Response to BMP4 signalling during ES cell differentiation defines intermediates of the ectoderm lineage

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Summary

The formation and differentiation of multipotent precursors underlies the generation of cell diversity during mammalian development. Recognition and analysis of these transient cell populations has been hampered by technical difficulties in accessing them in vivo. In vitro model systems, based on the differentiation of embryonic stem (ES) cells, provide an alternative means of identifying and characterizing these populations. Using a previously established mouse ES-cell-based system that recapitulates the development of the ectoderm lineage we have identified a transient population that is consistent with definitive ectoderm. This previously unidentified progenitor occurs as a temporally discrete population during ES cell differentiation, and differs from the preceding and succeeding populations in gene expression and differentiation potential, with the unique ability to form surface ectoderm in response to BMP4 signalling.

Key words: ES cells, Ectoderm, Differentiation, BMP4

Introduction

The ectoderm is established during mammalian gastrulation from the anterior-distal portion of the pre-streak primitive ectoderm. Although fated spatially, transplantation experiments demonstrate that at this early stage, the cells are pluripotent and retain the ability to contribute to tissues derived from other germ layers, such as mesoderm (Beddington, 1982; Lawson et al., 1991). As gastrulation proceeds, the ectodermal precursors move in a proximal and posterior direction (Lawson et al., 1991), so that at the completion of gastrulation they comprise the entire inner layer of the embryo. At this stage, the cells have lost pluripotence, and have a differentiation potential limited to the ectodermal lineages (Carey et al., 1995). In lower vertebrates, the formation of ectoderm proceeds via a bipotent intermediate, definitive ectoderm, which differentiates to form the two major ectodermal lineages: surface ectoderm and neurectoderm (Hemmati-Brivanlou and Melton, 1997). The transient and dynamic appearance of differentiation intermediates during mammalian gastrulation, and the complex differentiation environment created within the embryo, has precluded identification of definitive ectoderm in mammals. Fate-mapping studies of the embryo, however, have suggested the presence of a bipotent ectoderm progenitor during gastrulation (Lawson and Pedersen, 1992; Quinlan et al., 1995). The ability to form, recognize and manipulate the differentiation of definitive ectoderm is crucial to understanding the determination of cell fate in the ectoderm. The ability to progress via a definitive ectoderm intermediate will underpin the development of rational and efficient methodologies for the derivation of specific ectodermal cell populations from pluripotent cells in culture for use as experimental models or to produce cells with therapeutic applications.

The establishment of ectoderm can be modelled in vitro by directed differentiation of pluripotent embryonic stem (ES) cells in response to signals within HepG2 cell-conditioned medium (MEDII) (Rathjen et al., 2002; Rathjen et al., 1999). Aggregation and differentiation of ES cells in MEDII as embryoid bodies in MEDII (EBMs), results in the initial formation of a second pluripotent cell population, early primitive ectoderm-like (EPL) cells, followed by the emergence of a homogeneous population of neurectoderm (Rathjen et al., 2002). Analysis of differentiation potential and gene expression as differentiation proceeds in EBMs suggests that establishment of neurectoderm recapitulates embryonic development (Rathjen et al., 2002) and predicts formation of a cell population equivalent to the definitive ectoderm.

Studies in lower vertebrates have determined that the secreted molecule BMP4, a member of the bone morphogenic protein family, is an important factor in the patterning of definitive ectoderm and induces differentiation towards surface ectoderm (Hemmati-Brivanlou and Melton, 1997; Wilson and Hemmati-Brivanlou, 1995). In *Xenopus laevis*, BMP4 has been shown to suppress neural tissue and induce surface ectoderm from the definitive ectoderm (Wilson and Hemmati-Brivanlou, 1995). Later studies demonstrated that the molecules chordin and noggin, which are expressed in the dorsal blastopore lip and can induce neural tissue from animal pole explants, bound to and inhibited BMP4 (Piccolo et al., 1996; Zimmerman et al., 1996). Thus, in *X. laevis*

Journal of Cell Science

embryos, the ectoderm is patterned by the secretion of BMP antagonists from the dorsal blastopore lip, or Spemann's organizer: BMP signalling is active ventrally, and induces the formation of surface ectoderm, whereas dorsal BMP signalling is inhibited, and the cells adopt a neural fate (Hemmati-Brivanlou and Melton, 1997).

Gene-expression and gene-knockout studies have demonstrated that BMP4 is required for normal early development and has a multitude of functions (Hogan, 1996a; Hogan, 1996b; Zhao, 2003), including an involvement in the establishment of mesoderm at gastrulation (Winnier et al., 1995). More informatively, tissue-specific knockout of the BMP4 receptor *Bmpr1a* from the epiblast results in a reduction, but not complete loss, of surface ectoderm formation, suggesting that BMP signalling is involved in the patterning of this lineage during gastrulation (Davis et al., 2004).

Several approaches have been reported for the direction of ES cells to cells of the neural ectoderm or surface ectoderm lineages (Li et al., 1998; Rathjen et al., 2002; Rathjen and Rathjen, 2003; Troy and Turksen, 2005), but scant attention has been paid to the formation and characterization of the early progenitors of the ectoderm lineage within these protocols. Here, differentiation of ES cells to ectoderm in response to MEDII was used to identify and characterize transient cell populations that arise during establishment of the lineage. Three temporally distinct cell populations, defined by gene expression, differentiation potential and response to BMP4, were identified during ES cell differentiation in EBMs; the primitive ectoderm, definitive ectoderm and neurectoderm. The existence of a definitive ectoderm progenitor in mammals has not been demonstrated in vivo or during differentiation of pluripotent cells in vitro, although the existence of this intermediate has been previously implied (Rathjen et al., 2002). Here, the definitive ectoderm has been identified in EBMs as a population of cells that is present between the loss of pluripotence and upregulation of the neural markers; they comprise a transient population with the unique differentiation potential to form surface and neural ectoderm, but not mesoderm and endoderm.

Results

Gene expression demarcates four temporally distinct cell populations formed during differentiation of EBM

Differentiation of ES cells as aggregates in the presence of MEDII (embryoid bodies in MEDII; EBMs) results in directed differentiation to neurectoderm (Rathjen et al., 2002). Differentiation can be followed morphologically, with the initial formation of smooth and amorphous aggregates that develop a central cavity approximately 4 days after aggregation. This is followed by the elaboration of epithelial layers, which become progressively more stratified with time. Previous analysis has shown that expression of the primitive ectoderm marker *Fgf5* (Haub and Goldfarb, 1991; Pelton et al., 2002; Rathjen et al., 1999) increases between day 2 and day 5. Levels of the pluripotent marker *Oct4* drop between day 4 and day 6, and a near-homogenous population of neural progenitors form by day 9 (Rathjen et al., 2002). Northern blot and RT-PCR analysis of a number of genetic markers was used to map more precisely the temporal limits of the pluripotent primitive ectoderm-like and the neurectoderm populations and to reveal any additional intermediate populations that arise as differentiation proceeds in EBMs.

