Development 127, 1105-1114 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4296

A BMP pathway regulates cell fate allocation along the sea urchin animal-vegetal embryonic axis

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Accepted 16 December 1999; published on WWW 8 February 2000

SUMMARY

To examine whether a BMP signaling pathway functions in specification of cell fates in sea urchin embryos, we have cloned sea urchin BMP2/4, analyzed its expression in time and space in developing embryos and assayed the developmental consequences of changing its concentration through mRNA injection experiments. These studies show that BMP4 mRNAs accumulate transiently during blastula stages, beginning around the 200-cell stage, 14 hours postfertilization. Soon after the hatching blastula stage, BMP2/4 transcripts can be detected in presumptive ectoderm, where they are enriched on the oral side. Injection of BMP2/4 mRNA at the one-cell stage causes a dose-dependent suppression of commitment of cells to vegetal fates and ectoderm differentiates almost exclusively as a squamous epithelial tissue. In contrast, NOGGIN, an antagonist of BMP2/4, enhances differentiation of endoderm, a vegetal tissue, and promotes differentiation of cells characteristic of the ciliated band, which contains neurogenic ectoderm. These findings support a model in which the balance of BMP2/4 signals produced by animal cell progeny and opposing vegetalizing signals sent during cleavage stages regulate the position of the ectoderm/endoderm boundary. In addition, BMP2/4 levels influence the decision within ectoderm between epidermal and nonepidermal differentiation.

Key words: BMP2, BMP4, Animal-vegetal axis, Ectoderm, NOGGIN

INTRODUCTION

Classic experiments conducted more than 50 years ago demonstrated that the deuterostome sea urchin embryo elaborates its body plan along a maternally determined animal-vegetal (AV) axis and a more labile oral-aboral (OA) axis established after fertilization (Hörstadius, 1973). These were among the early findings in echinoderms that led to the generalization that eggs of many different species possess a maternally determined AV axis (Boveri, 1901), but the molecular basis for this initial asymmetry is not yet understood in any system. Many blastomere recombination experiments led to a model in which opposing animalizing and vegetalizing activities specify fates of cells along the primary A-V axis (for a review, see Runnström, 1975). More recently, Davidson (1989) proposed that cell-fate specification along the AV axis, as well as subsequent differentiation of ectoderm along the OA axis, rely primarily on a vegetal-to-animal cascade of inductive signals during cleavage. Recent work has demonstrated that cell-cell interactions are required along the AV axis, not only to conditionally specify fates during very early stages, but also continuously during early development through gastrulation to maintain those specifications (Ettensohn and McClay, 1988; Hardin et al., 1992; McClay and Logan, 1996).

The molecular pathways underlying these inductive events have remained undefined until recently. Work from several laboratories now provides strong evidence that components of the Wnt signaling pathway, GSK-3β (Emily-Fenouil et al., 1998), β-catenin (Wikramanayake and Klein, 1998; Logan et al., 1999) and TCF/LEF (Vonica et al., 1999; Huang et al., 1999) function during cleavage stages in specification of vegetal tissues, including endoderm and mesenchyme cells. This pathway is an excellent candidate component of the proposed vegetal signaling mechanisms that are initiated by the micromeres, because these cells both contain the highest levels of nuclear β-catenin and constitute the major signaling center. Micromeres have the capacity to induce a second axis if transplanted to ectopic positions (Hörstadius, 1973; Ransick and Davidson, 1993; Logan et al., 1999). The vegetal pole of the sea urchin embryo is therefore functionally similar to the organizing centers of vertebrate embryos that initiate gastrulation, a process that utilizes the same signaling pathway (for recent reviews, see DeRobertis and Sasai, 1996; Ferguson, 1996).

Although Davidson's model proposes that cell fates are specified along the AV axis of the sea urchin embryo primarily by a unidirectional cascade of signals, in other embryos such as those of *Xenopus* and *Drosophila*, positional information regulating cell differentiation along the dorsal-ventral (DV) axis

is controlled by the relative activities of two pathways converging from opposite poles, which lead to the production of BMP4 and its antagonists, NOGGIN and CHORDIN (or SOG) (reviewed by DeRobertis and Sasai, 1996). Here we present the first evidence that BMP2/4 signals also regulate cell fates in echinoderm embryos along the primary AV axis. We show that BMP2/4 is expressed during blastula stages primarily in animal blastomere progeny fated to become ectoderm. Perturbations of BMP2/4 expression shift the relative allocation of cells to ectoderm or endoderm and also affect the differentiation of different ectodermal cell types along the oral-aboral axis. Consequently, these data support a model in which an animalizing influence on cell fates in sea urchin embryos is provided, at least in part, by a BMP2/4 signaling pathway that counterbalances the vegetal-to-animal signaling cascade mediated at least in part by a β-catenin-dependent pathway. This view incorporates aspects of both the double gradient and sequential, unidirectional intercellular signaling theories and further suggests that cell-fate specification along the AV axis of the sea urchin embryo relies on the same pathways used to pattern the DV axes of frogs and flies.

MATERIALS AND METHODS

Isolation of BMP2/4 cDNAs from *Lytechinus variegatus* and *Strongylocentrotus purpuratus*

A 3 kb cDNA encoding the mature ligand region of LvBMP2/4 was obtained by screening a λgt11 cDNA library (Stratagene) representing mid-gastrula RNA sequences with a sequence from the highly conserved region of mature BMP4 that was isolated by RT-PCR with degenerate primers. The *S. purpuratus* homolog was isolated by a similar strategy using early blastula cDNA as a template. This sequence was extended into the less conserved prodomain region by 5′ RACE, using a kit from Gibco/BRL (Bethesda, MD) and sequences from this region were used for specific hybridization in RNase protection and in situ hybridization assays.

Preparation of synthetic mRNAs for microinjection

DNA templates for preparation of XBMP4 (*Xenopus*), LvBMP2/4 (*Lytechinus variegatus*) and NOGGIN (*Xenopus*) mRNAs were constructed as follows: XBMP4 cDNA (1950 bp) and NOGGIN cDNA (740 bp) were inserted into pSP64T at the *Bgl*II site (Dale et al., 1992; Wardle et al., 1999). *Lytechinus variegatus* BMP2/4 (LvBMP2/4) cDNA (3000 bp) was inserted at the *Eco*R1 site of a derivative of pSP64T (Tclone), altered by introducing additional restriction sites between the Sp6 promoter and poly(T) stretch. Capped, polyadenylated mRNAs were synthesized using the message Machine kit (Ambion) and truncated templates (*Xba*I for XBMP4 and LvBMP2/4 and *Eco*RI for NOGGIN). RNAs were purified according to the manufacturer's instructions, resuspended in 30% glycerol and filtered through 0.22 µm filters (Millex GV_4; Millipore, Inc.) prewashed with 30% glycerol. RNA concentrations and quality were analyzed by spectrophotometry and electrophoresis through formaldehyde-containing agarose gels.

