Int. J. Dev. Biol. 42: 917-925 (1998)

Using EC and ES cell culture to study early development: recent observations on *Indian hedgehog* and *Bmps*

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ABSTRACT Despite great technological advances in the study of mammalian development in the past two decades, certain problems in early development, such as how the extraembryonic lineages are established, have remained intractable. We suggest that teratocarcinoma (EC) and embryonic stem cells (ES) remain useful *in vitro* tools for studying some of these problems. We present a continuation of our studies on the role of IHH-based signaling in early development and demonstrate that the IHH N-peptide is expressed in the outer visceral endoderm cells of both the EC and ES-derived embryoid body. We also show that *Bmp2* is upregulated and *Bmp4* downregulated during the differentiation of F9 EC cells into embryoid bodies, whereas both genes are upregulated when J7 ES cells differentiate into embryoid bodies. We also examine the spatial localization of *Ihh, Bmp2,* and *Bmp4* in day 6.5-7.0 and 7.5-8.0 embryos by *in situ* hybridization analysis. These data support the EC temporal expression data in that all 3 genes are expressed in visceral endoderm. *Bmp4* expression appears to be limited to extraembryonic regions, where mesoderm as well as visceral endoderm are stained. *Ihh* and *Bmp2* are expressed in extraembryonic tissues and the embryo proper. Functional roles for the observed expression patterns are discussed.

KEY WORDS: *ES cells, EC cells, Indian hedgehog, Bmps, extraembryonic endoderm*

Introduction

In the 1970s, a student of developmental biology, experienced with a simple non-vertebrate or vertebrate model system, was easily daunted by the prospect of studying comparable phenomena in the mammalian embryo. Without the current array of technologies, including recombinant DNA technology and transgenic approaches, studying mammalian development beyond a descriptive effort was difficult. Despite the challenges, elegant chimera-based studies by a variety of researchers succeeded in establishing lineages for the embryonic and extraembryonic cell types in the mouse (Rossant and Papaioannou, 1977; Gardner, 1983). The introduction of mouse teratocarcinoma or embryonal carcinoma stem cells (EC) (see Graham, 1977 for review) and later embryonic stem cells (ES) (see Robertson and Bradley, 1986 for review) provided invaluable experimental material for studying certain events of early mammalian development *in vitro*. Although derived from quite distinct cell populations, a pluripotential teratocarcinoma in the case of EC cells and inner cell mass cells in the case of ES cells, both types of cell lines show comparable patterns of differentiation *in vitro*, generating a variety of lineages found in the early mouse embryo. There are many advantages to working with an *in vitro* system. Large amounts of experimental material representing specific tissues at specific stages of development can be isolated with relative ease. Conditions can be manipulated to test the role of proposed critical molecules and culture environments in specific morphogenic or differentiation events. Genetic approaches can also be taken to generate cell lines either deficient for specific gene products or overproducing specific gene products. Limitations to working *in vitro* include the need to restrict investigations to the time frame and cell types reflected in the model system and the obligation to return to the embryo, whenever possible, to verify and extend your observations.

A variety of EC stem cell lines with distinct *in vitro* differentiation properties have been characterized. Much of our work has focused on the F9 cell line (Hogan *et al.*, 1983; Grabel, 1992). As diagrammed in Figure 1, these cells grow as homogeneous undifferentiated stem cells, roughly equivalent to the cells of the inner cell

Abbreviations used in this paper: ES cells, embryonic stem cells; EC cells, embryonic carcinoma cells.

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0214-6282/98/$10.00
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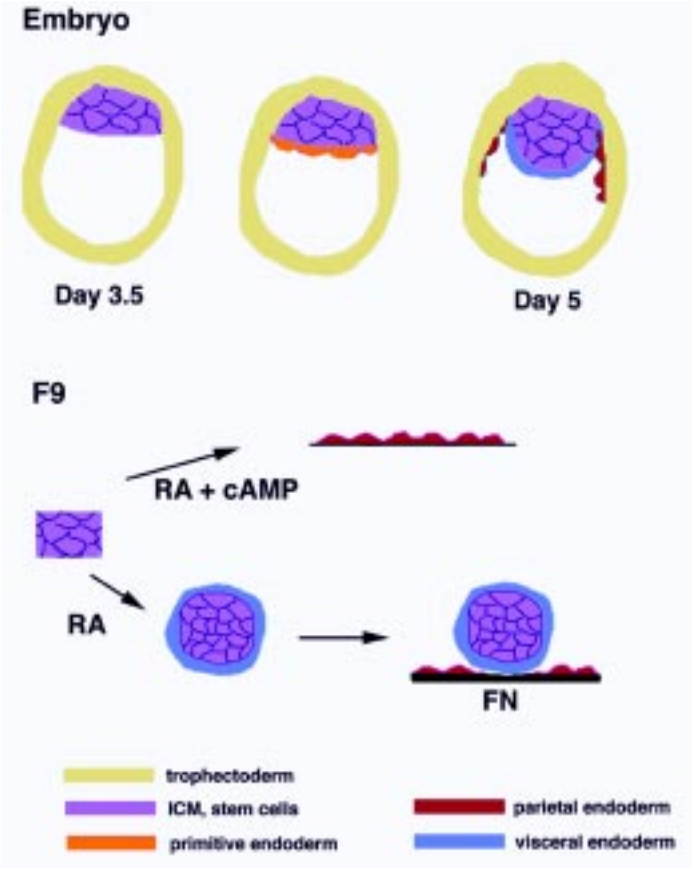