Sox2, which is a marker of the pluripotent lineage (Wood and Episkopou, 1999), was expressed in ES cells, then was reduced by about 15% in EBMs at day 1 (EBM1) and maintained at approximately 50% ES cell expression level until day 9 (EBM9) (Fig. 1A). *Oct4* expression closely followed the pattern of *Sox2* to EBM5, after which expression steadily decreased (Fig. 1A). *Rex1*, *Spp1* and *Nanog*, which are all markers of the inner cell mass

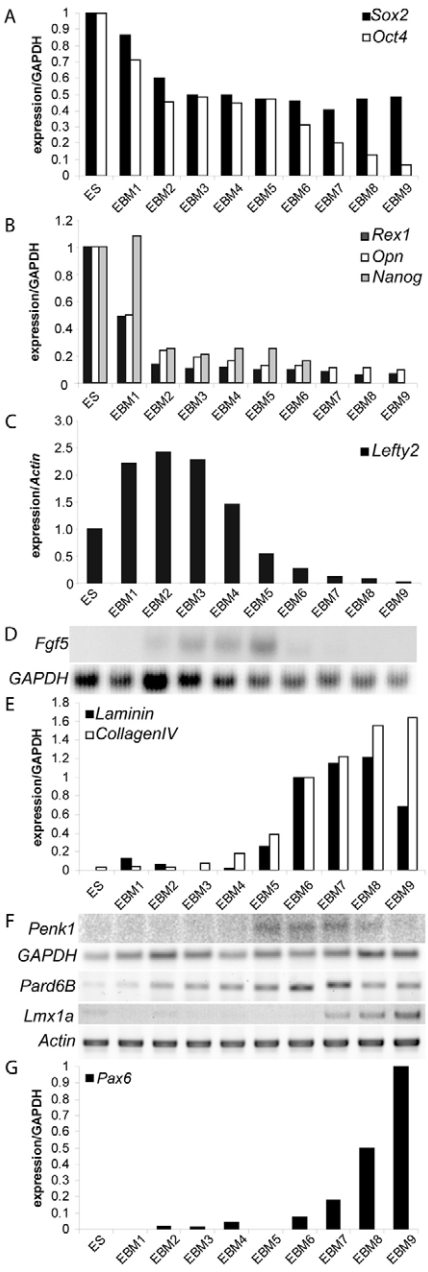


Fig. 1. Analysis of differentially expressed genes during differentiation within EBMs. RNA was isolated from ES cells and EBM1-EBM9 and analysed for the expression of the pluripotent cell marker *Sox2* and *Oct4* (**A**), the ICM markers *Opn*, *Nanog* and *Rex1* (**B**), the primitive ectoderm markers *Lefty2* (**C**) and *Fgf5* (**D**), the extracellular matrix proteins *laminin* and *Col4a4* (collagen IV) (**E**), prospective definitive ectoderm markers *Penk1* and *Pard6b*, the neural marker *Lmx1a* (**F**) and the neural marker *Pax6* (**G**). Expression levels were determined by either northern blot analysis (*Col4a4*, *Fgf5*, *laminin*, *Nanog*, *Opn*, *Pax6*, *Penk1*, *Rex1*, *Sox2*), RT-PCR (*Lmx1a*, *Pard6b*) or real-time PCR (*Lefty2*). The expression of *GAPDH* or *actin* was used as a loading control. Gene expression has been determined on at least two independent series and the expression shown is representative.

Journal of Cell Science

(ICM) (Botquin et al., 1998; Chambers et al., 2003; Mitsui et al., 2003; Rogers et al., 1991), were expressed in ES cells but downregulated by EBM2 (Fig. 1B). *Nanog* is re-expressed in the posterior primitive ectoderm during embryogenesis (Hart et al., 2004) but no re-expression of *Nanog* was seen in EBMs. As expected, expression of *Fgf5* was detected between EBM2 and EBM5 (Fig. 1D); this population was also marked by increased expression of *Lefty2* between day 1 and day 4 (Fig. 1C).

Expression of the extracellular matrix proteins laminin and collagen IV was upregulated in EBM6 and later (Fig. 1E). The upregulation of these markers is coincident with the downregulation of *Fgf5* and *Lefty2* and loss of primitive ectoderm-like cells. Our previous reports (Rathjen et al., 2002) and the expression of neural markers *Pax6* and *Lmx1a* (Chizhikov and Millen, 2004) here (Fig. 1F,G), suggest that neurectoderm formation initiates between EBM7 and EBM8. This gene-expression profile implies that a transient intermediate population is present after the loss of EPL cells, between EBM5 and EBM6, and before the upregulation of neurectoderm-specific markers in EBM7-EBM8. Microarray comparison of ES cells, EBM3, EBM6 and EBM9, identified a number of genes that were upregulated on EBM6 relative to both EBM3 and EBM9, and thus are potential markers of this population. Preproenkephalin1 (Penk1), a neuropeptide transmitter (Konig et al., 1996), and Pard6b, a protein involved in establishing cell polarity (Lin et al., 2000; Suzuki and Ohno, 2006), showed elevated expression in EBM5-EBM7, and EBM6-EBM7 populations, respectively (Fig. 1F). This is consistent with the presence of a temporally discrete population.

