Short-range Wnt5 signaling initiates specification of sea urchin posterior ectoderm

Daniel C. McIntyre1, N. Winn Seay2, Jenifer C. Croce3 and David R. McClay1,*

ABSTRACT

The border between the posterior ectoderm and the endoderm is a location where two germ layers meet and establish an enduring relationship that also later serves, in deuterostomes, as the anatomical site of the anus. In the sea urchin, a prototypic deuterostome, the ectoderm-endoderm boundary is established before gastrulation, and ectodermal cells at the boundary are thought to provide patterning inputs to the underlying mesenchyme. Here we show that a short-range Wnt5 signal from the endoderm actively patterns the adjacent boundary ectoderm. This signal activates a unique subcircuit of the ectoderm gene regulatory network, including the transcription factors IrxA, Nk1, Pax2/5/8 and Lim1, which are ultimately restricted to subregions of the border ectoderm (BE). Surprisingly, Nodal and BMP2/4, previously shown to be activators of ectodermal specification and the secondary embryonic axis, instead restrict the expression of these genes to subregions of the BE. A detailed examination showed that endodermal Wnt5 functions as a short-range signal that activates only a narrow band of ectodermal cells, even though all ectoderm is competent to receive the signal. Thus, cells in the BE integrate positive and negative signals from both the primary and secondary embryonic axes to correctly locate and specify the border ectoderm.

KEY WORDS: Wnt5, Sea urchin, VEGF, Ectoderm, Specification

INTRODUCTION

In an embryo, tissue boundaries form when cells of two different gene regulatory states are juxtaposed. These cells can interact with each other, and such interactions often have important consequences for development (Dahmann et al., 2011). For example, these interactions often prevent the mixture of the different cell types, thereby maintaining the boundary even in the face of cell proliferation and tissue morphogenesis (Mellitzer et al., 1999; Xu et al., 1999). In addition, cells at a boundary often act as a signaling center, directing patterning of other nearby tissues (Dahmann et al., 2011). One of the most basic, and curiously, least-studied boundaries in deuterostomes is the demarcation between the endoderm and ectoderm. Both germ layers arise from a single epidermal sheet, yet in deuterostomes, maintenance of other boundaries between somites requires signaling between the two juxtaposed tissues in order to prevent admixture and for initiation of subsequent developmental programs (Mellitzer et al., 1999; Sato et al., 2002; Watanabe et al., 2007; Watanabe et al., 2009; Xu et al., 1999). We sought to determine if similar mechanisms were working at the conserved boundary between ectoderm and endoderm.

Whereas the endomesoderm is patterned by a series of short-range signals in the posterior (vegetal) half of the embryo (Davidson, 1989; McClay, 2011), the ectoderm is primarily patterned by long-distance signals acting along the dorsoventral (oral-aboral) axis. With the exception of the neurogenic domain at the animal pole, territories within the endoderm are established by asymmetric TGFβ signaling (Angerer et al., 2000; Duboc et al., 2004). Initially, Nodal signaling is activated on the ventral (oral) side of the embryo. Nodal activates oral fates and activates BMP2/4, which is needed for aboral fates. Nodal and BMP act as positive regulators of oral and aboral ectoderm specification, respectively. Without these signals expression of markers in these territories is lost and most ectodermal cells assume a ciliary band fate (Angerer et al., 2000; Bradham and McClay, 2006; Saudemont et al., 2010).

