Available online at www.sciencedirect.com

ScienceDirect

Developmental Biology 302 (2007) 494–503

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Sp-Smad2/3 mediates patterning of neurogenic ectoderm by nodal in the sea urchin embryo

Shunsuke Yaguchi [1], Junko Yaguchi [1], Robert D. Burke *

Departments of Biology and Microbiology/Biochemistry, University of Victoria, Victoria, POB 3020, STN CSC, 3800 Finnerty Rd, Victoria, BC, Canada V8W 3N5

Received for publication 1 August 2006; revised 15 September 2006; accepted 5 October 2006
Available online 10 October 2006

Abstract

Nodal functions in axis and tissue specification during embryogenesis. In sea urchin embryos, Nodal is crucial for specification of oral ectoderm and is thought to pattern neurogenesis in the animal plate. To determine if Nodal functions directly in suppressing neuron differentiation we have prepared mutant forms of Sp-Smad2/3. Expressing an activated form produces embryos similar to embryos overexpressing Nodal, but with fewer neurons. In chimeras in which Nodal is suppressed, cells expressing activated Sp-Smad2/3 form oral ectoderm, but not neurons. In embryos with vegetal signaling blocked, neurons do not form if activated Smad2/3 is co-expressed. Expression of dominant negative mutants produces embryos identical to those resulting from blocking Nodal expression. In chimeras overexpressing Nodal, cells expressing dominant negative Sp-Smad2/3 form aboral ectoderm and give rise to neurons. In permanent blastula chimeras dominant negative Sp-Smad2/3 is able to suppress the effects of Nodal permitting neuron differentiation. In these chimeras Nodal expression in one half suppresses neural differentiation across the interface. Anti-phospho-Smad3 reveals that the cells adjacent to cells expressing Nodal have nuclear immunoreactivity. We conclude Sp-Smad2/3 is a component of the Nodal signaling pathway in sea urchins and that Nodal diffuses short distances to suppress neural differentiation.

Keywords: Cell fate specification; Neural development; Nodal signaling; Smad

Introduction

The TGF-β growth factors have emerged as a major family of paracrine signaling molecules regulating cell fate specification in early development. Among the TGF-β family members, Nodal is of particular interest because it has fundamental roles in establish asymmetries in embryos; either in establishing embryonic axes or left–right determination. Nodal was first identified in mice (Zhou et al., 1993) and plays important roles in induction of mesoderm and endoderm, neural patterning, and the left–right axis in vertebrate embryos (Feldman et al., 2000; Lowe et al., 2001; Vincent et al., 2003). Like all members of the TGF-β superfamily Nodal activates heteromeric receptors with intrinsic serine–threonine kinase activity (Shi and Massague, 2003). Nodal signaling is inherently complex because of the diversity of receptors, co-receptors, secreted antagonists and modulators (Schier, 2003). Nodal orthologues are not known from *C. elegans* and *Drosophila* making ascidians and sea urchins potentially useful invertebrate models for understanding the molecular mechanisms that underlie this important signaling pathway. In ascidians, Nodal is expressed in a single laterally positioned blastomere and functions in patterning the neural plate (Hudson and Yasuo, 2005). In sea urchin embryos, Nodal is an initiator of the gene regulatory network that specifies oral ectoderm (Duboc et al., 2004). Later in development, expression of Nodal inhibits the formation of the adult rudiment on the right side of larval body (Duboc et al., 2005). As well, Nodal has been proposed to function in patterning neurogenesis in the animal plate (Yaguchi et al., 2006). Thus, Nodal's roles in breaking embryonic symmetry and patterning nervous systems appear to be conserved deuterostome traits.

* Corresponding author. Fax: +1 250 721 7120.
E-mail address: rburke@uvic.ca (R.D. Burke).
[1] Current address: Developmental Mechanisms Unit, National Institute of Dental and Craniofacial Research, National Institutes of Health, 30 Convent Dr. MSC 4326, Bethesda, MD, USA.

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.10.010

Smad transcription factors are essential downstream targets of Nodal receptors. In mammals receptor-regulated Smads (R-Smads, Smad1, 2, 3, 5 and 8), Co-Smad (Smad4), and inhibitory Smads (I-Smad, Smad6 and 7) are substrates for receptor kinase activity and act cooperatively to initiate transcription of target genes. Because of receptor affinities, Smad2 and Smad3 mediate TGF-β/Activin/Nodal signaling and Smad1, Smad5 and Smad8 mediate BMP signaling. R-Smads have an amino-terminal MH1 domain, a linker region, an MH2 domain and at the carboxyl end is a Ser-X-Ser phosphorylation motif. The sequencing of the sea urchin genome has revealed a relatively simple set of TGF-β signaling molecules. Gene predictions for the sea urchin genome identify orthologues of Smad1/5/8, Smad2/3, Smad4 and Smad6 (Lapraz et al., 2006; Howard-Ashby et al., 2006).

In sea urchins embryonic neurogenesis in the animal plate is thought to be specified by a maternal program. Yaguchi et al. (2006) noted that the number and pattern of neurons in the animal plate depended on the adjacent ectoderm. Oral ectoderm suppresses serotonergic neuron formation, whereas aboral or ciliary band ectoderm appears permissive. Experiments with whole embryos and chimeras suggested this effect was mediated by Nodal suppressing neurogenesis. However, as Nodal is part of the initiation of oral specification, it is not clear if Nodal acts directly on neurons, or if there is a second paracrine factor involved. Here we report that Sp-Smad2/3 is a component of the Nodal pathway in *Strongylocentrotus purpuratus* where it mediates specification of oral ectoderm and acts directly on the animal plate to suppress formation of serotonergic neurons.

Materials and methods

Animals

Gametes of the sea urchin, *S. purpuratus*, collected locally, were obtained by intra-coelomic injection of 0.5 M KCl.

Amplification and mutagenesis of Sp-Smad2/3

PCR primer set for the predicted cDNA of Sp-Smad2/3 was designed from genomic sequences (Human Genome Sequencing Centre, Baylor College of Medicine, http://www.hgsc.bcm.tmc.edu/projects/seaurchin/) Smad-F1; ATGAGCACATTAAGCTTGCCT Smad-R1; TTAAGACACGCTGGAGCACTG. A single band of the predicted size was amplified from cDNA synthesized from 19-h hatching embryos. The amplified DNA was ligated into pTOPO-blunt vector (Invitrogen), and sequenced. To make a pseudophosphorylated form of Sp-Smad2/3 we designed PCR primers to change the C-terminal domain (Ser-Val-Ser) to Asp-Val-Asp; Smad-N; GAATTCATGAGCCACATTAAGC (EcoRI cloning site underlined) Smad-pp; CTCGAGTCAATCCACATCGGAGCACTGTTTATC (XhoI cloning site underlined). To delete or mutate this phosphorylation site, we designed the following reverse primer sets; Smad-dC CTCGAGTCAGGAGCACTGTTTATCAGG; Smad-mC CTCGAGTCATGCCACTGCGGAGCACTGTTT. After amplifying the modified forms of Sp-Smad2/3, inserts were subcloned into pCS2+ or derivative vectors at EcoRI-XhoI site. The diagram of Fig. 1A shows modified Sp-Smad2/3 used in this study.

Microinjection of modified Smad3 mRNAs

Eggs were prepared as described previously (Yaguchi et al., 2006). Messenger RNA (mRNA) was synthesized with mMessage mMachine kit (Ambion, TX) quantified spectroscopically (NanoDrop), and injected at a concentration of 1.0–3.0 μg/μl in a 22.5% glycerol. In some experiments, we injected one blastomere of a 2-cell stage following the injection of another reagent into the unfertilized egg. In some experiments we co-injected myc or DSRed mRNA (0.1 μg/μl) as a lineage tracer.