Egg injection and embryo immunostaining

Sea urchin eggs, dejellied in pH 4.3 artificial sea water (ASW), were injected with synthetic mRNAs (0.1-0. 2 pg *BMP* or 1-2 pg *Noggin*) in 2 pl of 30% glycerol. Only batches of eggs that yielded embryos with more than about 70% survival and normal development after injection of glycerol alone were analyzed. 3- to 4-day-old embryos were fixed with paraformaldehyde (2% in ASW, 5-10 minutes), washed several times in ASW and fixed again in cold (–20°C) methanol for 15 minutes (Malinda et al., 1995) to minimize deformation of embryos lacking normal skeletons during antibody staining procedures. For anti-Spec1 staining, embryos were fixed with 3-4% paraformaldehyde for 1 hour. Whole embryos were immunostained as follows: embryos were blocked with 5% goat serum or 3% BSA in ASW for 20 minutes, incubated for 1 hour with primary antibody: anti-Endo1 (1:10), anti-EctoV (1:10), anti-Spec1 (1:500) or anti UH2-95 (1:10), then washed three times in 5% goat serum in ASW, incubated with secondary antibodies conjugated to either fluorescein (goat anti-rabbit, 1:250; Zymed), Cy3 (goat anti-mouse, 1:500; Jackson Labs) or horseradish peroxidase (goat anti-mouse: 1:1000; Zymed) for 1 hour, and washed with ASW three times. Control immunostains with secondary antibody alone were negative. Most antibodies recognized epitopes in both species, but in our hands Spec1 and UH2-95 antibody stainings were only successful in *S. purpuratus* and *L. pictus*, respectively. Embryos were examined with a Nikon Diaphot TMD inverted microscope and photographed using Kodak Ektochrome P1600 Color Reversal or Elite 400 film with either differential interference contrast or fluorescence optics.

SpBMP2/4 RNase protection assays

Total RNA from embryos at selected stages was hybridized as described previously with two antisense RNA probes simultaneously (Gagnon et al., 1992): (1) a 559 nt sequence representing the prodomain sequence of SpBMP2/4 (2×10^5 dpm/ng) and (2) as a load control, a 360 nt transcribed ribosomal spacer sequence (1×10^4 cpm/ng) (Angerer et al., 1992).

SpBMP2/4 in situ hybridization

Adjacent 5 µm sections of *S. purpuratus* embryos at selected stages of development were hybridized as described previously (Angerer et al., 1987) with ^{33}P-labeled RNAs representing either the prodomain of SpBMP2/4 or the Spec2A sequence described previously (Hardin et al., 1988). The specific activities of the Spec2a (220 nt) and SpBMP2/4 (559 nt) probes were 2×10^5 and 3×10^5 dpm/ng, respectively. Stringent post-hybridization washes were a pre-RNAse incubation in 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA at 60°C for 10 minutes (approx. T_m–10°C) and a post-RNase incubation in 0.1×SSC at 50°C for 5 minutes.

RESULTS

Sea urchin BMP2/4 sequence

The inferred sequences of *Lytechinus variegatus* and *Strongylocentrotus purpuratus* BMP cDNAs are shown in Fig. 1. Searches using either prodomain or mature ligand region to query databases show that in each case the most similar sequences are vertebrate BMP2 and BMP4. The mature LvBMP2/4 sequence is 88% and 89% identical (asterisks above the first three sequences; 94% and 95% similar) to XBMP2 and XBMP4 sequences, respectively. Identity between the two sea urchin sequences is illustrated by vertical bars; the degree of identity is high not only in the ligand region but also in the generally poorly conserved prodomain region, indicating that these genes are homologous.

Sequence comparisons do not establish whether the sea urchin sequence is more closely related to BMP2 or to BMP4. Functional assays also do not distinguish between these peptides, since injection of mRNAs encoding any of them into *Xenopus* embryos causes ventralized phenotypes (LvBMP2/4: F. Wardle and L. Dale, unpublished observations; *Xenopus* BMP2: Clement et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; BMP4: Dale et al., 1992; Jones et al., 1992). Consequently, we refer to the sea urchin sequences as

LvBMP2/4 and SpBMP2/4. It is possible that duplication of the ancestral gene occurred near the time of divergence of echinoderms and chordates.

BMP2/4 mRNA accumulation is developmentally regulated

To determine the temporal expression pattern of BMP2/4 mRNA accumulation, RNase protection assays were carried out with RNAs isolated from *S. purpuratus* embryos of selected developmental stages. Labeled RNA encoding all but the ligand region of SpBMP2/4 sequence shown in Fig. 1 was used as probe. To control for load and recovery of RNase-resistant fragments, a probe representing ribosomal transcribed spacer was included in the reactions. The expected pattern was obtained with this control probe (Angerer et al., 1992), demonstrating that relative signals among samples obtained with the SpBMP2/4 probe are accurate. The results show that SpBMP2/4 transcripts are not detectable in ovaries or in mature eggs and first appear after cleavage, at about the 200-cell stage (14 hours; Fig. 2). mRNA levels decrease during gastrulation and are very low in the pluteus larva. Based on comparisons with similar assays of other target mRNAs whose abundances are known, we estimate that BMP4 mRNAs are rare, ≤5 copies/cell on average.

BMP2/4 mRNAs are spatially restricted in sea urchin blastulae

The distribution of BMP2/4 mRNAs was determined by in situ hybridization using the nonconserved prodomain sequence labeled with ^{33}P as a probe to achieve the high level of sensitivity required to detect these rare mRNAs. In agreement with the RNase protection data, BMP2/4 mRNA signals were only detectable above background during blastula and early gastrula stages; background levels were provided by sections of eggs and cleaving embryos which do not express detectable BMP2/4, as shown in Fig. 2. Signals were highest in the post-hatching blastula, whose AV axis can be identified by primary mesenchyme cells ingressing from the vegetal plate (veg; Fig. 3A,C). Fig. 3A,B and C,D illustrate two early mesenchyme blastulae in which the plane of sectioning passes close to this axis and the signal is located primarily over presumptive ectoderm cells. In most embryos at this stage of development, the grain densities are clearly higher on one side of the ectoderm (Fig. 3B and D, right side) and close to background level in the vegetal region. In some embryos, the restriction of signal to one side of the embryo is more pronounced (Fig. 3C,D), as is also observed later at mesenchyme blastula/early gastrula stages (Fig. 3E,F). Although the SpBMP2/4 mRNA signals are reduced in these older embryos, the same pattern is evident.

To determine whether BMP2/4 RNA concentration differences align with different ectoderm regions, we compared its distribution with that of Spec2A mRNA at mesenchyme blastula stage, which accumulates primarily in aboral ectoderm cells (ab) (Hardin et al., 1988). The four consecutive 5 µm sections (Fig. 3G-J), which were alternately hybridized with BMP2/4 and Spec2A probes, show that their spatial patterns of expression are largely reciprocal. This is also true at earlier stages, although the identification of presumptive aboral ectoderm is less clear because the Spec2A signal is lower and the distributions of the two mRNAs appear to be partially overlapping (not shown). We conclude that BMP2/4 mRNAs accumulate in ectoderm and are enriched on the oral side of the blastula.