Identification of a transient cell population consistent with definitive ectoderm: formation of early epidermal progenitors from EBM6 in response to BMP4

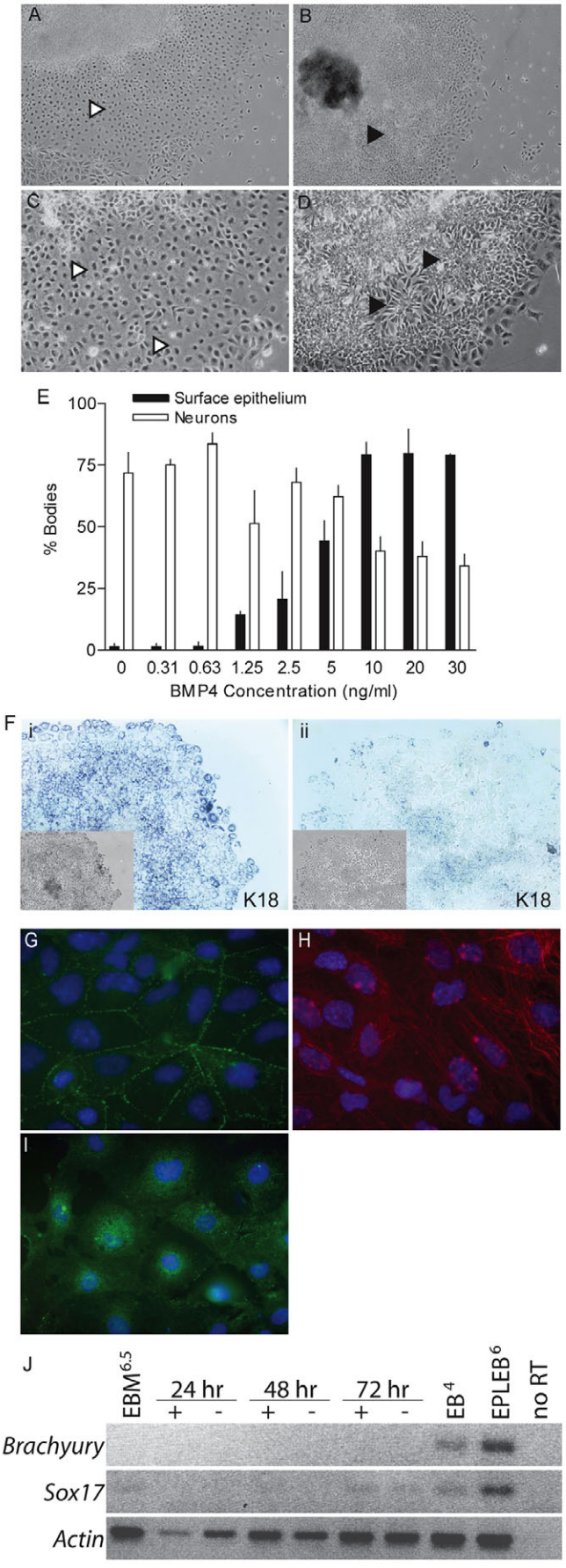
In *X. laevis*, neurectoderm and surface ectoderm are established from a common bipotent progenitor, the definitive ectoderm, with formation of surface ectoderm occurring in response to signalling by BMP4 (Wilson and Hemmati-Brivanlou, 1995). Similarly, human ES cells, which form primitive streak-like intermediates and mesoderm in response to BMP4, form surface ectoderm in response to BMP4 when the ability to differentiate to mesoderm is blocked by addition of DAPT, an antagonist of γ-secretase (Hughes et al., 2009). To identify a population capable of forming surface ectoderm, and consistent with definitive ectoderm, during EBM development we used an assay based on the differentiation response of cells to exogenously added BMP4. EBM5.5 were seeded into individual 2 ml wells and cultured in 50% MEDII for 12 hours to an EBM6 equivalent. At this point, the medium was changed to chemically defined medium supplemented with varying concentrations of BMP4, between 0 and 30 ng/ml. After 2 days, a distinct morphological difference was observed between EBMs cultured in 10 ng/ml BMP4 compared with untreated EBMs (Fig. 2A-D). Outgrowths derived from untreated EBMs comprised a sheet of tightly packed cells with a high nuclear-to-cytoplasmic ratio (Fig. 2B,D). Focal rosette formation could be identified, in keeping with early neural differentiation (Fig. 2B,D, closed arrow). With continued culture, neuron extensions were observed; these have previously been shown to express NeuN and tubulin βII (Rathjen et al., 2002). By contrast, BMP4-treated EBMs were surrounded by well-differentiated epithelial cells that were similar in appearance to early epidermal progenitor cells (Troy and Turksen, 2005). These cells were characterized by a low nuclear-to-cytoplasmic ratio and well-defined intercellular junctions (Fig. 2A,C, open arrows).

Individual EBMs were scored for the presence of the large, flat cell type and neural extensions 2 days and 6 days after the addition of BMP4, respectively (Fig. 2E). The morphologically distinct, large, flat outgrowths were barely detected in untreated EBMs, but were seen in 14% of EBMs cultured in 1.25 ng/ml BMP4, and up to 80% of EBMs cultured in 10-30 ng/ml BMP4. The increase in the formation of this cell type was accompanied by a decrease in the presence of neural extensions, from 75% of EBMs cultured in <1.25 ng/ml BMP4 to approximately 38% in EBMs cultured in 10-30 ng/ml BMP4.

Early in embryogenesis, around 8 d.p.c., the putative epidermis comprises a single layer of proliferating cells, the stratum germinatum, which is overlain by the periderm (Turksen and Troy, 1998). Both cell populations express keratin8 and keratin18, markers of simple epithelial cells (Turksen and Troy, 1998). In situ hybridization was used to detect the expression of *Krt18* in outgrowths from BMP4-treated and untreated EBM. As expected, *Krt18* expression was detected strongly in the outgrowths from BMP4-treated EBMs (Fig. 2Fi), but only poorly in the outgrowths from untreated aggregates (Fig. 2Fii), which is consistent with the formation of an early epidermal progenitor. As would be expected, fewer cells were positive for *Sox1* expression in outgrowths from BMP4-treated aggregates when compared with untreated controls (data not shown). Outgrowths from BMP4-treated aggregates were analyzed for the expression of additional early epidermal markers desmoplakin, E-cadherin and cytokeratin 8 (Troy and Turksen, 2005) by immunofluorescence. Cells within the large, flat outgrowths were positive for these markers, strongly suggesting the formation of an epidermal progenitor from EBM6 in response to BMP4 (Fig. 2G-J). Consistent with this conclusion, addition of BMP4 to EBM6.5 did not result in the upregulation of the primitive streak marker *brachyury* (*T*) or the endoderm marker *Sox17* (Fig. 2K).