Fig. 1. Comparison of early mouse embryogenesis and F9 differentiation.

mass. If treated in monolayer culture with retinoic acid plus cyclic AMP, they differentiate into parietal endoderm, the extraembryonic endoderm cells that migrate along the inner trophectoderm surface and contribute to the parietal yolk sac of the embryo (Strickland *et al.,* 1980). In contrast, if these cells are treated in suspension culture with retinoic acid alone, they differentiate into embryoid bodies, consisting of an outer layer of visceral endoderm, the extraembryonic endoderm cells that surround the epiblast prior to primitive streak formation, and contribute to the formation of the visceral yolk sac (Hogan *et al.,* 1981). When visceral endoderm-surrounded embryoid bodies are plated on extracellular matrix-coated substrates, such as fibronectin, parietal endoderm cells migrate onto the dish and away from the embryoid body (Grabel and Watts, 1987). Other teratocarcinoma cell lines such as PSA1 (Martin *et al.,* 1977), as well as ES cell lines (Doetschman *et al.,* 1985), are capable of undergoing more extensive differentiation *in vitro* following embryoid body formation. In both cases, a mesoderm layer initially appears between the endoderm and ectoderm cell layers, analogous to primitive streak formation, and subsequently generates a variety of mesoderm-derived cell types (Fig. 2). ES cell lines in particular have therefore been used extensively to study hematopoiesis and vasculogenesis (Doetschman *et al.,* 1993; Vittet *et al.,* 1996; Weiss and Orkin, 1996).

These simple *in vitro* model systems can be used to examine a number of events during early embryogenesis. We have used the F9 system to study how extraembryonic lineages are established in a position-dependent manner in the early embryo and how extracellular matrix receptors may control early morphogenesis, particularly parietal endoderm migration. Our work on the extracellular matrix receptors has determined that integrin receptor function is modulated in a variety of ways, including alternative mRNA processing (Jiang and Grabel, 1995) and cytoplasmic domain phosphorylation (Dahl and Grabel, 1989; Barreuther and Grabel, 1996) during early development. Our recent work on identifying specific genes and signal transduction pathways involved in establishing a functional visceral endoderm layer is the subject of this review.

What is known about how the extraembryonic endoderm lineage, parietal endoderm, visceral endoderm, and their common precursor primitive endoderm, is established in the early embryo? A long standing hypothesis supported by *in vitro* embryo recombination experiments, suggests that the establishment of this lineage, like the establishment of the trophoblast lineage, is position-specific (Hillman *et al.,* 1972). The cells on the outside of the inner cell mass, facing the blastocoel cavity, are destined to become primitive endoderm, the precursors for parietal and visceral endoderm, whereas the interior cells will become embryonic ectoderm or epiblast (Handyside, 1978). This specification may be the result of signaling between cells, but none of the increasingly well described signal transduction pathways used for such decision making has been implicated. We do know a number of genes whose expression and function appears to be critical for the formation of functional extraembryonic endoderm cells. A potential role for FGF4 has been suggested by some *in vitro* studies looking at differentiation of parietal endoderm in F9 teratocarcinoma cells and preimplantation embryos (Rappolee *et al.,* 1994). This role is substantiated by analysis of the phenotype of *fgf4* deficient mice generated by gene targeting (Feldman *et al.,* 1995). The embryos homozygous for the targeted mutation die around implantation, and blastocyst outgrowth cultures demonstrate reduced levels of parietal endoderm cells, a condition which can be reversed by the addition of recombinant FGF4. Mice deficient for the homeobox-containing transcription factor *evx1* are peri-implantation lethals, consistent with a role for this gene product in establishing extraembryonic lineages (Spyropoulos and Capecchi, 1994). In addition, targeted disruption of the gene for GATA-4 in ES cells inhibits the differentiation of visceral endoderm, suggesting a role for this transcription factor in differentiation of this extraembryonic endoderm lineage (Soudais, *et al*., 1995).

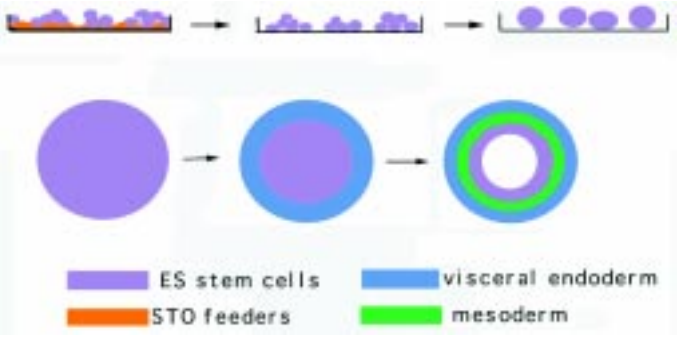

Fig. 2. ES cell embryoid body formation.