Although the mechanisms initially specifying both endoderm and ectoderm fate are understood, how the boundary between these two tissues is established is not clear. At a molecular level, endoderm can first be distinguished from endomesoderm by the expression of Brachury by 8 hours post fertilization (hpf) when the embryo is still an indistinct hollow ball of epithelial cells (Croce and McClay, 2010). By mesenchyme blastula stage, Brachury, FoxA and nuclear β-catenin are expressed at the boundary of the endoderm and ectoderm (Logan et al., 1999; Croce and McClay, 2010). Once established, the border with the ectoderm remains stable. How the precise position of this boundary is established remains unknown. At 12-14 hpf gastrulation begins, and the ensuing tissue rearrangements provide a massive challenge to boundary integrity. What mechanisms maintain the boundary in the face of these challenges? Although the endoderm-ectoderm boundary has not been studied previously in deuterostomes, maintenance of other boundaries such as the midbrain-hindbrain boundary and the boundaries between somites requires signaling between the two juxtaposed tissues in order to prevent admixture and for initiation of subsequent developmental programs (Mellitzer et al., 1999; Sato et al., 2002; Watanabe et al., 2007; Watanabe et al., 2009; Xu et al., 1999).
In this report we characterize the origin and specification of the posterior ectoderm-endoderm boundary in the urchin, showing that it requires inputs both from endodermal cells along the animal-vegetal axis and from Nodal and BMP2/4 on the oral-aboral axis. Cells in the ectoderm at the boundary, hereafter called border ectoderm (BE), express a group of basic helix-loop-helix (bHLH) and homeodomain transcription factors including IrxA, Nk1, Pax2/5/8 and Lim1. These genes have dynamic spatial expression profiles and many are restricted to subregions of the BE. Surprisingly, perturbations showed that Nodal and BMP2/4 restricted the expression of BE genes rather than activating them, indicating that the BE utilizes TGFβ signaling in a very different way from the rest of the ectoderm. Several lines of evidence then supported the hypothesis that the signal(s) necessary for activating the BE comes from the endoderm. A detailed examination of this signaling showed that endodermal Wnt5 is necessary for activation of BE specification. Our results demonstrate that Wnt5 acts as a short-range inter-blastomere signal, activating the BE subcircuit in a narrow band of adjacent ectodermal cells. The results show further that cells in the BE integrate different types of positional information from both the primary and secondary embryonic axes in order to correctly locate the production site of signals needed for skeleton formation to take place.

RESULTS

Dynamic expression of markers within the border ectoderm

The position of the border ectoderm was established with two-color in situ hybridization. Markers of the BE region were expressed in the ectoderm immediately adjacent to the endodermal markers FoxA and Hox11/13b (Fig. 1A-D). Depending on the stage, some partially colocalized with the ciliary band, which is orthogonal to and intersects the BE on the right and left side (Fig. 1E,F). IrxA was expressed opposite to Nk1 and Pax2/5/8, indicating that it was confined to the aboral BE, whereas Nk1 and Pax2/5/8 were expressed in the oral BE (Fig. 1G,H). These results were confirmed at later stages, when the morphology of the embryo could be used to determine oral/aboral position (supplementary material Fig. S1).

Expression of the six BE marker genes selected was dynamic. Fig. 1I graphically displays the expression domain of each gene at stages every 3 hours from mesenchyme blastula through gastrulation (supplementary material Fig. S1 shows the in situ data summarized in Fig. 1I). Preliminary expression profiles for several of these genes have been observed in other species of sea urchin, suggesting that the BE is a conserved region of the echinoderm ectoderm (Czerny et al., 1997; Duloquin et al., 2007; Saudemont et al., 2010; Su et al., 2009). The earliest marker expressed in the BE was Lim1. It was expressed throughout the BE beginning at 8 hpf and was diminished by gastrulation. Expression of four other genes in the BE was detected at 9 hpf. At that time, Pax2/5/8 and VEGF, two of the BE genes tracked, were broadly expressed throughout the BE. By contrast, Nk1 and IrxA were expressed on the oral and aboral sides of the BE, respectively. By 12 hpf gastrulation had begun, and genes in the BE exhibited more restricted expression patterns. Nk1 and IrxA remained on opposite sides of the embryo. VEGF was expressed, along with Pax2/5/8 at the BE-ciliary band intersection. At 12 hpf a sixth gene, Wnt5, was also expressed in the aboral BE. Before this time Wnt5 was expressed in the endoderm. At 15 hpf, IrxA expression was restricted to two lateral patches oral relative to Pax2/5/8. At prism stage (18 hpf), VEGF expression was reduced in the BE, and Wnt5 expression had become restricted to two lateral patches, located oral relative to IrxA.