Microinjection of XBMP4 or LvBMP2/4 mRNA into the one-cell zygote suppresses vegetal tissue differentiation and promotes epidermal cell fates in sea urchin embryos

To ask if perturbations of BMP4 signaling can alter cell fates

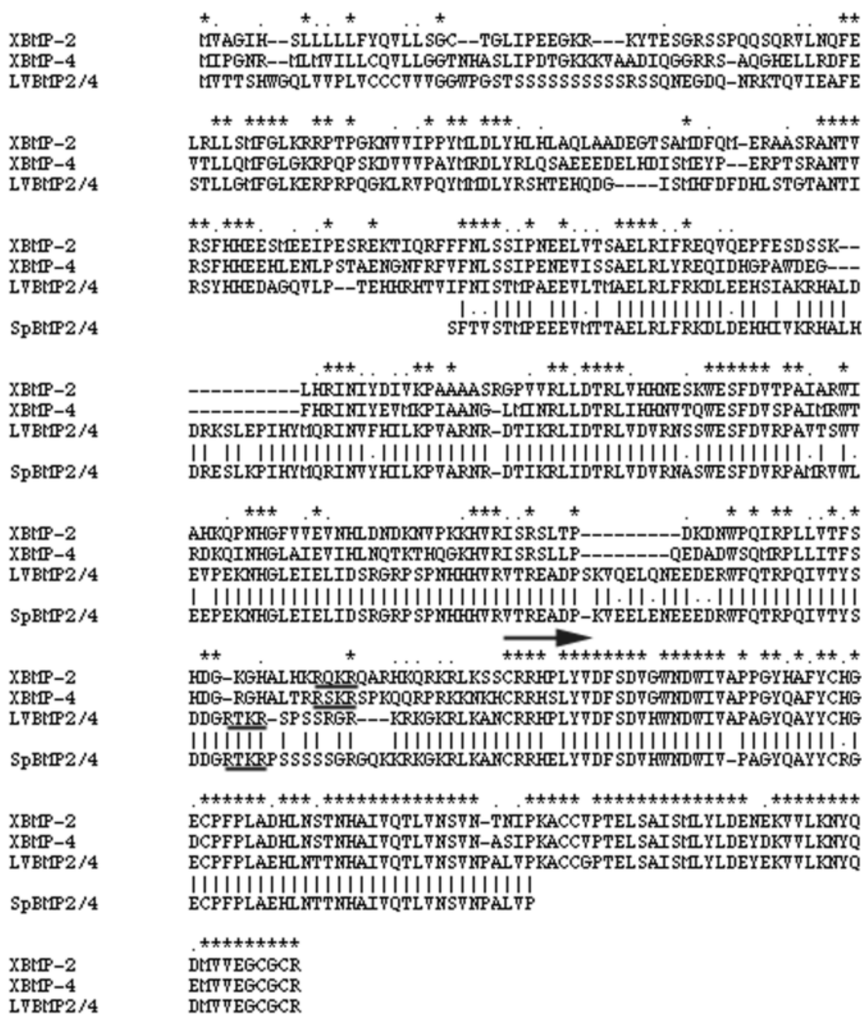

Fig. 1. Alignment of *Xenopus laevis* BMP2, BMP4 and LvBMP2/4 and a portion of SpBMP2/4 using ClustalW. Asterisks denote amino acid identity among the first three sequences and dots indicate similarities. Alignment of the two sea urchin sequences, LvBMP2/4 and SpBMP2/4, shows a high degree of similarity in both the nonconserved prodomain sequence upstream of the putative processing site (underlined) and the mature ligand domain downstream of the arrow. GenBank accession numbers of LvBMP2/4 and SpBMP2/4 sequences are AF119712 and AF119713, respectively.

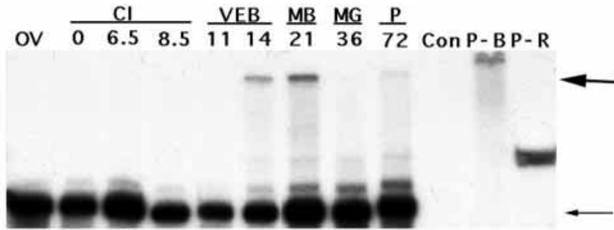

Fig. 2. SpBMP2/4 transcripts accumulate in sea urchin embryos transiently after cleavage stages. 10 µg of total RNA from ovary (OV), eggs (0) and embryos of selected developmental stages in hours post-fertilization (Cl, cleavage; VEB, very early blastula; MB, mesenchyme blastula; MG, midgastrula; P, pluteus) or control yeast tRNA (Con) were hybridized to a mixture of ^{32}P-labeled riboprobes containing either prodomain SpBMP2/4 sequence (BMP-P) or, as a load control, ribosomal transcribed spacer (RTS) sequence. Unhybridized probes are shown in the two right-hand lanes (P-B, P-R). The relative RTS signals (lower arrow) are as previously reported (Angerer et al., 1992); SpBMP4 signals (upper arrow) are highest in blastulae, absent in gastrulae and very low in plutei.

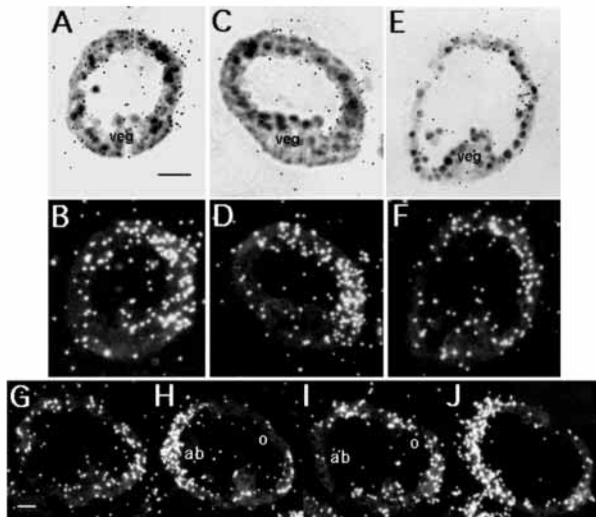

Fig. 3. In situ hybridization analysis with ^{33}P-labeled riboprobes shows that SpBMP2/4 RNAs are enriched in oral ectoderm. (A-F) Antisense RNA representing sequence from the prodomain of SpBMP2/4 was hybridized to sections of hatched blastula (A/B, C/D) or mesenchyme blastula (E/F); sections were autoradiographed for 3.5 weeks. Embryos are oriented with the vegetal pole (veg) down. (G-J) Alternate 5 µm sections of a mesenchyme blastula hybridized with antisense SpBMP2/4 (G,I) or Spec2A (H,J) riboprobes. Bars, 10 µm.

