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BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo

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SUMMARY

At E4.0 the inner cell mass of the mouse blastocyst consists of a core of embryonic ectoderm cells surrounded by an outer layer of primitive (extraembryonic) endoderm, which subsequently gives rise to both visceral endoderm and parietal endoderm. Shortly after blastocyst implantation, the solid mass of ectoderm cells is converted by a process known as cavitation into a pseudostratified columnar epithelium surrounding a central cavity. We have previously used two cell lines, which form embryoid bodies that do (PSA1) or do not (S2) cavitate, as an in vitro model system for studying the mechanism of cavitation in the early embryo. We provided evidence that cavitation is the result of both programmed cell death and selective cell survival, and that the process depends on signals from visceral endoderm (Coucouvanis, E. and Martin, G. R. (1995) *Cell* 83, 279-287). Here we show that *Bmp2* and *Bmp4* are expressed in PSA1 embryoid bodies and embryos at the stages when visceral endoderm differentiation and cavitation are occurring, and that blocking BMP signaling via expression of a transgene encoding a dominant negative mutant form of BMP receptor IB inhibits expression of the visceral endoderm marker, *Hnf4*, and prevents cavitation in PSA1 embryoid bodies. Furthermore, we show that addition of BMP protein to cultures of S2 embryoid bodies induces expression of *Hnf4* and other visceral endoderm markers and also cavitation. Taken together, these data indicate that BMP signaling is both capable of promoting, and required for differentiation of, visceral endoderm and cavitation of embryoid bodies. Based on these and other data, we propose a model for the role of BMP signaling during peri-implantation stages of mouse embryo development.

Key words: BMP2, BMP4, BMP receptor, Cavitation, Embryoid body, Programmed cell death, Visceral endoderm

INTRODUCTION

Morphogenesis of the peri-implantation mouse embryo involves the transformation of the solid inner cell mass (ICM) of the late blastocyst into a hollow structure known as the egg cylinder. At the time of implantation (~E4.5), the ICM consists of an inner core of embryonic ectodermal cells, which will give rise to all the cells of the embryo proper, surrounded by an outer layer of extraembryonic endoderm. Shortly after implantation (~E5.0), a cavity (the proamniotic cavity) begins to form in the core of the ICM and, by E6.0, the embryonic ectodermal cells that line the cavity have differentiated into a pseudostratified columnar epithelium. The trophectoderm-derived extraembryonic ectoderm undergoes a similar process of cavitation at a slightly later stage. The process by which these changes occur is known as cavitation, and this type of morphogenetic conversion also occurs during the formation of other hollow (tubular) structures that arise from solid primordia, such as the ducts of various exocrine glands. In early postimplantation mouse development, cavitation prepares the embryo for gastrulation, the process by which the three germ layers are formed.

Cavitation in the early embryo is difficult to study in vivo because of the small size and relative inaccessibility of the embryo at this stage of development. However, mouse embryonic stem (ES) or embryonal carcinoma (EC) cells that form cavitating embryoid bodies in vitro serve as a useful model system for investigating the mechanism of cavitation (Coucouvanis and Martin, 1995). Embryoid bodies are produced when appropriate ES or EC cells are grown as aggregates in suspension. These aggregates spontaneously differentiate in culture in a manner that mimics many key aspects of early mouse embryo development, including endoderm differentiation and cavitation (Martin et al., 1977; Coucouvanis and Martin, 1995). We have previously made use of the PSA1 EC cell line to investigate the mechanism of cavitation. Our results demonstrated that cavitation is initiated near the periphery of the embryoid body and proceeds inward. In addition, our data suggested that cavitation in embryoid bodies and embryos is the result of

the interaction of two signals, one that is produced by or depends on the presence of the outer endodermal layer and causes the death of inner ectodermal cells, and one that promotes survival of the single layer of columnar epithelial cells that lines the cavity. In the present study, we sought to identify the molecule(s) that serves as the death signal during cavitation by determining whether proteins that have been shown to cause cell death in other contexts are responsible for the cell death that is observed during cavitation of embryoid bodies and the pregastrulation mouse embryo.

Several members of the TGFβ superfamily of secreted signaling molecules have been implicated in the control of cell death. For example, TGFβ1 can cause apoptosis when added to cultures of primary rabbit epithelial cells (Rotello et al., 1991) and Mullerian Inhibiting Substance (MIS) is responsible for regression of the Mullerian duct in male embryos, a process that occurs by apoptosis (Price et al., 1977). Bone Morphogenic Protein 4 (BMP4), a member of the BMP subfamily of TGFβ-related proteins, has been shown to cause apoptosis in several developmental settings. For example, in the embryonic chick hindbrain, *Bmp4* is expressed in subpopulations of cells in rhombomeres 3 and 5 that undergo apoptosis as part of their normal developmental program. When these rhombomeres are explanted and cultured in vitro, *Bmp4* expression is downregulated and cell death does not occur. Addition of BMP4 protein to these explant cultures induces apoptotic cell death (Graham et al., 1994). Similarly, in the chick limb bud *Bmp4* and *Bmp2* are expressed specifically in the interdigital regions where PCD occurs (Lyons et al., 1990; Francis et al., 1994; Yokouchi et al., 1996), and application of beads containing BMP4 protein to regions in which death does not normally occur results in PCD (Ganan et al., 1996). Moreover, interference with BMP signal transduction has been shown to prevent normal interdigital PCD (Yokouchi et al., 1996; Zou and Niswander, 1996). These observations prompted us to explore the possibility that BMP4 and related proteins might play a role in promoting the PCD that occurs during cavitation of embryoid bodies and the early mouse embryo. Our results indicate that signaling by members of the BMP family is capable of promoting and is required for both differentiation of VE and cavitation. Based on these data, we have developed a model for possible functions of BMP signaling in pregastrulation mouse development.

MATERIALS AND METHODS

Cell culture

All cell lines were cultured and embryoid bodies were produced as previously described (Martin et al., 1977; Coucouvanis and Martin, 1995). Embryoid bodies were cultured in the presence of BMP proteins as follows: 12-20 embryoid bodies were selected from bulk culture dishes after 2 or 3 days of suspension culture and transferred to a 100 μl drop of culture medium to which recombinant human BMP2, BMP4 or BMP7 (generously provided by Genetics Institute, Cambridge, MA) had been added to a final concentration of 1 to 5 ng/μl. The drops were incubated under paraffin oil (Fluka, Ronkonkoma, NY) for 3-4 days, during which time the medium and BMP protein were replaced every 24-48 hours. Control cultures without added BMP were included in each experiment.

Dominant negative *Bmpr-Ib* transgene

We constructed an expression vector in which we placed a dominant negative mutant form of the mouse *Bmpr-Ib* gene (Zou and Niswander, 1996; kindly provided by Dr L. Niswander, Memorial Sloan Kettering Institute, New York) under the control of the mouse Phosphoglycerate kinase (*Pgk1*) promoter (Tybulewicz et al., 1991), upstream of an internal ribosome entry site (IRES) sequence (Mountford et al., 1994) followed by the selectable reporter gene *β-geo* (Friedrich and Soriano, 1991). The IRES-*β-geo* cassette was kindly provided by Dr A. Smith (University of Edinburgh). The resulting *Pgk*-dn*Bmpr-Ib*-IRES-*β-geo* construct was introduced into PSA1 cells by electroporation as previously described by Hébert et al. (1994). After 12-14 days of culture in selection medium (containing up to 400 μg/ml G418), resistant clones were isolated and analyzed for *lacZ* expression by X-GAL staining. Clones chosen for further analysis were routinely tested for continued expression of the transgene by staining with X-GAL.