TGFβ signals restrict expression of border ectoderm markers

As specification of most sea urchin ectoderm is activated along the oral-aboral (ventrodorsal) axis by TGFβ signaling (Angerer et al., 2000; Duboc et al., 2004; Duboc et al., 2010), the expression of BE genes was assessed in embryos where Nodal signaling through the
Alk4/5/7 receptor was disrupted by SB 431542 (Imman et al., 2002; Saudemont et al., 2010; Sethi et al., 2009). Surprisingly, when Nodal signaling was inhibited (Fig. 2A,B; supplementary material Fig. S1), BE gene expression expanded along the oral-aboral axis, indicating that, unlike more anterior ectoderm, BE is inhibited by TGFβ signaling. The territorial expansion persisted throughout gastrulation and was not simply a delay. Additionally, the late restriction of IrxA and Wnt5 to lateral patches also required Nodal signaling (supplementary material Fig. S2). To confirm these results, we examined IrxA expression in embryos injected with a Nodal morpholino antisense oligonucleotide (MO). Knockdown of Nodal led to expression of IrxA around the entire BE, consistent with the SB treatment results (Fig. 2C,C′). A second domain of IrxA expression was also observed in the greater aboral ectoderm at early gastrula. There, IrxA expression was lost in Nodal MO and SB treatments (Fig. 2D,D′), suggesting that at least two cis-regulatory modules control IrxA expression, one that is activated as a consequence of Nodal signaling (aboral anterior ectoderm region, indirectly through Nodal activation of BMP) and the other that represses IrxA in response to Nodal signaling (in the oral BE region).

To further challenge these results Nodal signaling was expanded to see if that would result in the loss of aboral BE genes. Sea urchin embryos treated with NiCl2 exhibit expanded Nodal signaling and develop with exclusively oral ectoderm fates (Hardin et al., 1992). As predicted, when Nodal expression was expanded as a result of NiCl2 treatment, expression of the aboral BE genes IrxA and Wnt5 was lost (compare Fig. 2A and 2E; supplementary material Fig. S1). Early expression of Wnt5 in the endoderm was not affected by expanded Nodal expression (supplementary material Fig. S1). The oral BE gene Nk1 expanded to a complete ring in these embryos. This is consistent with a lack of BMP signaling in oralized embryos (Duboc et al., 2004; Saudemont et al., 2010). Pax2/5/8 expression in the BE (Fig. 3E″) became unrestricted (12 hpf, vegetal view in C,C′, lateral view in D,D′). (E) Changes in BE gene expression after increasing Nodal signaling with NiCl2. Aboral BE gene expression was lost, whereas oral BE gene expression expanded. (F-G′) Nodal mRNA injection eliminated BE Wnt5 expression, and expanded Pax2/5/8 expression (15 hpf, vegetal views).

Endodermal inputs activate border ectoderm specification

As TGFβ signaling acts to restrict, rather than to activate, BE gene expression.

Endodermal inputs activate border ectoderm specification

As TGFβ signaling territorially restricted BE gene expression rather than activating it, we sought another source for the activating signal(s). We reasoned that those signals could: (1) originate from the endoderm; (2) originate from the animal pole; or (3) could be unrestricted, and BE gene expression might be restricted by later signals. The latter two hypotheses were unlikely, because both required isolated animal halves to express BE genes, and this was not observed (Fig. 3A−A″′; VEGF was expressed in vegetal but not animal halves). We therefore focused on endoderm as a potential source of a signal for BE specification. Hox11/13b is at or near the top of the endoderm GRN (Peter and Davidson, 2010), so we asked if the BE was affected when Hox11/13b was knocked down. In Hox11/13b morphant embryos, we observed a loss of both IrxA and Pax2/5/8 expression in the BE (Fig. 3B−C′). These results suggested that an endodermal signal, expressed downstream of Hox11/13b, was necessary for border formation.

A preliminary survey showed that the signaling ligand Wnt5 was expressed first in endoderm, before moving to BE after gastrulation began. Fig. 3D−D′″ shows the expression of Wnt5 from 7.5 to 18 hpf. Early Wnt5 expression colocalized with the endodermal marker FoxA (Peter and Davidson, 2010), and was adjacent to the BE marker IrxA (Fig. 3E,E′). After gastrulation began at 12 hpf, Wnt5 expression was adjacent to FoxA (Fig. 3E′). Wnt5 expression also changed from a full ring before gastrulation to an aboral crescent and then to two patches on either side of the border ectoderm (Fig. 3E″″, showing an enlargement of one patch). The movement from endodermal to ectodermal expression is diagrammed in Fig. 3F,F′. Wnt5 expression depended on Hox11/13b function. Both endodermal and ectodermal Wnt5 expression was lost in Hox11/13b-MO-treated embryos (Fig. 3G−G′).

