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Nodal and BMP2/4 pattern the mesoderm and endoderm during development of the sea urchin embryo

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SUMMARY

Nodal factors play fundamental roles in induction and patterning of the mesoderm and endoderm in vertebrates, but whether this reflects an ancient role or one that evolved recently in vertebrates is not known. Here, we report that in addition to its primary role in patterning the ectoderm, sea urchin Nodal is crucial for patterning of the endoderm and skeletogenic mesoderm through the regulation of the expression of key transcription factors and signalling molecules, including BMP2/4 and FGFA. In addition, we uncovered an essential role for Nodal and BMP2/4 in the formation and patterning of the non-skeletogenic mesoderm. By comparing the effects of misexpressing Nodal or an activated Nodal receptor in clones of cells, we provide evidence that Nodal acts over a long range in the endomesoderm and that its effects on the blastocoelar cell precursors are likely to be direct. The activity of Nodal and BMP2/4 are antagonistic, and although *bmp2/4* is transcribed in the ventral ectoderm downstream of Nodal, the BMP2/4 ligand is translocated to the dorsal side, where it activates signalling in the dorsal primary mesenchyme cells, the dorsal endoderm and in pigment cell precursors. Therefore, correct patterning of the endomesoderm depends on a balance between ventralising Nodal signals and dorsalising BMP2/4 signals. These experiments confirm that Nodal is a key regulator of dorsal-ventral polarity in the sea urchin and support the idea that the ventral ectoderm, like the Spemann organiser in vertebrates, is an organising centre that is required for patterning all three germ layers of the embryo.

KEY WORDS: Sea urchin embryo, Endomesoderm, Nodal, BMP2/4, Activin, FoxA, FoxD, *sm30*, GATA1/2/3, LMO2, *ese*, *gcm*, Pigment cells, Blastocoelar cells

INTRODUCTION

In vertebrates, Nodal factors play conserved roles in the induction of the mesoderm and endoderm, in the formation of a dorsally located organising centre and in the establishment of left-right asymmetry (Shen, 2007). Another conserved role of Nodal in vertebrates is in patterning of the mesoderm along the animal-vegetal and/or dorsal-ventral (D/V) axis. In *Xenopus*, higher levels of Nodal are required to induce dorsal mesoderm than ventral mesoderm, and Nodal signals on the dorsal side cooperate with stabilised β-catenin to induce formation of the Spemann organiser (Agius et al., 2000; Hashimoto-Partyka et al., 2003). In zebrafish, graded Nodal signalling is thought to pattern the mesoderm along the animal-vegetal axis, with high levels of Nodal being required for induction of prechordal plate precursors close to the margin and low levels of Nodal being required for specification of notochord progenitors located at a distance from the margin (Gritsman et al., 2000). Similarly, in mouse, analysis of Nodal signalling mutants indicates that higher levels of Nodal are required for induction of prechordal plate and anterior endoderm than for induction of the node and posterior mesoderm (Lowe et al., 2001; Meno et al., 2001). During gastrulation, the Spemann organiser is the source of antagonists of the BMP and Wnt families and the interplay between ventralising Wnt and BMP signals and their dorsally expressed antagonists is thought to further pattern the embryo along the D/V axis. Although the antagonism between BMP signals and their inhibitors appears to be an ancestral mechanism employed to pattern the D/V axis (DeRobertis and Sasai, 1996; Ferguson, 1996), it is presently unclear whether differential Nodal signalling has a similarly conserved function in mesoderm and endoderm patterning outside the vertebrates.

In the sea urchin, Nodal plays a central and early role in patterning of the ectoderm along the D/V axis (Duboc et al., 2004). Inhibition of Nodal function eliminates D/V polarity, resulting in radialised embryos with multiple spicule rudiments forming around a straight archenteron (Duboc et al., 2004; Yaguchi et al., 2006). Identical phenotypes are obtained following inhibition of Univin, a TGFβ related to Vg1 that is likely to function as a heterodimerisation partner for Nodal or by blocking the function of Alk4/5/7 that is required to transduce Nodal signals (Range et al., 2007). Moreover, rescue experiments with a synthetic *nodal* mRNA revealed that localised expression of Nodal into one blastomere at the 8-cell stage is sufficient to fully rescue the D/V polarity of embryos devoid of endogenous Nodal function, suggesting that Nodal-expressing cells behave as a D/V organising centre. Consistent with this proposal, Nodal induces BMP2/4 expression within the ventral ectoderm and BMP2/4 is translocated to the dorsal side and induces dorsal fates (Lapraz et al., 2009).

Although D/V polarity of the sea urchin embryo is most pronounced in the ectoderm, there are numerous indications that the other germ layers possess a D/V polarity in register with that of the ectoderm. For example, there is strong evidence that the vegetal plate, defined as the flattened region which surrounds the vegetal pole before invagination of the archenteron, is patterned along the D/V axis (Ruffins and Ettensohn, 1996; Sherwood and McClay, 1997). The vegetal plate contains precursors of the secondary mesoderm and endoderm arranged in concentric rings. Molecular evidence that the vegetal plate is patterned along the D/V axis was first provided by studies on the Notch receptor (Sherwood and

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McClay, 1997). Starting at late mesenchyme blastula stage, the Notch protein displays a strong dorsal localisation within the presumptive endoderm. This D/V gradient of Notch is abolished following treatment with the ventralising agent nickel chloride, which mimics ectopic Nodal signalling, suggesting that treatments that perturb the D/V polarity of the ectoderm also affect the polarity of the endoderm.

More recently, several gene markers, including *tbx2/3* (Croce et al., 2003; Gross et al., 2003), *foxB* (Luke et al., 1997; Minokawa et al., 2004), *foxD* (Tu et al., 2006), *krl* (Minokawa et al., 2004) and the *nemo-like kinase* (NLK), which in the sea urchin acts downstream of Notch/Delta signalling (Röttinger et al., 2006), were found to display a strong D/V bias of expression in the endoderm. Although the function of the D/V regionalised gene expression within the endoderm is not known, one possibility is that it might be important for mouth formation, which requires bending of the archenteron towards the presumptive ventral ectoderm and fusion of the two cell layers to form the stomodeum. Consistent with this idea, in Nodal morphants, the archenteron forms but remains straight and does not bend towards the ectoderm.

Patterning of the vegetal plate along the D/V axis is also prominent at the level of the secondary mesenchyme cell (SMC) precursors. Starting at the very early blastula stage, the presumptive SMCs, which derive from the macromere lineage, are induced by Notch/Delta signalling from the underlying micromeres (McClay et al., 2000; Sherwood and McClay, 1999; Sweet et al., 2002; Sweet et al., 1999). SMCs delaminate from the tip of the archenteron during gastrulation and give rise to a heterogeneous population of non-skeletogenic mesodermal cells that comprises muscles cells, precursors of the coelomic pouches (Ettensohn, 1992; Ruffins and Ettensohn, 1993), pigment cells (Calestani et al., 2003; Gibson and Burke, 1985) and blastocoelar cells (Tamboline and Burke, 1992). Fate mapping experiments revealed, unexpectedly, that blastocoelar cell and pigment cell precursors are distributed asymmetrically within the vegetal plate of the early gastrula with a striking D/V bias (Ruffins and Ettensohn, 1996). The pigment cell precursors, which express the transcription factor *gcm* (Ransick and Davidson, 2006; Rast et al., 2000), occupy a dorsal sector of the vegetal plate, whereas the blastocoelar cells, for which very few molecular markers are available (Sweet et al., 1999; Tamboline and Burke, 1992), are located on the opposite, ventral side.

Like the endoderm and the SMCs, the primary mesenchyme cells (PMCs), which build the skeleton of the larva, are also patterned along the D/V axis. At the beginning of gastrulation, bilateral thickenings of the ectoderm develop on the presumptive ventrolateral regions. Guided by VEGF and FGF signals emitted by these regions, a subpopulation of PMCs aggregates under these ectodermal thickenings and starts to form bilateral clusters (Duloquin et al., 2007; Röttinger et al., 2008a). PMCs belonging to the ventral clusters show an increased expression of marker genes, such as *sm30* and *fgfA*, compared with PMCs located on the dorsal side (George et al., 1991; Guss and Ettensohn, 1997; Röttinger et al., 2008). By contrast, the dorsal PMCs express a unique set of marker genes, including the transcription factor *tbx2/3* (Croce et al., 2003; Gross et al., 2003), indicating that the skeletogenic mesoderm, like the ectoderm and the SMC precursors, is regionalised along the D/V axis. The exact mechanism responsible for restricting the expression of these genes to the ventral or dorsal PMCs is not known, but in embryos with disrupted Nodal signalling the PMCs arrange circumferentially around the archenteron, suggesting that Nodal is required for establishing the D/V pattern of gene expression within the PMCs (Duboc et al., 2004).

Taken together, these observations indicate that not only the ectoderm, but also all three germ layers of the sea urchin embryo are patterned along the D/V axis, but the identity of the factors that regulate D/V patterning in these germ layers is not known. The goal of this study was to identify these factors.