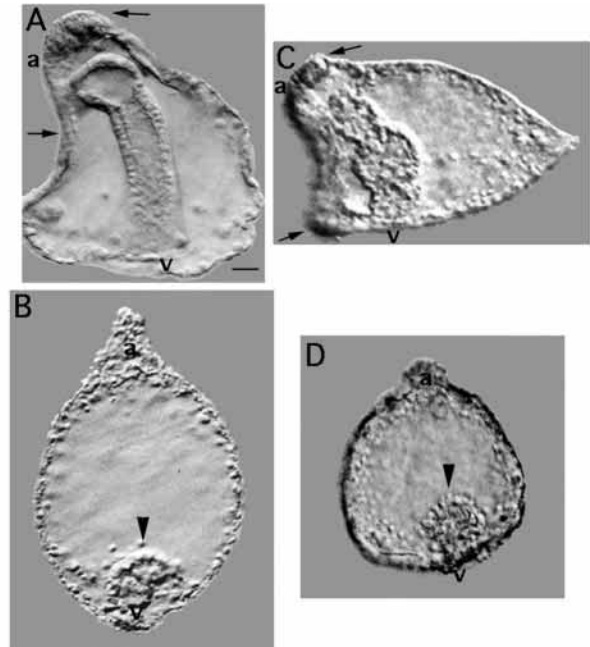

Fig. 4. Alterations in cell fate allocation resulting from injection of XBMP4 mRNA into sea urchin embryos. Glycerol (controls; A,C) or synthetic xBMP4 mRNA (B,D) was injected into eggs of either *L. pictus* (A,B) or *S. purpuratus* (C,D), which were then cultured for 3 days, corresponding to normal pluteus stage. (B) *L. pictus* embryos injected with XBMP4 mRNA are characterized by a reduced archenteron (arrowheads), absence of ectodermal polarization and extrusion of a small number of cells from the animal pole. (D) *S. purpuratus* embryos exhibit similar morphology, but have less elongation along the animal (a)-vegetal (v) axis. Arrows in A and C indicate the ciliary band. Bar, 15 µm.

during sea urchin embryogenesis, we first tested the effect of injecting either *Xenopus* BMP4 or LvBMP2/4 synthetic, capped, polyadenylated mRNA into eggs of *Lytechinus pictus* (Lp) or *Strongylocentrotus purpuratus* (Sp). 3 days after fertilization the phenotypes of XBMP4 mRNA-injected embryos (Fig. 4B,D) and glycerol-injected control embryos (Fig. 4A,C) were compared. Control embryos developed normally and contained a complete tripartite gut, two triradiate spicules elongated to form the larval skeleton (not shown), differentiated secondary mesenchyme derivatives and oral and aboral squamous epithelia separated by the ciliated band (Fig. 4A,C, arrows), a narrow strip of thickened cuboidal ectoderm that contains neural cells.

In contrast, the morphologies of embryos injected with either LvBMP2/4 or XBMP4 mRNA were indistinguishable (data not shown). Both are severely and reproducibly altered at this stage. Similar defects were obtained at doses ranging from approximately 5,000 to 30,000 of either LvBMP 2/4 or XBMP4 mRNA molecules (concentrations corresponding to those of rare to moderately rare endogeneous sea urchin embryo mRNAs) and with embryos of both species, the phenotype being slightly more severe in *L. pictus* embryos. The two major defects are (1) expansion of ectoderm that is squamous epithelial in character and (2) marked reduction of the gut located on the vegetal (v) side of the embryo (arrowhead, Fig. 4B, Lp; Fig. 4D, Sp). These embryos also lack morphological polarity in the ectoderm along the OA axis and no ciliated band is visible. A few cells form a narrow outpocketing at the animal pole (a), which would normally differentiate as the ciliated oral hood; this structure is especially pronounced in *L. pictus* embryos (Fig. 4B). Ectoderm differentiation is most sensitive to BMP4 misexpression since, at very low doses of mRNA injected, some embryos have a nearly normal archenteron but radialized squamous epithelium. The BMP4 phenotypes depend on the presence of a signal sequence since injection of mRNA encoding a XBMP4 peptide lacking amino acids 2-18 results

in normal development of both *L. pictus* and *S. purpuratus* embryos (data not shown). These results suggest that the phenotype obtained with mRNA encoding wild-type BMP4 depends on the peptides entering the secretory pathway.

The differentiation of different cell types in BMP4 mRNA-injected embryos was assayed with a panel of well-characterized antibodies (Fig. 5). All the embryos are oriented as in Fig. 4, with the animal pole at top and the vegetal pole at bottom; in glycerol-injected embryos, oral ectoderm is to the left and aboral ectoderm to the right. The major morphological defect appears to be a reduction in the number of endoderm cells that have invaginated into the blastocoel. Nevertheless, these cells express gut markers that appear relatively late in morphogenesis after gastrulation is complete. Furthermore, despite its greatly reduced size, the archenteron is patterned. For example, Endo1 antibody, which is specific for midgut and hindgut (Wessel and McClay, 1985; Fig. 5A,B), stains a small cluster of invaginated cells near the blastopore, although at a reduced level (Fig. 5C,D); EctoV antibody, a marker for foregut as well as oral ectoderm, which includes the stomodeum (s) and ciliated band (cb) (Coffman and McClay, 1990; Fig. 5E,F), stains adjacent cells distal from the blastopore in XBMP4 mRNA-injected embryos (Fig. 5G,H). Endo1 stains only invaginated cells, indicating that it is not the case that endoderm cells differentiate but fail to invaginate in these embryos. As discussed below, all of the cells that remain outside express the epithelial marker, Spec1 (Fig. 5K,L), suggesting that they are specified as ectoderm rather than endoderm. We conclude that late patterning of the archenteron occurs, despite suppression in the number of endoderm cells contributing to these tissues. This is consistent with the observations of McClay and Logan (1996) that ectoderm cells can be respecified at late stages to different gut cell fates upon transplantation or when part of the gut is removed.

Molecular assays also demonstrate that BMP4 mRNA injection alters patterning of ectoderm along the OA axis. In normal embryos, the ectoderm consists of two regions containing squamous epithelial cells, the oral facial epithelium and the aboral ectoderm, segregated by the thickened ciliated band of cuboidal cells that is specified via cell-cell interactions during gastrulation (Cameron et al., 1993). We have followed the differentiation of these cell types in BMP4 mRNA-injected embryos using three antibodies, α-Spec1, EctoV and UH2-95. Spec1 protein is a marker for epithelial regions of the ectoderm, both facial and aboral, at blastula/gastrula stages and becomes highly enriched in the aboral ectoderm at late stages (Carpenter et al., 1984; this paper, Fig. 5J). EctoV is a late marker for oral ectoderm. This epitope is found in both cuboidal and squamous epithelial cell types and appears at highest concentrations in cells of the ciliated band (Fig. 5F, CB) and around the stomodeum (Fig. 5F, S), and at lower levels in the facial epithelium that extends from the stomodeum to the ciliated band (Coffman and McClay, 1990; Fig. 5E). Thus, EctoV and Spec1 probes identify largely reciprocal regions of late embryo ectoderm. Lastly, the UH2-95 monoclonal antibody recognizes an epitope concentrated in the cuboidal ectoderm of the ciliated band, which also contains some neural cells (Adelson, 1985; Wikramanayake and Klein, 1997; Fig. 5M).

BMP4 mRNA-injected embryos stain strongly for Spec1 (Fig. 5L) at levels usually exceeding those in normal embryos (Fig. 5J). In contrast, the ectoderm of these embryos stains only weakly with anti-EctoV (Fig. 5H; the labeled cells in this embryo are in the invaginated endoderm, as described above). Finally, only a few BMP4 mRNA-injected embryos contain any UH2-95-positive cells. In these embryos, the few UH2-95-labeled cells in the ectoderm are located exactly at the animal pole (Fig. 5N, arrowhead). [In some embryos the mid/hindgut region also is labeled; the hindgut of some control embryos is also labeled at low levels (Fig. 5M, arrowhead). In both cases, this signal is of variable intensity and observed in only a small percentage of embryos; its relation to the signal in ciliated band is not known.] These results confirm the absence of the ciliated band in the ectoderm of BMP4 mRNA-injected embryos and support the observation that the ectoderm in these embryos is almost exclusively squamous epithelium.