To test the hypothesis that Wnt5 was the endodermal signal, a morpholino was designed to block the translation of Wnt5 mRNA (Wnt5 MO). At 24 hpf, control injected embryos had completed gastrulation and produced a normally patterned skeleton (n=48/50
mesenchyme cells were visible in the blastocoel (Fig. 4A). However, Wnt5-MO-injected embryos had not produced any skeleton, even though gastrulation had been completed and endodermal Wnt5 expression in the endomesoderm derived entirely from the vegetal half of the embryo containing the Wnt5 MO (Fig. 4C). As the endomesoderm derives entirely from the vegetal half (Davidson et al., 1998), this should separate the two Wnt5 domains. When the Wnt5 MO was confined to the animal half, a normal pluteus larva formed (compare Fig. 4D, D′ with 4E, E′) (n = 7/9). However, when the Wnt5 MO was confined to the vegetal half of the embryo, patterning at the boundary was disrupted (compare Fig. 4F, F′ with 4G, G′) (n = 7/7) and these embryos were indistinguishable from those containing the MO in all cells. Thus Wnt5 from the endoderm affects boundary formation.

Next, we showed that disruption of Wnt5 signaling blocked expression of the other BE ectoderm markers (Fig. 5). Pax2/5/8, VEGF and IrxA expression were lost in Wnt5-MO-treated embryos (Fig. 5A–C). Loss of VEGF probably explains the lack of skeleton in Wnt5-MO-injected embryos. Similarly, endodermal Wnt5 expression required input from endodermal Wnt5 (Fig. 5D, D′). Expression of Pax2/5/8 was also lost in chimeric embryos containing the Wnt5 MO in the vegetal, but not the animal, half (Fig. 5E, E′).

We hypothesized that the endodermal Wnt5 acted as a short-range signal. If this were true, we predicted that expansion of Wnt5 signaling would lead to an expanded boundary and broad activation of BE genes throughout the ectoderm. This hypothesis was tested by misexpressing Wnt5 mRNA, or a control mRNA, throughout the entire embryo. At 24 hpf control embryos were early pluteus larvae, with normal patterning of the boundary and associated skeletal structures (Fig. 6A). Embryos injected with Wnt5 mRNA exhibited an unusual phenotype, in which an ectopic chain of skeletogenic mesenchyme cells reached over the animal pole of the embryo, just underneath the ciliary band (Fig. 6A′; n = 43/46 embryos). We confirmed this novel phenotype by staining with a skeletogenic-mesenchyme-specific antibody 1D5 (Fig. 6B, B′). As predicted, the expression domains of BE genes expanded in Wnt5-misexpressing embryos. In control embryos Pax2/5/8 was expressed in two lateral patches of BE, sitting overtop of the lateral clusters of mesenchyme cells. In Wnt5-RNA-injected embryos Pax2/5/8 expression expanded in an arc over the animal pole of the embryo but remained narrowly restricted along the oral–aboral axis (Fig. 6C–C′). A similar expression pattern was seen for VEGF (Fig. 6D–D′′′, arrows indicating ectopic staining). Finally, expression of both IrxA (Fig. 6E–E′′′) and Nk1 (Fig. 6F–F′′′) expanded from the endoderm boundary throughout the ectoderm, but remained restricted to the dorsal and ventral territories, respectively.