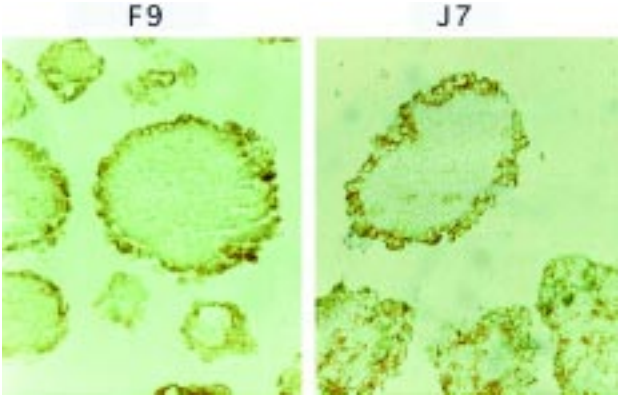

Fig. 3. Accumulation of *Bmp2* mRNA during differentiation of F9 embryoid bodies. *F9 cells were cultured in suspension in the absence (U) or presence of retinoic acid (RA) and the levels of mRNA for Bmp2, Ihh, AFP and actin were determined by Northern blot hybridization as previously described. Note increase in Bmp2 message concomitant with the increase in Ihh.*

The expression of additional genes, while not required to establish the primitive endoderm lineage, may be critical to generating a fully functional visceral endoderm. For example, the transcription factor HNF-4, which is expressed in visceral endoderm, appears to be required for normal gastrulation and subsequent expression of late mesoderm markers, but not for visceral endoderm differentiation, based upon gene targeting studies (Chen *et al.,* 1994). Using chimeric embryos consisting of *Hnf-4*$^{-/-}$ ES cells combined with tetraploid morulae that contribute exclusively to extraembryonic endoderm derivatives, it has been established that expression of HNF-4 in visceral endoderm alone is sufficient to overcome the early developmental arrest observed in *Hnf-4*-deficient embryos (Duncan *et al.,* 1997). In addition, the TGFβ family member *nodal* appears to be transiently expressed in visceral endoderm cells, and expression in this lineage appears to be essential for subsequent A-P patterning (Varlet *et al.,* 1997). Thus, in addition to generating components of the parietal and visceral yolk sacs, the primitive endoderm derivatives apparently play key roles in inducing the proper differentiation of epiblast-derived tissue as well. The potential inducing capability of the visceral endoderm layer is not surprising given its proximity to the epiblast and the role of the hypoblast in the chick embryo in influencing the primitive streak (Azar and Eyal-Giladi, 1981).

Given the proposed importance of the extraembryonic endoderm in influencing development of the embryo proper as well as generating the yolk sacs, there is surprisingly little information on how this lineage is established. Since these events take place just subsequent to implantation, they are quite difficult to study *in vivo*. Thus the *in vitro* EC and ES systems can be exploited to provide relevant information.

We have recently established that a member of the *hedgehog (hh)*gene family, *Indian hedgehog (Ihh)* is expressed by extraembryonic endoderm cells (Becker *et al.,* 1997). The *Drosophila hh* gene plays a role in pattern formation during embryogenesis in both segment and imaginal disc morphogenesis (Mohler, 1988; Lee *et al.,* 1992; Mohler and Vani, 1992; Tabata *et al.,* 1992). Three homologs have been identified in the mouse, *Ihh, Sonic hedgehog (Shh),* and *Desert hedgehog (Dhh)* (Echelard *et al.,* 1993), and additional related genes have been identified in other vertebrates (Krauss, *et al.,* 1993; Riddle *et al.,* 1993; Chang *et al.,* 1994; Roelink *et al.,* 1994; Ekker *et al.,* 1995; Lai *et al.,* 1995). *Shh*, the best studied vertebrate gene, plays a role in anterior/posterior pattern formation in the limb and dorsal/ventral pattern formation in the neural tube and somites and influences cell proliferation and differentiation at numerous other sites during embryogenesis (see Hammerschmidt *et al.* (1997) and Tabin and McMahon (1997) for recent HH reviews. *Dhh* is made in the Sertoli cells and appears to be essential for spermatogenesis (Bitgood *et al.,* 1996). *Ihh* is involved in cartilage formation and gut morphogenesis (Echelard *et al.,* 1993; Bitgood and McMahon, 1995; Vortkamp *et al.,* 1996; Lanske *et al.,* 1996).

The protein products of these *hh* genes undergo a unique series of modifications to form the active, secreted peptide. Following cleavage of the signal sequence, a domain within the carboxy-terminus of the protein with homology to serine proteases promotes the autocatalysis of the protein into amino (N) and carboxy-peptides (Lee *et al.,* 1994; Bumcrot *et al.,* 1995; Porter *et al.,* 1995). During the proteolytic cleavage event, the N-peptide is covalently modified by the addition of cholesterol, which then keeps the N-peptide tethered to the membrane, facilitating the build up of high local concentrations of HH adjacent to its site of synthesis (Porter *et al.,* 1996a,b). The N-peptide appears to be responsible for the signaling activity and can act in a concentration-dependent manner to induce the differentiation of distinct cell types (Marti *et al.,* 1995a,b; Roelink *et al.,* 1995). HH peptides play a role in long range as well as short range signaling (Johnson and Tabin, 1995), and evidence exists to support both a direct model, in which cells respond at some distance to decreasing concentrations of hh, and an indirect model, in which other signaling molecules are responsible for the long range effects (Yang *et al.,* 1997).