Histological analysis and in situ hybridization

Cavitation of embryoid bodies was assayed by histological analysis of samples embedded in glycol methacrylate (GMA; Polysciences, Warrington, PA) as previously described (Coucouvanis and Martin, 1995). X-GAL staining to assay for β-GAL activity was performed essentially as described by Sanes et al. (1986) prior to GMA embedding. In situ hybridization analysis was performed on embryos obtained by mating random-bred CD1 mice obtained from Charles River Laboratories (Hollister, CA). The day on which a vaginal plug was detected was considered day 0 of gestation. There was considerable variation in developmental stage within and among litters. Embryos were fixed within the uterine decidua, dehydrated and embedded in paraffin and sectioned. Embryoid bodies to be analyzed by in situ hybridization were harvested at various stages of culture and were similarly fixed, embedded and sectioned.

In situ hybridization was performed as previously described (Frohman et al., 1990) with the following modifications: probes were labeled with ^{33}P, sections were not dehydrated prior to hybridization, a 3 hour prehybridization step was always included, hybridization was performed at 65-68°C for all probes, and the high-stringency wash was for 2 hours at 65°C. Probes used included mouse *Bmp2* and *Bmp4* (Wozney et al., 1988), *Bmp7* (Furuta et al., 1997), *Hnf4* (Duncan et al., 1994) and *Bmpr-Ib (Alk6*; Zou and Niswander, 1996). Hybridized slides were dipped in NTB3 emulsion (Eastman Kodak, Rochester, NY) and exposed for 14-30 days. For each probe, no specific hybridization was observed with a sense strand control (data not shown).

RT-PCR analysis

An RT-PCR analysis of gene expression was performed on embryoid bodies harvested after culture in drops as described above or on an equivalent number of embryoid bodies isolated from bulk cultures. Total RNA was harvested from embryoid bodies using the RNAeasy kit (Qiagen, Valencia, CA) and cDNA was prepared using the Superscript system (Gibco BRL, Gaithersburg, MD). These cDNAs provided templates for PCRs using the primer pairs described by Duncan et al. (1997) for the specific amplification of Hepatocyte nuclear factor 4 (*Hnf4)*, α-fetoprotein (*Afp*) and Transthyretin (*Ttr*) sequences. To control for the relative amount of cDNA used in each PCR assay, we also used primers that amplify β- and γ-cytoskeletal actin sequences (Hoppe et al., 1992), which are expressed ubiquitously at relatively constant levels. The conditions of the PCRs were the same for all primer pairs (annealing temperature 63°C, 28 cycles) except *Ttr* (annealing temperature 60°C, 30 cycles).

RESULTS

BMP gene expression in embryoid bodies and peri-implantation embryos

As a first step in determining whether BMP signaling might be responsible for the cell death that occurs in the course of cavitation, we sought to determine whether the functionally related BMP genes, *Bmp4*, *Bmp2*, and *Bmp7*, are expressed in embryoid bodies and embryos prior to and/or during cavitation. Embryoid bodies were produced by seeding undifferentiated PSA1 cells at high density in tissue culture dishes, and then detaching the resulting cell aggregates after three days and transferring them to suspension culture. During the next 1-6 days of culture >95% of the aggregates differentiate, giving rise first to simple embryoid bodies with an outer layer of endoderm surrounding a solid core of ectodermal cells and then to cavitated embryoid bodies in which many of the inner cells have undergone apoptosis and the remaining ones have differentiated into a single layer of columnar epithelial cells surrounding a central cavity. An RNA in situ hybridization analysis using probes for *Bmp2*, *Bmp4* and *Bmp7* was performed on sections of embryoid bodies harvested on successive days of development beginning with the day of detachment (D+0).

We detected *Bmp4* RNA in PSA1 cell aggregates on D+0 and D+1 (Fig. 1A,A′ and data not shown). At these stages, the aggregates consisted primarily of undifferentiated ectodermal cells, although by D+1 some of the embryoid bodies had begun to form a morphologically distinct layer of endoderm on the outer surface (Fig. 1A). *Bmp4* expression was always found to be specific to the ectodermal core cells (Fig. 1A′ and data not shown). On D+3, when some of the embryoid bodies had cavitated and others had not, *Bmp4* RNA was not detected in the cavitated embryoid bodies but was detected in the ectodermal cells of most non-cavitated embryoid bodies (data not shown). By D+4, when most of the embryoid bodies had cavitated (Fig. 1B), *Bmp4* RNA was no longer detected (Fig. 1B′). These data indicate that *Bmp4* is expressed in undifferentiated ectodermal cells and that expression of the gene is downregulated during the process of cavitation.

We also assayed for *Bmp4* expression in embryoid bodies produced by S2 EC cells. The S2 EC cell line was derived from the same teratocarcinoma as the PSA1 cell line, but S2 embryoid bodies differ from PSA1 embryoid bodies in the type of endoderm that they form (they develop parietal endoderm (PE), whereas the endodermal layer in PSA1 embryoid bodies contains a mixture of visceral endoderm (VE) and PE, with VE predominating) and S2 embryoid bodies fail to cavitate (Fig. 1C,D; see Martin et al., 1977). Interestingly, *Bmp4* RNA was not detected in S2 embryoid bodies at any of the stages of development examined (D+ 0 to D+ 5; Fig. 1C′, D′ and data not shown).

In contrast to the results obtained for *Bmp4*, *Bmp2* RNA was not detected in ectodermal core cells of PSA1 or S2 embryoid bodies. However, *Bmp2* RNA was detected at low levels in the endoderm of PSA1 and S2 embryoid bodies, at all stages of their development (Fig. 1E,E′,F,F′ and data not shown). *Bmp7* RNA was not detected in either undifferentiated PSA1 or S2 cells, or in the embryoid bodies that they produce (data not shown).

To determine whether the expression patterns observed for *Bmp4*, *Bmp2* and *Bmp7* in embryoid bodies reflect their normal patterns of expression in the pregastrulation embryo, we performed a gene expression analysis in precavitation and postcavitation stage mouse embryos. The undifferentiated aggregates of PSA1 cells and the endoderm-containing precavitation PSAl embryoid bodies in which *Bmp4* expression was detected most closely resemble the ICM of the early (E3.5) and late (E4.5) blastocyst, respectively, whereas the cavitated embryoid bodies in which *Bmp4* expression was not detected most closely resemble the embryonic portion of the E5.5 conceptus (see Fig. 2A). When we assayed for *Bmp4* expression in the embryo, *Bmp4* RNA was detected in ICM cells and the polar trophectoderm of the E3.5 blastocyst (Fig. 2B,B′). At E4.5, after the blastocyst has implanted in the uterus, *Bmp4* RNA was also detected in the ICM and polar trophectoderm, but not in the primitive endoderm cells covering the blastocoelic surface of the ICM (data not shown). A subset of the E4.5 embryos examined were clearly more developmentally advanced, in that extraembryonic ectoderm (which develops from the polar trophectoderm of the blastocyst) was evident. In such embryos, *Bmp4* expression was detected in the extraembryonic ectoderm but not the ICM-derived embryonic ectoderm (data not shown). By E5.5, the embryonic ectoderm has undergone cavitation, and *Bmp4* RNA was not detected in those cells, although it was still detected in the cells of the as yet uncavitated extraembryonic ectoderm (Fig. 2C,C′). The latter undergoes cavitation at a slightly later stage than the embryonic ectoderm. By E6.5, when cavitation of the extraembryonic ectoderm is complete, *Bmp4* RNA becomes restricted to a circumferential band of extraembryonic ectoderm just proximal to the boundary between the embryonic and extraembryonic regions of the conceptus (Fig. 2D,D′; see Waldrip et al., 1998). When we performed assays for *Bmp2* and *Bmp7* expression, we found that *Bmp2* RNA was detectable at low levels in the endoderm of embryos at E5.0 to E6.5 (Fig. 2E,E′,F,F′), whereas *Bmp7* RNA was not detected in the conceptus, but was detected at high levels in maternal decidual tissue surrounding it (Fig. 2G,G′). Expression of *Bmp2* and *Bmp4* was also detected in uterine tissue, but in cells located at a considerable distance from the conceptus (data not shown).