The pattern observed was consistent with an expansion of Wnt5 signaling assuming the continued presence of the repressive effects of Nodal and BMP2/4 seen earlier. We therefore hypothesized that BE markers should be expressed throughout the ectoderm in embryos with both expanded Wnt5 activity and disrupted TGFβ signaling. We tested this by examining Pax2/5/8 expression in embryos both injected with Wnt5 mRNA and treated with SB431542. Pax2/5/8 was expressed normally in control embryos, expressed in an arc over the animal pole in Wnt5-mRNA-injected embryos, and expressed throughout the ectoderm in embryos treated with both Wnt5 mRNA and SB (Fig. 7B–B′). This result further demonstrated the independent effects of these two signaling systems on posterior ectoderm specification.
DISCUSSION
These results suggest a model in which initially all ectodermal cells are competent to receive the Wnt5 signal. In normal embryos, endodermal Wnt5 acts as a short-range signal, activating BE genes in a narrow band of posterior ectodermal cells. TGFβ signaling then restricts expression of these genes to subregions of the BE (Fig. 7A, left). When extra Wnt5 is provided, all ectodermal cells receive the activating input for BE. However, because the activation of Nodal and BMP are unaffected by expanded Wnt5 signaling, BE gene expression remains restricted along the secondary axis of the embryo (Fig. 7A, center). When TGFβ signaling is also disrupted, BE markers are expressed throughout the ectoderm (Fig. 7A, right). The role played by TGFβ signaling in restricting BE gene expression is similar to the role these signals play in limiting ciliary band specification along the oral-aboral axis (Yaguchi et al., 2010). Our data indicate that a subcircuit of the GRN is activated in BE cells in response to Wnt5 signaling (Fig. 7C). This circuit is activated by endodermal Wnt5, which acts through an as yet unknown Wnt receptor to induce the expression of genes in the BE including Nk1, Pax2/5/8, Vegf, IrxA and Wnt5. The expression of each BE gene is then restricted to specific subterritories by Nodal and BMP2/4. Because these signals are expressed before BE restriction occurs, repressors must exist downstream of these signals to mediate their effects on transcription factors in the BE [one such repressor is likely to be Not1 (Li et al., 2012)]. Those modeled repressors or other components of the circuit then stabilize the border ectoderm territories following the later inactivation of Nodal and BMP signaling.

The GRN subcircuit(s) active in the BE may function to refine the expression boundaries of genes that are initially more broadly expressed. BE genes, including Pax2/5/8, IrxA and VEGF, are first expressed throughout the BE but then rapidly become restricted to subdomains of the BE. In other systems genes of the Iroquois, NxK, Pax, Lim, Msx and Fox families function as repressors, often working to establish boundaries between differently fated populations of cells within contiguous tissues. A good example of this is found in the dorsoventral patterning system at work in the vertebrate neural tube. In vertebrates, the neural tube is patterned on the dorsoventral axis by opposing gradients of BMP and SHH (Liem et al., 1995; Roelink et al., 1994). In response, a series of homeodomain (HD) and bHLH transcription factors are activated in specific subregions of the neural tube. Adjacent pairs of transcription factors act as mutual repressors, creating the sharp expression boundaries observed along the dorsoventral axis of the neural tube (Briscoe and Ericson, 2001). A similar regulatory logic appears to apply to the sea urchin BE as well. Our results indicate a broad activation signal is combined with a series of spatially restricted transcriptional repressors that subdivide the different regions within the BE. Detailed GRN analysis has not yet been done, but one prediction is that at least some of these transcription factors work as mutual repressors in the BE to restrict territorial expression.

Perturbations to Wnt5 suggest that it signals directly to the border ectoderm, and an important question is how that signal is transduced. In other systems, Wnt5 is thought to signal primarily through the noncanonical, or β-catenin-independent, Wnt signaling pathway, where it plays a role in directing cell migration and convergent extension (Nishita et al., 2010). However, Wnt5 has also been reported to activate the canonical pathway in certain contexts (Mikels and Nusse, 2006). In the sea urchin, nuclear β-catenin is necessary for endoderm, but not ectoderm, formation (Cameron and Davidson, 1997; Logan et al., 1999). Thus, Wnt5 probably acts through a β-catenin-independent pathway to activate BE genes.

A second question logically follows—what is the receptor(s) active in the ectoderm through which Wnt5 signals? Wnt ligands are known to signal through Frizzled (Fzd) receptors, and the sea urchin genome contains four Fzds comprising four of the five deuterostome frizzled families (Bhanot et al., 1996; Croce et al., 2006a). Perturbations to two of these genes, Fzd5/8 and Fzd1/2/7, lead to defects in mesoderm and endoderm specification, respectively (Croce et al., 2006b; Lhomond et al., 2012). Frz1/2/7 is also expressed in the ectoderm, and perturbations to Fzd1/2/7 cause defects in skeleton formation. However, the phenotype of these embryos is less severe than that of Wnt5 perturbations, suggesting that if Fzd1/2/7 conducts the Wnt5 signal, other co-receptors may also function.