The downstream events initiated in response to HH appear to be well conserved between flies and vertebrates. Recent analysis has revealed that HH binds to PTC, a 12-transmembrane protein with homology to transporters (Chen and Struhl, 1996; Marigo *et al.,* 1996; Stone *et al.,* 1996). The PTC protein is associated in the

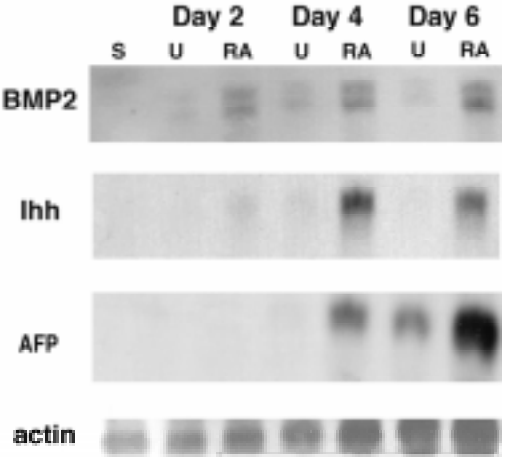

Fig. 4. Downregulation of *Bmp4* mRNA during differentiation of F9 embryoid bodies. *Northern hybridization was done as described for Figure 3, except ethidium bromide staining was used for a loading control and only retinoic acid treated samples are shown. Note, levels of Bmp4 mRNA decrease substantially at day 3, when Bmp2 and Ihh mRNA levels begin to increase.*

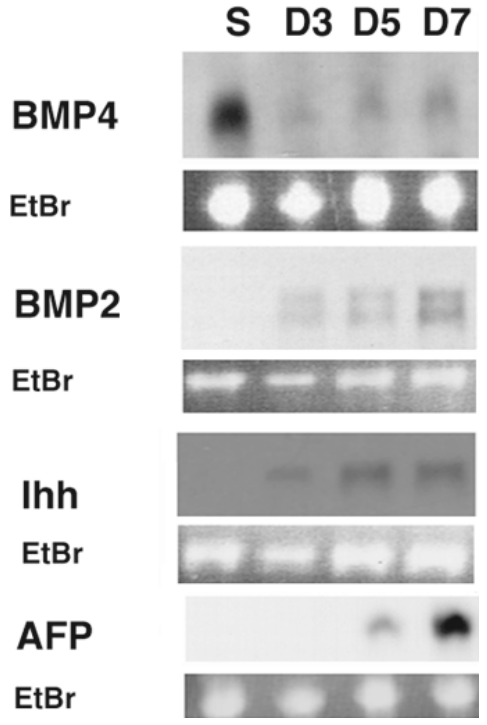

Fig. 5. Accumulation of *Bmp2* and *4* during differentiation of J7 ES embryoid bodies. *ES cell culture and Northern hybridization were performed as previously described. Note the increase in all 3 transcripts at day 7.*

plasma membrane with SMO, a G-protein-linked serpentine receptor (Alcedo *et al.,* 1996; van den Heuvel and Ingham, 1996). HH binding relieves PTC-mediated repression of downstream events, presumably by allowing SMO-mediated signaling. Activation of downstream HH targets is mediated, at least in part, via the action of the cubitus interruptis (Ci) transcription factor in *Drosophila* and its vertebrate relatives the Gli genes (Alexandre *et al.,* 1996; Dominguez *et al.,* 1996). Actual downstream targets include members of the TGFβ family; DPP in *Drosophila* (Basler and Struhl, 1994; Capdevila and Guerrero, 1994) and BMP2 and BMP4 (Laufer *et al.,* 1994; Roberts *et al.,* 1995) in vertebrate systems and PTC.

The observation that retinoic acid induces the differentiation of F9 stem cells into extraembryonic endodermal derivatives and can mimic the same patterning events signaled by HH action in some vertebrate tissues, suggested that it acts in the F9 system by inducing expression of a *hh* gene. We have determined that expression of *Ihh* is upregulated upon retinoic acid-induced differentiation of F9 cells (Becker *et al.,* 1997). Differentiating ES cells also upregulate *Ihh* expression as they form embryoid bodies, which they do in the absence of retinoic acid, suggesting that *Ihh* expression is linked to extraembryonic endoderm differentiation and not to retinoic acid treatment. In blastocyst outgrowth cultures, levels of *Ihh* mRNA increase dramatically as parietal endoderm appears, suggesting that the gene is upregulated during embryogenesis. *In situ* hybridization analysis indicates that *Ihh* mRNA localizes to the outer visceral endoderm layer in F9 embryoid bodies and to the outer visceral endoderm layer of the visceral yolk sac. In our initial report we demonstrated that the active N-peptide form of IHH appears in differentiating F9 cultures just subsequent to the appearance of the mRNA. We now show that this peptide also preferentially localizes to the outer visceral endoderm layer of F9 and ES embryoid bodies.