MATERIALS AND METHODS

Animals, embryos and treatments

Sea urchin (*Paracentrotus lividus*) embryos were cultured essentially as described (Lepage and Gache, 1989; Lepage and Gache, 1990). Treatments with SB431542 (5 μM) were performed by adding the chemical diluted from stocks in DMSO in 24-well plates protected from light.

RNA and morpholino injections

The Nodal and BMP2/4 morpholinos are as described (Duboc et al., 2004). Cloning of *P. lividus alk4/5/7*, construction and injection of capped mRNA coding for the activated form of *P. lividus* Alk4/5/7, and the Alk4/5/7 and Univin morpholino oligonucleotides are as described (Range et al., 2007). The Activin morpholino is 5′-CATGAACGACAGGTACAGAGAACTC-3′. Morpholino oligonucleotides were injected at 1 mM for Mo-Activin, 0.5 mM for Mo-nodal1 and 0.4 mM for Mo-BMP2/4. Experiments involving injection at the egg stage were repeated two to four times on a batch of 100-200 embryos per experiment. Experiments involving injection into one blastomere at the 4- or 8-cell stage were repeated twice with 15-20 embryos analysed per experiment. Only representative phenotypes observed in more than 80% of the embryos are presented. For the clonal analysis, each mRNA was injected in parallel into the egg at the same concentration as that used in the clonal analysis and expression of *gcm* and *gata1/2/3* was analysed at mesenchyme blastula/early gastrula stages. pSmad1/5/8 immunostaining was performed as described (Lapraz et al., 2009).

Gene regulatory interactions deduced from perturbation analysis were used to build a gene regulatory network with Biotapestry (Longabaugh et al., 2009). The accession numbers of the mRNA sequences used in this study are: *gata1/2/3* (GQ377404), *gcm* (DQ666827), *lmo2* (GQ377406), *ese* (GQ377405) and *activin* (GQ896540).

RESULTS

Perturbation of Nodal and BMP2/4 signalling dramatically affects morphogenesis of the endoderm and disrupts patterning of the skeletogenic and non-skeletogenic mesoderm

In the course of analysing the function of Nodal and BMP2/4 in sea urchin, we repeatedly noticed that morphogenesis of all three germ layers was profoundly affected upon misexpression of these factors. For example, whereas in control embryos the archenteron normally bends towards the presumptive ventral side at the end of gastrulation, inhibition of Nodal or BMP2/4 signalling resulted in embryos with a straight archenteron and, in the case of Nodal, no mouth (Fig. 1). Severe patterning defects were also apparent in the skeletogenic mesoderm following perturbation of Nodal or BMP signalling (Fig. 1), as reported previously (Duboc et al., 2004; Lapraz et al., 2009). One of the most striking patterning defects caused by Nodal and BMP2/4 perturbation concerned the non-skeletogenic mesoderm. For example, when mRNA encoding Nodal (Fig. 1C) or an activated form of the Nodal receptor (Fig. 1D) was overexpressed, or when the embryos were treated with Activin (Fig. 1E) or with the ventralising agent nickel chloride (data not shown), the resulting embryos were always completely devoid of pigment cells but contained a dense network of mesenchymal cells that resembled blastocoelar cells in morphology (Fig. 1B-E and data not shown). By contrast, inhibition of Nodal, Univin or Alk4/5/7 signalling by microinjection of morpholino oligonucleotides directed against the corresponding transcripts consistently produced embryos with a large excess of pigment cells (Fig. 1G) (see also Duboc et al., 2004; Range et al.,

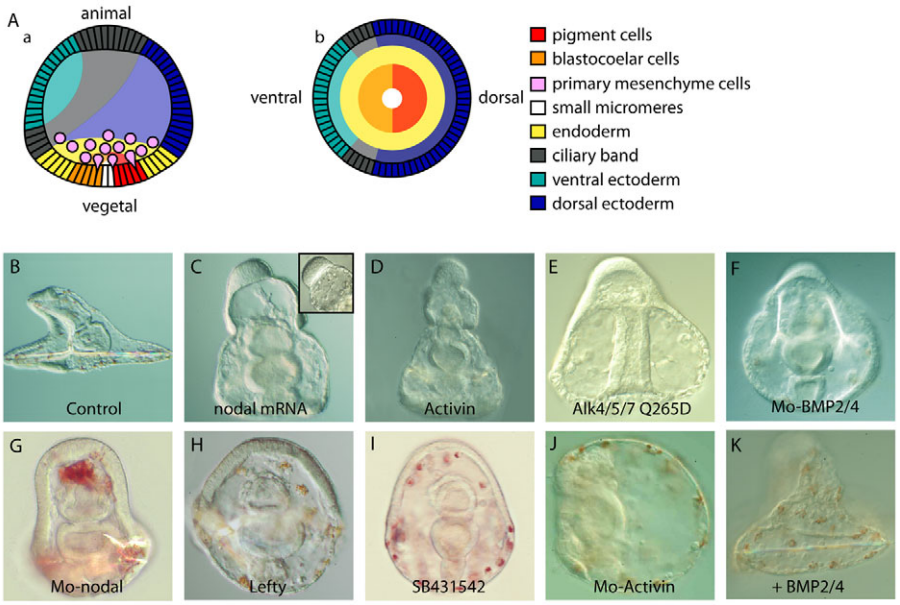

Fig. 1. Perturbation of the Nodal and BMP2/4 signalling pathways affects morphogenesis and patterning in all three germ layers of the sea urchin embryo. (**A**) Fate maps at mesenchyme blastula stage (see also Ruffins and Ettensohn, 1996). (**a**) Side view. (**b**) View from the vegetal plate. (**B-K**) DIC images of 48-hour plutei larvae obtained by the indicated treatments. Note that the embryos overexpressing *nodal* (C) or activated Alk4/5/7 (Alk4/5/7 Q265D) (E) lack pigment cells but contain a network of mesenchymal cells, the morphology of which is very similar to that of blastocoelar cells, as shown in the inset in C. Cell counts indicated that control embryos contain an average of 25 pigment cells. Embryos treated with SB431542 (I) show an 80% increase in the number of pigment cells (n=46±15 pigment cells), whereas embryos injected with the Activin morpholino (J) show a 40% increase (n=35±12 pigment cells).

2007). In these embryos, most of the pigment cells gathered into clusters at the tip of the archenteron where they formed typical red aggregates, or they populated the sub-anal ectoderm, which is the most vegetal thin epithelium surrounding the blastopore. Highly pigmented embryos were also obtained upon overexpression of the Nodal antagonist Lefty (Antivin) (Fig. 1H), after treating embryos with the Nodal receptor inhibitor SB431542 (Fig. 1I) or by injecting a morpholino oligonucleotide directed against *activin* (Fig. 1J). Interestingly, reciprocal phenotypes were obtained by manipulating BMP2/4 signalling. Inhibition of BMP2/4 signalling always produced albino embryos that completely lacked pigment cells, suggesting that pigment cell formation was blocked in these embryos (Fig. 1F), whereas overexpression of BMP2/4 always produced highly pigmented embryos (Fig. 1K). These observations strongly suggested that in the sea urchin embryo, Nodal and BMP2/4 exert opposite effects on specification and/or differentiation of the pigment cells.

Nodal and BMP2/4 signalling regulate D/V patterning of the skeletogenic mesoderm and endoderm

To test whether Nodal and/or BMP2/4 signalling are implicated in patterning of the endomesoderm along the D/V axis, we first examined the expression of genes that are expressed asymmetrically in the PMCs following misexpression of Nodal or BMP2/4 pathway components. In control embryos, *fgfA* is expressed strongly in the bilateral clusters of PMCs during gastrulation (Fig. 2A). In Nodal morphants, *fgfA* was expressed radially in the PMCs (Fig. 2B), whereas it was abrogated by overexpression of Nodal (Fig. 2C). In BMP2/4 morphants, *fgfA* expression expanded to the dorsal PMCs (Fig. 2D), whereas in BMP2/4-overexpressing embryos it expanded to all the PMCs (Fig. 2E). In control embryos at gastrula stage, *sm30* is expressed predominantly in the PMC clusters and in the dorsal PMCs downstream of FGFA signalling (Fig. 2F) (Röttinger et al., 2008). In Nodal morpholino-injected embryos, the ventral restriction of *sm30* expression was abolished, consistent with the radial expression of *fgfA* in the ectoderm of the Nodal morphants (Fig. 2G). Conversely, overexpression of *nodal* eliminated *sm30* expression, consistent with the absence of *fgfA* expression and lack of PMC clusters in these embryos (Fig. 2H). This suggests that *sm30* expression is repressed by Nodal signalling in the ventral PMCs. Inhibition of BMP2/4 signalling did not block *sm30* expression (Fig. 2I), whereas overexpression of BMP2/4 caused *sm30* to be expressed radially in all the PMCs (Fig. 2J). Similarly, expression of *tbx2/3*, which at gastrula stage is restricted to the dorsal PMCs and dorsal ectoderm, became radially symmetrical within the PMCs in Nodal morphants, whereas it was abolished in embryos overexpressing *nodal* (Fig. 2K-M). This suggests that Nodal ligands emitted from the ventral ectoderm might act as ventralising signals on the PMCs by repressing *tbx2/3* expression. Inhibition of BMP2/4 signalling abolished expression of *tbx2/3* in the dorsal ectoderm but did not prevent expression of *tbx2/3* in the dorsal PMCs (Fig. 2N), whereas overexpression of BMP2/4 caused *tbx2/3* expression to become radial within both the ectoderm and the PMCs (Fig. 2O).