In addition to fewer endoderm cells, BMP4 mRNA-injected embryos contain fewer pigment cells, another vegetal derivative. Because the number of pigment cells/embryo was highly variable, this observation is illustrated by the histogram shown in Fig. 6. On average, the number was about half that of glycerol-injected embryos (cf. red and black bars) and remains reduced even after prolonged embryo culture. Similar decreases are likely to occur among other secondary mesenchyme cell types, such as circumesophageal muscle and blastocoelar cells, but this has not been verified.

In contrast to the alterations in ectoderm, endoderm and pigment cell mesenchyme in BMP4 mRNA-injected embryos, the development of primary mesenchyme cells (PMCs) is relatively normal. The last round of PMC division is delayed, but the eventual number of these cells is essentially unchanged. They differentiate normally, producing skeletal spicules and expressing a PMC-specific epitope (Fig. 7A). The accompanying drawing illustrates that this embryo is oriented with the animal pole tilted back about 45° to illustrate the vegetal ring of PMCs that are evenly spaced. Ventrolateral PMC clusters characteristic of normal embryos at this stage do not form and, as a consequence, the spicules are straight (Fig. 7B, STR), not triradiate, and often their number is less than 2 (R<2). As shown in Fig. 7C, the ring of PMCs tends to be positioned more vegetally (–), consistent with expansion of the animal region (Fig. 7C, red bars). In this experiment, the position of the ring in XBMP4 mRNA-injected embryos was compared to that in normal sibling embryos at gastrula stage. Injection of approximately 15,000 molecules of XBMP4 mRNA causes a vegetal shift of the PMC ring in 80% of the embryos. This phenomenon has been observed in all experiments, but in some batches of eggs it was clearly detectable in a lower percentage of embryos (35-80%).

The phenotypes of NOGGIN- and BMP4 mRNA-injected embryos are largely reciprocal

It is likely that the primary defects elicited by injection of BMP4 mRNA, i.e. suppression of vegetal differentiation and cuboidal ciliated band ectoderm, result from a combination of precocious and ectopic BMP4 expression, since endogenous SpBMP2/4 mRNA is not detectable until the blastula stage and is more concentrated in presumptive oral ectoderm. It was therefore of interest to determine the effects of loss of BMP2/4 function during early sea urchin embryogenesis. NOGGIN has been shown to bind BMP4 (Zimmerman et al., 1996) and antagonize its function in *Xenopus* embryos and to inhibit signaling by the *Drosophila* homologue, DPP (Holley et al.,

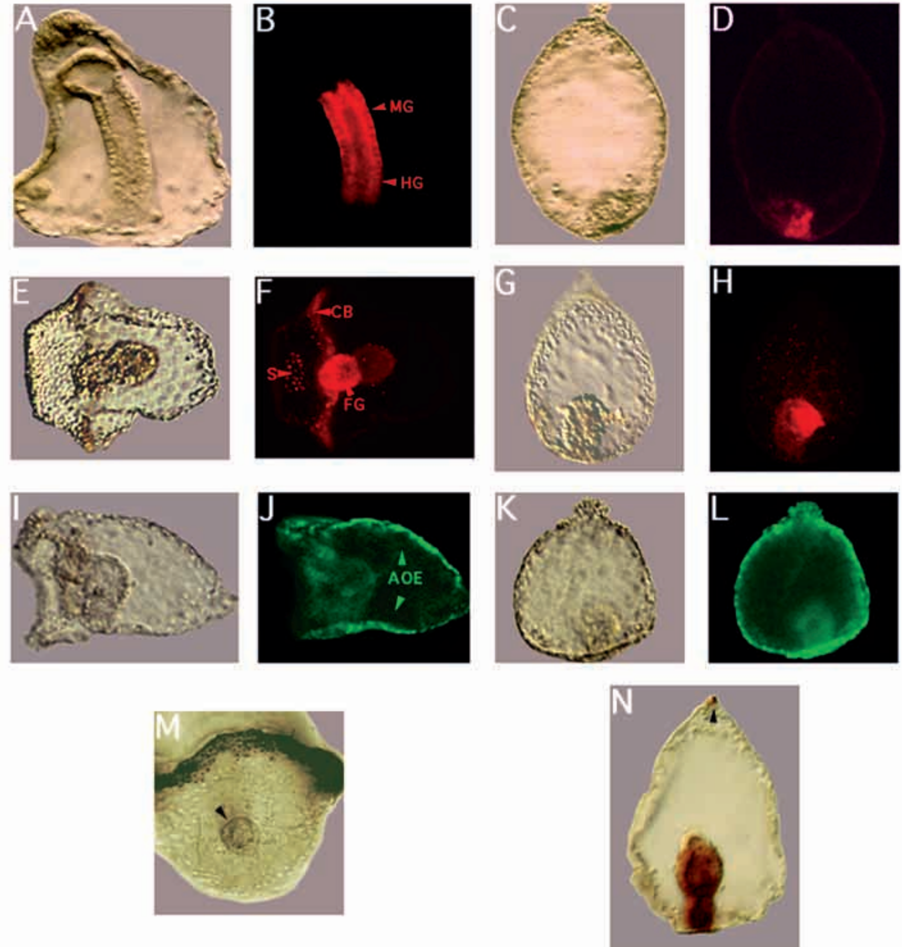

Fig. 5. Staining of tissue-specific antigens in glycerol- and XBMP4 mRNA-injected embryos. Normal plutei (A,E,I,M, Nomarski; B,F,J, epifluorescence) and XBMP4 mRNA-injected embryos of the same age (C,G,K,N) were stained with antibodies having the indicated tissue specificities in normal embryos; in M and N, a horseradish peroxidase-coupled secondary antibody was used. (B,D) Endo1 staining, mid- and hindgut (B, arrowheads). (F,H) EctoV staining, foregut (FG), ciliated band (CB) and stomodeum (S). (J,L) Spec1 staining of the aboral ectoderm (J, AOE) of a normal pluteus (I) and an XBMP4 mRNA-injected embryo (K,L). (M,N) UH2-95 staining. The ciliated band is the predominantly labeled region in normal embryos (M); variable levels of staining are observed in the hindgut of some embryos (arrowhead). The XBMP4 mRNA-injected *L. pictus* embryo (N) contains several positive cells at the animal pole (arrowhead) and exhibits the highest level of gut staining observed for any embryo. In some batches of both normal and XBMP4-expressing embryos, the gut is not labeled (see text).

1996). Because sea urchin BMP2/4 is so similar in sequence and function to XBMP4 (Fig. 1), we reasoned that *Xenopus* NOGGIN might also interfere with its function.

As shown in Fig. 8, in NOGGIN mRNA injected-embryos, the gut is enlarged and either protrudes through the animal pole ectoderm (top) [70-80% of *S. purpuratus* (Fig. 8A) and all *L. pictus* (Fig. 8C) embryos] or everts as the result of exogastrulation, a defect classically associated with vegetalization (Fig. 8E; 20-30% of *S. purpuratus* embryos). Staining with Endo1 (Fig. 8B; mid- and hindgut) and EctoV (Fig. 8D; foregut and oral ectoderm) antibodies shows that these embryos accumulate both markers in the appropriate regions of the expanded archenteron to levels comparable to those in normal embryos (see Fig. 5B and F, respectively).

