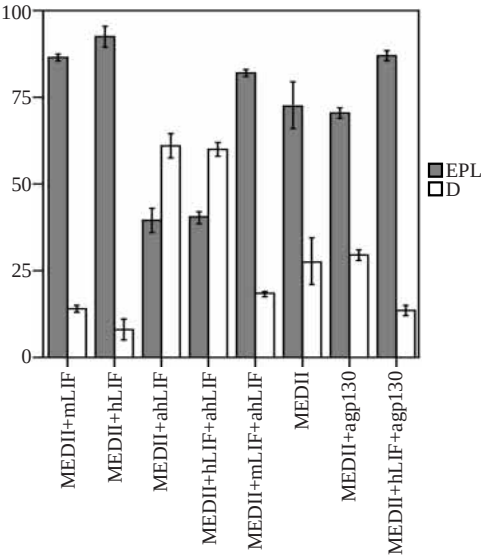

Fig. 5. hLIF is required for the maintenance of EPL cells in culture. ES cells were seeded at a density of 250 cells/cm^2 into medium containing 50% MEDII or 50% MEDII+mLIF (1000 units/ml), hLIF (1000 units/ml), anti-hLIF antibodies (10 μg/ml), hLIF (1000 units/ml) and anti-hLIF antibodies (10 μg/ml), mLIF (1000 units/ml) and anti-hLIF antibodies (10 μg/ml), anti-gp130 antibodies (10 μg/ml) or hLIF (1000 units/ml) and anti-gp130 antibodies (10 μg/ml). After 5 days in culture colonies were stained for alkaline phosphatase and the percentage of EPL cell containing colonies (EPL) and differentiated colonies (D) determined. Plating efficiencies (%) for each condition were 32.5±1.5 (50% MEDII); 39.8±1.3 (50% MEDII+mLIF); 35.0±0.2 (MEDII + hLIF); 25.5±1.0 (MEDII + anti-hLIF antibodies); 28.8±3.8 (MEDII + hLIF + anti-hLIF antibodies); 40.0±0.5 (MEDII + mLIF + anti-hLIF antibodies); 36.8±3.3 (MEDII + anti-gp130 antibodies); 36.3±2.3 (MEDII + hLIF + anti-gp130 antibodies).

in MEDII, but not mLIF expressed by the cells, was important EPL cell maintenance. The addition of both anti-hLIF antibodies and 1,000 units of mLIF (ESGRO) reduced the levels of differentiation to those seen in EPL cell cultures formed in MEDII+mLIF, demonstrating that gp130 signalling was able to restore EPL cell stability in culture.

ES cells were seeded into medium containing MEDII, anti-hLIF antibodies (10 μg/ml) and mLIF at concentrations between 10 and 1000 units/ml (ESGRO; Amrad). EPL cell maintenance was achieved with the addition of between 50 and 100 units/ml of mLIF (data not shown), 10- to 20-fold less than the 1000 units/ml required for the maintenance of ES cells (Williams et al., 1988: J. Rathjen and J. Lake, unpublished).

DISCUSSION

MEDII was identified as a conditioned medium which caused the transition of ES cells to a novel cell population we have termed early primitive ectoderm-like, or EPL, cells. Two separable factors contained within MEDII were required for both the formation and maintenance of EPL cells. Maintenance of EPL cells also required LIF, at concentrations 10- to 20-fold lower than required by ES cells. The ES cells used in this paper were shown to be comparable with cells of the ICM in gene expression and ability to contribute to chimera formation. In contrast, characterisation of EPL cells showed them to be a cell population which express pluripotent cell markers but which were distinct from ES cells in terms of cell morphology, gene expression, cytokine requirements and the inability of EPL cells to contribute to chimera development following blastocyst injection.