Differential response to BMP4 defines four populations of cells during EBM development

The time that definitive ectoderm was present within EBM was defined by analysing the response of cells to BMP4 throughout EBM development. EBMs between day 4 and day 5.5 cultured with BMP4 differentiated to a population of cells that were morphologically distinct from surface epithelium, and contained areas of beating cardiomyocytes (Fig. 3Ai). The formation of beating cardiomyocytes was not seen from EBM4 cultured without BMP4 (Fig. 3Aii,iii). The ability of cells to give rise to mesoderm in response to BMP4 was lost abruptly between EBM5.5 and EBM6 (Fig. 3Ai), corresponding to the downregulation of *Fgf5*. This suggested that mesoderm formation in response to BMP4 was a characteristic of pluripotent EPL cells. This response also distinguishes these cells from ES cells that are maintained in the presence of BMP4 signalling (Ying et al., 2003). Outgrowths from EBM4 cultured in 10 ng/ml BMP4 were analysed by in situ hybridization for *brachyury* and compared with untreated EBM4. Consistent with the formation of terminally differentiated mesodermal derivatives late in differentiation in aggregates treated with BMP4, *brachyury*-expressing cells were detected in these bodies 2 days after BMP4 addition (data not shown). To test whether the formation of mesoderm in response to BMP4 is a general characteristic of EPL cells, EBM2-EBM4 were formed from a cell line in which *GFP* had been knocked-in to one allele of the *brachyury* locus (Fehling et al., 2003). These were analyzed after treatment with BMP4 for the upregulation of GFP expression



and compared with untreated controls. GFP upregulation was detected from BMP4-treated EBM2-EBM4, but not in the untreated controls (Fig. 3B), correlating EPL cell gene expression and BMP4 induction of mesoderm.

The loss of EPL cells between days 5 and 6 correlated with the ability of cells to differentiate to neural cells, and surface ectoderm in the presence of BMP4 (Fig. 3Ci,ii), suggesting that before this point, BMP4 effectively suppressed the formation of the ectodermal lineages (Fig. 3Aiii). The presence of neural extensions in BMP4-treated EBM5-EBM7 was reduced in comparison to EBM8 and EBM9, potentially reflecting the ability of these cells to form surface epithelium in response to BMP4. Surface epithelium formation was low, but not absent, in EBM4.5 and EBM5, and increased between EBM5 and EBM5.5. Surface epithelium formation peaked from EBM5.5 to EBM6.5 and was then gradually lost. Together, these results suggest that definitive ectoderm: (1) was present at a low level from day 5, coincident with late EPL cells; (2) comprised the major population after the loss of pluripotence; and (3) was gradually more committed to the neural lineage after day 7. The differential response to BMP4 suggests that EPL cells persist as the majority population up to day 5 and are lost by day 6.

BMP4 induces Smad phosphorylation during in vitro ectoderm differentiation

BMP4 signals are most commonly transduced by a type 1 receptor, primarily ALK3 or ALK6, in a complex with BMPRII and result in the phosphorylation of Smad1, Smad5 or Smad8 (Shi and Massague, 2003). To confirm that this pathway was active in our system, EBM4 and EBM6 were exposed to BMP4 and assayed after 15 and 30 minutes, for Smad protein phosphorylation, by western blot using an antibody that specifically recognizes only phosphorylated forms of Smad1, Smad5 or Smad8 (Fig. 4A). Without addition of exogenous BMP4, the level of phosphorylated Smad proteins was very low (EBM4), or not detected (EBM6).

Fig. 2. Addition of BMP4 to EBM6 results in the formation of surface ectoderm. (**A-D**) EBM5.5 were seeded for 12 hours before changing to chemically defined medium with (A,C) or without (B,D) 10 ng/ml BMP4. Bodies were cultured for a further 2 days. Open arrows indicate the large, flat cells that surround BMP4-treated EBMs. Closed arrows indicate the stellate-like cell structures reminiscent of neural progenitors that form from untreated aggregates. Photomicrographs were taken under phase contrast on day 2 at 4× (A,B) and 20× magnification (C,D). (**E**) EBM5.5 were seeded for 12 hours before changing to chemically defined medium supplemented with 0, 0.31, 0.63, 1.25, 2.5, 5, 10, 20 and 30 ng/ml BMP4. Bodies were cultured for a further 6 days and scored for the presence of surface epithelial outgrowths (on day 2) or neuronal extensions (on day 6) by morphology. n=3, approximately 24 bodies per treatment, error bars denote s.e. (**F**) EBM5.5 were seeded for 12 hours before changing to chemically defined medium with (i) or without (ii) 10 ng/ml BMP4. After culture for a further 2 days, the bodies were fixed in 4% PFA and analyzed for the expression of the surface ectoderm marker *Krt18* by whole-mount in situ hybridization. Photomicrographs were taken under bright-field (main pictures) or phase-contrast (insets) illumination. (**G-J**) EBM6 were seeded onto gelatinized glass coverslips in chemically defined medium with 10 ng/ml BMP4. Cells were cultured for 2 days and analyzed for the expression of desmoplakin (G; green), E-cadherin (H; red) and cytokeratin 8 (I; green) by immunofluorescence. Cell nuclei stained with DAPI. (J) EBM6.5 and EBM6.5 that had been cultured in chemically defined medium with (+) or without (–) BMP4 for 24, 48 and 72 hours were analyzed for the expression of the primitive streak marker *brachyury* and the definitive endoderm marker *Sox17* by RT-PCR. RNA isolated from embryoid bodies on day 4 and EPLEBs (Lake et al., 2000) on day 6 was used as positive controls.