In contrast, the number of pigment cells that are derived from blastomeres located more vegetally than endoderm precursors does not increase in NOGGIN mRNA-injected embryos (cf. the distributions indicated by the blue and black bars in Fig. 6). This is the expected result if there is little or no endogenous BMP2/4 in this region of the embryo, which is consistent with its mRNA distribution (Fig. 3).

The number of PMCs derived from vegetal-most blastomeres also does not change, but skeletogenesis is altered. The number of spicules increases from 2 to as many as 8, and these are almost always small and triradiate (Fig. 7B; I(>2)). The fact that the spicules are spaced uniformly in a ring indicates that the

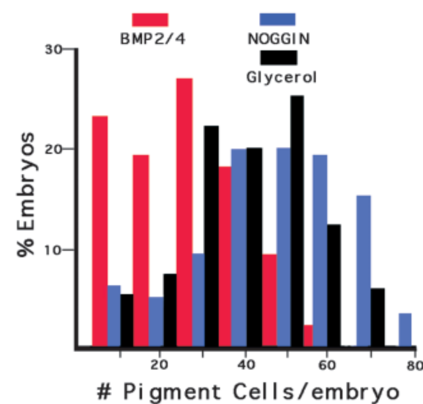

Fig. 6. Comparison of pigment cells/embryo in embryos injected with XBMP4 mRNA (red bars), glycerol (black bars) and NOGGIN mRNA (blue bars). For each sample, 100 embryos were analyzed.

ectoderm in NOGGIN mRNA-injected embryos also lacks oral-aboral polarity. The position of the PMC ring in NOGGIN mRNA-injected embryos often shifts toward the animal pole, an effect that is reciprocal to that elicited by XBMP4. This is most clearly demonstrated by the experiment shown in Fig. 7C. When a constant number of XBMP4 mRNAs (15,000) are coinjected with increasing doses of NOGGIN mRNA (from

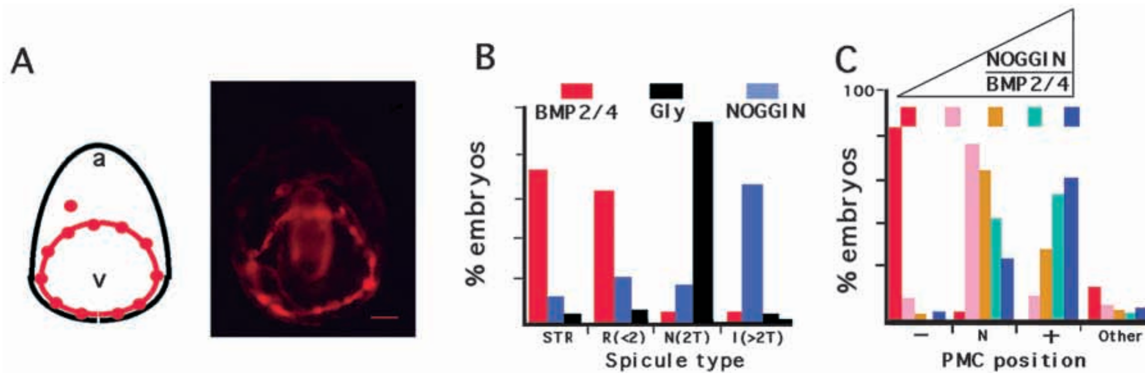

Fig. 7. (A) Immunostaining of XBMP4 mRNA-injected embryos with 6e10 monoclonal antibody (a kind gift from Dr C. Ettensohn), which recognizes primary mesenchyme cells (PMC). (A) As indicated by the diagram (left), this embryo is tilted back approximately 45° to allow visualization of the vegetal ring of PMCs, which are uniformly distributed, reflecting the radialized ectoderm in these embryos. Bar, 20 µm. (B) Spiculogenesis is altered in XBMP4- and XNOGGIN -expressing embryos (red and blue, respectively) compared to glycerol-injected controls (black). Three major defects are tabulated from four separate experiments, each including approximately 100 *L. pictus* or *S. purpuratus* embryos. Embryos exhibiting spicules of more than one morphology have been included in each category. STR, spicules that are primarily straight, but sometimes contain irregular small branches; R (<2), embryos containing either 0 or 1 spicule; N (2T), two normal triradiate spicules; I (>2T), an increased number of triradiate spicules. (C) The position of the PMC ring of gastrula-stage embryos injected with approximately 15,000 XBMP4 mRNA molecules (red bars) or coinjected with increasing NOGGIN mRNA molecules: 0 (red), 7000 (pink), 14,000 (orange), 35,000 (green), 70,000 (blue). N, the normal position (±5% of the distance between animal and vegetal poles) of the ring along the animal-vegetal axis; –, a more vegetal position and +, a more animal position.