Whereas the expression of *fgfA*, *sm30* and *tbx2/3* was affected in a similar manner by perturbation of Nodal or BMP2/4, a different behaviour in response to these treatments was observed with GATA1/2/3. Expression of *gata1/2/3*, which is restricted to the dorsal chain of PMCs at late gastrula stages, was eliminated from the PMCs in Nodal morphants or Nodal-overexpressing embryos (Fig. 2P-R). Since *nodal* expression is restricted to the ventral side of the embryo, this result suggested that the effects of the Nodal morpholino on *gata1/2/3* expression were likely to be indirect. Indeed, blocking the function of the Nodal target gene *bmp2/4* mimicked the effects of blocking Nodal function and prevented *gata1/2/3* expression in the dorsal PMCs, whereas overexpression of BMP2/4 caused ectopic expression of *gata1/2/3* in the ventral PMCs (Fig. 2S,T). This indicates that BMP2/4 acts downstream of Nodal as a dorsalising signal for the PMCs.

We next examined whether the polarised D/V gene expression within the endoderm is regulated by Nodal and BMP2/4 signals. At the mesenchyme blastula stage, *foxA* is expressed in a ventral-dorsal gradient within the presumptive endoderm territory (Fig. 1U) (Oliveri et al., 2006). Both inhibition of Nodal translation and overexpression of BMP2/4 strongly affected the expression of *foxA*, causing an approximately twofold reduction in the number of cells expressing this gene (Fig. 2U,V,Y). By contrast, both overexpression of Nodal and inhibition of BMP2/4 signalling caused radially symmetrical expression of *foxA* (Fig. 2W,X). Similarly, expression of *foxD*, which is normally restricted to the ventral precursors of the hindgut (Tu et al., 2006) (Fig. 2Z), was abolished in the embryos

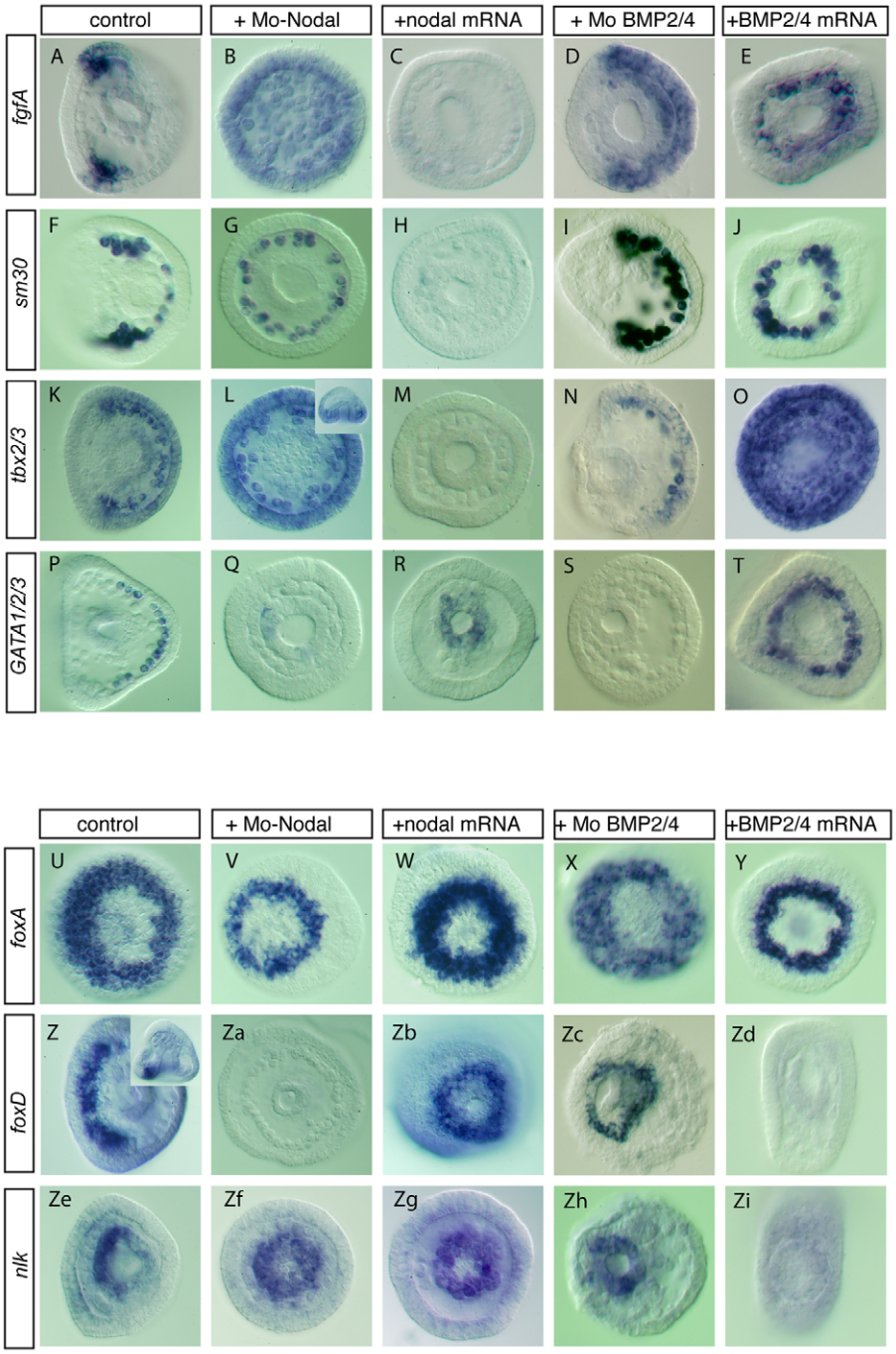

Fig. 2. Nodal and BMP2/4 signalling regulate D/V patterning of the skeletogenic mesoderm and endoderm. (**A-Zi**) Expression of sea urchin *fgfA* (A-E), *sm30* (F-J), *tbx2/3* (K-O), *gata1/2/3* (P-T), *foxA* (U-Y) *foxD* (Z-Zd) and *nlk* (Ze-Zi) following perturbation of Nodal and BMP2/4 signalling. Embryos are at the gastrula or prism stage, except those in U-Y, which are at the mesenchyme blastula stage. Insets in L and Z show side views of the embryo.

microinjected with the Nodal morpholino (Fig. 2Za) or in embryos overexpressing BMP2/4 (Fig. 2Zd), but became radially symmetrical in embryos overexpressing *nodal* (Fig. 2Zb) or injected with a BMP2/4 morpholino (Fig. 2Zc). Finally, we analysed the expression of *nlk*, which, in the sea urchin embryo, is expressed with a strong ventral bias within the endoderm (Fig. 2Ze) and is a downstream target of Notch/Delta signalling (Röttinger et al., 2006). *nlk* was expressed radially in the archenteron following injection of *nodal* mRNA or of the Nodal or BMP2/4 morpholinos (Fig. 2Zf-h), and was expressed at low and uniform levels along the D/V axis in the BMP2/4-overexpressing embryos (Fig. 2Zi). This result suggests that the differential expression of *nlk* is a downstream consequence of Nodal and/or BMP2/4 signalling and that *nlk* expression is repressed by BMP2/4 on the dorsal side of the archenteron.

Taken together, these experiments indicate that in addition to its essential role in patterning of the ectoderm, Nodal signalling is also necessary for establishing the D/V pattern of gene expression, both in the PMCs and in the endoderm, either directly or through the regulation of the expression of key signalling factors such as FGFA and BMP2/4.

The dynamics of gene expression within the SMCs reveals progressive determination of the blastocoelar cell and pigment cell lineages

To better understand how the different lineages of SMC precursors are specified on the ventral and dorsal sides of the vegetal plate, we examined the expression of transcription factors that are expressed early within this territory, including the zinc-finger transcription