In addition, we investigate the temporal and spatial pattern of expression of *Bmp2* and *Bmp4* in EC (F9) and ES (J7) embryoid bodies and in early postimplantation embryos. We are particularly interested in how the expression patterns of *Bmp2* and *Bmp4* compare to expression patterns of *Ihh* and how these genes may be coordinately regulated during early embryogenesis. Previous studies have indicated that *Bmp2* is upregulated and *Bmp4* downregulated during the differentiation of F9 stem cells in monolayer culture into parietal endoderm (Rogers *et al.,* 1992). Previous *in situ* hybridization analysis on day 6.5-7.5 embryos (egg cylinder to primitive streak stage) indicates that *Bmp2* is expressed first in extraembryonic mesoderm associated with the proamniotic canal and later amnion and chorion, (Winnier *et al.,* 1995; Zhang and Bradley, 1996) while *Bmp4* is expressed initially in the posterior primitive streak and extraembryonic mesoderm as well (Winnier *et al.,* 1995). Both genes show subsequent expression in the neural folds and continued expression in mesoderm derivatives (Winnier *et al.,* 1995). These previous reports give no indication that either *Bmp* gene is expressed in extraembryonic endoderm, in contradiction to the F9 Northern hybridization data for *Bmp2,* which suggest upregulation as parietal and visceral endoderm differentiate. The phenotypes of *Bmp2* and *Bmp4*-deficient mice (Winnier *et al.,* 1995; Zhang and Bradley, 1996), as well as the phenotype of mice deficient for the BMP receptor gene, *Bmpr* (Mishina *et al.,* 1995) are consistent with the importance of extraembryonic mesoderm expression of these genes.

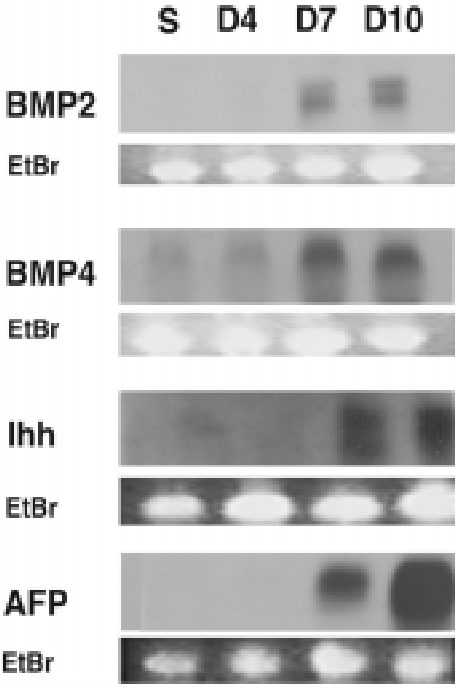

Fig. 6. Localization of IHH peptide in F9 and J7 embryoid bodies. *Immunocytochemistry was performed as previously described using the 5E1 monoclonal antibody and an HRP-linked secondary antibody. Note that the peptide is restricted to the outer visceral endoderm cell layer.*

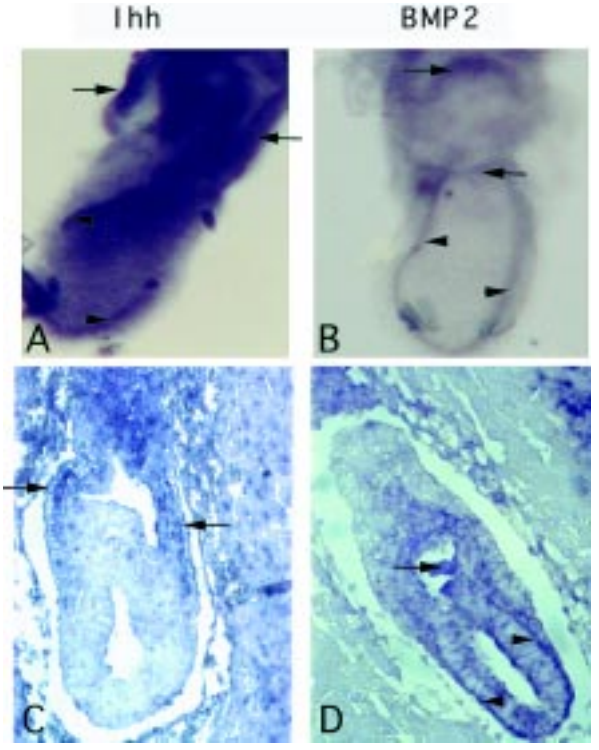

Fig. 7. Localization of Ihh and Bmp2 mRNA in day 6.5/7.0 embryos. *In situ hybridization was performed in whole-mount* **(A** *and* **B)** *or in section* **(C** *and* **D)** *as previously described. Arrows in A and C show visceral endoderm staining; arrowheads show staining between visceral endoderm and mesoderm layers in the distal portion of the embryo. Arrows in B and D show extraembryonic mesoderm staining (allantoic bud in D) and arrowheads show interface staining between visceral endoderm and mesoderm.*

We demonstrate here that, as described for other emerging tissues later in development, temporal patterns of expression of *Ihh* and *Bmp* are coincident and spatial domains of expression are adjacent and/or overlapping. We also show that *Ihh, Bmp2,* and *Bmp4* are all expressed in the visceral endoderm, as well as previously reported tissues.