Whole-mount immunohistochemistry

Three-day old embryos were fixed with 4% paraformaldehyde in filtered seawater (FSW) for 10 min at ambient temperature (AT). They were washed with phosphate-buffered saline with Tween (PBST; 0.8 mM Na_2HPO_4–$12H_2O$, 0.15 mM KH_2PO_4, 14 mM NaCl, 0.27 mM KCl, 0.1% Tween-20, pH 7.0) three times, blocked with 5.0% lamb serum in PBST for 1 h (AT), and then they were incubated with primary antibodies overnight at 4°C. Primary antibodies were detected with Alexa 488 (1:800 dilution in PBST; Molecular Probes, Eugene, OR)- or Alexa 564-conjugated secondary antibodies (1:800 dilution in PBST) for 2 h at AT. After washing three times with PBST, specimens were examined with a confocal microscope (Zeiss 410) or with epifluorescence (Leica DM600). The primary antibodies we used were: rabbit anti-serotonin antibody (Sigma, 1:500 dilution), rabbit anti-Sp-Nk2.1 antibody (Takacs et al., 2004 1:800 dilution), mouse anti-synaptotagmin (1E11) (Nakajima et al., 2004, 1:100 dilution), rabbit anti-Spec1 (Wikramanayake et al., 1995, 1:800 dilution), mouse anti-myc (9E10, Evan et al., 1985 1:800 dilution), rabbit anti-phosphoSmad3 (Cell Signaling #9514 1:500 dilution).

Whole-mount in situ hybridization

Whole-mount in situ hybridization to detect Sp-Hnf6 mRNA expression was performed as previously described (Yaguchi and Katow, 2003). Hybridization was for 1 week at 47.5°C. After in situ hybridization, we stained the sample with anti-Sp-Nk2.1 or anti-myc antibodies as described.

Results

Sp-Smad2/3 is regulated post-transcriptionally

We amplified Sp-Smad2/3 (SPU_017642) from the predicted start codon to terminal codon from cDNA synthesized from total RNA of 19-h hatching blastulae. The cDNA is 1284 nt long and the deduced amino acid sequence (427 amino acids) confirms gene predictions (NCBI Accession: AB267397). The predicted protein has Smad family-specific MH1 and MH2 domains.

Previous studies indicate that Nodal can induce oral ectodermal features in presumptive oral and aboral ectoderm, and that the animal plate has the capability of receiving Nodal signaling (Duboc et al., 2004; Yaguchi et al., 2006). This suggests that Nodal receptors and signal pathway components are present throughout the ectoderm. Gene tiling array data and EST data indicate that Smad2/3 is expressed throughout early cleavage stages, in primary mesenchyme cells and into early larval life (Samanta et al., 2006; Poustka et al., 2003; Zhu et al., 2001). QPCR analysis of Sp-Smad2/3 indicates mRNA is present in unfertilized eggs and expressed until at least 48 h (data not shown). It has been reported that the activity of Smad2 or Smad3 in other organisms is regulated by its phosphorylation by TGF-β receptors. In the sea urchin embryo, overexpression of wild-type Sp-Smad2/3 has no developmental effect (data not shown), whereas expression of mutant forms (see below) invariably altered development. This suggests that the activity of Sp-Smad2/3 is not regulated by tissue specific expression and, as in other organisms, post-transcriptional mechanisms control the function of Sp-Smad2/3.