Taken together, these results indicate that the expression patterns of these BMP genes are similar in PSA1 embryoid bodies and normal embryos in vivo, and that PSA1 cells therefore provide a valid model system in which to study cavitation in the peri-implantation embryo. The data on BMP gene expression are consistent with a role for these molecules in cavitation-associated programmed cell death. Moreover, *Bmp4* RNA is detected in the ectoderm of PSA1 embryoid bodies and embryos prior to cavitation, and is not detected in S2 embryoid bodies, which do not cavitate.

Blocking BMP signaling prevents cavitation of PSA1 embryoid bodies

To determine whether interfering with BMP signaling prevents cavitation, we examined the effects of expressing a dominant negative (dn) mutant BMP receptor transgene in embryoid bodies that normally cavitate. The strategy that we used is based on current knowledge of the mechanism of BMP signaling, which occurs through heterodimeric complexes of two different types of serine/threonine kinase receptors (type I and type II). Upon ligand binding to a type

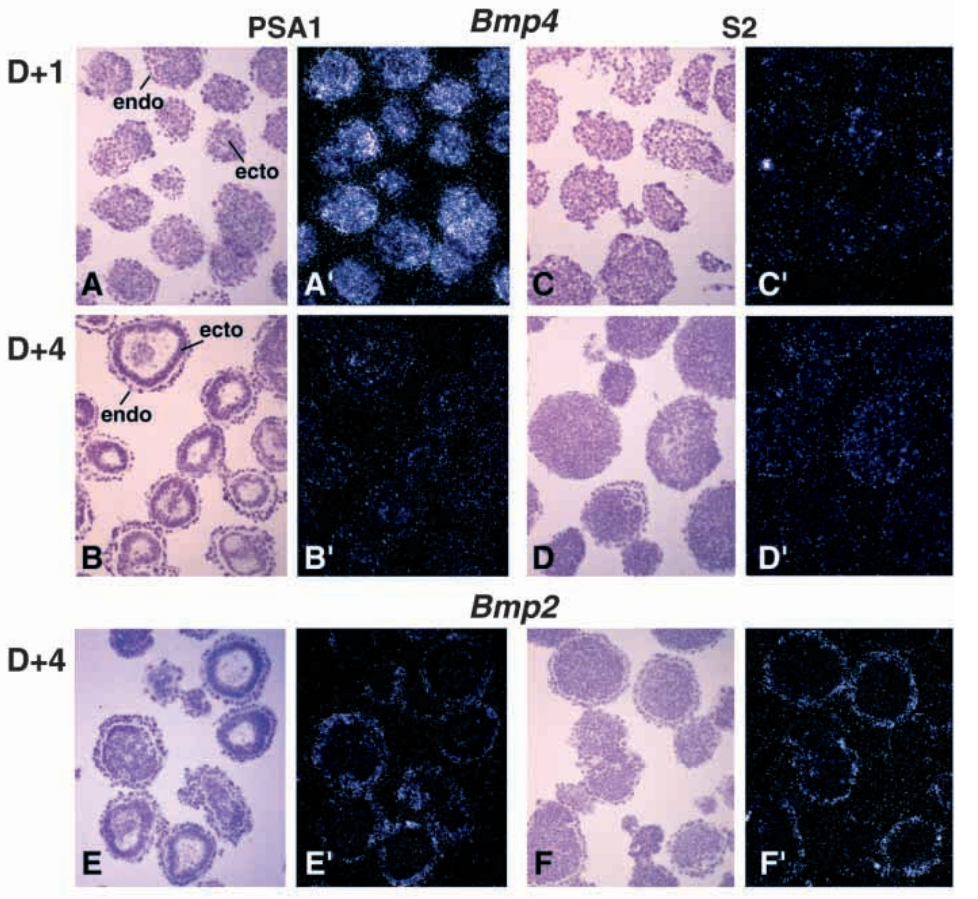

Fig. 1. Expression of *Bmp2* and *Bmp4* in embryoid bodies. In situ hybridization using antisense probes to detect *Bmp4* (A,A′-D,D′) and *Bmp2* (E,E′,F,F′) RNA in embryoid bodies harvested after either 1 day (A,C) or 4 days (B,D-F) of suspension culture. (A-F) Bright-field images, (A′-F′) corresponding dark-field views. (A,B,E) Embryoid bodies (cavitating) produced by the PSA1 cell line; (C,D,F) embryoid bodies (non-cavitating) produced by the S2 cell line. Abbreviations: ecto, ectoderm; endo, endoderm. Radioautographic exposure time: (A′) 16 days; (B′,C′) 20 days; (D′) 24 days; (E′,F′) 14 days.

II receptor, a type I receptor is recruited to the complex and its kinase domain is activated, leading to signal transduction through intracellular proteins known as SMADs (reviewed by Baker and Harland, 1997; Heldin et al., 1997). The BMP type II receptor (BMPR-II) can bind BMP2, BMP4 or BMP7, and then associate with and activate signaling through any of three type I receptors: BMPR-IA, BMPR-IB or ActR-I (reviewed by Massague and Weis-Garcia, 1996). One way to inhibit signaling by these three BMP ligands is to express at high levels a mutant transgene that encodes a non-functional BMPR-IB protein. This can result in the recruitment of all ligand-bound BMPR-II receptors into non-functional complexes and thus prevent them from interacting with and activating any wild-type BMPR-IA, BMPR-IB or ActR-I proteins that may be present.

PSA1 cells were transfected with an expression vector (see Fig. 3A) that includes a mutated mouse *Bmpr-Ib* gene with a point mutation in the region that encodes the ATP-binding domain of the BMPR-IB kinase. This mutation has been shown to abolish kinase activity of the receptor protein and render it inactive in a signaling assay (Zou and Niswander, 1996). When expressed in the chick limb bud in ovo, this dominant negative mutant receptor gene interferes with BMP signaling and prevents PCD (Zou and Niswander, 1996). To provide a means of monitoring expression of the transgene, we placed an internal ribosome entry site (IRES) downstream of the stop codon in the mutant *Bmpr-Ib* gene, followed by the *β-geo* reporter gene. This configuration results in production of a single, dicistronic message encoding both the dominant negative receptor and *β-geo* genes (Mountford et al., 1994). *β-geo* is a fusion gene between *lacZ* and neo^R. Its expression can be detected by staining for β-galactosidase (β-GAL) activity with X-GAL, and provides a selectable marker (neomycin resistance) to facilitate the isolation of cells that express the dominant negative receptor transgene (Friedrich and Soriano, 1991).