A novel cell population with properties equivalent to the primitive ectoderm

The expression patterns of pluripotent cell markers *Oct4*, alkaline phosphatase and *uvomorulin* identified EPL cells as equivalent to pluripotent cells of the early embryo, distinct from primordial germ cells. Consistent with this, EPL cells did not express marker genes associated with cell lineages determined at gastrulation or the extraembryonic lineages of the embryo. Conversion from ES to EPL cells was found to be accompanied by an increase in *Fgf5* expression and a decrease in both *Gbx2* and *Rex1* expression. *Fgf5* is not expressed by the pluripotent cells of the ICM, but has been detected within cells of the epiblast later, at 5.25 d.p.c., where it persists until differentiation (Haub and Goldfarb, 1991; Hébert et al., 1991). *Fgf5* is also expressed by P19 EC cells, which have been proposed to be similar to the primitive ectoderm by virtue of their morphology, gene expression (van den Eijnden-van Raaij et al., 1991; Rogers et al., 1991) and differentiation potential (Rossant and McBurney, 1982; Rudnicki and McBurney, 1987). *Rex1* is expressed by the ICM of 4.5 d.p.c. embryos and ES cells, but not in the primitive ectoderm of 6.0 d.p.c. embryos or P19 EC cells (Rogers et al., 1991). The embryonic expression pattern of *Rex1* has been further refined and the down regulation in expression shown to have occurred by 5.25 d.p.c. (T. Pelton and P. D. Rathjen, unpublished). Similarly, expression of *Gbx2* has been detected in the ICM of the 4.0 d.p.c. blastocyst but not in the primitive ectoderm of embryoid bodies or the 6.5 d.p.c. embryo (Bulfone et al., 1993; Chapman et al., 1997). *Gbx2* expression is reinitiated in the three primary germ layers following gastrulation (Bulfone et al., 1993; Bouillet et al., 1995). The up regulation of *Fgf5* exhibited by EPL cells suggests an equivalence with cells of the primitive ectoderm post 5.25 d.p.c. Likewise, down regulation of *Rex1* and *Gbx2* expression during the formation of EPL cells supports the identification of EPL cells as primitive ectoderm-like. Taken together the gene expression pattern suggests the embryonic equivalent of EPL cells occurs around 5.25 d.p.c., or immediately after cavitation of the pluripotent cell mass, coinciding with the appearance of early primitive ectoderm.

The acquisition of primitive ectoderm-like gene expression by EPL cells was not immediate but occurred gradually over time. The progressive increase in *Fgf5* expression in EPL cell cultures was similar to the kinetics of *Fgf5* up regulation in embryoid bodies, where *Fgf5* expression has been observed to increase as the primitive ectoderm matures (Hébert et al., 1991). The similarities with primitive ectoderm formation in embryoid bodies suggest that the progressive maturation of primitive ectoderm gene expression during EPL cell formation may be reflected in vivo. A similar gradual alteration in pluripotent cell identity has been proposed for cells of the ICM, which show a progressive restriction in ability to form extraembryonic tissues, such as the trophectoderm, when differentiated in vitro (Smith, 1992).

The establishment of *Fgf5*, *Gbx2* and *Rex1* gene expression patterns was delayed in EPL cells formed and maintained in the presence of MED+mLIF. Several lines of evidence suggest that LIF can delay the development of pluripotent cells both in vitro and in vivo. The formation of primitive ectoderm during embryoid body differentiation of ES cells has been shown to be inhibited by the presence of exogenous LIF (Shen and Leder, 1992). Furthermore, the pluripotent cells of chimeric mice overexpressing the M-LIF cDNA showed abnormal proliferation and did not undergo differentiation into mesoderm (Conquet et al., 1992). The ability of LIF to inhibit the development of EPL cell characteristics further underlines the relationship between the primitive ectoderm and EPL cells.

The assignation of EPL cells as primitive ectoderm-like is supported by the inability of EPL cells to contribute to the development of chimeric mice when introduced into a host blastocyst. This deficiency does not appear to be attributable to a loss of pluripotence as reverted EPL cells readily contributed to mouse development. Experiments carried out by Beddington (1983) have demonstrated a similar limitation in ability of pluripotent cell populations of the post-implantation embryo to contribute to the formation of chimeric mice. These data suggest that development of the ICM to the primitive ectoderm, which occurs at the time of implantation, alters the pluripotent cells such that they can no longer assimilate with the cells of the ICM or partake in development when introduced at this developmentally inappropriate stage.

MEDII action parallels primitive ectoderm formation

Formation of the primitive ectoderm in the mouse embryo commences at the time of implantation of the blastocyst into the uterine wall and results in the formation of a cup shaped pseudo-stratified epithelium of pluripotent cells lining the proamniotic cavity. Primitive ectoderm formation is a pivotal developmental event resulting in the establishment of a population of cells which act as a substrate for further differentiation. In vitro, as in

vivo, the formation of primitive ectoderm appears to be an obligatory intermediate in the differentiation of ES cells into embryonic cell lineages (Shen and Leder, 1992).

Although several gene ablation experiments have identified genes involved in proliferation and maintenance of the primitive ectoderm (Chen et al., 1994; Spyropoulos and Capecchi, 1994; Feldman et al., 1995; Mishina et al., 1995; Winnier et al., 1995; Hakem et al., 1996; Hogan, 1996; Liu et al., 1996; Duncan et al., 1997) little is known of the origin or biochemical identity of the signals responsible for induction of primitive ectoderm in the embryo.