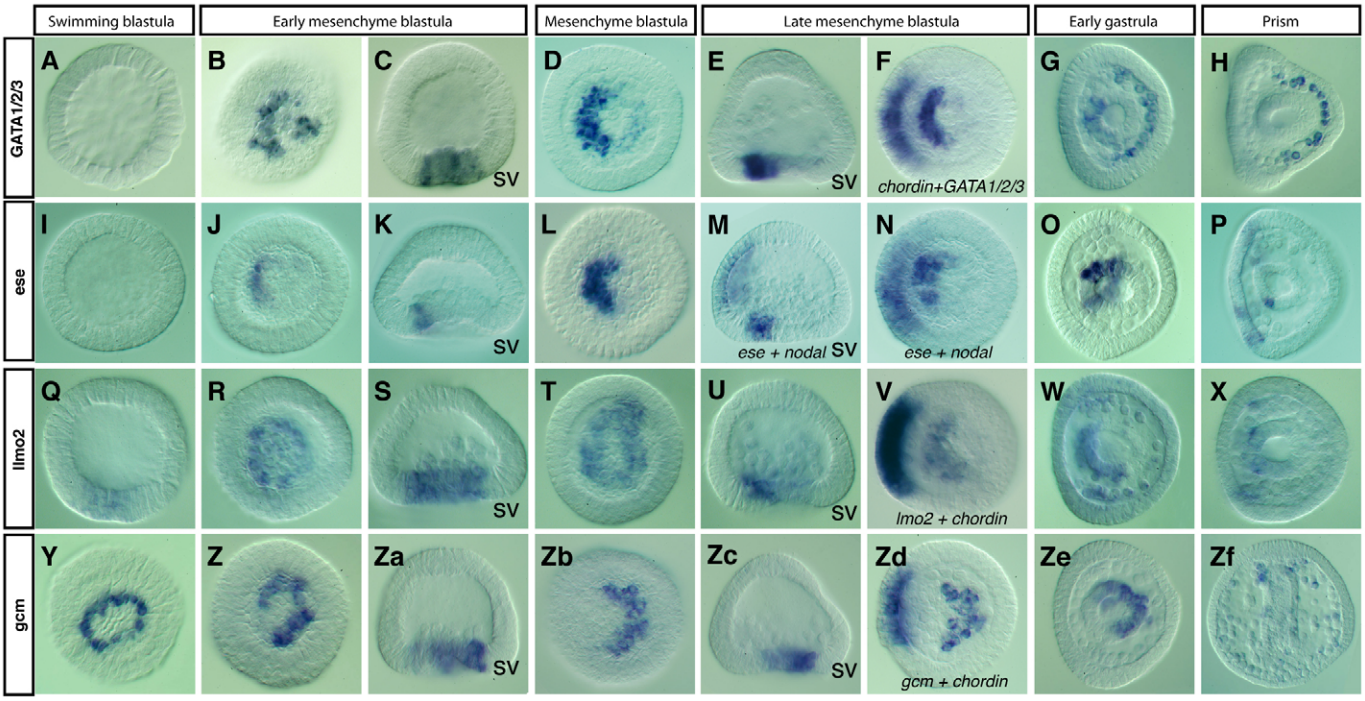

Fig. 3. Progressive determination of the blastocoelar cell and pigment cell progenitors revealed by the dynamic expression of four transcriptional regulators. Time course analysis of the expression of sea urchin *gata1/2/3*, *ese*, *lmo2* and *gcm*. Embryos are viewed from the vegetal pole unless otherwise indicated. SV, side view.

factor *gata1/2/3* (Davidson et al., 2002b; Howard-Ashby et al., 2006), the Ets gene *ese* (Rizzo et al., 2006) and the pigment cell gene marker *gcm* (Ransick and Davidson, 2006; Ransick et al., 2002). In addition, we analysed the expression of the gene that encodes the transcription factor LIM domain only (LMO2), which we identified as an SMC-specific marker in the course of an in situ screen (our unpublished results) (Fig. 3).

This analysis revealed that three of these genes – *lmo2*, *gata1/2/3* and *gcm* – are initially expressed radially, then later become restricted to the ventral or dorsal side at the late mesenchyme blastula stage (Fig. 3). Furthermore, the onset of *gata1/2/3*, *lmo2* and *ese* expression at the early mesenchyme blastula stage is concomitant with the abrupt restriction of *gcm* expression to the dorsal side (Fig. 3B,J,R,Z). These observations suggest that allocation of the blastocoelar and pigment cell fates occurs at the onset of PMC ingression, i.e. at the late blastula/early mesenchyme blastula stage. Furthermore, the initial broad expression and progressive restriction of *gata1/2/3*, *gcm* and *lmo2* expression suggests that the SMC precursors might be initially specified as bipotential precursor cells by Delta signalling and that blastocoelar and pigment cell fates are subsequently segregated by inductive events at the beginning of gastrulation.

Overexpression of Nodal, but not of Delta, disrupts D/V patterning of the SMCs

Since SMCs are induced by Delta signalling from the micromeres (see Fig. S2 in the supplementary material) (Sherwood and McClay, 1999; Sweet et al., 1999; Sweet et al., 2002), we first tested whether overexpression of Delta could perturb the D/V pattern of the SMCs. Injection of *delta* mRNA (400 μg/ml) into the egg produced partially radialised embryos with an excess of mesoderm as described previously (Fig. 4B) (Röttinger et al., 2006; Sweet et al., 2002) and a dramatic increase in the number of SMC precursors expressing *gata1/2/3* (Fig. 4F-H), *gcm* (Fig. 4K-M) or *papss* (Fig. 4P-R) (Röttinger et al., 2006). However, overexpression of Delta did not significantly change the spatial distribution of blastocoelar cell and pigment cell precursors as *gata1/2/3* expression remained mostly localised to the ventral side, whereas *gcm* expression remained largely restricted to the dorsal sector of the SMC territory. Similar results were obtained following overexpression of *nlk* (data not shown), which in the sea urchin acts downstream of Notch signalling (Röttinger et al., 2006). Therefore, differential activation of the Notch pathway along the D/V axis is unlikely to be responsible for D/V patterning of the SMCs.

Strikingly, injection of *nodal* mRNA (200 μg/ml) into one blastomere at the 4-cell stage in embryos overexpressing Delta completely reversed the effects of Delta overexpression and resulted in formation of normal-looking pluteus larvae (Fig. 4A-E). Furthermore, injection of *nodal* mRNA caused strong ectopic expression of *gata1/2/3* on the dorsal side (Fig. 4I,J). Nodal overexpression also largely counteracted the effects of Delta overexpression on the number of pigment cell precursors (Fig. 4N,O). This suggested that local expression of Nodal promoted formation of blastocoelar cell precursors and repressed specification of pigment cell precursors. Taken together, these results strongly suggest that a Nodal/Activin signalling regulates D/V patterning of the SMCs and that Nodal produced in the ventral ectoderm can signal directly and over a long range to mesodermal cells located on the dorsal side of the embryo.

TGFβ signalling is required at the late blastula stage to restrict *gcm* expression

To test whether TGFβ signalling is required to pattern the SMCs, we treated embryos with the Alk4/5/7 inhibitor SB431542 at different stages after fertilisation (Fig. 5A). In control embryos at the late mesenchyme blastula stage, *gcm* expression was always restricted

to the dorsal side (Fig. 5B), whereas in embryos treated with SB431542 at the 16-cell, 128-cell, prehatching blastula or swimming blastula stage, *gcm* expression was radialised (Fig. 5C-E). By contrast, in most embryos treated with SB431542 starting at the late blastula/early mesenchyme blastula stage, *gcm* expression was restricted normally (Fig. 5F). This suggested that TGFβ signalling between the swimming blastula and early mesenchyme blastula stages is required to restrict *gcm* expression.

Nodal was a good candidate for this signal because its expression peaks at blastula stages. To estimate more precisely the distance between *nodal*-expressing cells and SMC progenitors, we performed a double in situ hybridisation with *gcm* and *nodal* probes. At the swimming blastula stages, *nodal*-expressing cells were separated from the dorsal-most *gcm*-expressing cells by seven to nine rows of cells (Fig. 5I,L), which is within the known range of Nodal signalling in vertebrates (Chen and Schier, 2001; Schier, 2004).

Nodal promotes, whereas BMP2/4 antagonises, specification of blastocoelar cells

To further investigate whether TGFβ signalling is responsible for patterning of the SMCs along the D/V axis, we examined the expression of *gcm* and *gata1/2/3* following perturbation of Nodal, Activin and BMP2/4 function (Fig. 6). In embryos overexpressing *nodal*, either by injecting *nodal* mRNA (Fig. 6B) or a Lefty morpholino (Fig. 6C), or in those injected with an mRNA coding for the activated form of Alk4/5/7 (Fig. 6D), *gcm* expression was abolished, consistent with the albino phenotype of these embryos. The same treatments resulted in ectopic expression of *gata1/2/3*, *ese* and *lmo2* in the dorsal sector of the presumptive SMC territory (Fig. 6F-I and data not shown). Similar results were obtained for *profilin*, *ets1* and *erg*, which display a strong ventral bias of expression (see Fig. S1 in the supplementary material). By contrast, overexpression of BMP2/4 caused *gcm* to be expressed in all the SMCs (Fig. 6E). As predicted, overexpression of BMP2/4 abolished expression of *gata1/2/3* in the SMCs but, unexpectedly, it caused precocious and ectopic expression of *gata1/2/3* in the PMCs (Fig. 6J, see also Fig. 2O). Reciprocally, in embryos injected with the Nodal morpholino (Fig. 6K), treated with the Nodal receptor inhibitor SB431542 (Fig. 6L) or injected with *lefty* mRNA (Fig. 6M), the D/V restriction of *gcm* expression was abolished and *gcm* was instead expressed strongly and radially in all the SMC precursors. The same perturbations strongly downregulated the expression of *gata1/2/3*, indicating that transcription of *gata1/2/3* strongly depends on Nodal (Fig. 6P-R). Similar results were obtained for *ese* and *lmo2* (data not shown) and for *profilin*, *ets1* and *erg* (see Fig. S1 in the supplementary material), the expression of which is enriched in the ventral SMCs.