We isolated numerous stably transfected neo^R stem cell clones and assayed the undifferentiated cells for β-GAL activity. From these clones, we selected nine for further analysis, five that were resistant to the levels of G418 used for selection, but did not produce enough *β-geo* to display detectable β-GAL activity, and four that produced β-GAL activity at high levels. We then produced embryoid bodies from these nine clones and assayed for cavitation after 4-6 days of suspension culture. Whereas the embryoid bodies formed by the five β-GAL-negative clones cavitated normally (Fig. 3B and data not shown), those produced by the four clones that stained strongly for β-GAL activity failed to undergo cavitation. Although occasional embryoid bodies derived from the four positive clones displayed both a loss of β-GAL activity and evidence of cavitation, we never observed cavitation in embryoid bodies that were strongly positive for β-GAL activity (Fig. 3C and data not shown). To confirm that the high levels of β-GAL activity observed reflected expression of the dn*Bmpr-Ib* gene, we performed an in situ hybridization analysis using a probe for *Bmpr-Ib*, which detects both mutant transgene and endogenous wild-type RNA. In the embryoid bodies that expressed high levels of β-GAL activity, we

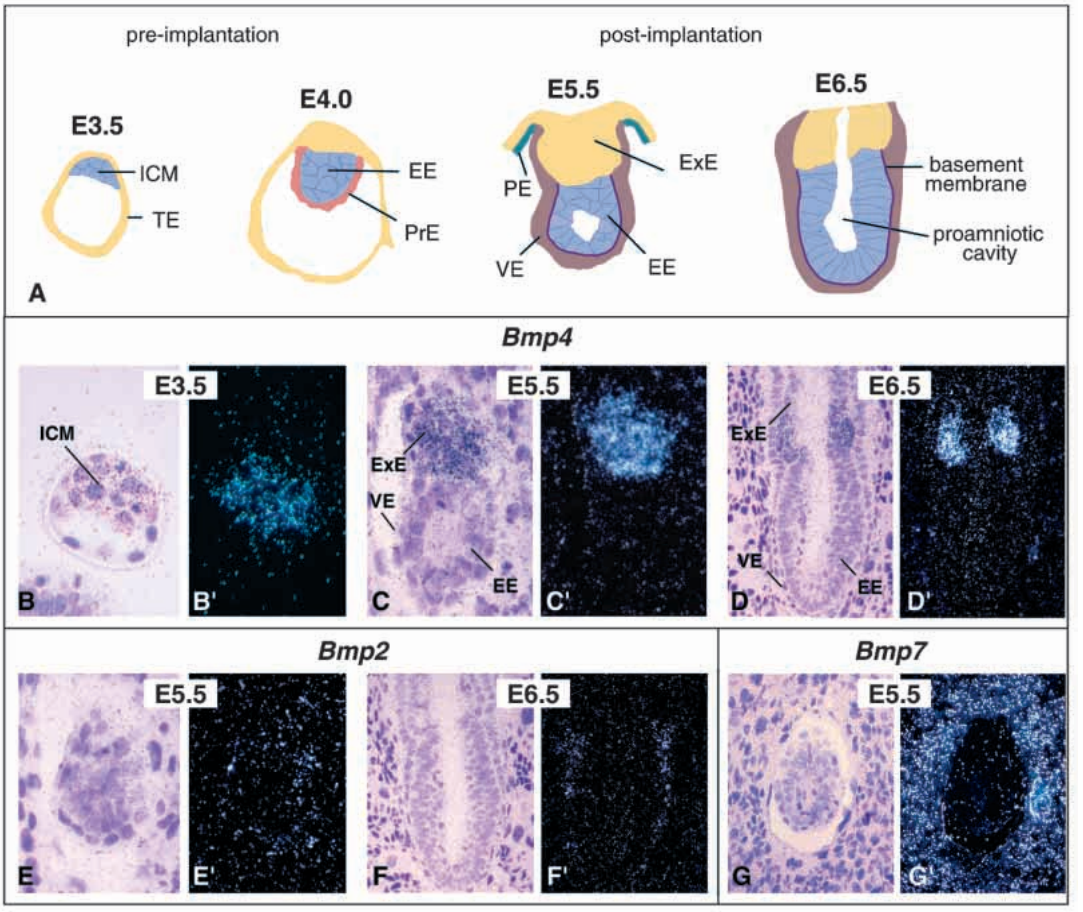

Fig. 2. Expression of BMP genes in peri-implantation embryos. (A) Schematic diagrams of the mouse embryo at E3.5 through E6.5, illustrating the development of the extraembryonic endoderm and the process of cavitation leading to the formation of the proamniotic cavity. (B-G) Hybridization of antisense probes for *Bmp4* (B,B′-D,D′), *Bmp2* (E,E′,F,F′), or *Bmp7* (G,G′) to sections through embryos at the stages indicated. (B-G) bright-field images; (B′-G′) corresponding dark-field views. (C,D,F) Sagittal sections; (E) the embryonic portion of the conceptus sectioned in a plane that is midway between transverse and sagittal. The proamniotic cavity is present in this embryo but is not evident due to the oblique plane of section. The plane of section in G is transverse, but slightly oblique. Abbreviations: EE, embryonic ectoderm; ExE, extraembryonic ectoderm; ICM, inner cell mass; PE, parietal endoderm; PrE, primitive endoderm; TE, trophectoderm; VE, visceral endoderm. Radioautographic exposure time: (B′,C′,E′) 24 days; (D′,F′) 30 days; (G′) 20 days.

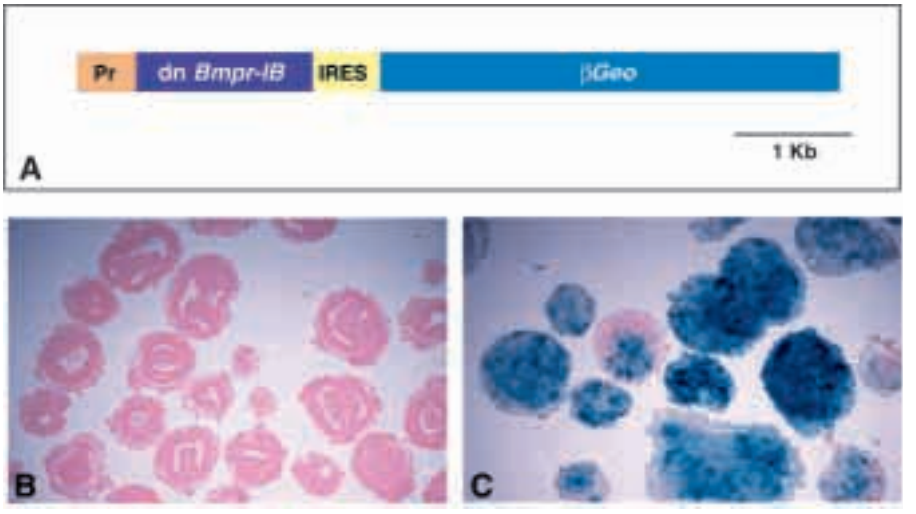

Fig. 3. Expression of a dominant negative mutant *Bmpr-Ib* transgene prevents cavitation. (A) Schematic diagram of the construct used to express a dominant negative mutant form of BMP receptor IB in PSA1 cells. The promoter from the mouse *Pgk1* gene (orange box) drives expression of the coding sequence of the mouse *Bmpr-Ib* gene (purple box), which contains a point mutation in the ATP-binding domain. Downstream of the mutant *Bmpr-Ib* gene is an internal ribosome entry site (IRES; yellow box) followed by the *β-geo* fusion gene (blue box). (B,C) Embryoid bodies produced by clonal lines isolated following stable transfection of PSA1 cells with the construct shown in A. The embryoid bodies, which were fixed and stained with X-GAL after 4 days in suspension culture, were produced by (B) a clonal line in which expression of the transgene is low or absent (as indicated by the lack of blue stain); note that these embryoid bodies cavitate normally, although at the stage shown they have not yet completed cavitation, or (C) a clonal line that expresses high levels of the construct; note that these embryoid bodies fail to cavitate (<5% of embryoid bodies displayed evidence of cavitation).

detected *Bmpr-Ib* RNA at much higher levels than in the embryoid bodies formed by control (non-transfected) PSA1 cells (data not shown).

Interestingly, two of the four clones that initially produced high levels of β-GAL activity displayed a substantial decrease in transgene expression in successive passages (although still under selection), as evidenced by low or undetectable levels of β-GAL activity, which was paralleled by a dramatic decrease in *Bmpr-Ib* expression as assayed by in situ hybridization. Embryoid bodies produced by these lines after this decrease in transgene expression appeared to have regained the ability to cavitate normally. These data indicate that the initial failure to cavitate seen in the embryoid bodies produced by these lines was reversible. Importantly, in embryoid bodies produced by the two clones that continued to express the transgene at high levels, line A2 and line B4, cavitation failed to occur (see below). Taken together, these results demonstrate a close correlation between high levels of dn*Bmpr-Ib* transgene expression and a lack of cavitation in embryoid bodies.

