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 morphogenetic 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 mesenchymal 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.*
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 trophoderm 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 blastocoeal 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).
The expression of additional genes, while not required to establish the primitive endodermal 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 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 endodermal 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 serum 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 (Martí 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). 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 serum 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 (Martí 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
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 interruptus (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. 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.

**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.
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...
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 (arrowheads 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 (arrowheads), 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 in situ hybridization studies (Winnier et al., 1995; Zhang and Bradley, 1996). 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 expression in visceral endoderm conflicts 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 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 exocoelic cavity, where Ihh, Bmp2, and Bmp4 are expressed, is the initial site of vacuolization 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 (Bielsinska 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.

References