Since, in addition to Nodal and BMP2/4, *activin* transcripts are expressed during blastula stages, and as Alk4/5/7 transduces both Nodal and Activin signals, we analysed the consequences of blocking Activin function on patterning of the SMCs. Inhibition of Activin signalling affected pigment cell formation as reported previously (Fig. 1J) (Sethi et al., 2009), and resulted in a broader

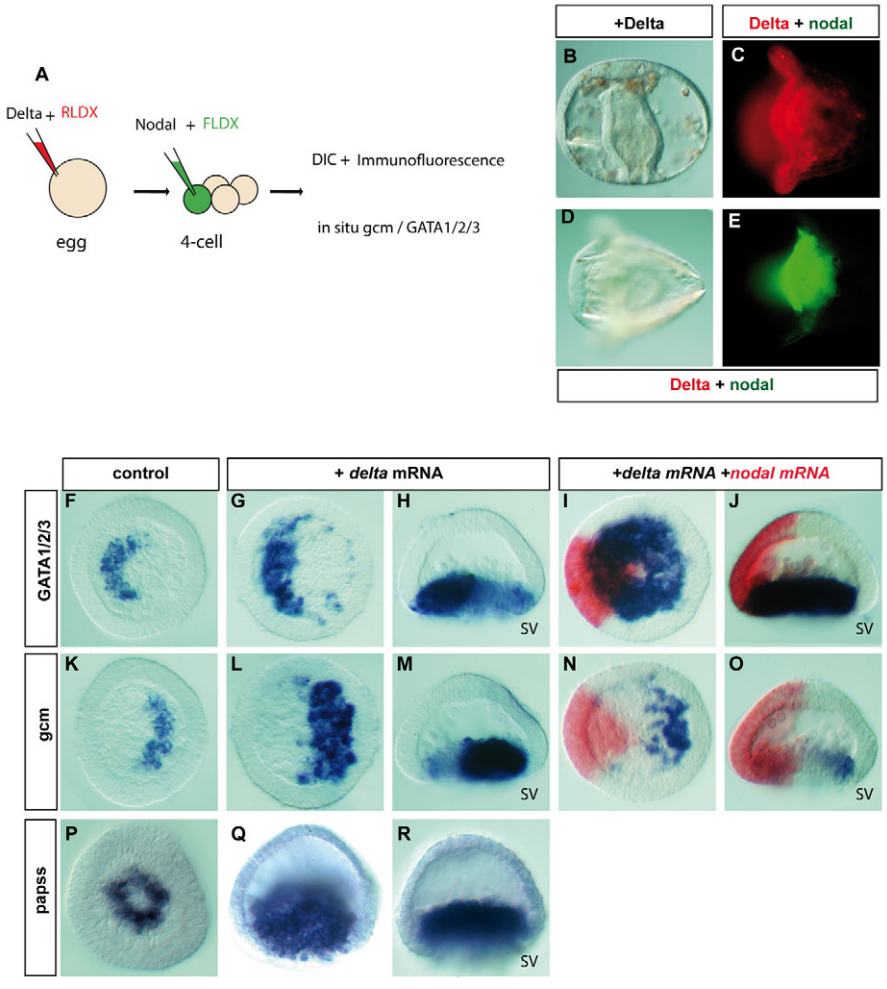

Fig. 4. Overexpression of sea urchin Nodal, but not of Delta, affects D/V patterning of SMCs precursors. (**A**) Scheme of the experiment. (**B-R**) Morphological and molecular phenotypes resulting from the experiment. Embryos are viewed from the vegetal pole unless otherwise indicated. (I,J,N,O) The progeny of the injected cell are labelled in red. SV, side view.

crescent of *gcm* and *gata1/2/3* expression than in control embryos, but it did not cause ectopic expression of either of these genes outside the dorsal or ventral regions where they are normally expressed (Fig. 6N,S). Remarkably, in BMP2/4 morpholino-injected embryos, *gcm* expression was abolished at mesenchyme blastula and gastrula stages, consistent with the albino phenotype of these embryos (Fig. 6O), whereas *gata1/2/3* was radially expressed within the SMCs (Fig. 6T).

The finding that BMP2/4, produced on the ventral side, regulates *gcm* expression on the dorsal side prompted us to analyse BMP signalling within the SMC territory. Starting at early mesenchyme blastula stage, strong nuclear phosphorylated (p) Smad1/5/8 immunoreactivity was detected in half of the embryo in the dorsal ectoderm, in the dorsal PMCs, and in a crescent within the vegetal plate that most likely corresponds to the dorsal SMCs. Double labelling for *gcm* and pSmad1/5/8 in control embryos confirmed that this pSmad staining was located in the dorsal sector of the SMCs that express *gcm* (Fig. 6W,X). Immunostaining in BMP2/4 morphants further demonstrated that nuclear translocation of pSmad1/5/8 in dorsal SMCs is BMP2/4 dependent (Fig. 6Y). This result strongly suggests that the effects of BMP2/4 on dorsal SMCs are direct and, therefore, that despite its ventral transcription, BMP2/4 is translocated to the dorsal sector of the SMCs where it promotes pigment cell formation. Taken together, these results show that the ventral expression of *gata1/2/3*, *ese* and *lmo2* is dependent on Nodal, whereas the dorsal expression of *gcm* is dependent on the Nodal target gene *bmp2/4*. They also suggest that proper regionalisation of the SMCs into pigment cells and blastocoelar cells depends on the balance between Nodal and BMP signalling.

Expression of *gata1/2/3* and restriction of *gcm* require Alk4/5/7 signalling in the vegetal hemisphere

We next examined whether signalling through the Nodal receptor is required on the ventral side of the vegetal pole region to induce blastocoelar cell fates and to repress pigment cell fates. We injected a morpholino directed against *alk4/5/7* (Range et al., 2007) into one blastomere at the 4- and 8-cell stages together with a lineage tracer and examined the expression of *gcm* and *gata1/2/3* at the early gastrula stage (Fig. 7A). In all the embryos in which translation of the receptor was inhibited in a clone of cells, *gcm* was expressed cell-autonomously within the clone, indicating that inhibition of Nodal signal transduction caused derepression of *gcm* on the ventral side (Fig. 7D-F). Conversely, in all the embryos injected with Mo-Alk4/5/7, the expression of *gata1/2/3* was either strongly downregulated or eliminated within the clone (Fig. 7I,J). Therefore, inhibiting signalling from the Alk4/5/7 receptor in a clone of cells of the vegetal plate triggered the cell-autonomous expression of *gcm* and the cell-autonomous repression of *gata1/2/3*. Taken with our previous finding that preventing translation of Nodal, but not of Activin or of TGFβ sensu stricto (data not shown), disrupts D/V patterning of the SMCs, these results strongly suggest that Nodal activates Alk4/5/7 at the vegetal pole region to regulate *gata1/2/3* and *gcm* expression.

Nodal may act directly on blastocoelar cell precursors rather than through a relay

To further test whether Nodal acts directly on cells of the vegetal plate, or if it requires a relay in either the ectoderm or the endoderm, we compared the effects of misexpressing Nodal or an activated form of its receptor (Lapraz et al., 2009) in one blastomere at the 8-cell stage (Fig. 8). Since Nodal signalling activates *nodal* expression, these experiments were performed in a Nodal morpholino context to prevent Nodal autoregulation and with a synthetic *nodal* mRNA immune to the morpholino. Eggs were first injected with a morpholino against the 5′UTR of the *nodal* transcript to block endogenous Nodal signalling, and then, at the 8-cell stage, one blastomere was injected with mRNA encoding either the diffusible Nodal ligand or the activated form of its receptor.

In control embryos injected with the Mo-Nodal alone, *gcm* was expressed radially, whereas in all the embryos overexpressing Nodal in endomesodermal clones, *gcm* expression was restricted to the side

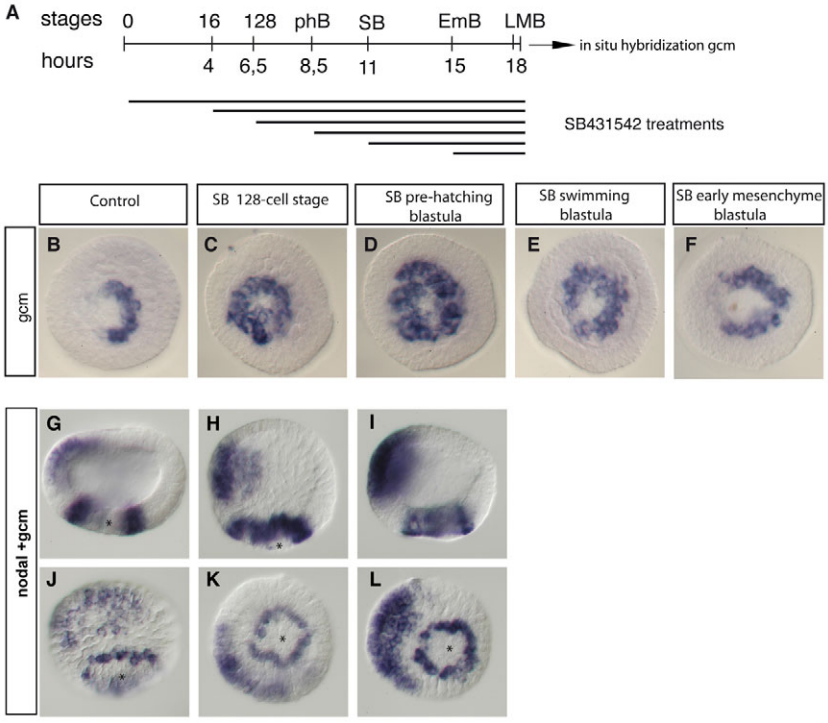

Fig. 5. TGFβ signalling is required at late blastula stage to restrict *gcm* expression. (**A**) Scheme of the experiment. (**B-F**) *gcm* expression in sea urchin embryos treated with the Nodal receptor inhibitor SB431542 at the indicated period. (**G-L**) Double in situ hybridisation with *nodal* and *gcm* probes at the early blastula (G,J); pre-hatching blastula (H,K) or swimming blastula (I,L) stages. (G-J) Side views. (K,L) Vegetal pole views. *gcm*-expressing cells are identified as a ring of stained cells that surround a population of unstained primary mesenchyme cell precursors (asterisk).