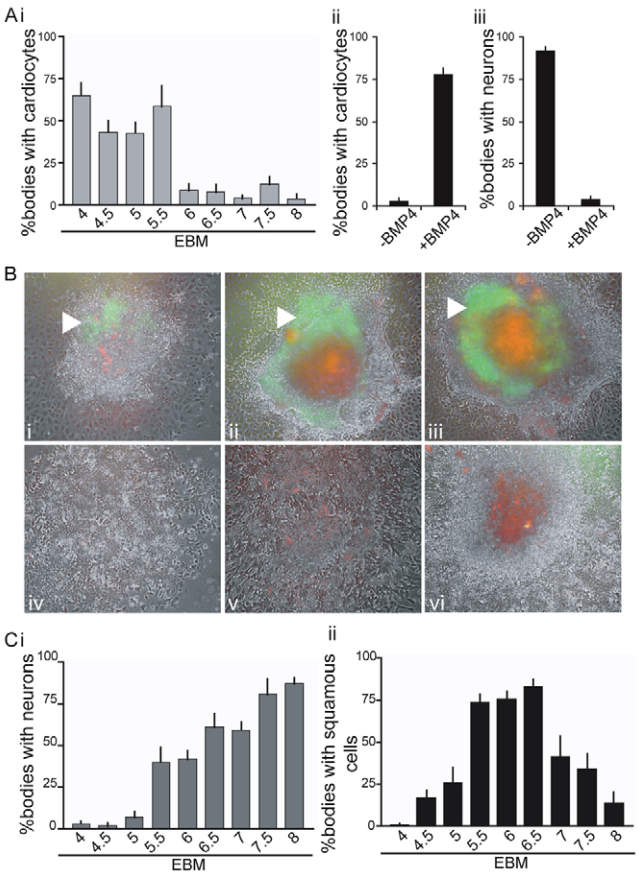


Fig. 3. Addition of BMP4 to differentiating EBMs distinguishes the intermediate cell populations. (**A**) EBM3.5-EBM7.5 were seeded at 12 hourly intervals (i), or EBM3.5 were seeded (ii,iii) and cultured for 12 hours before changing to chemically defined medium with or without 10 ng/ml BMP4 (approximately 24 bodies per treatment). Bodies were cultured for 6 days and scored for the presence of beating cardiomyocytes (i, ii) and neural extensions (iii). *n*=3, error bars denote s.e. (**B**) EBMs formed from GFP-Bry ES cells were seeded on day 1.5 (i, iv), day 2.5 (ii, v) and day 3.5 (iii, vi) of differentiation and cultured for 12 hours before changing to chemically defined medium with (i, ii, iii) or without (iv, v, vi) 10 ng/ml BMP4. Bodies were cultured for a further 3 days before being analyzed for the expression of GFP by fluorescent microscopy (green, indicated by arrowheads) or autofluorescence (red). Fluorescence are overlaid onto photomicrographs taken using phase-contrast illumination. (**C**) Approximately 24 EBM3.5-EBM7.5 were seeded at 12 hourly intervals and cultured for 12 hours before changing to chemically defined medium containing 10 ng/ml BMP4. Bodies were cultured for 6 days and scored for the presence of surface epithelial outgrowths (on day 2) and neural extensions (on day 6). *n*=3, error bars denote s.e.

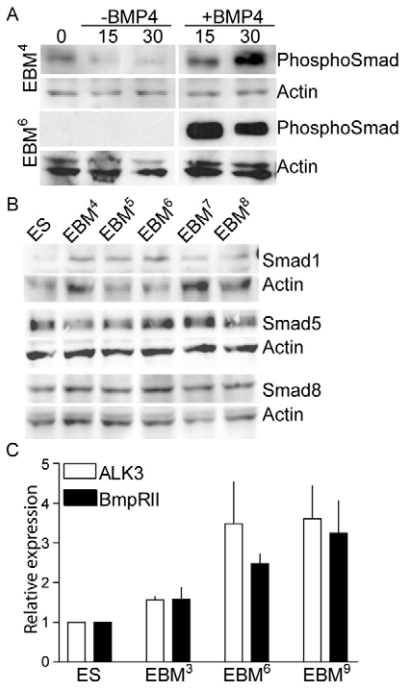


Fig. 4. EBMs are capable of responding to BMP4 signalling. (**A**) EBM4 and EBM6 were placed into chemically defined medium with or without BMP4 for 15 and 30 minutes. Protein extracts were analyzed by western blot for the presence of phosphorylated Smad proteins. Actin was used to normalize for protein loading. (**B**) Western blot analysis of protein isolated from ES cells and EBM4-EBM8 for the presence of Smad1, Smad5 and Smad8. Actin was used to normalize for protein loading. (**C**) Real-time RT-PCR analysis of RNA isolated from ES cells, EBM4, EBM6 and EBM9 for the expression of the BMP receptors ALK3 and BmpRII.

The low level of phosphorylated Smad proteins detected in EBM4 in serum (Fig. 4A, time 0) might reflect the response of these cells to the presence of a BMP within the serum. Smad protein phosphorylation was readily detectable in both populations after exposure to BMP4, confirming that BMP4 is signalling via Smad protein phosphorylation.

Components of this signalling pathway were analyzed in ES cells and EBM4-EBM8 to determine whether variation in expression levels or protein usage occurred during differentiation. Protein extracts from ES cells and EBM4-EBM8 were analyzed by western blot for the presence of total Smad1, Smad5 and Smad8 (Fig. 4B). The levels of Smad5 and Smad8 proteins were unchanged in all the cell samples tested. Smad1 was expressed at lower levels in ES cells compared with other cell populations tested.

Expression of the receptors was analyzed by qRT-PCR in ES cells, EBM4, EBM6 and EBM9. Transcripts encoding ALK3 and BMPRII (Fig. 4C), but not ALK6 (data not shown), were detected, suggesting that an ALK3-BMPRII receptor complex was present in all populations. Although the mRNA levels of ALK3 and BMPRII increased with differentiation, such that EBM9 expressed approximately threefold higher levels than ES cells, the ratio of ALK3 to BMPRII mRNA in each cell population remained approximately equivalent.

ES and EPL cells can be distinguished molecularly by their response to BMP4

We compared the levels of phosphorylated Smad proteins after BMP4 stimulation between ES cells and EPL cells. Without BMP4 addition, very little phosphorylated Smad protein was detected in ES cells or EBM on the day of analysis (Fig. 5; data not shown). In comparison, the addition of BMP4 resulted in an upregulation of phosphorylated Smad levels in all samples. A comparison of BMP4-treated ES cells with EBM2, EBM3 and EBM4, showed that levels of phosphorylated SMAD were higher in the EBMs (Fig. 5A,Bi); quantification of EBM4 and ES cell levels showed that this difference was significant (Fig. 5Bii). Smad protein phosphorylation was not consistently elevated in BMP4-treated

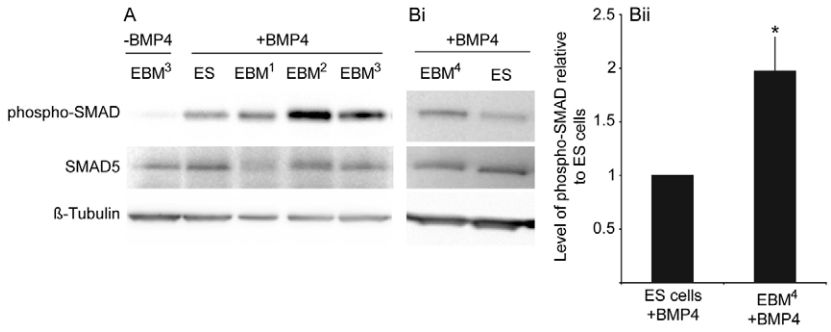


Fig. 5. ES and EPL cells respond differently to BMP4. (**A**) Western blot analysis of phosphorylated Smad proteins in protein extracted from ES cells and EBM1-EBM3 (i), and ES cells and EBM4 (ii) that had been exposed to 10 ng/ml BMP4 for 30 minutes, as denoted. SMAD5 and β-tubulin were used to normalize for protein loading. Western blot was probed with Smad1, but protein levels were below detection (data not shown). (**B**) The levels of phosphorylated Smad proteins were determined and expressed relative to β-tubulin for ES cells and EBM4 cells after exposure to 10 ng/ml BMP4 for 30 minutes. n=3, error bars denote s.e.; *P<0.05, Student's paired t-test.