Cavitation of S2 embryoid bodies is induced by BMP proteins

The data described above indicate that BMP signaling is required for cavitation. Moreover, the observation that S2 embryoid bodies (which fail to cavitate) do not express *Bmp4* raises the possibility that lack of BMP4 is responsible for the lack of cavitation in S2 embryoid bodies. To test this hypothesis, we examined the effect of recombinant BMP4 protein on S2 embryoid bodies. In each experiment (n=8), we cultured 12-20 embryoid bodies with 1 to 5 ng/μl BMP4 and an equal number in control medium. By analyzing serial sections of these samples, we found that in contrast to control S2 embryoid bodies, which never exhibited any features of cavitation (Fig. 4A and data not shown), 20-60% of BMP4-treated S2 embryoid bodies in each experiment contained one or more small cavities located near the periphery of the embryoid body, in which apoptotic cells were apparent, and in which the cells surrounding these cavities had formed a columnar epithelium (Fig. 4B). However, the complete cavitation typical of PSA1 embryoid bodies (see Fig. 1B), which initiates near the periphery and progresses inward until the core cells are completely gone, was not observed in any of the BMP4-treated S2 embryoid bodies, even when they were cultured in the presence of BMP4 for 6-8 days (data not shown). Thus addition of BMP4 induces cavitation near the periphery of S2 embryoid bodies.

We also tested the ability of BMP2, BMP7 and a variety of related and unrelated signaling molecules to induce cavitation of S2 embryoid bodies. Both BMP2 and BMP7 had the same effect as BMP4. At the same concentrations used for BMP4, BMP2 (n=5 experiments) and BMP7 (n=2 experiments) treatment caused the appearance of small cavities lined with columnar epithelium and containing apoptotic cells in 20-60% of the embryoid bodies in each experiment (data not shown). In experiments in which all three proteins were compared directly, no differences in their effects were observed with respect to number of embryoid bodies affected or extent of cavitation. In contrast, addition of human Activin (provided by National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, and found to be active in Xenopus animal cap assays [Dr T. Musci, UCSF, personal communication]), human TGFβ1 (kindly provided by Drs. A. Erlebacher and R. Derynk, UCSF), human IGFII (Sigma, St. Louis, MO), human FGF4 (kindly provided by Genetics Institute, Cambridge, MA), or human EGF (R&D Systems, Minneapolis, MN) at 5 ng/μl had no effect on S2 embryoid bodies (data not shown). These results demonstrate that the ability to induce cavitation in S2 embryoid bodies is displayed by certain BMPs but not other types of signaling molecules (including the TGFβ superfamily members Activin and TGFβ1).

BMP signaling promotes the differentiation of visceral endoderm

In the protein addition experiments described above, we noted that the outer endodermal cells of S2 embryoid bodies treated with BMPs differed from those of control S2 embryoid bodies. Specifically, endoderm of untreated S2 embryoid bodies consists of individual cells that do not appear to be organized into a cohesive epithelium (Fig. 4A). In contrast, endoderm on the surface of BMP-treated S2 embryoid bodies is more highly organized into an epithelial layer and the cells appear to be more tightly adherent to one another. In addition, the endodermal cells of BMP-treated S2 embryoid bodies contain numerous vacuoles that are not evident in the endoderm of untreated embryoid bodies (Fig. 4B and data not shown). On the whole, we noted that the endoderm of BMP4-treated S2 embryoid bodies strongly resembles the VE of cavitating embryoid bodies and normal embryos, rather than the PE found on the surface of untreated S2 embryoid bodies.

To determine whether these BMP-induced morphological changes are accompanied by changes in endoderm gene expression, we used an RT-PCR assay to test for the expression of the VE-specific molecular markers *Hnf4* (Duncan et al., 1994), *Afp* (Dziadek and Adamson, 1978) and *Ttr* (Makover et al., 1989). *Hnf4* has been shown to be specific to VE as early as E4.5 (Duncan et al., 1994), whereas *Afp* and *Ttr* are markers for VE at later stages of development (Dziadek and Adamson, 1978; Makover et al., 1989). Expression of all three genes was detected in PSA1 but not control S2 embryoid bodies (Fig. 4C), consistent with the conclusion from previous studies that the endodermal layer of S2 embryoid bodies does not normally contain VE cells (Martin et al., 1977). Similar results were obtained irrespective of whether the embryoid bodies were cultured in small drops or in bulk culture (data not shown). In contrast to the lack of VE gene expression observed in control S2 cultures, expression of these three genes was detected in S2 embryoid bodies treated with BMP4 (Fig. 4C).

To confirm that the gene expression detected in BMP-treated S2 embryoid bodies is specific to the endoderm, we also examined expression of *Hnf4* by in situ hybridization. *Hnf4* RNA was detected in the VE of embryos at E4.5- E6.5 (Fig. 4D,D′ and data not shown; see also Duncan et al., 1994) and also in the endoderm of PSA1 embryoid bodies prior to and during cavitation (Fig. 4E,E′ and data not shown). Expression of *Hnf4* is not homogeneous throughout the endoderm of PSA1 embryoid bodies, presumably due to the presence of PE cells intermingled with the VE cells (Martin et al., 1977). In agreement with the results of the RT-PCR assay described above, *Hnf4* RNA was not detected in control S2 embryoid

bodies at any of the stages assayed (Fig. 4F,F′ and data not shown). However, *Hnf4* RNA was detected in the outer endodermal cell layer of S2 embryoid bodies that had been treated with BMP4 or BMP2 (Fig. 4G,G′ and data not shown). In some cases, the region in which *Hnf4* RNA was most abundant coincided with areas of underlying ectoderm in which cavitation was occurring (see inset in Fig. 4G′). These data indicate that one effect of BMP proteins on S2 cells is to promote the differentiation of VE.

Additional evidence in support of this conclusion came from analysis of the embryoid bodies produced by one of the PSAl cell lines expressing the dn*Bmpr-Ib* transgene (line B4), which fail to cavitate. β-GAL activity was detected at high levels in the endoderm of those embryoid bodies (Fig. 5A), and *Hnf4* expression in the endoderm was substantially reduced or absent (Fig. 5B′). In contrast, *Hnf4* RNA is strongly expressed throughout the endoderm of control PSA1 embryoid bodies (compare Fig. 5B′ with Fig. 4E′). Thus, expression of the dominant negative receptor transgene at high levels in the endoderm of PSA1 embryoid bodies appears to greatly diminish the expression of at least one marker of VE, suggesting that BMP signaling in embryoid bodies is required for VE differentiation. Taken together, these results provide evidence that BMP signaling is both capable of promoting and required for the differentiation of VE.

Cavitation may also require BMP signal reception in the ectoderm

The data described above, indicating that BMPs promote differentiation of VE, raise the possibility that this is their only function in cavitation and that once differentiated, the VE cells then produce some other (non-BMP) signal that is more directly involved in initiating the cavitation process. If this were the case, then the presence of VE should be the only requirement for cavitation to occur, and an embryoid body expressing high levels of the dn*Bmpr-Ib* transgene throughout the ectoderm should still cavitate, as long as the endodermal layer is negative for dn*Bmpr-Ib* expression so that VE differentiation can occur. However, the results of an analysis of the embryoid bodies formed by one of the dominant negative mutant receptor transgene-expressing PSAl cell lines lead us to propose that BMP signaling may also play a more direct role in cavitation.