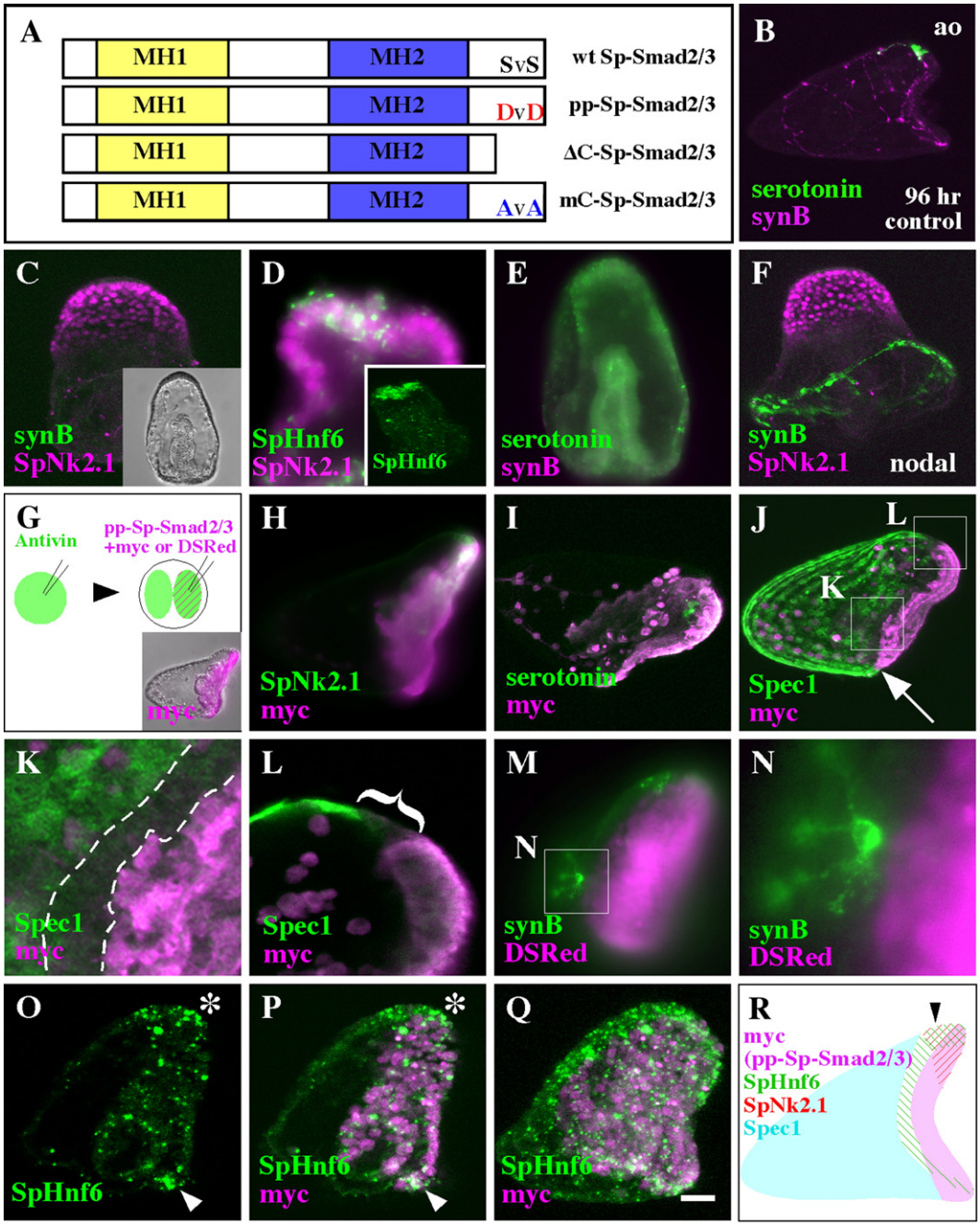


Fig. 1. Overexpression of pseudophospho-Sp-Smad2/3 (pp-Sp-Smad2/3) dominantly induces oral ectoderm. (A) Mutated or truncated form of Sp-Smad2/3 used in this study. MH1 and MH2 domain shows Mad-homology1 and Mad-homology 2, respectively. (B) Serotonergic neurons and ciliary band neurons (synB) in 96-h control embryo. ao; apical organ. (C–E) Embryos injected with pp-Sp-Smad2/3 mRNA. (C) Sp-Nk2.1 proteins are present at the animal plate and sub-region of radialized oral ectoderm. There are no synB-positive neurons. Inset shows the DIC image. (D) Sp-Hnf6 and Sp-Nk2.1 co-localized animal plate is restricted to the animal pole domain. Inset shows the Sp-Hnf6 mRNA localization in the entire embryo. (E) Neither serotonergic nor synB neurons are detected. (F) The location of Sp-Nk2.1 proteins in the Nodal injected embryo is similar to that of pp-Sp-Smad2/3 injected embryo. However, well-developed plexus of synB neurons is detected at the ciliary band region in Nodal injected embryo. (G–R) Chimeras in which the endogenous Nodal activity is blocked throughout the entire embryo by Antivin injection and one half of the embryo is misexpressing pp-Sp-Smad2/3 and lineage tracer. (G) The scheme for making chimeras with Antivin injection into the unfertilized egg and pp-Sp-Smad2/3 injection into one blastomere of 2-cell stage. Inset shows chimeras that have a pluteus shape form and the pp-Sp-Smad2/3 co-injected half always becomes the oral surface. (H) Sp-Nk2.1 protein is detected in cells expressing and cells not expressing pp-Sp-Smad2/3. (I) Serotonergic neurons are detected only in cells not expressing pp-Sp-Smad2/3. (J–L) Aboral marker Spec1 and pp-Sp-Smad2/3 injected half are separated by a region lacking both markers (arrow in panel J). These chimeras fail to form a mouth at 72 h. (M, N) Ciliary band (SynB) neurons are present in the Spec1 and pp-Sp-Smad2/3-negative region. (O–Q) Double staining of Sp-Hnf6 in situ hybridization and myc lineage marker in chimeras. Animal plate (asterisk) and a part of ciliary band between post-oral arms (white arrowhead) express *Sp-Hnf6* genes even though these cells include pp-Sp-Smad2/3, whereas lateral ciliary bands develop from pp-Sp-Smad2/3-negative half. (R) The scheme for summary of the ectodermal region of the chimera. Black arrowhead shows the place where serotonergic neurons are differentiated. Scale bar = 20 μm.

Pseudophosphorylated Sp-Smad2/3 induces oral ectoderm and inhibits neurogenesis

Smad2 or Smad3 mediates TGF-β/Activin/Nodal signaling and is activated by phosphorylation of conserved serine residues at the carboxyl end of the protein (Ser-X-Ser; Massague and Wotton, 2000). It has been shown that pseudophosphorylated R-Smads by the substitution of negatively charged amino acids for carboxyl serines can result in an activated form that functions independently of ligands and receptors (Chacko et al., 2001). We prepared a pseudophosphorylated form of Sp-Smad2/3 (pp-Sp-Smad2/3) by site-directed mutagenesis of the serine residues at the carboxy end (S425D, S427D; Fig. 1A). Embryos developing from eggs injected with RNA encoding pp-Sp-Smad2/3 are radially symmetric and bell-shaped. Animal plate ectoderm is a unique region of thickened ectoderm that co-expresses the transcription factors Sp-Nk2.1 and Sp-Hnf6 and gives rise to the apical organ (Yaguchi et al., 2006). Embryos expressing pp-Sp-Smad2/3 have thickened ectoderm at the animal pole and the domain of expression of Sp-Nk2.1 is radially symmetric and broader than the animal plate (Fig. 1C). Embryos expressing pp-Sp-Smad2/3 double labeled for Sp-Nk2.1 and Sp-Hnf6 indicate that the animal plate is restricted to the thickened ectoderm portion of the Sp-Nk2.1 domain (Fig. 1D). Neurons could not be detected in 72-h embryos with either anti-serotonin or anti-synaptotagmin (synB, Fig. 1E). In 96-h pp-Sp-Smad2/3 injected embryos a small number of anti-synaptotagmin immunoreactive neurons could be detected. Spec1, a late aboral ectoderm-specific marker (Wikramanayake et al., 1995), is restricted to a small region of ectoderm surrounding the anus in pp-Sp-Smad2/3 injected embryos (data not shown). The form of the embryos and the distribution of tissue-specific markers indicate that expression of pp-Sp-Smad2/3 produces a similar phenotype to expression of Nodal (Fig. 1F; Duboc et al., 2004). A difference between embryos expressing Nodal and those expressing pp-Sp-Smad2/3 is that in embryos expressing Nodal, anti-synaptotagmin immunoreactive neurons are present in 72 h where they form tracts of ciliary band associated axons, whereas there are fewer neurons in embryos expressing pp-Sp-Smad2/3 (Fig. 1F).

To further characterize the effect of expression of pp-Sp-Smad2/3, we prepared chimeras in which endogenous Nodal activity is blocked by expression of Antivin throughout the embryo, and in one half of the embryo pp-Sp-Smad2/3 is expressed with a lineage tracer (myc or DSRed). Overexpression of Antivin produces the same phenotype as Nodal-morpholino antisense oligo injection (Duboc et al., 2004). These chimeras have a typical pluteus shape (Fig. 1G), whereas embryos injected with Antivin alone are radially symmetric (Duboc et al., 2004 and data not shown). The half of the embryo expressing pp-Smad2/3 invariably formed the oral ectoderm, indicating pp-Sp-Smad2/3 induces oral ectoderm (Fig. 1G, 99% of 196 chimeras). However, most of the chimeras lacked a mouth at 72 h (Fig. 1J). Preparation of chimeras with anti-Sp-Nk2.1 indicates that the thickened animal plate is composed of myc-positive and myc-negative ectoderm (Fig. 1H). Serotonin containing neurons are present in the animal plate, but invariably they are derived from cells not expressing myc and pp-Smad2/3 (Fig. 1I). In chimeras prepared with anti-Spec1, most of the half of the embryo expressing Antivin alone became aboral ectoderm (Fig. 1J). At the interface of the chimeric embryo there is a narrow band, 3–4 cell diameters wide that is derived from the half of the embryo expressing Antivin alone (myc negative) that does not express Spec1 (Fig. 1K). Anti-synaptotagmin immunoreactive neurons derived from cells not expressing pp-Sp-Smad2/3 are detected at this narrow band (Figs. 1M, N). This band of ectoderm expressing Antivin, but not Spec1 crosses the animal plate, and in this region the cells express Sp-Nk2.1 protein and some of these cells are immunoreactive to anti-serotonin (Fig. 1L). Sp-Hnf6 mRNA serves as a marker for ciliated cells of the ciliary band (Figs. 1O–R). In the animal plate of chimeras, Sp-Hnf6 mRNA is expressed throughout the animal plate in myc-positive and myc-negative cells (Figs. 1O and P, asterisk). In the ciliary band lateral to the mouth between the pre-oral and post-oral arms, Sp-Hnf6 mRNA is in cells adjacent to the myc-positive oral ectoderm, in a position consistent with the strip of cells that are derived from the half of the embryo expressing Antivin alone (myc negative) that does not express Spec1 (Figs. 1J, K). However, Sp-Hnf6-positive ciliary band between post-oral arms is derived from the myc-positive half, indicating that these cells are differentiated into the ciliary band cells despite the expression of pp-Sp-Smad2/3 (Fig. 1P, arrowhead). Endomesodermal tissues appear to be unaffected by the expression of pp-Sp-Smad2/3 (Figs. 1C, H, and P). These experiments indicate that ciliary band cells are derived from both halves of the embryo, but neurons do not form from cells expressing pp-Sp-Smad2/3.