EBM1; interestingly the level of Smad5 protein appeared diminished on this day compared with ES cells and EBM2. The ability to respond to BMP4 with Smad phosphorylation was acquired between 24 and 48 hours after exposure of ES cells to MEDII (Fig. 6Aii), and correlates with the loss of ICM markers. This response provides an early functional readout of EPL cell formation.

Discussion

During development, the presence and potential of the differentiation intermediates that occur in the ontogeny of many differentiated cell populations is poorly understood. The differentiation of ES cells in culture to populations representative of embryonic lineages has been postulated to recapitulate early embryonic development, providing paradigms for the identification and characterization of cell populations that arise transiently during cell type formation (Choi et al., 1998; Kennedy et al., 1997; Rathjen et al., 2002; Yamashita et al., 2000). Differentiation of ES cells within EBMs appears to recapitulate the embryonic processes that lead to the establishment of the ectodermal lineages on the anterior side of the gastrulating embryo (Rathjen et al., 2002), providing a model for the identification and characterization of ectoderm intermediates. In previous work, we hypothesized that formation of neurectoderm within EBMs proceeded via the establishment of transient differentiation intermediates that are equivalent to intermediate populations postulated to arise within the embryo (Rathjen et al., 2002). In this report, we provide genetic and functional evidence for the existence of these populations, and demonstrate that one of these has properties that are consistent with definitive ectoderm, a population of cells in mammals that has proven refractory to characterization, both in vivo and in vitro.

Gene expression demarcates three temporally distinct cell populations that arise during neurectoderm establishment

We have refined and extended our earlier description (Rathjen et al., 2002) of pluripotent cell populations that precede establishment of neurectoderm gene expression in the EBM system. The starting population of ES cells was shown to be equivalent to the pluripotent cells of the inner cell mass, expressing the pluripotent markers *Oct4* and *Sox2*, and the ICM markers *Rex1*, *Opn* and *Nanog*. Differentiation of this population occurred within 24 hours of aggregation and exposure to MEDII, as demonstrated by reduced transcript levels of *Rex1*, *Lefty1* and *Opn*. However, 48 hours was required for the complete downregulation of ICM-specific genes, acquisition of the differential response to BMP4, and transition to a population of EPL cells expressing *Fgf5*, *Oct4* and *Sox2*. Elevated expression of *Lefty2* was also correlated with the presence of EPL cells within EBM.

Loss of pluripotence, as defined by an alteration in differentiation outcome between EBM5 and EBM6, with EBM5.5, but not EBM6, capable of forming beating cardiomyocytes after BMP4 treatment, coincided with downregulation of *Fgf5* and upregulation of genes encoding laminin and collagen IV. The EBM system therefore comprises a population of EPL or primitive ectoderm cells between days 2 and 5.5. Loss of pluripotence appeared to be sharp and rapid, with alterations occurring to completion within 24 hours or 1-2 cell cycles. This suggests a high degree of developmental synchrony within the population. The trigger for the loss of pluripotence during EBM development is not known, but is likely to be cell autonomous, because no alterations to the culture conditions occur at this time. A cell-autonomous trigger for primitive ectoderm differentiation during gastrulation would enforce loss of pluripotence and prevent inappropriate maintenance of potentially teratogenic cells within the developing embryo. Within the embryo, active signalling that arises from adjacent populations and signalling centres, such as the extraembryonic endoderm and node, is likely to modulate the differentiation outcome. Interestingly, loss of pluripotence was not reflected in a rapid loss of *Oct4* transcripts, suggesting that *Oct4* is not precisely correlated with pluripotence during gastrulation (Mossman et al., 2005; Rathjen et al., 2002).

The expression of neurectoderm markers such as *Pax6* and *Lmx1a* did not immediately follow loss of pluripotence, but was delayed for 1-2 days. EBMs at this stage expressed low levels of markers that characterize pluripotent (*Rex1*, *Opn*, *Nanog* and *Fgf5*) and neural (*Pax6*, *Lmx1a* and *Sox1*) (Rathjen et al., 2002) lineages, and acquired the expression of novel genetic profile that included *Penk1* and *Pard6b*. A population of cells with this genetic signature has not been previously identified during pluripotent cell differentiation. This work defines a third intermediate cell population that is formed temporally between the EPL or primitive ectoderm cell and the establishment of neurogenesis, between day 5.5 and day 7 of the EBM system.

The definition of clear discrete intermediary populations during the differentiation of ES cells within EBMs is described here using gene expression and functional markers. A recent examination of this differentiation system using changes in replication timing and chromatin conformation also resulted in the definition of distinct cell populations occurring during differentiation (Hiratani et al., 2009). Moreover, both techniques – cell biology used by us, and epigenetics used by Hiratani and colleagues – defined the same number of distinct cell populations, and both outcomes aligned well.

Responsiveness to BMP4 provides evidence for functional differences between differentiation intermediates

Changes in gene expression provide a temporal description of successive progenitor populations in the EBM system, which can

Journal of Cell Science

Journal of Cell Science

in some cases be correlated with cell types formed during embryonic development. The function of progenitor populations can be assessed by evaluation of their potency and the repertoire of cell lineages that can be formed during differentiation. Evidence for functional relevance of the changes in gene expression seen here was established by evaluating the variation in differentiation outcome from these progenitor populations following exposure of cells to BMP4.

Consistent with the observations of others (Winnier et al., 1995; Ying et al., 2003), the ES and EPL cell states, at day 0 and day 2-5.5 of differentiation, respectively, were distinguished by induction of mesoderm in response to BMP4 in the latter, but not the former. Formation of mesoderm was manifest in the upregulation of *brachyury* expression and in further differentiation to cell types of representative of mesoderm. Neural cell fates were almost completely suppressed from late EPL cells (EBM4-EBM5.5) cultured in the presence of BMP4.

Treatment of the differentiation intermediate present on day 6 with BMP4 induced a different response. Mesoderm was not induced, the formation of neural lineages was suppressed, and a novel cell population, which is not normally observed at significant levels in either the EBM or embryoid body system, was induced. Morphology and gene expression within this population were indicative of the formation of surface ectoderm. This work is consistent with similar observations in human ES cells. Human ES cells are induced to form primitive streak-like intermediates and mesoderm in response to BMP4. When the ability to form mesoderm is blocked by addition of an agonist of γ-secretase, the presence of BMP4 induces the formation of surface ectoderm (Hughes et al., 2009).