Line A2 was initially found to produce embryoid bodies with high levels of β-GAL activity throughout, but after numerous passages it was found that the embryoid bodies produced by these cells displayed high levels of β-GAL activity in the ectodermal cells, but little or no activity in the endoderm (Fig. 5C). Because the endodermal cells express low levels of the dominant negative receptor transgene, BMP signaling in those cells should not be blocked. Therefore, they should be able to respond to endogenous BMPs and produce VE. Indeed, we found that *Hnf4* RNA could be detected in many (but not all) of the endodermal cells of line A2 embryoid bodies, suggesting that VE had formed (Fig. 5D,D′). Importantly, despite the apparent presence of VE, the embryoid bodies failed to cavitate (Fig. 5C,D). These data suggest that, in the presence of VE, cavitation does not occur unless ectodermal cells are capable of BMP signal reception and thus are consistent with a requirement for BMP signaling in that cell type, as well as in the endoderm, for normal cavitation.

DISCUSSION

This study was initiated to explore the possibility that BMPs might serve as the signal for the programmed cell death that is a key feature of the cavitation that occurs in embryoid bodies and the early postimplantation mouse embryo. Although the results of our experiments have not resolved the question of whether BMPs function as the death signal per se, they do provide strong evidence that BMP signaling is required for cavitation. Specifically, we have shown that *Bmp2* and *Bmp4* are expressed in a temporally and spatially restricted pattern that is consistent with a role for these factors in the cavitation of both PSA1 embryoid bodies and normal embryos, and we have demonstrated that interfering with BMP signaling via expression of a dominant negative mutant receptor transgene abolishes cavitation of PSA1 embryoid bodies. Furthermore, we showed that treatment of S2 embryoid bodies, which normally fail to cavitate, with BMP2, BMP4 or BMP7 induces cavitation near the periphery of the embryoid bodies. Analysis of the expression of molecular markers of VE in both PSA1 embryoid bodies that express a dominant negative mutant BMP receptor transgene and S2 embryoid bodies treated with BMPs revealed that BMP signaling is required for differentiation of VE in embryoid bodies.

Based on these results, and on accumulated evidence demonstrating that embryoid bodies formed by certain EC and ES cell lines provide a valid model system in which to study the mechanisms of normal early mouse embryogenesis (see for example, Coucouvanis and Martin, 1995; Duncan et al., 1997; Choi et al., 1998), we propose a model for peri-implantation mouse development in which BMP signaling is required to promote differentiation of primitive endoderm to VE and cavitation of the ectoderm (Fig. 6). According to this model, BMP2 and BMP4 are functionally redundant with respect to these two proposed activities. Thus, as depicted in Fig. 6, during endoderm development, BMP4 produced in the ectoderm or BMP2 produced in the endoderm can promote VE differentiation. Likewise, during cavitation, BMP2 produced in the endoderm or BMP4 produced in the ectoderm can act on the ectodermal core cells, possibly in conjunction with another molecule(s) produced by VE, to cause the cell death and columnar epithelial differentiation that constitute cavitation. This proposed functional redundancy is a key aspect of our model and is consistent with the observation that embryos homozygous for a null allele of either *Bmp2* or *Bmp4* cavitate normally (Winnier et al., 1995; Zhang and Bradley, 1996).

BMP signaling and the differentiation of visceral endoderm

In the normal embryo, VE derives from the extraembryonic endodermal lineage that is first established at ~E4.0, when cells in the outer layer of the ICM of the blastocyst delaminate to form the primitive endoderm; the remaining inner cells of the ICM form the embryonic ectoderm. Primitive endoderm cells that maintain contact with the ectoderm differentiate into VE, whereas those that migrate away from the ICM along the inner surface of the trophectoderm form parietal endoderm (Nadijcka and Hillman, 1974; Enders et al., 1978; see Fig. 2A). Although it has been established through studies of chimeric embryos that an initial precursor cell population (primitive endoderm) gives rise to both VE and PE (Gardner, 1982), the

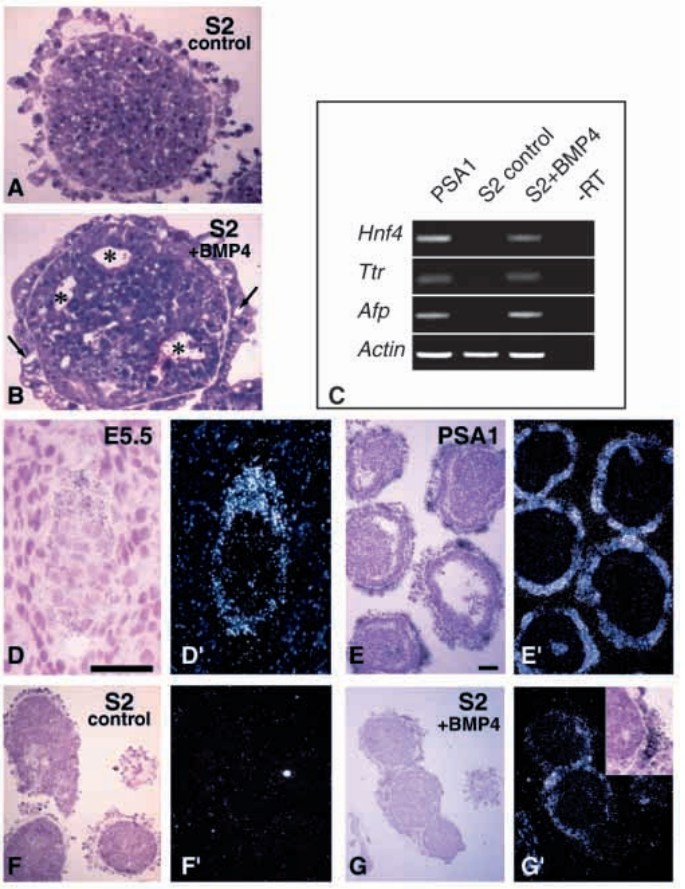

Fig. 4. S2 embryoid bodies treated with BMP4 undergo partial cavitation and express markers of visceral endoderm. (A,B) Sections through S2 embryoid bodies harvested after 4 days of culture in control medium (A) or in medium with BMP4 at 5 ng/μl (B). Note the cavities surrounded by a columnar epithelium (marked with asterisks) at the periphery of the embryoid body. Arrows point to vacuoles in endodermal cells. (C) Expression analysis of VE marker genes by RT-PCR. RNA was isolated from PSA1 or S2 embryoid bodies cultured in control medium, or S2 embryoid bodies cultured in medium containing BMP4 (3 ng/μl). Following reverse transcription, the cDNA templates were assayed by PCR using primer pairs specific for *Hnf4, Ttr, Afp* and *Actin.* Control samples included templates prepared in the absence of reverse transcriptase using RNA isolated from S2 embryoid bodies cultured in medium containing BMP4, as well as PSA1 and S2 embryoid bodies cultured in control medium (–RT lane and data not shown). (D,E) *Hnf4* expression in an E5.5 embryo (D) and in PSA1 embryoid bodies (E). In the embryoid bodies shown, cavitation is occurring normally but is not yet complete in most of them. (F,G) Bright-field views of S2 embryoid bodies assayed by in situ hybridization for *Hnf4* RNA following culture in control (F) or BMP4-containing (G) medium. (D′-G′) The dark-field views corresponding to D-G. The scale bars in D and E illustrate the relative sizes of the E5.5 embryo and embryoid bodies. Radioautographic exposure time: (D′) 28 days; (E′-G′) 21 days.