Expression of Δ-cadherin in sea urchin embryos blocks nuclearization of β-catenin and vegetal signaling, and produces permanent blastulae with an expanded animal plate (Logan et al., 1999). Co-injection of Δ-cadherin and pp-Smad2/3 enables us to observe the effect of pp-Sp-Smad2/3 in the animal plate region without endogenous vegetal signaling (Yaguchi et al., 2006). The co-injected embryos are morphologically identical to embryos injected with Δ-cadherin alone. However, the entire region of thickened ectoderm have Sp-Nk2.1 protein in their nuclei and no serotonergic neurons could be detected (Figs. 2A, B). This phenotype was similar to that of Δ-cadherin and Nodal co-injected embryos (Yaguchi et al., 2006). In addition, we prepared chimeras in which vegetal signaling is blocked throughout the entire embryo and half of the embryo co-expressed pp-Sp-Smad2/3. The half co-expressing Δ-cadherin and pp-Sp-Smad2/3 had thicker animal plate region and thinner vegetal region and no serotonergic neurons formed. The half expressing Δ-cadherin alone produced large numbers of serotonergic neurons (Fig. 2D). Serotonergic neurons formed directly at the interface with cells co-expressing pp-Sp-Smad2/3 and Δ-cadherin, indicating that suppression of serotonergic neuron formation is cell autonomous.

Truncated or mutated Sp-Smad2/3 acts as a dominant-negative

To further investigate the role of Sp-Smad2/3 we prepared cDNAs in which the carboxyl-terminal serine residues were

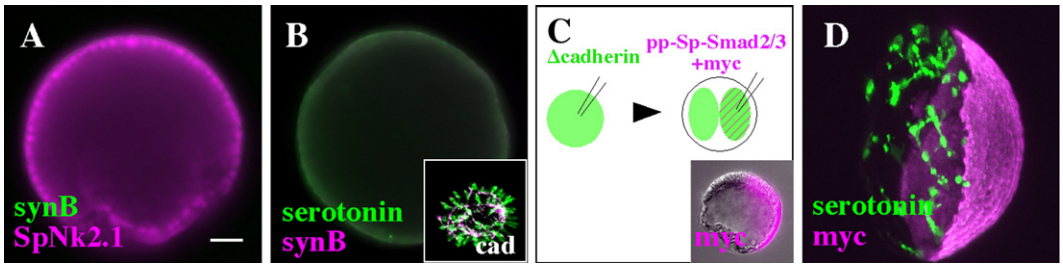


Fig. 2. pp-Sp-Smad2/3 suppresses the differentiation of serotonergic neurons in the animal plate. (A) Sp-Nk2.1 proteins are present at the entire ectoderm and no synB signals are detected in the Δ-cadherin and pp-Sp-Smad2/3 co-injected embryo. (B) No serotonergic neurons differentiate in the co-injected embryo. Inset shows the serotonergic neurons in Δ-cadherin injected control embryo. (C) The scheme for making chimeras with Δ-cadherin injection into the unfertilized egg and pp-Sp-Smad2/3 injection into one blastomere of 2-cell stage. Inset shows DIC image of the chimera in which the vegetal signaling is blocked in the entire embryo and one half of the embryo was injected with pp-Sp-Smad2/3 (myc positive). (D) Stack of confocal optical sections showing differentiation of serotonergic neurons is suppressed only in the co-injected half of the chimera. Scale bar=20 μm.

deleted (S425Stop; ΔC-Sp-Smad2/3, Fig. 1A) or substituted to alanine residues (S425A, S427A; mC-Sp-Smad2/3, Fig. 1A). Embryos injected with ΔC-Sp-Smad2/3 are radially symmetric, bell-shaped, with somewhat thicker lateral ectoderm and a complete gut (Fig. 3A). The expression of Sp-Nk2.1 is restricted to the central portion of the thickened epithelium of the animal plate. A ring of serotonergic neurons differentiate around the perimeter of the animal plate (Figs. 3B, D). The lateral ectoderm has a large number of anti-synaptotagmin immunoreactive neurons scattered throughout (Fig. 3C). The ΔC-Sp-Smad2/3 injected embryos are identical to embryos that result from blocking Nodal expression, by expression of Antivin RNA, Nodal-morpholino injection, or SB431542 treated (Figs. 3I, J, Duboc et al., 2004; Yaguchi et al., 2006). Expression of mC-Sp-Smad2/3 produces embryos that are identical to those expressing ΔC-Sp-Smad2/3 (Figs. 3E–H). These results indicate that terminal truncations of Sp-Smad2/3 or mutations that prevent phosphorylation are able to block endogenous Nodal signaling.

The test the ability of ΔC-Sp-Smad2/3 to block Nodal signaling, we produced chimeras in which Nodal RNA is expressed throughout the entire embryo and after the first cleavage division, half of the embryo was co-injected ΔC-Sp-Smad2/3 with myc lineage tracer. In these chimeras oral and aboral ectoderm domains form and the half of the embryo expressing exogenous Nodal and ΔC-Sp-Smad2/3 always developed into the aboral ectoderm (Fig. 3L). Serotonergic neurons with bilateral symmetry form in these chimeras and the neurons are always derived from the co-injected half of the embryo (Fig. 3L). These results indicate that ΔC-Sp-Smad2/3 is able to block Nodal signaling in the co-injected half and form aboral ectoderm and serotonergic neurons.

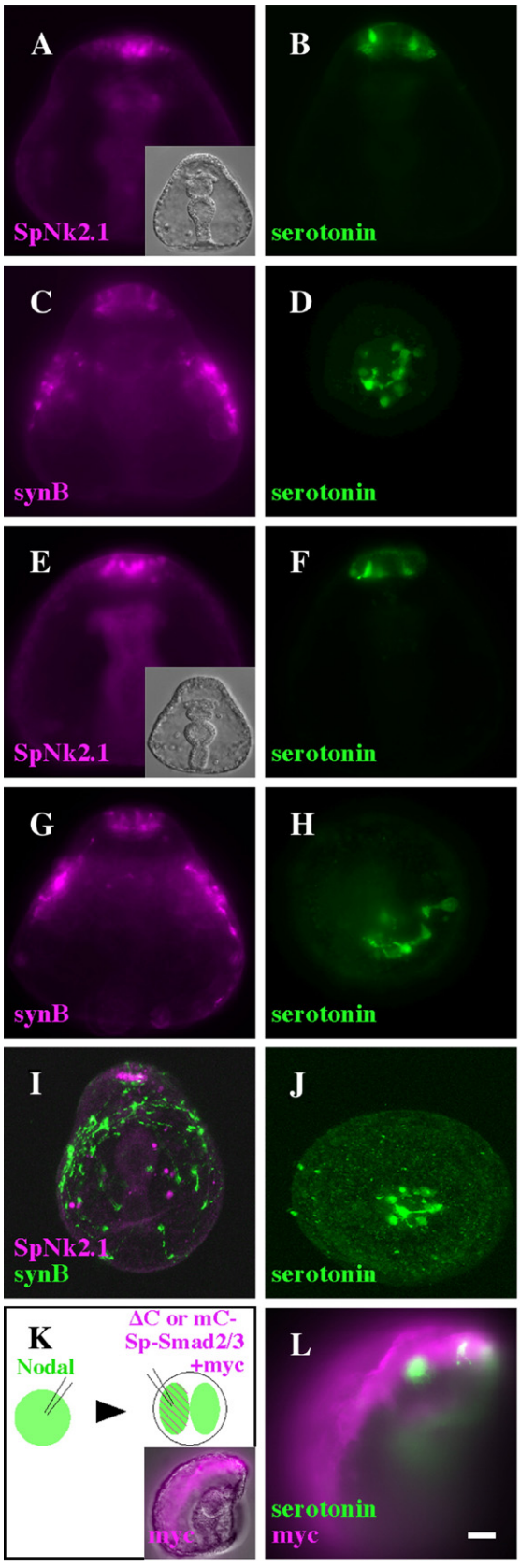
To examine the function of Sp-Smad2/3 in the neurogenesis, we made chimeras in which the vegetal signaling and serotonergic neuron differentiation were blocked throughout the entire embryo by the expression of Δ-cadherin and Nodal, in addition, half of the embryo was co-injected ΔC-Sp-Smad2/3. In these chimeras the co-injected half formed a large number of serotonergic neurons in the thickened epithelium, whereas the Δ-cadherin and Nodal injected only rarely produced neurons (Figs. 4A–D, H). Using mC-Sp-Smad2/3, phenotypes identical to those with ΔC-Sp-Smad2/3 resulted (Figs. 4E–G). Thus, mutated and truncated Smad2/3 are able to rescue the suppression of serotonergic neuron differentiation produced by Nodal.