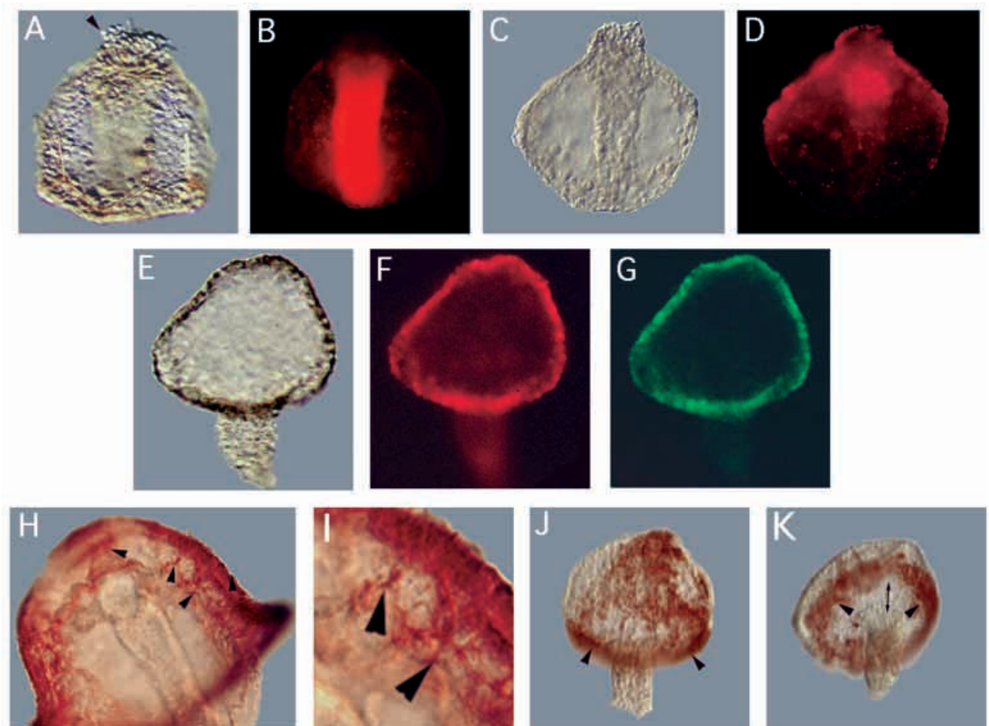

Fig. 8. Morphological and cell-fate alterations resulting from injection of NOGGIN mRNA into sea urchin 1-cell embryos. (A,C,E,I,J) DIC images of embryos expressing NOGGIN. Embryos are oriented with animal pole at top and vegetal pole at bottom. *L. pictus* embryos (A-C) are radialized and have enlarged guts that protrude at the animal pole (arrowhead); *S. purpuratus* embryos (E-G) are similarly affected but exogastrulate more frequently. (B) Endo1 is expressed in the enlarged mid- and hindgut. (C,F) EctoV is expressed in the foregut, and at increased levels in the ectoderm. (G) Expression of Spec1 is variable, but usually similar or less than observed in normal embryos. (H-K) UH2-95 epitope, expressed in ciliated band of normal embryos including some neuron-like cells [arrowheads, H, I (higher magnification)], and distributed in patches of cells throughout the ectoderm of NOGGIN-expressing embryos (J) and/or in a widened, irregular band (K). Arrowheads in J and K refer to UH2-95-positive cells that are located near the vegetal pole, often separated by only a few unlabeled cells (double-headed arrow, K).

7000 to 70,000 molecules), the PMC ring shifts toward the animal pole (+). These observations suggest that the position of the ectodermal cues that specify PMC location along the AV axis is sensitive to changes in BMP4 levels.

The radialized ectoderm of NOGGIN-expressing embryos does not expand and stains intensely with anti-EctoV (Fig. 8D). Staining with anti-Spec1 antibody (Fig. 8G) is positive, but reduced compared to BMP4 mRNA-injected embryos and sometimes less than in normal embryos. Of particular interest is the observation that the number of ectoderm cells expressing the ciliated band epitope is increased and their distribution reflects the lack of O-A ectoderm polarity in these embryos. Labeled cells can be found in variably sized patches dispersed throughout the ectoderm (Fig. 8I) instead of the single

continuous narrow band of cells found in normal embryos (Figs 8H or 5M). In addition to dispersed clusters of positive cells, many embryos also have a band of stained cells that is wider than the normal ciliated band (Fig. 8H,I, arrowheads). The orientation of this band is often orthogonal to the AV axis (Fig. 8J) and variable in position along the axis among embryos, ranging from close to the vegetal border to equatorial. In the most severe phenotypes, much of the ectoderm stains (Fig. 8I) except cells close to the vegetal blastopore (Fig. 8J, double arrow). Examination of embryos doubly stained for PMCs and ciliated band shows that the PMC ring is located either immediately adjacent to the UH2-95-positive region in mildly affected embryos or within it in severely affected embryos (data not shown). We conclude that, in the ectoderm, NOGGIN causes differentiation of ectopic ciliated band cells and alters the location of PMC positioning cues. Because the ciliated band marker is expressed by at least some of the embryo's nerve cells (Wikramanayake and Klein, 1997; indicated by the arrowheads in Fig. 8H), it is possible that a neurogenic cell population also is expanded in NOGGIN-expressing sea urchin embryos. Similar effects have been observed by expressing NOGGIN in both *Xenopus* and *Drosophila* embryos (Lamb et al., 1993; Holley et al., 1996).

The effects of misexpressing BMP4 and NOGGIN in sea urchin embryos are largely reciprocal in most tissues, except the PMCs, whose fate does not depend on interactions with other blastomeres of the embryo. These observations strongly support the interpretation that *Xenopus* NOGGIN can reduce the level of active endogenous sea urchin BMP2/4 and that maintenance of correct BMP signaling levels is critical for normal allocation of cell fates along the AV axis and within the ectoderm.

DISCUSSION

In this report, we have provided evidence that a sea urchin BMP gene (LvBMP2/4) closely related to vertebrate BMP2 and BMP4 is active in the progeny of animal blastomeres during blastula and early gastrula stages when the fates of these cells are being determined. Perturbation of BMP signaling levels by injection of mRNAs encoding either LvBMP2/4 or *Xenopus* BMP4 or that of a BMP4 antagonist, NOGGIN, causes specific alterations in differentiation of ectodermal cell types, in oral/aboral ectodermal polarity and in the allocation of cells to ectoderm versus endoderm territories. In all these regions of the sea urchin embryo, cell-fate specification depends heavily on cell-cell interactions; in contrast, the cells whose differentiation is unaffected by perturbing BMP4 signaling are the maternally determined skeletogenic PMCs, derived from vegetal micromeres that differentiate cell autonomously. Together these observations support the conclusion that a BMP4 signaling pathway is important in mediating communication among cells that regulates cell fate decisions.

Ectoderm differentiation

Within the ectoderm, we have detected and characterized two major alterations when BMP4 or NOGGIN mRNA is injected: (1) a change in the allocation of cells to different cell types and (2) a loss of polarity along the oral/aboral axis. BMP2/4 mRNA injection results in embryos whose ectoderm is almost exclusively squamous epithelium, while reduction of BMP2/4 by NOGGIN elicits differentiation of ectopic cuboidal, ciliated band cells. Since the ciliated band includes the neurogenic cells of the embryo that are also recognized by UH2-95 (Wikramanayake and Klein, 1997), it is likely that BMP signaling is required for epidermal cell differentiation, while reduced levels are required for neural cell development, as is observed in other embryos, e.g. ascidians (Miya et al., 1997) or vertebrates (reviewed by Hemmati-Brivanlou and Melton, 1997; Pera et al., 1999).

In both BMP4 and NOGGIN mRNA-injected embryos, the oral/aboral polarity of the ectoderm is absent. PMCs, which are normally positioned by ectodermal cues (Ettensohn, 1990) are distributed radially rather than clustering on the oral side of the embryo, and the embryos are morphologically symmetric about the AV axis. In NOGGIN mRNA-injected embryos, the ciliated band is frequently reoriented or clusters of cells are randomly dispersed. The facts that both exogeneously supplied BMP4 and sequestration of endogeneous ligand lead to loss of polarity suggest that a difference in BMP signaling levels is required to generate or maintain oral/aboral polarity. Therefore, the higher concentration of BMP2/4 mRNA on the oral side of the ectoderm may be relevant in establishing or maintaining oral/aboral axis specification.

The sea urchin BMP2/4 gene is preferentially expressed in presumptive ectoderm, and within it on the oral side, a region that contains several cell types, including the squamous facial epithelium and the ciliated band. Levels of BMP2/4 mRNA are much lower in aboral ectoderm, a squamous epidermal tissue. Suppression of BMP2/4 signaling via NOGGIN mRNA injection leads to an excess of cuboidal ciliated band cells and a transfating of many presumptive epidermal cells to ciliated band fate. The simplest explanation of these results is that, in the normal embryo, higher local BMP2/4 signaling levels promote epithelial fate while lower levels lead to differentiation of ciliated band. If the concentration of BMP2/4 protein reflects the distribution of its mRNA in the embryo, then this simple model is not sufficient to explain why the ciliated band is restricted to a narrow band of cells at the oral-aboral border. BMP2/4 signaling might be sufficient on the oral side to restrict the domain of the ciliated band, but processes or factors other than or in addition to BMP2/4 must function on the aboral side. It is clear that the molecular phenotypes of oral and aboral epidermal cells are different, as is their requirement for vegetal signals (Wikramanayake and Klein, 1997). One of these signals is likely to depend on nuclear β-catenin since treatments of animal caps that promote its function as a transcription factor induce aboral ectoderm marker expression and oral-aboral polarity, including an intervening narrow, ciliated band (Wikramanayake and Klein, 1997, 1998).