Genetic signatures of differentiation intermediates in the EBM system therefore reflect the formation of progenitor populations with alternative functional properties. BMP4 induced non-neural cell fates from pluripotent and committed populations with alternative developmental choices.

Identification of a transient cell population with properties consistent with the formation of definitive ectoderm

Mammalian definitive ectoderm has been proposed to exist by analogy with *X. laevis* (Wilson and Hemmati-Brivanlou, 1995); however, the potential transient and dynamic nature of an equivalent population in the mammalian embryo and in EBs, coupled with a lack of markers, has precluded identification and formal characterization of this cell. Fate-mapping studies of the embryo have suggested that surface and neural ectoderm arise from adjacent zones of the anterior primitive ectoderm (Lawson and Pedersen, 1992), and marking of single or small groups of cells has demonstrated the presence of cells within the proximal anterior of the early streak embryo with the potential to contribute to both the surface and neural ectoderm lineages, consistent with the presence of a bipotent progenitor (Lawson and Pedersen, 1992; Quinlan et al., 1995).

A differentiation intermediate present in EBM6 is temporally consistent with definitive ectoderm, arising between primitive ectoderm expressing *Oct4* and *Fgf5* and neural progenitors expressing *Pax6*, *Lmx1a* and *Sox1*. This cell population expresses a unique set of markers that is not associated with pluripotence or neuroectoderm, and has a differentiation potential that includes both neurectoderm and surface ectoderm. The cells within this population do not form significant levels of mesoderm, endoderm or extraembryonic lineage under any of the conditions that we have assessed. Downregulation of neural differentiation and upregulation of a surface ectoderm equivalent in response to BMP4 is a differentiation profile shared by definitive ectoderm in vertebrate embryos. BMP suppression of neural differentiation in vivo (Wilson and Hemmati-Brivanlou, 1995; Wilson and Edlund, 2001) and during ES cell differentiation (Tropepe et al., 2001; Ying et al., 2003) has been well documented, whereas definitive ectoderm in *X. laevis* has been shown by genetic approaches to form surface ectoderm in response to BMP4 (Wilson and Hemmati-Brivanlou, 1995). Although EBM6 cells align with definitive ectoderm in terms of ontogeny, genetic signature and differentiation potential, the persistence of cells capable of forming neural projections in BMP4-treated aggregates beyond day 5 does not allow us to preclude the formation of a second, dedicated neural progenitor during differentiation within EBMs.

BMP4 signalling activates Smad phosphorylation in differentiation intermediates

Differences in the response of distinct, closely related differentiation intermediates to BMP4 prompted an investigation of the molecular basis for this pleiotropy. BMP receptors and Smad proteins were present at all stages of differentiation, with no obvious differences in levels that could explain diverse responses to the ligand. Treatment with BMP4 was shown to result in phosphorylation of Smad proteins in each of the differentiation intermediates tested. The most striking difference between the populations in terms of the molecular response to BMP4 was in the levels of phosphorylated Smad proteins, which were significantly higher in BMP-treated EPL cells than in BMP-treated ES cells, providing a molecular marker for EPL cells in culture.

Although these data suggest that BMP4 signalling in differentiation intermediates of the ectoderm includes Smad phosphorylation, the differential response of alternative progenitor populations points to a greater complexity. This might be manifest in differential activation of alternative signalling pathways that are characteristic of each cell type in response to BMP4 or other molecules within the differentiation milieu, or the induction of distinct subsets of genes in response to a common Smad-based pathway.

Conclusions

The ability to control differentiation in culture will rely on the ability to form and precisely differentiate intermediate progenitor states. Characterization of the differentiation intermediates that occur as ES cells differentiate to the ectoderm lineage provides a description of the presence and persistence of the differentiation intermediates. This includes the formation and subsequent differentiation of a population consistent with an immediate post-pluripotent definitive ectoderm progenitor, which arises as pluripotent cells differentiate to an early neural progenitor. We hypothesize that these intermediates will underpin the development of rational differentiation protocols and supply material for the analysis of the differentiation machinery, providing a system for the analysis of the genetic and epigenetic control of lineage establishment from pluripotent cells.

Materials and Methods

Cell culture

The D3 ES cell line (Doetschman et al., 1985) and GFP-Bry ES cells (Fehling et al., 2003) were used. The routine culture of ES and EPL cells, formation of cellular aggregates (EBMs) and the production of MEDII were as described previously (Rathjen et al., 2002; Rathjen and Rathjen, 2003). The differentiation potential of

Journal of Cell Science

cells within EBMs was analysed by seeding aggregates onto gelatin-treated tissue culture grade plasticware (Falcon) for approximately 12 hours in 50% MEDII (Rathjen and Rathjen, 2003) before the medium was replaced with chemically defined medium (Rathjen and Rathjen, 2003) with or without BMP4, as noted in the text. Outgrowths were examined microscopically 2, 4 and 6 days after seeding and scored for the presence of neural projections, flattened epithelial sheets and beating cardiomyocytes. Cells with neural projections have been shown previously to express tubulin-βIII and NeuN (Rathjen et al., 2002). Generally, for each experimental condition, 24 individual wells were seeded with randomly selected cellular aggregates and experiments were repeated three times. At all differentiation points tested, the morphology of the cellular aggregates was highly consistent and in accordance with that described previously (Rathjen et al., 2002). Alkaline phosphatase activity was detected using the alkaline phosphatase activity detection kit (Sigma) modified as described (Rathjen et al., 1999).

Northern blot analysis

Total cytoplasmic RNA was isolated using RNAzol™ (TelTest). Northern blot analysis was performed essentially as described in Breeden Lab Methods (http://www.fhcrc.org/labs/breeden/Methods/Northern_blot). Filters were rinsed in 2×SSC, crosslinked with 120 mJ at 254 nm in a UV Stratalinker 1800 (Stratagene), washed in 1×SSC, 0.1% SDS at 65°C for 30 minutes before prehybridization and hybridization in Church hybridization buffer (Church and Gilbert, 1984). Filters were washed for 2×20 minutes in 1×SSC, 0.1% SDS, exposed to an imaging plate (Fujifilm) and signal detected on a Bio-Rad FX imager (Bio-Rad). Band quantification was performed using Bio-Rad Quantity One software. Radioactive DNA probes were synthesized using the DECAprime™ II Randomly Primed DNA Labeling Kit (Ambion). DNA fragments used for analysis of *Fgf5*, *Rex1* and *GAPDH* were as described previously (Lake et al., 2000; Rathjen et al., 1999). cDNA fragments to be used as probes for mRNA encoding of Opn, laminin, collagen IV, Sox2 and Penk1 were cloned into pGEM®-T Easy (Promega) and released by digestion with *Eco*RI. Bands were detected with a Fuji Imaging Plate, which was scanned with a Molecular Imager FX™ (Bio-Rad) and analyzed with Quantity One™ software (Bio-Rad).