Diffusion of nodal activates Sp-Smad2/3

Antibodies that bind phosphorylated forms of Smads have been useful in determining the timing and location of endogenous signaling (Faure et al., 2000). We have found that an antibody that was prepared against the human Smad3 carboxyl end (SPSIRCSSVS; AAL68976) appears to recognize sea urchin Smad2/3 (PPDKQCSSVS) and Smad1/5/8 (SPHNPISSVS; SPU_020722, SPU_023107). In embryos fixed throughout early cleavage, the antibody did not localize to nuclei. However, in embryos fixed at 11 h (13°C), there is a clear nuclear immunoreactivity on one surface of the embryo (Figs. 5A–C). By 24 h, when primary mesenchyme cells begin to ingress, one half of the embryo has strong immunoreactivity and the other half is weak (data not shown). SB431542 is an inhibitor of TGF/Activin type IB receptor (Duboc et al., 2005; Yaguchi et al., 2006) and is useful in distinguishing activation of Sp-Smad2/3 from Sp-Smad1/5/8. The initial phase of nuclearization of Smad on one surface of the embryos is suppressed applying SB431542 for 1 h (Figs. 5A–F). However, after PMC ingression, the stronger nuclear immunoreactivity is insensitive to SB431542. This indicates that the initial activation of Smad to one surface of the embryo in early blastulae is mediated by Type 1B receptors, whereas the later strong signals involve receptors that are not inhibitable with SB431542.

There are no clear markers for the animal plate ectoderm at this stage, so we are unable to determine if any of the cells in early blastulae responding to Nodal signaling are neural progenitors. However, we are able to address this using chimeras, in which the vegetal signaling is blocked in the entire embryo by injecting Δ-cadherin and one half of the embryo is injected with Nodal. These chimeras lack serotonergic neurons in the co-injected half and in the strip of cell adjacent to exogenous Nodal expressing cells. We have previously suggested that Nodal diffuses from co-injected half and suppresses the differentiation of serotonergic neurons (Yaguchi et al., 2006). In embryos injected with Δ-cadherin embryo, there is no nuclear anti-phospho-Smad2/3 immunoreactivity at 24 h,

consistent with Nodal expression being dependent on vegetal signaling (Duboc et al., 2004; Figs. 5G–I). However, all of the nuclei are immunoreactive to anti-phospho-Smad2/3 in embryos co-injected with Δ-cadherin and Nodal RNA (Figs. 5J–L). In chimeras, the half expressing Δ-cadherin and Nodal have anti-phospho-Smad3 immunoreactive nuclei. Moreover, in the half of the embryo expressing Δ-cadherin alone there is nuclear staining of phospho-Sp-Smad2/3 only in the cells that are within 2 to 3 cell diameters of the interface (panels M–R). This indicates that Nodal is able to diffuse a short distance to activate Sp-Smad2/3.



Discussion

Effects of nodal signaling

In sea urchin embryos, Nodal specifies oral ectoderm and unilaterally suppresses adult rudiment formation (Duboc et al., 2004, 2005). The expression of an activated form of Sp-Smad2/3 produces embryos that have a similar phenotype to embryos overexpressing Nodal (Duboc et al., 2004; Yaguchi et al., 2006). Expressing activated Sp-Smad2/3 in whole embryos and chimeras enables us to determine directly the effects of Nodal signaling. In chimeras the half of the embryo expressing activated Sp-Smad2/3 forms the oral ectoderm but curiously they are unable to form a mouth. The endoderm appears normal and divides into three parts, but the tip fails to fuse with the ectoderm. In *Paracentrotus lividus*, Nodal expression at oral ectoderm disappears between late gastrula and prism stage (Duboc et al., 2005); suggesting that down-regulation of Nodal in oral ectoderm is necessary for formation of the mouth.

Expression of activated Sp-Smad2/3 also results in a reduction in the number of ciliary band neurons that form. The effect of Nodal signaling on serotonergic neurons in the animal plate had previously been noted (Yaguchi et al., 2006); however, when Nodal was expressed throughout the embryo there was no apparent effect on ciliary band neurons. In chimeras it was clear that the ciliary band neurons formed from the half not expressing activated Sp-Smad2/3. This suggests that Nodal may have a role in regulating neuron formation in the

Fig. 3. C-terminus deleted (dC) or mutated (mC) Sp-Smad2/3 function as dominant-negative forms. (A–D) dC-Sp-Smad2/3 injected embryos. (A) The animal plate recognized with Sp-Nk2.1 is restricted to the small animal pole region. Inset shows the DIC image. (B) The serotonergic neurons are differentiated at the small animal plate. (C) Ciliary band neurons occupy the lateral thickened ectoderm. (D) The bilateral symmetry of serotonergic neuron network is missing in the injected embryo. (E–H) mC-Sp-Smad2/3 injected embryos. (E) The animal plate is restricted to the small animal pole region. (F–H) The serotonergic neurons as well as ciliary band synB neurons are distributed like those in the dC-Sp-Smad injected embryos. (I, J) Antivin injected embryos. (I) The animal plate is restricted to the animal pole region and the lateral thickened epithelium contains ciliary band (synB) neurons. (J) The serotonergic neuronal network in the Antivin injected embryo is radially symmetrical. (K, L) Chimeras in which Nodal is overexpressed throughout the embryo and one half of the embryo is co-injected dC-Sp-Smad2/3 mRNA with myc lineage tracer. (K) The scheme for making chimeras with Nodal injection into the unfertilized egg and ΔC or mC-Sp-Smad2/3 injection into one blastomere of 2-cell stage. Inset shows DIC image of the chimera. (L) The co-injected half always develops to the aboral side in this chimera. The serotonergic neurons are derived from the co-injected half. Scale bar=20 μm.