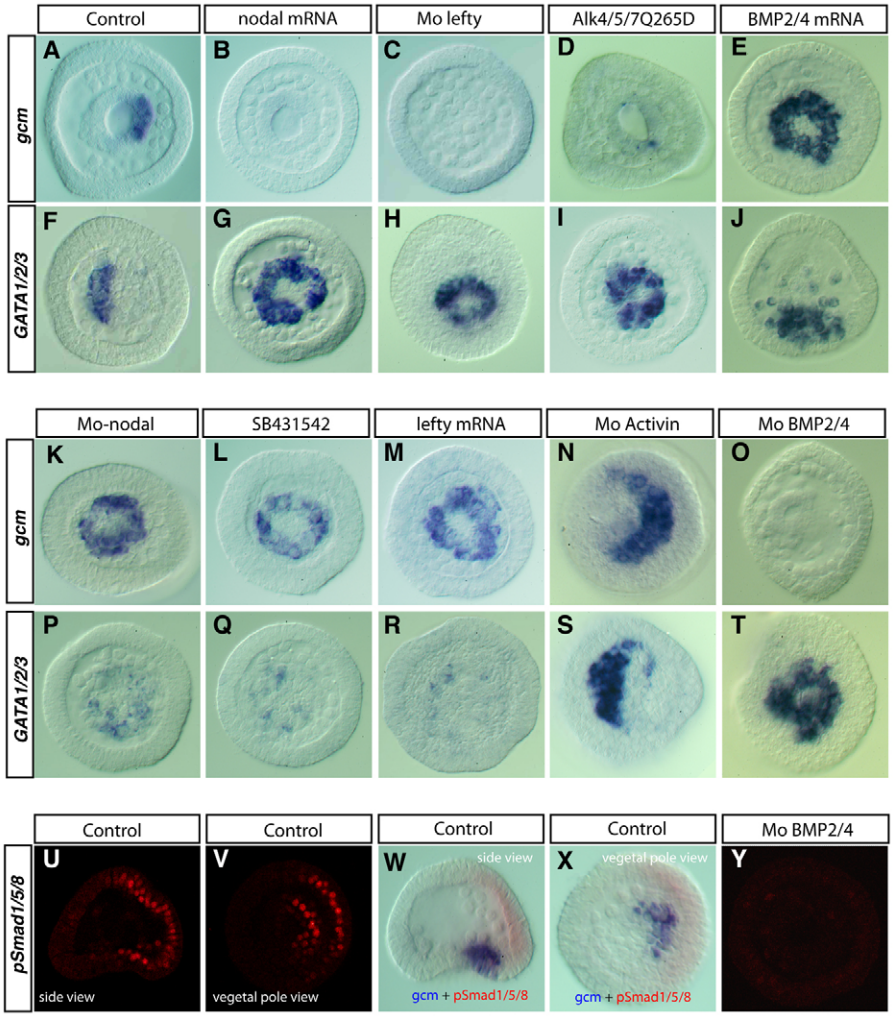

Fig. 6. Nodal and BMP2/4 signalling regulate D/V patterning of the SMC progenitors. (**A-T**) Expression of sea urchin *gata1/2/3* and *gcm* at the mesenchyme blastula/early gastrula stages following disruption or overactivation of the Nodal, BMP or Activin signalling pathways. (**U**,**V**,**Y**) Confocal sections of pSmad1/5/8-immunostained embryos at early mesenchyme blastula stage. (**W**,**X**) Double pSmad1/5/8 immunostaining (red) and in situ hybridisation with a *gcm* probe (blue) at mesenchyme blastula stage.

opposite to the clone (Fig. 8B,C). Furthermore, in these endomesodermal clones, the territory expressing *gcm* was often separated from the clone by a few *gcm*-negative cells, suggesting that Nodal was acting outside the clone to repress *gcm* expression. Similarly, in most of the endomesodermal clones overexpressing the activated Nodal receptor, *gcm* expression was excluded from the clone (Fig. 8C,D), but, in this case, the *gcm*-expressing territory was closely juxtaposed to the territory expressing the activated Nodal receptor, consistent with the cell-autonomous repression of *gcm* expression observed previously. Finally, in most of the endomesodermal clones overexpressing the activated Nodal receptor, *gata1/2/3* expression was rescued within the clone (Fig. 8E,F). Whereas overexpression of both the ligand and of the activated receptor were able to rescue the restriction of *gcm* expression in endomesodermal clones, a strikingly different result was observed in the case of ectodermal clones. Expression of the Nodal ligand in ectodermal clones rescued the D/V restriction of *gcm* expression in all the injected embryos (Fig. 8G,H), but expression of the activated receptor failed to restore the normal pattern of *gcm* expression (Fig. 8I,J) and failed to restore strong expression of *gata1/2/3* on the ventral side of the SMCs (Fig. 8K,L) (see Fig. S3 in the supplementary material). Even when mRNA encoding the activated receptor was injected at high concentration (1 mg/ml) into an animal blastomere (Fig. 8A), *gcm* remained radially expressed within the vegetal plate. Taken together, these results strongly suggest that Nodal diffuses from the ventral ectoderm and acts directly on cells of the ventral vegetal plate to regulate *gata1/2/3* and *gcm* expression.

DISCUSSION

Nodal, BMP2/4 and D/V patterning of the skeletogenic mesenchyme

We showed previously that Nodal signalling is required to restrict the signals that attract the PMCs to the lateral ectoderm and that in the absence of Nodal, *fgfA* is expressed radially in most of the ectoderm, consistent with the presence of multiple spicule rudiments in these embryos (Röttinger et al., 2008). Here, we extended these observations by showing that during gastrulation, Nodal and BMP2/4 are required to pattern gene expression within the PMCs. We showed that Nodal is required for repressing *fgfA*, *sm30* and *tbx2/3* expression in the ventral PMCs, whereas BMP2/4 is required for activating *gata1/2/3* in the dorsal PMCs. Indeed, these experiments do not distinguish between a direct role of Nodal or BMP2/4 in the PMCs and an indirect role through patterning of the ectoderm. In the case of *sm30*, the effects of perturbing Nodal signalling are probably indirect and mediated by repression of *fgfA*, as *sm30* is regulated by FGFA signalling (Röttinger et al., 2008). However, the activating function of BMP2/4 on *gata1/2/3* is probably direct, as pSmad1/5/8 is restricted to the PMC clusters and dorsal PMCs starting at the mesenchyme blastula stage (Lapraz et

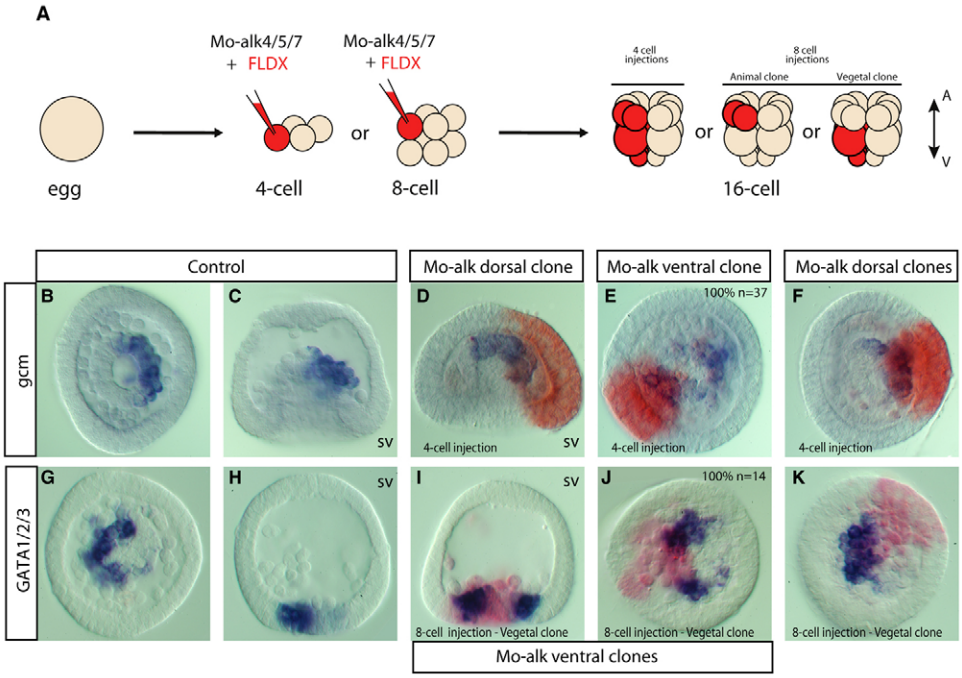

Fig. 7. An Alk4/5/7-mediated signalling pathway is required at the sea urchin vegetal pole of the sea urchin embryo to pattern the SMC progenitors along the D/V axis. (**A**) Scheme of the experiment. (**B-K**) Unless otherwise indicated, embryos are viewed from the vegetal pole to show the endogenous expression of *gcm* or *gata1/2/3*. For the 8-cell stage injections, only vegetal clones are informative and presented here. SV, side view.

al., 2009). Chimera experiments, in which PMCs derived from embryos injected with morpholinos against the Nodal (Alk4/5/7) or BMP2/4 (Alk3/6) receptors are recombined with wild-type ectoderm, should help to determine whether the functions of these receptors are required within the PMCs or within the ectoderm for normal PMC patterning.