Results

Localization of the Ihh N-peptide in embryoid bodies

Our previous *in situ* hybridization data suggest that *Ihh* mRNA accumulates in the differentiating visceral endoderm cells (Becker *et al.,* 1997). Using a monoclonal antibody 5E1(myeloma cells obtained from the Developmental Studies Hybridoma Bank, University of Iowa) directed against the N-peptide of Shh which cross-reacts with IHH (Ericson *et al.,* 1996), we now demonstrate that the peptide is also expressed in visceral endoderm cells. We previously demonstrated that *Ihh* is the only hedgehog gene expressed in F9 embryoid bodies, indicating that the cross-reacting antibody is recognizing only IHH and not DHH or SHH peptide in these cultures (Becker *et al.,* 1997). Figure 3 shows that the outer visceral endoderm layer of both F9 embryoid bodies and embryoid bodies derived from the J7 ES cell line express the IHH peptide. These data support the observation that secreted HH peptides accumulate close to their site of synthesis, perhaps due to the cholesterol modification that tethers the peptide to the plasma membrane. If the peptide is made and secreted by the differentiating visceral endoderm cells, responding cells may be the visceral endoderm cells themselves, and/or cell types that derive from the underlying embryonic ectoderm or epiblast. As a first step towards identifying the responding cell type, we have examined the temporal and spatial pattern of expression of *Bmp2* and *Bmp4*, prospective downstream genes in a HH-induced cascade, relative to *Ihh.*

Temporal expression of Bmp2 and Bmp4 in F9 and ES-derived embryoid bodies

Previously described Northern hybridization experiments have indicated that levels of *Bmp2* increase when F9 stem cells are treated in monolayer culture with retinoic acid plus cyclic AMP and differentiate into parietal endoderm (Rogers *et al.,* 1992). In contrast, levels of *Bmp4* message are quite high in F9 stem cells and decrease to low levels under these conditions (Rogers *et al.,* 1992). We examined the expression of these two genes when F9 and J7 ES cells are cultured in suspension to generate visceral endoderm-containing embryoid bodies. Figure 4 shows that *Bmp2* mRNA is detectable at 2 days in retinoic acid treated F9 cultures, at the same time *Ihh* message is observed. *Ihh* message levels continue to increase through day 6 in treated cultures, while *Bmp2* levels remain elevated (Fig. 4). mRNA for AFP, the visceral endoderm marker, is first observed at day 4. In contrast, Figure 5 shows that *Bmp4* message is abundant in untreated F9 stem cells, and levels

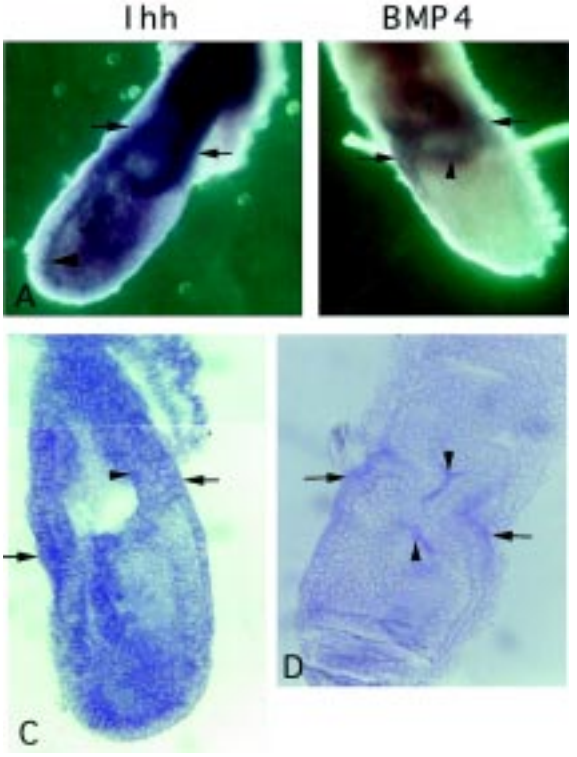

Fig. 8. Localization of *Ihh* and *Bmp4* mRNA in day 6.5/7.0 embryos. *In situ hybridization was performed in whole-mount* **(A** *and* **B)** *and these same embryos sectioned* **(C** *and* **D)**. *Arrows in A and C show visceral endoderm staining; large arrowhead, the staining between visceral endoderm and mesoderm layers in the distal portion of the embryo (A). Arrows in B and D show visceral endoderm staining at the embryonic/extraembryonic border, arrowheads show extraembryonic mesoderm staining.*