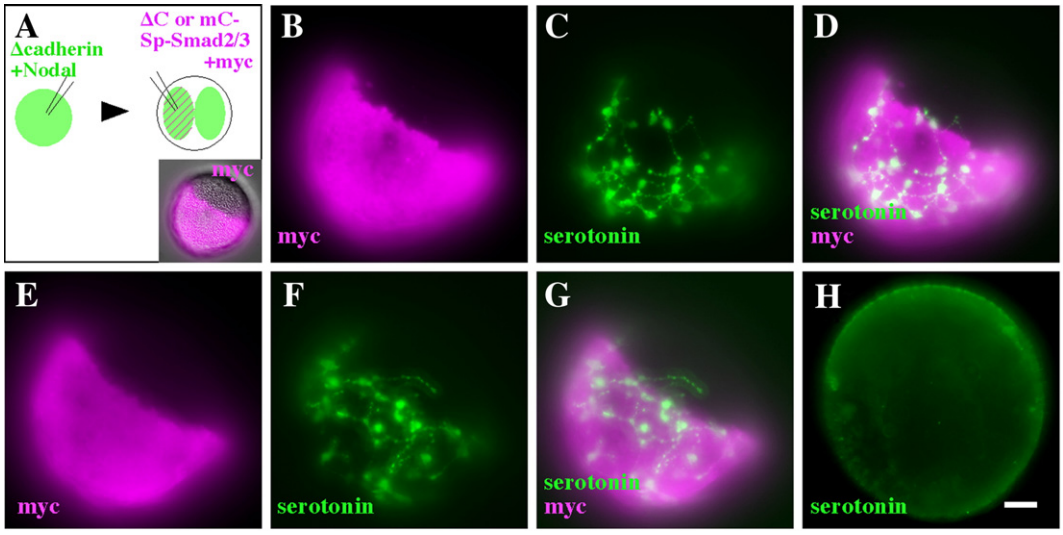


Fig. 4. Dominant-negative form of Sp-Smad2/3 rescues the suppression of serotonergic neurogenesis by the overexpression of Nodal. The chimera in which the endogenous vegetal signaling and serotonergic neurogenesis are blocked by injection with Δ-cadherin and Nodal, and one half of the embryo is co-injected with dC-Sp-Smad2/3 (A–D) or mC-Sp-Smad2/3 (E–G) and myc mRNA. (A) The scheme for making chimeras with Δ-cadherin and Nodal injection into the unfertilized egg and ΔC or mC-Sp-Smad2/3 injection into one blastomere of 2-cell stage. Inset shows animal view of the chimera. (B) Magenta indicates the half of the embryo expressing dC-Sp-Smad2/3 and myc mRNA. (C) The serotonergic neurons in the chimera. (D) Merged image of panels B and C shows the serotonergic neurons have differentiated only in the co-injected half. (E) Half of the embryo is injected with mC-Sp-Smad2/3. (F) The serotonergic neurons in panel E. (G) Merged image of panels E and F shows that the serotonergic neurons are differentiated in the co-injected half. (L) The control embryo injected with Δ-cadherin and Nodal contains almost no neurons (72 h). Scale bar=20 μm.

ciliary band and is not restricted to patterning serotonergic cells in the animal plate. The ciliary band is derived from the domains that give rise to oral and aboral ectoderm (Cameron et al., 1993). Ciliary band specification is though to precede specification of oral and aboral ectoderm (Duboc et al., 2004). This is in part based on embryos that have Nodal expression blocked and fail to specify oral and aboral ectoderm, yet most of the ectoderm differentiates as ciliary band (Duboc et al., 2004; Yaguchi et al., 2006). The expression of activated Sp-Smad2/3 in chimeras indicates that the lateral regions of the ciliary band differ from the pre-oral and post-oral transverse regions of the ciliary band. The lateral ciliary bands are derived from the domain that will form aboral ectoderm and the pre- and post-oral ciliary band from the domain that will form oral ectoderm. Because Nodal signaling inhibits specification of ciliary band neurons and ciliary band specification precedes oral and aboral specification, there appears to be a mechanism that protects the ciliary band from Nodal signaling.

Expression of Sp-Smad2/3 with deletions of carboxy-terminal residues or site-directed mutations that prevent phosphorylation produces embryos identical to those over-expressing Antivin or treated with SB431542 (Duboc et al., 2004; Yaguchi et al., 2006). As in other organisms, these forms of Sp-Smad2/3 appear to function as dominant negative mutations (Wang et al., 2005). Expression of dominant negative forms of Sp-Smad2/3 enables us to determine the effects of an absence of Nodal signaling. Expression throughout the embryo confirms Nodal/Sp-Smad2/3 signaling is not necessary for the specification of ciliary band ectoderm (Duboc et al., 2004; Yaguchi et al., 2006). As well, expression in chimeras indicates that an absence of Nodal signaling permits the formation of neurons. The abundance of neurons in embryos expressing dominant negative Sp-Smad2/3 is nearly normal; suggesting a lack of Nodal is permissive for neuron formation.

Nodal/Sp-Smad2/3 directly suppresses serotonergic neuron differentiation

Nodal expression in the oral ectoderm is proposed to have a role in patterning neurogenesis in the adjacent animal plate ectoderm by suppressing serotonergic neuron differentiation (Yaguchi et al., 2006). Previous studies were unable to distinguish Nodal acting directly on animal plate ectoderm or if another diffusible ligand, produced as a consequence of oral specification is responsible. The chimeras in which the endogenous vegetal signaling or Nodal was blocked throughout the embryo and half the embryo expressed either activated or dominant negative Sp-Smad2/3 indicate that activation of Sp-Smad2/3, is necessary and sufficient for suppressing the serotonergic neuron differentiation in the animal plate. These experiments indicate that Nodal acts directly on the animal plate cells to suppress the neurogenesis.

Our data indicate that Nodal acting through Sp-Smad2/3 inhibits the formation of serotonergic neurons. However, because we lack details of the types of cells that comprise the animal plate, we do not know if Nodal inhibits all neuron specification or if Nodal promotes formation of another set of cells. It is crucial for patterning of the animal plate that Nodal suppresses the serotonergic neuron differentiation only on the oral side of the animal plate (Fig. 6). Yet, it is unclear how Nodal activity is restricted to one part of the animal plate ectoderm. Duboc et al. (2004) proposed that Antivin diffuses further than