Nodal signalling and endoderm formation

In the sea urchin, the primary inducer of endoderm is the canonical Wnt pathway (Emily-Fenouil et al., 1998; Logan et al., 1999; Wikramanayake et al., 1998). Maternal determinants localised at the vegetal pole and zygotic Wnt signals are thought to cause β-catenin to be stabilised in the endoderm precursors. β-catenin, in turn, activates a gene regulatory network that leads to specification of the endoderm (Davidson et al., 2002a). It has been suggested that the role of β-catenin in specification of the endomesodermal germ layer might reflect an ancestral function because it is conserved in other deuterostomes and in cnidarians (Kawai et al., 2007; Momose and Houliston, 2007; Wikramanayake et al., 2003). In vertebrates, however, TGFβ signals, not Wnts, appear to be the primary inducers of the endoderm. Loss-of-function experiments have demonstrated that Nodal signals are essential for specification of the endoderm in mouse, fish, frog and chick (Schier, 2004; Shen, 2007). In the sea urchin, a recent study reported that in addition to Wnt signalling, TGFβ signalling is required before gastrulation for micromere-dependent endomesoderm specification (Sethi et al., 2009). When Activin or Alk4/5/7 signalling is blocked in *S. purpuratus* embryos, gastrulation is delayed and the early expression of at least three transcription factors (*foxA*, *eve* and *z13*) is reduced. We showed here that two other TGFβ signalling components, Nodal and BMP2/4, are required for normal patterning of the endoderm. Nodal is required for upregulating the expression of *foxA* on the ventral side of the presumptive endoderm territory and for inducing *foxD* on the ventral side of the blastopore, whereas BMP2/4 is required for the downregulation of *foxA*, *foxD* and *nlk* on the dorsal side of the archenteron. Therefore, in the sea urchin, as in vertebrates, TGFβ signalling plays an important role in endoderm formation. However, in the sea urchin, Nodal appears to be mainly involved in endoderm patterning rather than in endoderm specification. Again, it is not known whether the effects of Nodal and BMP2/4 on patterning of the endoderm are direct or indirect. Nevertheless, the restricted activation of pSmad1/5/8 in the dorsal endoderm strongly suggests that the effects of BMP2/4 in this germ layer are indeed direct (Lapraz et al., 2009).

An additional role for Nodal in patterning of SMC progenitors in the sea urchin embryo

In this study, we provided several lines of evidence that Nodal signals emanating from the ventral ectoderm act non-cell-autonomously on the vegetal plate to pattern the SMC progenitors and that these effects require diffusion of the Nodal ligand. First, we showed by gain- and loss-of-function experiments that perturbation of Nodal signalling affects specification of blastocoelar cells and of pigment cells in opposite ways: increasing Nodal signalling promotes specification of blastocoelar cell fates and antagonises pigment cell fates, whereas decreasing Nodal signalling interferes with specification of blastocoelar cell fates and promotes formation of pigment cells. Second, we showed that the Alk4/5/7 receptor is required in the vegetal hemisphere for expression of the blastocoelar cell marker *gata1/2/3* and for repression of the pigment cell marker *gcm*. Third, in an assay for rescue of SMC patterning, we showed that misexpression of the diffusible Nodal ligand into one ectodermal precursor, but not misexpression of the non-diffusible activated Alk4/5/7 receptor, is able to rescue restriction of *gcm* expression at a distance from the expressing cell. Therefore, in addition to its important role in patterning of the ectoderm, Nodal has a key function in patterning of mesodermal derivatives in the vegetal hemisphere.

Two different modes of action of Nodal along the animal-vegetal and D/V axes

One interesting finding of this study is that Nodal appears to act as a long-range signal in patterning of the endomesoderm. How does this novel activity of Nodal compare to its activity during D/V

patterning of the ectoderm? We showed previously that injection of *nodal* mRNA into one blastomere at the 8-cell stage fully rescues the dorsal and ventral ectodermal regions of Nodal morpholino-injected embryos. Thus, Nodal-expressing cells also have a patent long-range organising activity along the D/V axis (Duboc et al., 2004). There is, however, a major difference in the mechanism employed by Nodal during these two patterning events. In the case of D/V patterning of the ectoderm, the long-range organising activity of Nodal relies on its ability to induce BMP2/4, which in turns acts as a relay to induce dorsal fates. Accordingly, ectopic expression of an activated Nodal receptor rescues D/V patterning of the ectoderm when re-injected into one blastomere just as efficiently as the Nodal ligand (Lapraz et al., 2009). This contrasts with the results presented here that indicate that ectopic expression of the Nodal ligand, but not of the activated Nodal receptor, rescues patterning of the SMCs. If Nodal were acting through a relay from the ectoderm to the vegetal plate to restrict the expression of *gcm*, then both ectopic expression of the activated receptor and ectopic expression of the ligand should have been capable of inducing this relay to restore restriction of *gcm* expression. By contrast, if Nodal diffuses from the ectoderm to act directly on cells of the vegetal pole region, then only ectopic expression of the ligand, and not of the activated receptor, should be able to restrict *gcm* expression, which is what we observed. Furthermore, the finding that *gata1/2/3* and *foxD* expression depend on Nodal signalling and expand to the dorsal side in the BMP2/4 morphants strongly suggests that Nodal can signal over seven to nine cell diameters up to the dorsal side of the SMCs and endoderm precursors. This raises novel questions regarding the range of Nodal signalling and the reaction diffusion mechanism that operates along the D/V axis (Duboc et al., 2008). Why does Lefty not prevent Nodal from acting on the vegetal pole cells? In other words, if Lefty limits the range of action of Nodal activity along the animal-vegetal axis as it does along the D/V axis, then how can Nodal overcome the inhibitory action of Lefty in the vegetal hemisphere? One possibility is that the function of Lefty is antagonised in the endomesoderm by an unknown factor, or, alternatively, that these cells express a factor that facilitates long-range Nodal signalling. Future studies should resolve these issues.

Role of BMP2/4 signalling in patterning of the secondary mesoderm

In addition to Nodal, BMP2/4 also appears to play an important role in patterning of the SMC precursors in the sea urchin embryo. However, unlike in vertebrates, in the sea urchin BMP2/4 signalling is not a positive regulator of *gata1/2/3* expression but a negative regulator, as overexpression of BMP2/4 abolishes *gata1/2/3* expression. By contrast, BMP2/4 appears to be a positive regulator of *gcm* expression. In the absence of BMP2/4 signalling, *gcm*