Wnt5 also has been reported to have a highly conserved interaction with the orphan tyrosine kinase receptor ROR, which acts in many developmental contexts, including skeletal and nervous
system development (Green et al., 2008). ROR1/2 double-knockout mice exhibit a nearly identical phenotype to Wnt5a knockout mice, indicating that RORs are likely to be the key mediator of Wnt5a signaling in that system (Ho et al., 2012). Wnt5-ROR signaling is believed to be a noncanonical Wnt signaling pathway; however, the phenotypes of ROR double-knockout mice do not substantially overlap with Van Gogh double-knockout mice, indicating that it does not act through the planar cell polarity pathway (Ho et al., 2012; Song et al., 2010). One ROR receptor is encoded in the sea urchin genome, but the gene has otherwise not been studied in sea urchins. It will be important to determine if the interaction with Wnt5 is conserved and if it does indeed mediate Wnt5 activation of BE genes.

Sea urchin endomesoderm is segregated into nonskeletogenic mesenchyme and endoderm by Delta/Notch signaling (Croce and McClay, 2010; Sherwood and McClay, 1997; Sherwood and McClay, 1999; Sweet et al., 2002). Prolonged exposure of Veg2 progeny that remain in contact with Delta-expressing skeletogenic mesoderm cells activates specification of the nonskeletogenic mesoderm and separates these cells from the endoderm (Croce and McClay, 2010). Because Delta/Notch acts as a short-range signal, it can effectively segregate the two neighboring groups of cells. Similarly, activation of the BE, which is at most four cells wide, requires a signal that acts over a relatively short range. Our data from Wnt5-misexpressing embryos indicate that Wnt5 acts in such a manner. Indeed, the entire ectoderm appears competent to receive this signal, and in Wnt5-misexpressing embryos BE genes are activated in the ectoderm without restriction along the animal-vegetal axis. Wnt ligands are known to be lipid modified, and this dramatically reduces their diffusion and activity as long-range signals (MacDonald et al., 2009). In systems where Wnts act in this manner, other accessory proteins such as Reggie-1 are required, possibly to package the ligands into a less hydrophobic form (Katanaev et al., 2008). Consistent with these results, endogenous Wnt5a appears to not be released into the media in mammalian cell culture systems and wild-type cells are unable to rescue Wnt5a−/− cells even when cultured in close proximity (Ho et al., 2012). These authors conclude that Wnt5a signaling may require close contact for effective signaling. That is consistent with the conclusion of this work, in which endodermal Wnt5 acts as a short-range signal to activate BE fates in a narrow band of ectodermal cells.

Much of the current understanding of ectoderm specification in vertebrates has been gleaned from studying amphibians, where dorsoventral specification distinguishes between neural and epidermal fates. Ectodermal cells receiving BMP become epidermis. The dorsal ectoderm, however, is protected from these signals by
secreted inhibitors such as Noggin and Chordin (Hemmati-Brivanlou and Melton, 1994; Sasai et al., 1994; Smith et al., 1993). As a result, a region of the dorsal ectoderm assumes a neural fate. Wnt signaling then further refines neural fates along the anteroposterior axis, distinguishing brain from spinal cord (Bouwmeester et al., 1996). This model appears true for other vertebrates and sea urchins (Muñoz-Sanjuán and Brivanlou, 2002; Range et al., 2013). Our results suggest that an additional layer of complexity exists in this system. Signaling from the endoderm distinguishes the BE from the remaining ectoderm in the sea urchin, and in mammals signaling from the anterior endoderm distinguishes the anterior neurectoderm from more posterior neurectoderm (Beddington and Robertson, 1999). In both cases Wnt signaling is involved.