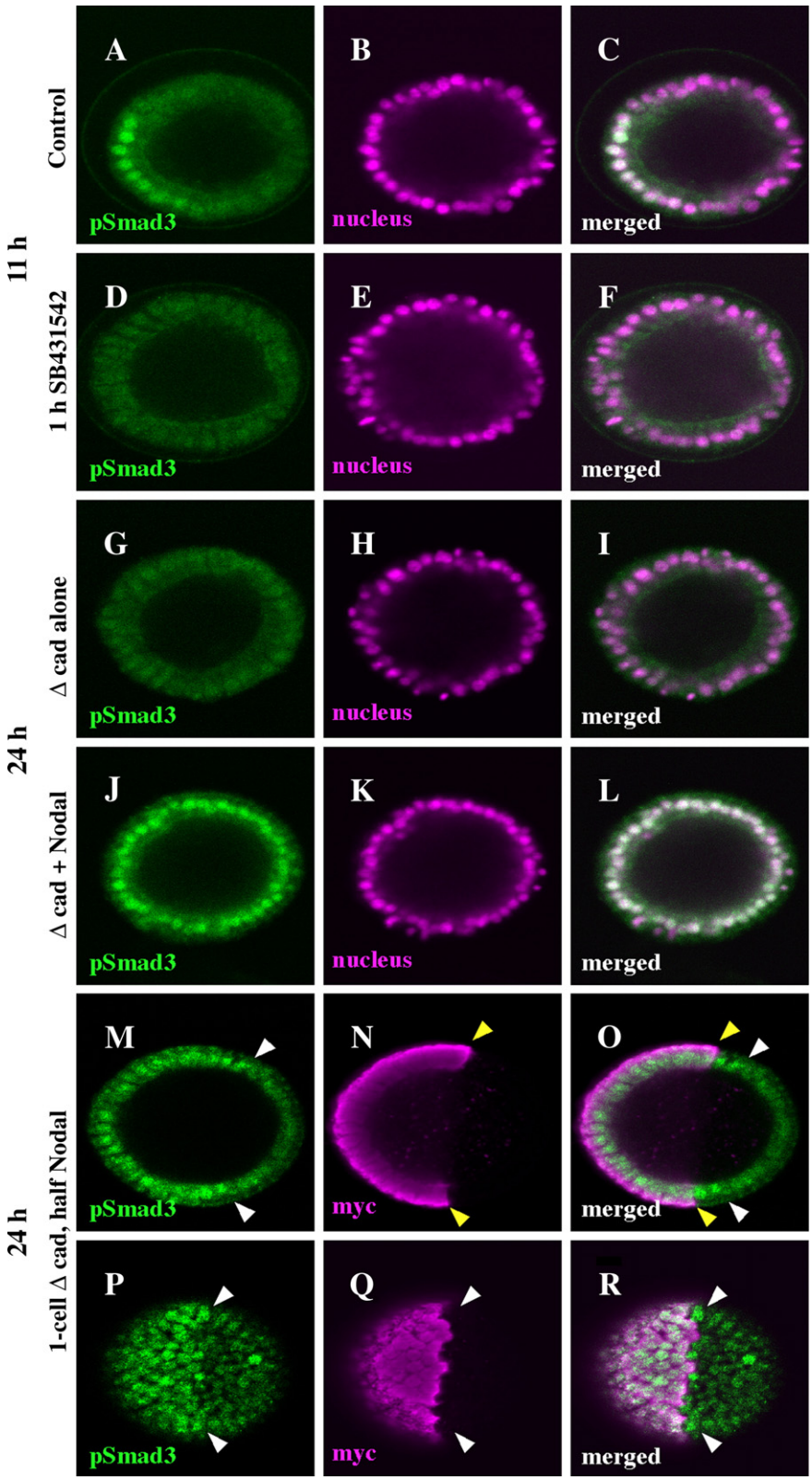


Fig. 5. Nodal diffuses a short-range and induces the phosphorylation of Sp-Smad2/3. (A–C) Control early blastula (11 h) stained with anti-phospho-Smad3 (pSmad3) antibody. (A) pSmad3 signals are detected only on one surface of the embryo. These signals disappear after 1 h treatment of SB431542 (D–F). (G–I) Δ-cadherin injected embryo has no anti-pSmad3 immunoreactivity in the nuclei at 24 h. (J–L) Δ-cadherin and Nodal co-injected embryo has strong anti-pSmad3 staining in the nuclei throughout the entire ectoderm. (M–R) The single optical section of chimera in which the vegetal signaling is blocked throughout the entire embryo by injection of Δ-cadherin and one half of the embryo is co-injected with Nodal. (M–O) Middle part of the chimera shows the pSmad3-immunoreactive region (white arrowheads) is broader than Nodal injected half (yellow arrowheads). (P–R) Stack of three optical sections of the chimera. The strong, nuclear anti-pSmad3 signals are detected at the interface with the Nodal expressing half (white arrowheads). Scale bar = 20 μm.

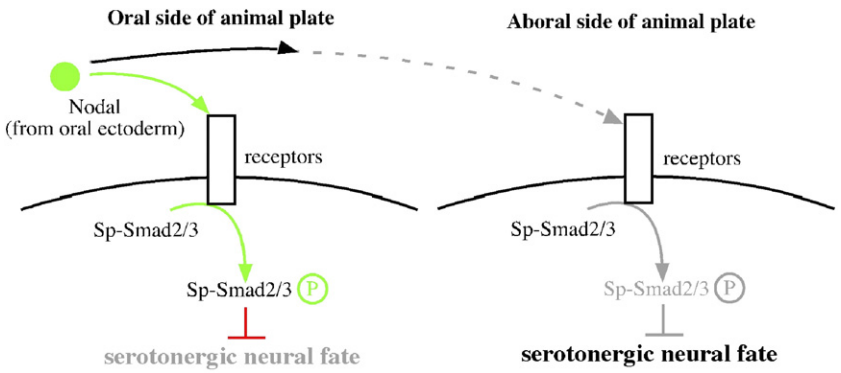


Fig. 6. Summary diagram of the effects of Nodal on neurogenesis in the animal plate. Nodal is expressed in the oral ectoderm and diffuses to interact with cells only in the adjacent region of the animal plate. In the animal plate cells it induces phosphorylation of Sp-Smad2/3, resulting in the suppression of serotonergic neurogenesis. On the aboral side of the animal plate, which is out of the diffusion range of Nodal, cells differentiate as serotonergic neurons.

Nodal to protect the ciliary band and aboral ectoderm from Nodal activity. Our results indicate that Nodal is able to induce phosphorylation of Sp-Smad2/3 only with a few cells diameters from cells expressing Nodal. It remains unclear why Nodal's effects remain localized and why Nodal does not induce the expression of Antivin in animal plate cells (Duboc et al., 2004).

In vertebrates, Nodal patterns neurogenesis by specifying posterior neuroectoderm. When Nodal activity is blocked, the anterior neuroectoderm expands (Londin et al., 2005; Feldman et al., 2000). Conversely, when Nodal activity is enhanced, the posterior neuroectoderm expands and the anterior neuroectoderm is reduced (Silva et al., 2003). In the ascidian, blocking of Nodal activity expands the medial neural plate fate to the lateral neural plate (Hudson and Yasuo, 2005). In all of these treatments, Nodal does not change the overall size of the neuroectoderm. In sea urchins, Nodal signaling does not restrict the size of the neurogenic animal plate but suppresses serotonergic neuron differentiation in the animal plate. These observations suggest that, Nodal has a role in patterning neuroectoderm that is a conserved feature of deuterostomes.

Antibodies that bind only phosphorylated forms of Smads have proven useful in identifying where and when cells are responding to TGF-β signaling. The anti-phospho-Smad3 antibody, in combination with the inhibitor SB431542, indicates there is signaling through TGF/Activin type IB receptors on one surface of the embryo. This observation is consistent with the time and location of Nodal expression and specification of oral ectoderm (Duboc et al., 2004). Analysis of neural genes indicates that the pro-neural genes, Sp-*paired-class*, Sp-*achaete-scute* and Sp-*Zic2* are core components of a metazoan neurogenic gene regulatory network that is expressed in the animal plate ectoderm of early blastulae (Burke et al., 2006). The timing and location of Nodal signaling indicate that Nodal, acting through Sp-Smad2/3, is likely to impinge on this regulatory network and mediate patterning of the animal plate ectoderm by regulation of proneural genes and their targets.

In sea urchin embryos four domains are specified in the ectoderm, each of which gives rise to a specific larval tissue. The animal plate neurogenic ectoderm, the oral ectoderm, the aboral ectoderm and the ciliary band are probably like the endomesoderm in that each is specified by a gene regulatory network (Davidson et al., 2002a,b; Angerer et al., 2001, 2000; Amore et al., 2003). Neural specification in urchin embryos is a unique model that has the potential to give new perspectives on the interplay of localized maternal determinants with signaling pathways that are activated during cleavage. Examining the specification of a small number of neurons by conserved signaling pathways will provide insights into the molecular basis of neuron specification.