It is not yet clear whether BMP4 mRNA injection affects sea urchin embryo ectoderm differentiation directly or indirectly by altering vegetal cell functions. Evidence supporting a direct effect is our observation that ectoderm mispatterning occurs at very low BMP4 doses that have little, if any, detectable effect on vegetal development. Similarly, the influence of BMP4 on epidermal differentiation in *Xenopus* appears to be direct. For example, ectopic administration of BMP4 to dissociated *Xenopus* animal cap cells can shift them from a neuralized fate to an epithelial phenotype (Wilson and Hemmati-Brivanlou, 1995). Conversely, expression of dominant negative versions of *Xenopus* BMP4 in animal caps promotes neural fates

(Hawley et al., 1996). We cannot, however, exclude the possibility that, in BMP4-expressing sea urchin embryos, vegetal inductive signals required for differentiation of ectoderm cell types may be reduced. Wikramanayake and Klein (1997) have argued from studies on embryoids derived from 8-cell animal blastomere tiers that differentiation of squamous epithelium and neurogenic ectoderm require both positive and negative signals from vegetal cells.

The ectoderm/endoderm border

BMP4 and NOGGIN misexpression cause reciprocal shifts in the allocation of cell fates along the animal-vegetal axis. BMP4 compresses the endodermal field and the ectoderm/endoderm border shifts vegetally, as does the PMC ring, whereas NOGGIN has the opposite effects. This argues that precisely controlled levels of BMP signaling are required for correct patterning along the AV axis of sea urchin embryos, as is the case for the DV axes of both *Drosophila* and *Xenopus* embryos. In the latter systems, BMP4 is thought to behave as a morphogen, and that the fates cells adopt are sensitive to BMP4 concentration (Ferguson and Anderson, 1992; Dosch et al., 1997). Although, in *Xenopus* embryos, the most thoroughly studied effects of BMP signaling are on dorsal-ventral patterning of mesoderm, and epidermal versus neural specification of ectoderm, BMP4 also affects endoderm differentiation, since either CHORDIN or NOGGIN can induce expression of an endodermal marker in animal caps (Sasai et al., 1996). In normal sea urchin embryos, the ectoderm/endoderm boundary is not conditionally specified until late blastula stages and not determined until late gastrula stage. This decision depends on cell-cell signaling, since the boundary does not conform to lineage boundaries and can regulate after removal of the archenteron (Logan and McClay, 1997). Here we show that BMP2/4 expression occurs in progeny of animal blastomeres at an appropriate time to participate in ectoderm/endoderm boundary decisions.

Both loss- and gain-of-function experiments have recently demonstrated that a nuclear β-catenin-dependent pathway functions in endoderm specification in sea urchin embryos and also plays a key role in establishing the ectoderm/endoderm boundary (Emily-Fenuoil et al., 1998; Wikramanayake and Klein, 1998; Logan et al., 1999; Vonica et al., 1999; recently reviewed in Davidson et al., 1998; Angerer and Angerer, 2000). In the unperturbed embryo, β-catenin enters nuclei of vegetal cells in a vegetal-to-animal wave beginning with the micromeres at the 16-cell stage, progressing to the veg_2 tier at the 64-cell stage and finally to a vegetal subset of veg_1 progeny. It is among veg_1 derivatives that the position of the ectoderm-endoderm boundary is finally determined. Thus, sea urchin BMP2/4 is likely to function after the primary vegetal signaling center becomes active and during the transition from conditional specification of the ectoderm/endoderm boundary to its irreversible determination, which is a gradual process during blastula and gastrula stages of development (Logan and McClay, 1997). This is similar to the case in *Xenopus* embryos, in which BMP4 signaling functions during gastrulation, after specification of the dorsal/anterior organizing center (Jones et al., 1996).

The range of sea urchin BMP2/4 signaling is not known, but our results suggest that it probably does not extend to the secondary mesenchyme lineages. BMP2/4 mRNA is only detectable in nonvegetal blastomeres and reduction of endogenous BMP2/4 via NOGGIN mRNA injection has no effect on the number of pigment cells, which are derived from the more vegetal regions of veg_2 blastomeres. Consistent with this interpretation, misexpression of BMP4 in vegetal cells by means of mRNA injection at the 1-cell stage reduces the number of pigment cells on average about twofold. In contrast, increasing the level of nuclear β-catenin, which normally is restricted to vegetal blastomeres, results in a large increase in the number of pigment cells (Wikramanayake and Klein, 1998) by diverting presumptive ectoderm to vegetal fates. An interesting possibility is that β-catenin in vegetal cells activates production of a BMP4 antagonist such as NOGGIN (Zimmerman et al., 1996) or CHORDIN (Piccolo et al., 1996).

Both the normal BMP2/4 expression pattern and the developmental effects of perturbing that pattern support a modification of the original double gradient model (Runnström, 1975). Instead of an early discrete center of diffusible animalizing activity, we suggest that sea urchin BMP2/4 activity provides a later, broadly distributed animalizing influence**.** We suggest that its vegetal border is regulated by signals emanating from vegetal blastomeres that may include BMP2/4 inhibitors. In this view, micromeres function in a manner analogous to the primary organizing centers in vertebrate embryos, which also depend on a β-catenin signal transduction pathway (Fagotto et al., 1997 and references cited therein; Laurent et al., 1997).

The conservation of the BMP signaling pathway in early patterning along the developmental axes of embryos with distinctly different body plans is striking. Echinoderms are on the evolutionary line leading to vertebrates because the larval forms are bilaterally symmetric, enterocoelous deuterostomes. This work, as well as recent studies on the nuclear β-catenin-dependent vegetal signaling mechanisms in sea urchin embryos, shows that cell-fate specification along the animal-vegetal axis uses some of the same biochemical pathways that are central to specification of the dorsal-ventral axes of well-studied vertebrate systems, such as frogs and zebrafish. While this raises the obvious possibility that the sea urchin AV and vertebrate DV axes are homologous developmental coordinate systems, it is also important to consider the fundamental differences between the fate maps of the primary germ layers in echinoderm and vertebrate embryos. In particular, mesenchymal lineages are derived from the most vegetal regions in echinoderms while endodermal anlage occupy this position in vertebrates. Furthermore, the cellular mechanisms of mesoderm formation are also quite different. Instead of interpreting the conserved use of BMP4 and β-catenin-dependent pathways solely in terms of embryonic axis specification, we suggest that the common theme in many different types of embryos, vertebrate and invertebrate, deuterostome and protostome, is that cells producing BMP4 signals are distal from the sites from which the first morphogenetic movements are initiated. These sites include the fly ventral midline, the frog dorsal lip of the blastopore, the zebrafish dorsal shield, the mouse primitive streak and the sea urchin vegetal pole.

We would like to acknowledge support of this work by NIH grants GM25553 (R.C.A.), HD14483 (D.R.M.) and the Medical Research Council (L.D.). We are grateful to Drs Athula Wikramanayake and William Klein for immunological reagents and stimulating discussions throughout the course of this work.

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