The BE region appears to have at least two major functions in the embryo. First, because their specification history is unique, cells in the BE have the potential for providing territory-specific patterning information. The BE is a rich source of patterning information for the skeletogenic mesenchyme (Armstrong et al., 1993; Duloquin et al., 2007; Guss and Ettensohn, 1997; Röttinger et al., 2008), and perturbations to BE specification disrupt skeleton patterning. Second, specification of the BE probably includes regulatory components necessary to enforce the ectoderm-endoderm boundary, which remains distinct through gastrulation and beyond. Immiscibility at a boundary is frequently achieved via signaling (Dahmann et al., 2011). For example, alternate rhombomeres in the zebrafish hindbrain are segregated by repulsive Eph-Ephrin signaling (Xu et al., 1999). In the sea urchin, a clean boundary between the ectoderm and endoderm is maintained throughout gastrulation and beyond, based on marker expression. How this boundary remains stable has yet to be shown. In the future, the ease of embryological intervention and advanced imaging will allow the BE to be studied in detail and make the sea urchin an attractive system in which to address formation and function of this conserved boundary.

**Materials and Methods**

**Embryos, surgery and drug treatments**

Adult *Lytechinus variegatus* were collected at the Duke University Marine Lab (Beaufort, NC, USA) or were purchased from Sea Life (Tavernier, FL, USA). Adult sea urchins were spawned by intracoelomic injection of 0.5 M KCl. Once fertilized, embryos were cultured in artificial seawater (ASW) at 20-23°C. Microsurgical manipulations including mesomere transplants and animal/vegetal recombinations were performed at the 16-cell or 32-cell stage. Donor embryos were injected with either a rhodamine or fluorescein dextran lineage tracer so that the different clones could be followed later in development. Microsurgery was performed with fine glass needles and Narishige micromanipulators. SB 431542 (Sigma) was used at 10 to 50 μM and added to cultures after fertilization. NiCl₂ was used at 50-100 μM and added to cultures after fertilization. For both SB and NiCl₂ treatments, minimal effective doses were used and these were determined by dose-response experiments.

**MO and mRNA microinjection**

MOs were obtained from GeneTools. MO sequences are as follows: Wnt5-MO (5′-CCCTGCGACAAAGGCCACTCGA-3′), Nodal-MO (5′-TGGATGTTAAAGGCTTCTAGAT-3′), BMP2/4-MO (5′-GACC-CAATGTGGTGAACATCACTCAG-3′), Hox11/13b-MO (5′-AAGCCTGT-TCCATACTATCTCGCAT-3′), Control-MO (5′-TCTTACCTACGTTACATTATAA-3′) (Bradham and McClay, 2006; Bradham et al., 2009; Peter and Davidson, 2010). MOs were used at the following concentrations: Wnt5, 1.0 mM; Nodal, 0.3 mM; BMP2/4, 0.5 mM; Hox11/13b, 0.75 mM; Control, at concentration equal to experimental. Wnt5 CDS, Wnt5-DN and mem-RFP were cloned in pCS2 and mRNA was synthesized *in vitro* using the mMessage mMachine kit (Ambion). Nodal mRNA was made as previously described (Bradham and McClay, 2006). For injection, mRNAs were diluted in 20% glycerol in diethylpyrocarbonate-treated H₂O with or without a fluorescent lineage marker as indicated in the text. Concentrations used were as follows: Wnt5 mRNA, and mem-RFP mRNA were injected at 300-500 ng/μl; Nodal mRNA was injected at 100-250 ng/μl. The precise concentration was slightly different depending on the batch of urchins, and was determined by dose-response experiments. Control mRNA (mem-RFP) was used at the same concentration as the corresponding experimental
mRNA. GenBank accession numbers for the mRNAs cloned for this work are as follows: Wnt5, KC133515; Pax2/5/8, KC133516; IrxA, KC133517; Nk1, KC133518; VEGF, KC133519.

RNA in situ hybridization

RNA in situ hybridization was performed using standard methods. Briefly, embryos were fixed in 4% paraformaldehyde for 60 minutes and stored in methanol at −20°C. Digoxigenin-11-UTP- and fluorescein-12-UTP-labeled RNA probes were synthesized in vitro and used at 1 ng/µl. Hybridization took place at 60-65°C. For chromogenic in situ hybridizations, probes were visualized using alkaline phosphatase conjugated anti-DIG antibody (Roche, 1:1500) and the yeast unigene ASH1554. (1989). Characterization of novel Pax genes of the sea urchin and Drosophila reveals an ancient evolutionary origin of the Pax2/5/8 subfamily. Mech. Dev. 67, 179-192.