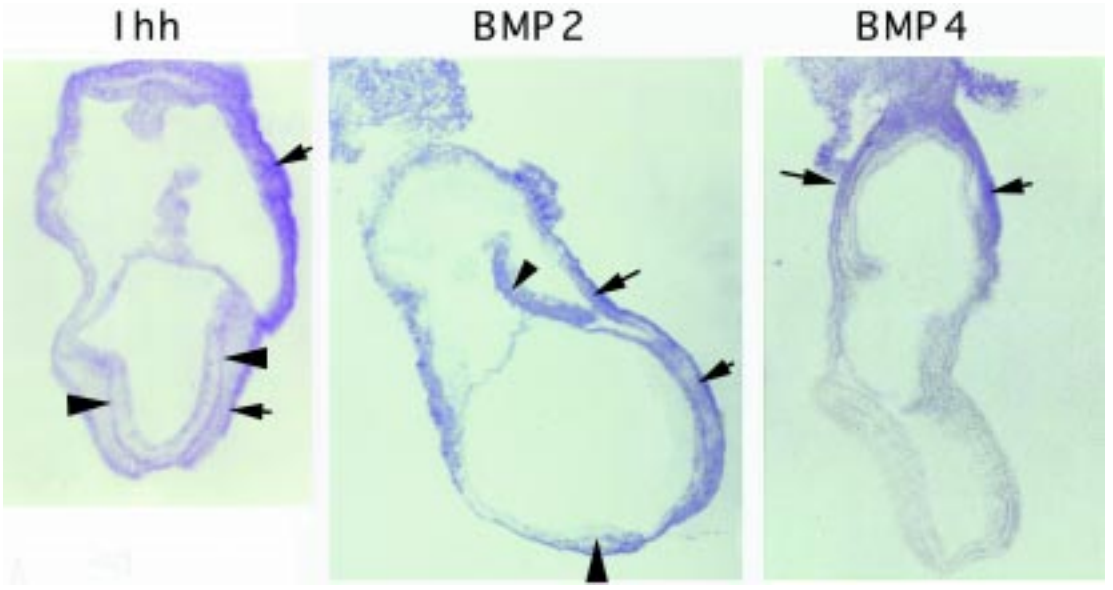

Fig. 9. Localization of *Ihh* and *Bmp2* and *Bmp4* mRNA in day 7.5/8.0 embryos. *In situ hybridization was performed in whole-mount and the embryos sectioned. Anterior is to the left and posterior to the right. Arrows highlight visceral endoderm staining, small arrowhead the prominent allantois staining with the Bmp2 probe, and the large arrowhead notes localization of Ihh and Bmp2 mRNA to the interface between embryonic ectoderm and mesoderm.*

decrease upon suspension culture in the presence of retinoic acid, just as levels of *Bmp2* and *Ihh* are increasing. Examining J7 embryoid body cultures for the expression of these genes indicates that *Bmp2* and *Ihh* are upregulated in suspension culture, reaching high levels by day 7 (Fig. 6). *Bmp4* message is present at modest levels in the untreated ES cells and is also upregulated by day 7. Thus, all 3 of these genes are upregulated coincidentally during ES culture, suggesting coordinate regulation.

Embryo in situ hybridization studies for Ihh and Bmp expression

To establish the relative localization of *Ihh*, *Bmp2*, and *Bmp4* mRNAs during early embryogenesis, *in situ* hybridization analysis was performed on day 6.5/7.0 embryos. Figure 7 shows embryos probed in whole mount (A and B) or as sections (C and D) for *Ihh* (A and C) or *Bmp2* mRNA (B and D). Arrows in A and C note the prominent signal for *Ihh* observed in the visceral endoderm. The section (C) shows that the ectoplacental cone and extraembryonic ectoderm cells are also prominently stained, as previously reported (Becker *et al.,* 1997). Additional staining is observed in the embryonic regions of the whole-mount embryo (arrow heads in A), particularly at what appears to be the interface between visceral endoderm and embryonic ectoderm in the distal region of the embryo. *Bmp2* expression is clearly seen in the chorion and amnion (arrows in B) as well as in the outer visceral endoderm and extraembryonic endoderm layers (see base of top arrow in B). This extraembryonic mesoderm staining can be seen in the section, which also shows expression in the allantoic bud (arrow in D), as previously described (Zhang *et al.,* 1997). Additional staining can be observed in whole-mount (B) and in the section (D) at what appears to be the interface between visceral endoderm and embryonic ectoderm in the distal portion of the embryo (arrow heads), as was observed for *Ihh* expression.

Figure 8 compares the localization of *Ihh* (A and C) and *Bmp4* (B and D) in whole-mount embryos (A and B) and sections derived from these identical embryos (C and D). *Ihh* is expressed in the pattern described above for Figure 7. *Bmp4* is expressed prominently at the junction between embryonic and extraembryonic regions of the embryo in the visceral endoderm layer adjacent to the amnion (arrows in B and D) and in amnion and chorion as well (arrowheads in B and D). No *Bmp4* message can be seen in the distal, embryonic portion of the embryo at this stage.

Figure 9 shows the localization of these 3 messages at the early head fold stage (day 7.5-8.0) in sections of embryos probed as whole-mounts. Arrows in each panel demonstrate the prominent visceral endoderm localization for each mRNA. Additional staining in the embryonic, distal portion of the embryo can once again be visualized for *Ihh* and *Bmp2,* but not for *Bmp4* at this stage (large arrowheads). Prominent allantois staining for *Bmp2* is also shown (small arrowhead). Note that the visceral endoderm in posterior side of the embryo, the site of primitive streak formation, appears to express significantly higher levels of both *Ihh* and *Bmp2* mRNA than the anterior side of the embryo.

Discussion

We describe here the use of EC and ES *in vitro* systems to study the molecular cues modulating differentiation of extraembryonic endoderm in the early mouse embryo. Our previous study implicated a role for *Ihh* in early development (Becker *et al.,* 1997). We demonstrated that *Ihh* mRNA is expressed as both F9 and ES cells (D3 cell line) differentiate *in vitro*. The message localizes preferentially to the outer visceral endoderm layer of these embryoid bodies. As a continuation of these studies, we now show that the IHH N-peptide is localized to the outer differentiating visceral endoderm cells of both F9 and ES embryoid bodies. At earlier days in culture, peptide is also localized to the outer cells of the differentiating embryoid body, although in a patchy, non-uniform pattern (data not shown). These results are consistent with previous reports documenting the localization of the secreted, processed HH peptide within and adjacent to the cells responsible for its synthesis (Marti *et al.,* 1995a). The restricted mobility of the peptide is supported by the recent reports documenting the covalent attachment of cholesterol to the peptide as a potential tethering

device, but inconsistent with the proposed role of HH peptides in direct long range signaling (Porter *et al.,* 1996). A recent report attempts to reconcile these observations in the limb bud by suggesting that long range signaling is mediated by intervening relay molecules, BMPs, and not directly by HH (Yang *et al.,* 1997).