Several lines of evidence suggest that signalling from the visceral endoderm is required for primitive ectoderm induction. Disruption of the *Evx1* gene (Spyropoulos and Capecchi, 1994) results in disruption of primitive ectoderm formation at the time of implantation. At this time, *Evx1* expression is restricted to visceral endoderm (Spyropoulos and Capecchi, 1994), suggesting that the loss of *Evx1* expression within the visceral endoderm prevented primitive ectoderm induction. Coucouvanis and Martin (1995) investigated formation of the proamniotic cavity and primitive ectoderm in early rodent embryogenesis using embryoid bodies as an in vitro model. They proposed that cavitation within the epiblast was a result of apoptosis in response to a 'death' signal emanating from the visceral endoderm. Survival of the outer cells, which proliferate to form the primitive ectoderm, was ensured through contact with a 'survival' signal located on the basement membrane between the pluripotent cells and visceral endoderm. These experimental systems implicate signals emanating from the neighbouring visceral endoderm cell layer, possibly located within the intervening basement membrane in the establishment and maintenance of primitive ectoderm.

In this regard it is interesting that EPL cells arise from ES cells in response to factors expressed by a hepatic cell line. Although visceral endoderm and liver are derived from different cell lineages, the gene expression profiles of the two cell types have been found to be similar (Meehan et al., 1984). A preliminary characterisation of sfMEDII showed the presence of two active components within the media which could be readily separated by standard separation techniques. Further characterisation of the factors suggests they comprise a large molecular mass protein (retained fraction; M. Bettess and P. D. Rathjen, unpublished) and a small peptide (eluted fraction; J. Washington and P. D. Rathjen, unpublished). The formation and maintenance of EPL cells required both of these factors. A more detailed understanding of the active factors within MEDII may provide an alternative route for the identification factors involved in primitive ectoderm induction within the embryo.

The relationship between the inductive factors found in MEDII, and factors previously described in the literature to have a role in primitive ectoderm, biology is unclear. However, parallels can be drawn between the two factor model proposed by Coucouvanis and Martin (1995), which postulates the existence of small, diffusible signal derived from the visceral endoderm and a second signal associated with the extracellular matrix.

The ES to EPL cell transition reveals distinct, interconvertible cell states

The transition between ES cells and EPL cells was a reversible process as ES cells could be generated from EPL cells by the withdrawal of MEDII and the maintenance of LIF within the culture. Reverted EPL cells grew as compact domed colonies, equivalent to ES cell colonies in morphology, gene expression and ability to contribute to the formation of chimeric mice following blastocyst injection. The ability of EPL cells to revert to ES cells suggests that the formation of EPL cells is not an irreversible differentiation event but a process in which closely related and interchangeable cell states are adopted in response to specific signals. The existence of distinct cell states has been evidenced by differential gene expression, but more importantly by developmental reprogramming of the cells both in vitro and in vivo. A similar phenomenon has been described for other pluripotent cell types in vitro. Primordial germ cells, from both the migratory germ cell population and the gonadal population, require a combination of LIF, stem cell factor and bFGF for initial in vitro culture but extended culture can be achieved as ES cell-like cells in LIF alone (Matsui et al., 1992; Resnick et al., 1992). Based largely on these observations, Rossant (1993) has predicted that all the pluripotent cells of the mammalian embryo can be cultured as a 'basal' ES/EC pluripotent cell population. The interconvertibility of pluripotent cell populations may underlie the ability of the early mouse embryo to reprogram development in response to environmental insult (Snow and Tam, 1979; Smith, 1992).

The initial events leading to primitive ectoderm formation from the ICM occur between 4.75 and 5.5 d.p.c. of mouse embryogenesis, at a time when analysis of the embryo is hampered by the small size and inaccessibility of the embryo within the uterine environment. The elucidation of many of the molecular processes involved at this critical stage of embryogenesis requires in vitro models of both the primitive ectoderm and gastrulation. ES cells to date have proved an intractable model for the understanding of many of these processes. The formation of EPL cells from ES cells is the first demonstration of uniform differentiation of ES cells in response to biological factors. Resembling closely the primitive ectoderm, EPL cells demonstrate the ability of pluripotent cells to adopt distinct, interchangeable states determined by the presence and absence of factors. EPL cells can be manipulated and studied in vitro and will allow more precise modelling of the processes of early mammalian embryogenesis, in particular the processes of pluripotent cell development and the subsequent events of gastrulation. MEDII provides an alternative route for the biochemical identification of factors involved in the induction and maintenance of the primitive ectoderm in vivo. Further, EPL cells represent a first step in the development of lineage specific differentiation protocols for ES cells.

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