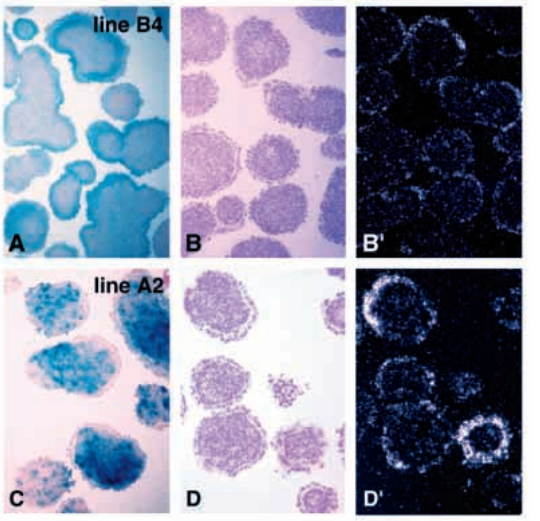

Fig. 5. Dominant negative BMP receptor expression prevents VE differentiation. (A) Assay by X-GAL staining for expression of the dn*Bmpr-Ib* transgene in embryoid bodies formed by clonal line B4. Note that expression is detected at high levels predominantly in the endoderm and peripheral ectoderm, and at lower levels throughout the ectodermal core. (B,B′) *Hnf4* expression in B4 embryoid bodies. (C) X-GAL assay for transgene expression in embryoid bodies formed by clonal line A2. Note that expression is detected predominantly in the ectoderm. (D,D′) *Hnf4* expression in A2 embryoid bodies. Note the negative correlation between expression of the dn*Bmpr-Ib* transgene in the endoderm and the level of *Hnf4* expression. Radioautographic exposure time: (B′,D′) 26 days.

precise timing, as well as the nature of the transition from primitive endoderm to VE or PE is poorly understood. The available evidence suggests that interactions between endodermal cells and embryonic ectoderm or trophectoderm cells promote the development of VE or PE, respectively (Hogan et al., 1981; Hogan and Tilly, 1981). Several studies have recently demonstrated that VE plays an essential role in patterning the anterior embryonic ectoderm (Thomas and Beddington, 1996; Varlet et al., 1997; Rhinn et al., 1998), and also that embryos with defective VE fail to gastrulate normally (Duncan et al., 1994; Sirard et al., 1998; Waldrip et al., 1998). Despite the importance of VE in early development, the mechanisms governing its formation and differentiation remain largely unknown. There is evidence from genetic studies suggesting that BMP signaling may be involved in VE development. In embryos homozygous for a null allele of *Bmpr-Ia*, the VE appears morphologically different from wild-type VE at E7.0 (Mishina et al., 1995). Moreover, embryos and embryoid bodies homozygous for a null allele of *Smad4*, a gene that encodes a component that appears to be common to all TGFβ-related signal transduction pathways (including the BMP pathway), display abnormalities in VE morphology and gene expression (Sirard et al., 1998).

Here we provide evidence that BMP signaling is both capable of promoting and is required for the differentiation of VE. We have shown that addition of BMP4, which is normally produced in the ectoderm, to S2 embryoid bodies (which fail to express *Bmp4*) induces morphological and molecular changes in the endodermal cells indicative of VE differentiation, including the expression of *Hnf4*, a marker for VE in the embryo as early as E4.5. Furthermore, we have shown that interfering with BMP signaling in the endoderm of PSA1 embryoid bodies apparently inhibits the expression of *Hnf4*. Because our studies demonstrated that BMP2 and BMP4 have similar effects on S2 embryoid bodies, we hypothesize

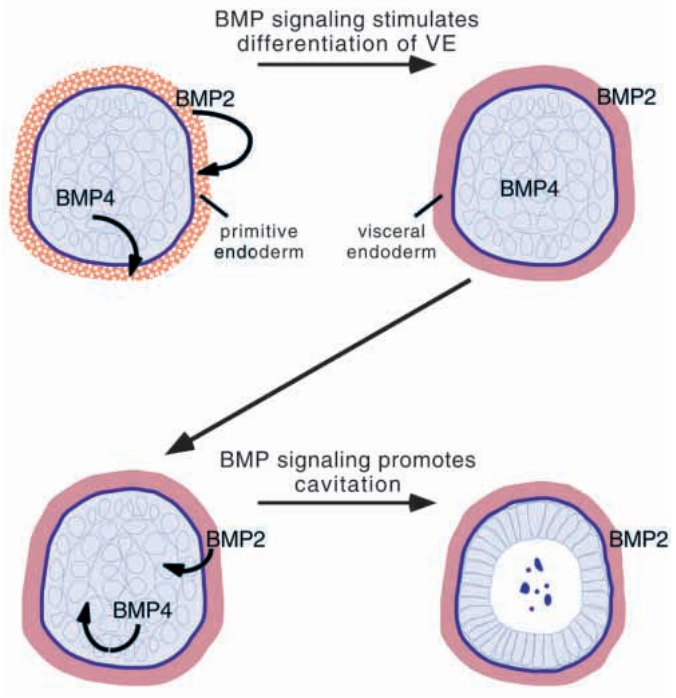

Fig. 6. A model illustrating proposed roles of BMP signaling in development of peri-implantation embryos and embryoid bodies. Schematic diagram depicting the proposed role of BMP signaling in VE differentiation and cavitation. The top row shows sections through the endoderm and ectoderm of a precavitation embryo or embryoid body. BMP4 produced in the ectoderm acts on the primitive endoderm to promote VE differentiation. BMP2 produced in the endoderm may also play a role in the process. The bottom row shows similar sections immediately prior to and after cavitation. BMP4 produced in the ectoderm and/or BMP2 produced in the endoderm act(s) on the ectoderm (perhaps in conjunction with other, VE-derived signals) to promote PCD of the inner cells and differentiation of the outer layer of columnar cells.

that BMP2, which is normally produced in the endoderm, may also promote VE differentiation. Consistent with our model, we have found that *Bmp4* is expressed in the ectoderm of embryoid bodies and the ICM of embryos prior to, during, and for a short time after primitive endoderm formation, and might therefore influence *Hnf4* expression in the nascent VE. However, it is unclear whether *Bmp2* is expressed in primitive endoderm or nascent VE prior to *Hnf4*, in part because the levels of *Bmp2* detected are low. If *Bmp2* is first expressed after *Hnf4* expression commences, then BMP2 would be unlikely to promote early stages of VE differentiation under normal circumstances, but might do so when other BMP signals are absent, as for example in *Bmp4*-deficient embryos.

One finding that appears to be inconsistent with the hypothesis that VE can be induced by BMP2 produced in the endoderm is that the endodermal cells of S2 embryoid bodies express *Bmp2* RNA yet they morphologically resemble PE and do not express *Hnf4*. One possible explanation for this apparent inconsistency is that the *Bmp2* RNA produced by S2 embryoid bodies does not produce functional BMP2 protein, and therefore S2 embryoid bodies, which do not express *Bmp4*, fail to form VE due to the lack of a BMP signal. To address this issue experimentally, we attempted to determine directly whether BMP2 protein is produced in S2 embryoid bodies using anti-BMP2 antibodies. Unfortunately, the antibodies available proved unsuitable for such studies. We also compared the coding sequences of PCR-amplified *Bmp2* cDNAs derived from S2 and PSA1 cells, but found no differences between them (data not shown). Another possibility is that functional BMP2 protein is produced in S2 endoderm, but some aspect of BMP signal reception is abnormal in S2 cells, resulting in BMP signal transduction when large amounts of protein (such as the amounts that were added to the culture medium) are present, but not in response to lower, endogenous levels of BMP protein.

The role of BMP signaling in cavitation

Our results demonstrate that the PCD and columnar epithelial differentiation of ectodermal cells that constitute cavitation are dependent on BMP signaling. However, they do not exclude the possibility that this is an indirect effect, and the requirement for BMP signaling might be met by the BMP-mediated effects on endoderm discussed above. According to this model, VE differentiation in response to BMP signaling would result in the production of other molecules required for cavitation. Such molecules would presumably be produced by early rather than late stage VE, since cavitation is normal in *Hnf4*$^{-/-}$ or *Smad4*$^{-/-}$ embryos, in which early but not mature VE is apparently formed (Chen et al., 1994; Duncan et al., 1997; Sirard et al., 1998).