Given the potential role of BMPs in HH mediated signaling events, we investigated the temporal pattern of expression of the *Drosophila Dpp* homologs *Bmp2* and *Bmp4,* during F9 and ES embryoid body differentiation. A previous study demonstrated that *Bmp2* mRNA is upregulated while *Bmp4* is downregulated when F9 stem cells differentiate into parietal endoderm in monolayer culture in response to retinoic acid plus cyclic AMP (Rogers *et al.,* 1992). We observe the same pattern of expression for these 2 genes when F9 cells differentiate in suspension culture to form embryoid bodies in response to retinoic acid alone. Thus, differentiation of either parietal or visceral endoderm is associated with increased levels of *Bmp2* message and decreased levels of *Bmp4* message. In contrast, both *Bmp2* and *Bmp4* mRNA are upregulated when ES cells differentiate spontaneously in suspension into embryoid bodies.

Our new *Bmp2 in situ* hybridization data, indicating expression in visceral endoderm, provide an explanation for the apparent discrepancy between the F9 Northern hybridization data and the previously reported localization studies (Winnier *et al.,* 1995; Zhang and Bradley, 1996). It remains unclear why *Bmp4* is expressed at high levels in F9 stem cells, and why its expression decreases as F9 cells differentiate. The upregulation of both *Bmp* genes in ES culture at a time when extraembryonic endoderm and mesoderm differentiate, appears to accurately reflect the expression of these genes in the embryo. This suggests that ES cultures may be appropriate models for examining the interaction of *Ihh* and *Bmp* genes during early development.

Our description of *Bmp2* and *Bmp4* expression in visceral endoderm contradicts previous reports in which *Bmp2* is first observed in extraembryonic mesoderm, and *Bmp4* in the primitive streak and extraembryonic endoderm (Winnier *et al.,* 1995; Zhang and Bradley, 1996). The absence of expression in the visceral endoderm at day 6.5-7.5 in the previous reports may be attributed to a lack of sensitivity in the *in situ* hybridization protocol. The prominent expression of both *Bmp2* and *Bmp4* in extraembryonic mesoderm cells is consistent with previously reported *in situ* hybridization data and with the phenotypes observed in the deficient mice (Winnier *et al.,* 1995; Zhang and Bradley, 1996). *Bmp2*-deficient embryos are unable to form amnion and chorion layers and the transient proamniotic canal persists. Although the phenotype of *Bmp4*-deficient embryos is more variable, they show a clear reduction in extraembryonic mesoderm and its derivatives, including blood islands. The expression of these genes in visceral endoderm as well supports the idea that this cell type may be key to proper induction of mesoderm and its derivatives during early post-implantation embryogenesis.

The region of the 7.5 day embryo surrounding the exocoelomic cavity, where *Ihh, Bmp2,* and *Bmp4* are expressed, is the initial site of vaculogenesis and hematopoiesis in the embryo resulting in blood island formation (Palis *et al.,* 1995). The differentiation of blood islands appears to involve the induction of mesoderm, which forms both endothelium and blood cells, by the adjacent visceral endoderm layer (Bielinska *et al.*, 1996). Support for a role for visceral endoderm comes from the observation that ES embryoid bodies formed from cells deficient for the transcription factor GATA4 do not make visceral endoderm and do not form blood islands (Soudais *et al.,* 1995). Data from a variety of sources, including this report, are consistent with a role for HH and BMPs in this key induction event (Johansson and Wiles, 1995; Farrington *et al.,* 1997). One report based on RT-PCR analysis of isolated tissues, suggests that in the day 10.5 yolk sac, *Ihh* expression is restricted to the visceral endoderm layer, while *Bmp2* and *Bmp4* are expressed in the mesoderm layer. Our *in situ* hybridization data reported here suggest that at earlier stages this restricted expression is not yet in place. As ES cells have been used to study both vasculogenesis and hematopoiesis, and appear to mimic the pattern of expression of *Ihh, Bmp2,* and *Bmp4* observed in the embryo, this system can be used to resolve the coordinate regulation of these genes and their role in yolk sac maturation.

We show that at 6-8 days, both *Bmp2* and *Ihh* are expressed in the distal region of the embryo, at the apparent interface between visceral endoderm and epiblast tissue. Previous reports localize *Bmp2* to the neural folds at the head fold stage (Winnier *et al.,* 1995) and our preliminary *in situ* analysis suggests this is the case for *Ihh* as well. The presence of *Ihh* in the embryo proper at these early stages suggests that *Ihh* as well as *Shh* may play a role in patterning the embryo proper at these early stages.

Our observation that *Ihh, Bmp2* and *Bmp4* are expressed in adjacent and overlapping cell types at this stage suggests that the establishment and differentiation of extraembryonic endoderm and mesoderm may be triggered by HH-based signaling. Our temporal studies examining the expression of *Ihh, Bmp2* and *Bmp4* in the EC and ES cell systems suggest, however, that their regulation is coincident and that *Ihh* may not initiate *Bmp* expression. Studies examining the effect of added exogenous IHH and BMP peptides on accumulation of these genes, as well as studies employing ES cell lines deficient for each of these genes will help to establish the regulatory cascade.

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