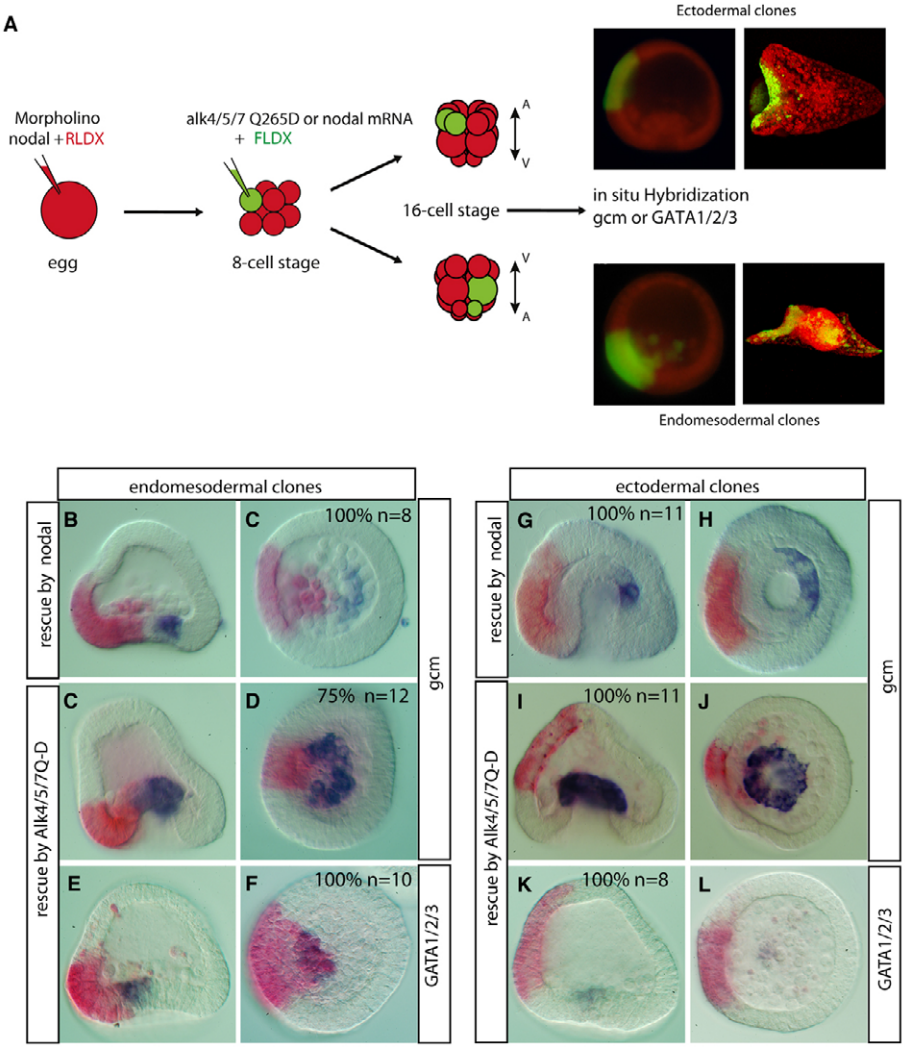

Fig. 8. Diffusion of Nodal is necessary for correct D/V patterning of the SMC progenitors. (**A**) Experimental design. (**B-L**) Expression of sea urchin *gcm* and *gata1/2/3* in endomesodermal (B-F) or ectodermal (G-L) clones following injection of mRNA encoding the Nodal ligand (B,C,G,H) or the activated non-diffusible Nodal receptor (C-F,I-L).

expression is initiated normally but is not maintained after the mesenchyme blastula stage and, as a consequence, pigment cells are replaced by blastocoelar cells (Fig. 9A). Paradoxically, although pigment cells do not form in BMP2/4 morphants, in Nodal morphants, which do not express BMP2/4, pigment cells are produced in excess at the expense of blastocoelar cells. An explanation for this apparent paradox is that BMP signalling may not be required for specification of pigment cells per se, but that it might be required to counteract the effect of Nodal signalling on the dorsal side. Our findings suggest that BMP2/4 represses blastocoelar cell fates in the dorsal SMC progenitors by downregulating *gata1/2/3* expression in these cells. Therefore, in Nodal morphants, *gcm* expression is initiated normally in all the SMC precursors but, as *gata1/2/3* expression is expressed at a very low level in these embryos, *gcm* is not restricted to the dorsal side after the mesenchyme blastula stage and all the SMC progenitors consequently differentiate as pigment cells (Fig. 9A).

We showed previously that although BMP2/4 is expressed in the ventral ectoderm, its function is required on the dorsal side of the embryo for expression of dorsal ectodermal genes such as *tbx2/3* and *29D* (Duboc et al., 2004; Lapraz et al., 2009). Here, we extended these observations to the secondary mesoderm by showing that BMP2/4 is required for expression of the dorsal mesodermal marker *gcm*. Furthermore, we showed that phosphorylation of Smad1/5/8 is activated in a very restricted manner in the pigment cell progenitors by BMP2/4 signals, indicating that although the *bmp2/4* gene is

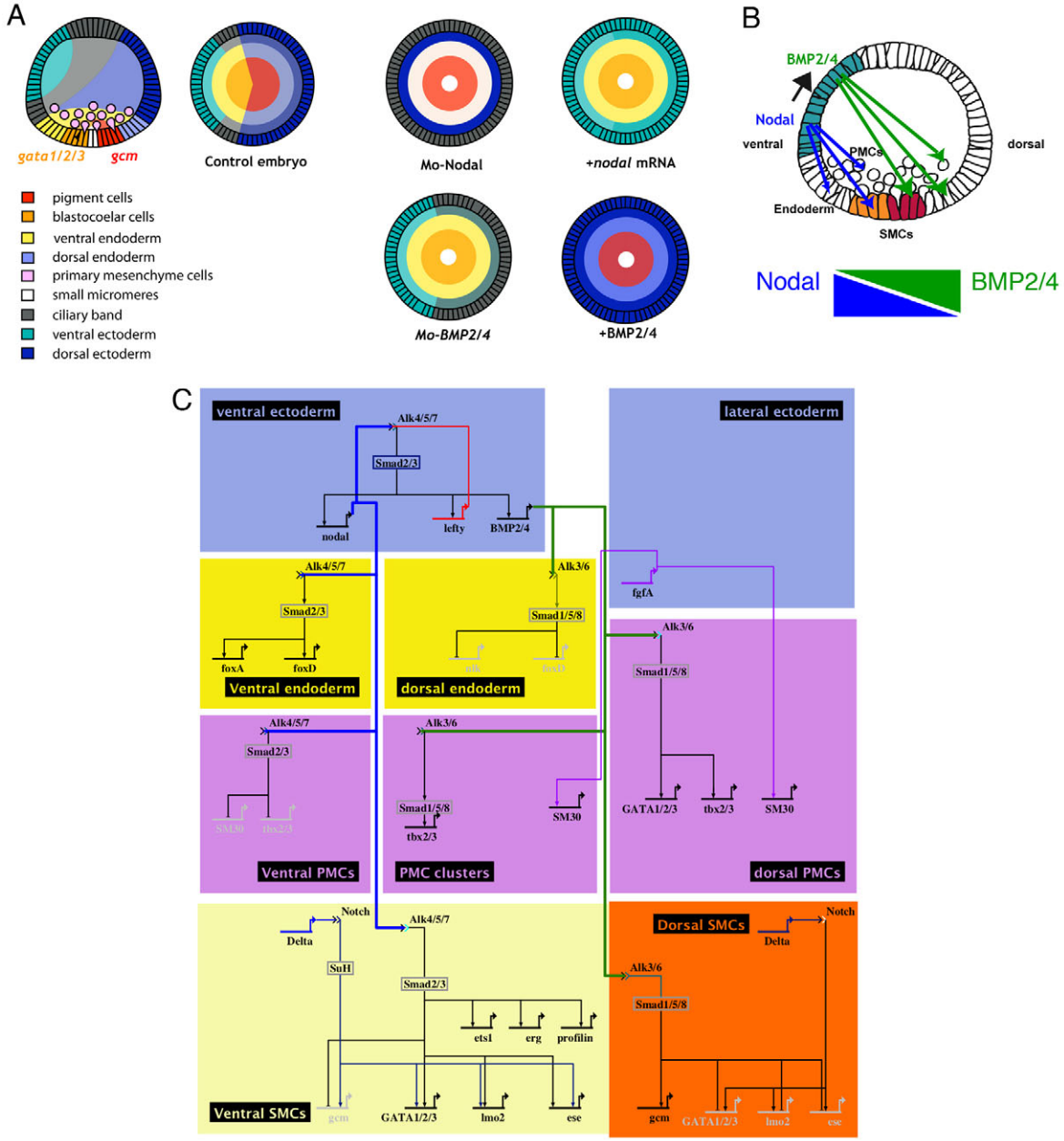

Fig. 9. Nodal and BMP2/4 pattern all three germ layers of the sea urchin embryo. (**A**) The fates of the SMCs and endoderm following perturbation of Nodal or BMP2/4 signalling pathways. (**B**) Summary of the ventralising role of Nodal and the dorsalising role of BMP2/4 in patterning the endomesoderm. (**C**) Biotapestry diagram summarising the signalling events and novel gene regulatory interactions downstream of Nodal and BMP2/4 uncovered in this study.

transcribed in the ventral ectoderm downstream of Nodal, the BMP2/4 protein is translocated to the dorsal side of the embryo where it activates BMP signalling in all three germ layers including the dorsal SMCs. The finding that BMP2/4 produced in the ventral ectoderm induces BMP signalling in the dorsal SMCs, but not in the ventral SMCs, suggests that either an inhibitory factor, such as Chordin, prevents BMP signalling on the ventral side of the SMCs, or that a factor required for BMP signalling is present only on the dorsal side. Future studies should resolve these issues.

To summarise, this study shows that the ectoderm and endomesoderm gene regulatory networks are interconnected through Nodal and BMP2/4 (Fig. 9C). Our results are consistent with a model in which D/V patterning of the endomesodermal precursors depends on a balance between ventralising Nodal and dorsalising BMP2/4 signals (Fig. 9B). Nodal is required to regionalise the PMCs and endoderm along the D/V axis, to induce blastocoelar cells on the ventral side of the SMCs, and to restrict pigment cell precursors to the dorsal side. In addition, Nodal induces the dorsalising factor BMP2/4, which diffuses from the ventral ectoderm to the dorsal PMCs, endoderm and SMCs, where it is required to counteract the ventralising activity of Nodal in these germ layers. Our results are therefore consistent with the idea that the ventral ectoderm, like the Spemann organiser of vertebrates, is a signalling centre that patterns all three germ layers of the embryo.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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