Although it is possible that BMP signals are necessary only for differentiation of VE, and VE subsequently causes cavitation via a BMP-independent mechanism, our data suggest that BMP signaling may also play a more direct role in cavitation. Our observation that embryoid bodies formed by line A2 (which express the dominant negative mutant receptor transgene at high levels exclusively in the ectoderm) fail to cavitate is consistent with a requirement for downstream components of BMP signaling in ectodermal cells in order for cavitation to occur, thus suggesting that BMP might be the death signal per se. It should be noted, however, that a direct role for BMP signaling in the PCD and/or differentiation of the columnar epithelial cells does not preclude a possible requirement for some other molecule(s) produced by VE that works in conjunction with a BMP signal (either VE-derived BMP2 or ectoderm-derived BMP4) to promote cavitation.

There have been two reports demonstrating that BMP4 induces cell death in P19 EC cells. Glozak and Rogers (1996) found that, in combination with retinoic acid (RA), BMP4 caused apoptosis in aggregates or monolayer cultures of P19 cells. In their assay, BMP4 alone had only a minimal effect on cell death. In contrast to those findings, Marazzi et al. (1997) reported that addition of BMP4 (with or without RA) increases cell death in aggregates, but not monolayers, of P19 cells. The relevance of these results to our study is unclear, since P19 aggregates neither form an outer layer of endoderm nor undergo cavitation.

One potential argument against a role for BMP signaling in either the early stages of VE differentiation or cavitation is based on the assumption that SMAD4 is required for all forms of TGFβ signaling, and the finding that early markers of VE differentiation are expressed and cavitation occurs in *Smad4* null mutant embryos (Sirard et al., 1998). However, there is

mounting evidence that SMAD4 may not be absolutely required, either because there might be additional SMADs (as yet unidentified) that can functionally substitute for it, or because there might exist TGFβ family signaling mechanisms in which SMAD4 function is not required. For example, in the presence of wild-type extraembryonic tissues, development of *Smad4* null mutant embryos appears relatively normal until E9.0 (Sirard et al., 1998), but it seems unlikely that signaling by all TGFβ family members in the embryo proper is dispensable until that stage (see Varlet et al., 1997). Thus, we favor the view that the ability of *Smad4* mutant embryos to cavitate normally is a manifestation of functional redundancy of this type of SMAD in the BMP signal transduction pathway.

One interesting question is why addition of BMPs to S2 cultures causes cavitation only at the periphery of the embryoid bodies. One possible explanation of this observation is based on the assumption that BMP protein added to the culture medium is not able to diffuse very far into the core of the S2 embryoid bodies. Thus, in S2 embryoid bodies, which do not express *Bmp4* in the ectoderm and may not produce functional BMP2 in the endoderm, the added BMP might be available only to cells in the periphery of the embryoid bodies. If this is the case, it raises the possibility that the effective range of BMP2 produced in the endoderm of PSA1 embryoid bodies may also be limited, and that cavitation occurs throughout those embryoid bodies because BMP2 activity is complemented by BMP4 produced throughout the ectoderm. In the normal mouse embryo, BMP2 produced in the endoderm may be sufficient for complete cavitation of the embryonic ectoderm because at the stage when cavitation occurs, the embryo is only 6-8 cell widths in diameter.

Functional redundancy of BMPs during peri-implantation development

An important feature of the model discussed above is the concept that BMPs produced at peri-implantation stages of development are redundant with respect to their proposed functions in VE differentiation and cavitation. There is substantial evidence from other types of studies for functional overlap among members of the BMP family. For example, BMP2 and BMP4 both promote apoptosis in chick leg mesenchyme cells in culture (Yokouchi et al., 1996) and both factors can induce a dorsomedial phenotype when added to telencephalic neuroectoderm explants (Furuta et al., 1997). Consistent with those results, we observed identical effects on cavitation of S2 embryoid bodies with BMP2, BMP4 and BMP7. Such functional overlap is presumably related to the reported ability of these different ligands to interact with the same receptor, BMPR-II, which has been shown to be expressed in the late blastocyst and from E6.0 onwards (Roelen et al., 1997). The BMPR-II-ligand complex can associate with and activate signaling via any of three type I receptors: BMPR-IA, BMPR-IB or ActR-I (reviewed by Massague and Weis-Garcia, 1996). If multiple receptor interactions and co-expression occur in the precavitation embryo, these might provide an explanation for the observation that embryos homozygous for a null allele of *Bmpr-Ia* cavitate normally (Mishina et al., 1995).

Limited data are available on the expression of BMP type I receptors during peri-implantation mouse development. *Bmpr-Ia* has been detected by RT-PCR assays at the late blastocyst stage, and again at E7.0, but not at E6.0; other stages between late blastocyst and E6.0 were not examined (Roelen et al., 1997). However, the observation that embryos homozygous for a null allele of *Bmpr-Ia* appear normal at E5.5 but display a phenotype by E7.0 (Mishina et al., 1995) suggests that *Bmpr-Ia* is expressed between E4.5 and E7.0. *Bmpr-Ib* expression has also been detected by RT-PCR assays at the early morula stage, but not in the compacted morula or blastocyst. Furthermore, as was the case for *Bmpr-Ia*, *Bmpr-Ib* expression was not detected at E6.0, but was detected at E7.0 (Roelen et al., 1997). In apparent conflict with those data, we detected *Bmpr-Ib* expression by in situ hybridization at low levels throughout the embryo at E5.5 and E6.5 (data not shown). *Actr-I* expression has also been detected by RT-PCR assays at the blastocyst stage (Roelen et al., 1997) and at E6.0 and E6.5 (Roelen et al., 1994).

We have shown that *Bmp2*, *Bmp4* and *Bmp7* are expressed in temporally and spatially restricted patterns that are consistent with a role for the products of these genes in promoting VE differentiation and cavitation. Indeed, our data demonstrate that *Bmp2* and *Bmp4* are expressed earlier in the mouse embryo than has been previously reported (Jones et al., 1991; Lyons et al., 1995; Winnier et al., 1995). However, since embryos homozygous for null alleles of either *Bmp2* or *Bmp4* are reported to develop normally up to approximately E7.5 and E6.0, respectively, it appears that neither gene alone is required for normal peri-implantation development and cavitation (Winnier et al., 1995; Zhang and Bradley, 1996). In accord with our model, we speculate that functional redundancy between BMP2 and BMP4, or perhaps rescue by maternally derived BMP7, accounts for the lack of a cavitation phenotype in the mutant embryos. Furthermore, we would predict that *Bmp2/4* double homozygous null embryos and embryoid bodies would fail to cavitate in vitro, although that might not be the case in vivo, where maternal BMP7 might complement the lack of BMP2 and BMP4 in the double mutant embryos.

Concluding remarks

The results of our previous studies led us to propose that cavitation depended on VE as the source of a signal that causes death of cells in the ectoderm, and that a concurrent survival signal prevented the death of any cells adhering to the basement membrane separating the ectoderm from the endoderm (Coucouvanis and Martin, 1995). In the present study, we have provided further evidence for a connection between the presence of VE and the occurrence of cavitation and we have shown that BMP signaling promotes differentiation of VE. We also identified BMPs as possible mediators of PCD during cavitation. Further studies will be required to determine whether their role in cavitation is indirect, via an effect on the differentiation of VE, or whether they function as the death signal per se. In addition, appropriate experiments with mutant embryos or embryoid bodies should help to determine the extent to which BMP2, BMP4 and perhaps BMP7 are able to functionally substitute for one another during cavitation of embryos in vivo.

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