Semi Quantitative RT-PCR

cDNA was synthesized from total RNA with the Omniscript® RT kit (Qiagen). PCR reactions contained 10 μl Platinum® PCR Supermix (Invitrogen), 200 nM of each primer and 50-100 ng of cDNA. Products were separated on a 2% (w/v) agarose gel, and stained with SYBR® Gold (Invitrogen). Product bands were scanned with a Molecular Imager FX (Bio-Rad) or captured on a BioDoc-It Imaging system. The sequences, length of amplified products, annealing temperatures and cycle numbers were: *Pard6b* (447 bp, 60°C, 24 cycles): 5′-ACTACCACAAGGCGGTTTCC-3′ and 5′-TCCCGGACACTTCTATACCG-3′; *Actin* (501 bp, 60°C, 26 cycles): 5′-ATGGATGACGATATCGCTG-3′ and 5′-ATGAGGTAGTCTGTCAGGT-3′; *Lmx1a* (500 bp, 60°C, 28 cycles): 5′-ACTCAGGCAAAAGCGATGAT-3′ and 5′-ACCATAGGGGTGCATGTGAT-3′; *Brachyury* (143 bp, 60°C, 28 cycles): 5′-TGCTGCCTGTGAGTCATAAC-3′ and 5′-GCCTCGAAAGAACTGAGCTC-3′; *Sox17* (211 bp, 60°C, 30 cycles): 5′-GCCAAAGACGAACGCAAGCGGT-3′ and 5′-TCATGCGCTTCACCTGCTTG-3′.

Real-Time PCR (qRT-PCR)

RNA and cDNA was prepared as previously described. The PCR reaction mix comprised 12 μl SYBR® Green PCR reaction mix (Invitrogen), 1 μl ROX reference mix (Invitrogen), 50 ng cDNA and 200 nM of each primer made up to a total volume of 25 μl with water. Samples were heated to 50°C for 2 minutes, then 95°C for 10 minutes, before being cycled 40 times through the following sequence on a 9600 thermocycler (ABI Prism): 95°C for 15 seconds, 56°C for 1 minute. The raw data was analyzed using the Q-Gene software package (Muller et al., 2002; Simon, 2003). Primers were designed using Primer Express® software (Applied Biosystems). The sequences and length of amplified products were: *ALK3* (120 bp): 5′-TTTCAATGCAAGGATTCACCG-3′ and 5′-AAAGAACGGACCTATAACAACAGGG-3′; *ALK6* (79 bp): 5′-GGCGGGTTAACTTCGGC-3′ and 5′-TTGCTCCTTTAACTTTTGTTTGC-3′; *BmpRII* (96 bp): 5′-AGAATGTTGACAGGAGACCGG-3′ and 5′-CTTCAGGTTATCCAGGTCAAGGG-3′; *Lefty2* (360 bp): 5′-TGTATTCTCAGTGAGCTT-3′ and 5′-GCAGTCCCTGACATGGTA-3′; *Actin* (89 bp): 5′-CTGCCTGACGGCCAGG-3′ and 5′-GATTCCATACCCAAGAAGGAAGG-3′.

In situ hybridization analysis

Whole-mount in situ hybridization analysis of cellular aggregates was performed using the method of Rosen and Beddington (Rosen and Beddington, 1993) with modifications (Lake et al., 2000; Rathjen et al., 1999). Antisense and sense probes were synthesized using a DIG RNA Labeling Kit (Roche). Plasmid details for *Sox1* and *brachyury* were as described previously (Lake et al., 2000; Rathjen et al., 2002). An 1165 bp *Hin*dIII fragment of *Krt18* cDNA was a kind gift from Robert Oshima (Singer et al., 1986). This fragment was cloned into the *Hin*dIII site of pBluescript® KS. Antisense probes were generated by restriction with *Bam*HI and transcription with T3 RNA polymerase, sense probes were generated by restriction with *Xho*I and transcription with T7 RNA polymerase.

Cellular protein extraction

Adherent cells were collected by incubating with 0.5-1.0 ml TEN buffer (40 mM Tris-HCl, pH 7.4, 10 mM EDTA, 150 mM NaCl) for 5-10 minutes at 4°C. Cells in suspension culture were simply collected by centrifugation and washed with PBS. A volume of ice-cold lysis buffer [50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% (v/v) NP-40, 1 mM EDTA, 1 mM $NaVO_4$, 50 mM NaF, 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche) per 10 ml buffer, added immediately before use] approximately equal to the volume of the cell pellet was added, and incubated for 30-60 minutes at 4°C on a rotating platform. Cellular debris was removed by centrifugation for 10 minutes, also at 4°C. Total protein concentration was determined using the Bio-Rad protein assay procedure

SDS-PAGE and western blot analysis

SDS-PAGE and western blotting were carried out as previously described (Kavanagh et al., 2005), with the following modifications. When AP-conjugated secondary antibodies were being used Tris-buffered saline with 0.1% (v/v) Tween-20 (TBST) was used in the place of phosphate-buffered saline. When the anti-phosphorylated Smad antibody was being used as the primary antibody, blocking was performed with 5% (w/v) bovine serum albumin (BSA, Sigma) in TBST as per the manufacturer's instructions. When AP-conjugated secondary antibodies were used, membranes were developed by incubation with ECF™ substrate (Amersham Pharmacia Biotech) reagents in the dark for 5 minutes, before being scanned with a Molecular Imager FX™ (Bio-Rad) and analyzed with Quantity One™ software (Bio-Rad). Statistical analysis was performed using Microsoft Excel software.

Immunofluorescence

EBMs were grown in suspension culture for 6 days before being seeded onto gelatin-treated glass coverslips in chemically defined medium with or without 10 ng/ml BMP4. Medium was refreshed daily. After 48 hours, coverslips were fixed in ice cold acetone for 5 minutes and stored in 1× PBS at 4°C. Fixed cells were blocked with 1% BSA. Primary antibodies were sourced from AbCam and applied at 1:200.

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