Acknowledgments

This research was supported by Discovery Grants from NSERC to RDB. We are grateful to David McClay, Eric Davidson, Thierry Lepage, Bill Klein, Kevin Peterson and Yoko Nakajima for providing essential reagents. Lynne Angerer provided helpful comments on the manuscript.

References

Amore, G., Yavrouian, R.G., Peterson, K.J., Ransick, A., McClay, D.R., Davidson, E.H., 2003. SpDeadringer, a sea urchin embryo gene required separately in skeletogenic and oral ectoderm gene regulatory networks. Dev. Biol. 261, 55–81.

Angerer, L.M., Angerer, R.C., 2000. Animal–vegetal axis patterning mechanisms in the early sea urchin embryo. Dev. Biol. 218, 1–12.

Angerer, L.M., Oleksyn, D.W., Levine, A.M., Li, X., Klein, W.H., Angerer, R.C., 2001. Sea urchin goosecoid function links fate specification along the animal–vegetal and oral–aboral embryonic axes. Development 128, 4393–4404.

Burke, R.D., Angerer, L.M., Elphick, M.R., Humphrey, G.W., Yaguchi, S., Kiyama, T., Liang, S., Mu, X., Agca, C., Klein, W.H., Brandhorst, B.P., Rowe, M., Wilson, K., Churcher, A.M., Taylor, J.S., Chen, N., Murray, G., Wang, D., Mellot, D., Olinski, R., Hallbook, F., Thorndyke, M.C., 2006. A genomic view of the sea urchin nervous system. Dev. Biol., doi:10.1016/j.ydbio.2006.08.007.

Cameron, R.A., Britten, R.J., Davidson, E.H., 1993. The embryonic ciliated band of the sea urchin, *Strongylocentrotus purpuratus* derives from both oral and aboral ectoderm. Dev. Biol. 160, 369–376.

Chacko, B.M., Qin, B., Correia, J.J., Lam, S.S., de Caestecker, M.P., Lin, K., 2001. The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. Nat. Struct. Biol. 8, 248–253.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C.T., Livi, C.B., Lee, P.Y., Revilla, R., Rust, A.G., Pan, Z., Schilstra, M.J.,

Clarke, P.J., Arnone, M.I., Rowen, L., Cameron, R.A., McClay, D.R., Hood, L., Bolouri, H., 2002a. A genomic regulatory network for development. Science 295, 1669–1678.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C.T., Livi, C.B., Lee, P.Y., Revilla, R., Schilstra, M.J., Clarke, P.J., Rust, A.G., Pan, Z., Arnone, M.I., Rowen, L., Cameron, R.A., McClay, D.R., Hood, L., Bolouri, H., 2002b. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. Dev. Biol. 246, 162–190.

Duboc, V., Rottinger, E., Besnardeau, L., Lepage, T., 2004. Nodal and BMP2/4 signaling organizes the oral–aboral axis of the sea urchin embryo. Dev. Cell 6, 397–410.

Duboc, V., Rottinger, E., Lapraz, F., Besnardeau, L., Lepage, T., 2005. Left–right asymmetry in the sea urchin embryo is regulated by nodal signaling on the right side. Dev. Cell 9, 147–158.

Evan, G.I., Lewis, G.K., Ramsay, G., Bishop, J.M., 1985. Isolation of monoclonal-antibodies specific for human C-myc proto-oncogene product. Mol. Cell. Biol. 5, 3610–3616.

Faure, S., Lee, M.A., Keller, T., ten Dijke, P., Whitman, M., 2000. Endogenous patterns of TGFbeta superfamily signaling during early *Xenopus* development. Development 127, 2917–2931.

Feldman, B., Dougan, S.T., Schier, A.F., Talbot, W.S., 2000. Nodal-related signals establish mesendodermal fate and trunk neural identity in zebrafish. Curr. Biol. 10, 531–534.

Howard-Ashby, M., Materna, S.C., Brown, C.T., Chen, L., Cameron, R.A., Davidson, E.H., 2006. Gene families encoding transcription factors expressed in early development of *Strongylocentrotus purpuratus*. Dev. Biol., doi:10.1016/j.ydbio.2006.08.033.

Hudson, C., Yasuo, H., 2005. Patterning across the ascidian neural plate by lateral Nodal signalling sources. Development 132, 1199–1210.

Lapraz, F., Rottinger, E., Duboc, V., Range, R., Duloquin, L., Walton, K., Wu, S., Bradham, C.A., Loza, M.A., Hibino, T., Wilson, K., Poustka, A.J., McClay, D.R., Angerer, L.M., Gache, C., Lepage, T., 2006. RTK and TGF-β signaling pathways genes in the sea urchin genome. Dev. Biol., doi:10.1016/j.ydbio.2006.08.048.

Logan, C.Y., Miller, J.R., Ferkowicz, M.J., McClay, D.R., 1999. Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. Development 126, 345–357.

Londin, E.R., Niemiec, J., Sirotkin, H.I., 2005. Chordin, FGF signaling, and mesodermal factors cooperate in zebrafish neural induction. Dev. Biol. 279, 1–19.

Lowe, L.A., Yamada, S., Kuehn, M.R., 2001. Genetic dissection of nodal function in patterning the mouse embryo. Development 128, 1831–1843.

Massague, J., Wotton, D., 2000. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. 19, 1745–1754.

Nakajima, Y., Kaneko, H., Murray, G., Burke, R.D., 2004. Divergent patterns of neural development in larval echinoids and asteroids. Evol. Dev. 6, 95–104.

Poustka, A.J., Groth, D., Hennig, S., Thamm, S., Cameron, A., Beck, A., Reinhardt, R., Herwig, R., Panopoulou, G., Lehrach, H., 2003. Generation, annotation, evolutionary analysis, and database integration of 20,000 unique sea urchin EST clusters. Genome Res. 13, 2736–2746.

Samanta, M.P., Tongprasit, W., Istrail, S., Cameron, R.A., Tu, Q., Davidson, E.H., Stolc, V., 2006. A High-resolution transcriptome map of the sea urchin embryo. Science (in press).

Schier, A.F., 2003. Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol. 19, 589–621.

Shi, Y., Massague, J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685–700.

Silva, A.C., Filipe, M., Kuerner, K.M., Steinbeisser, H., Belo, J.A., 2003. Endogenous Cerberus activity is required for anterior head specification in *Xenopus*. Development 130, 4943–4953.

Takacs, C.M., Amore, G., Oliveri, P., Poustka, A.J., Wang, D., Burke, R.D., Peterson, K.J., 2004. Expression of an NK2 homeodomain gene in the apical ectoderm defines a new territory in the early sea urchin embryo. Dev. Biol. 269, 152–164.

Vincent, S.D., Dunn, N.R., Hayashi, S., Norris, D.P., Robertson, E.J., 2003. Cell fate decisions within the mouse organizer are governed by graded Nodal signals. Genes Dev. 17, 1646–1662.

Wang, J., Mohler, W.A., Savage-Dunn, C., 2005. C-terminal mutants of *C. elegans* Smads reveal tissue-specific requirements for protein activation by TGF-beta signaling. Development 132, 3505–3513.

Wikramanayake, A.H., Brandhorst, B.P., Klein, W.H., 1995. Autonomous and non-autonomous differentiation of ectoderm in different sea urchin species. Development 121, 1497–1505.

Yaguchi, S., Katow, H., 2003. Expression of tryptophan 5-hydroxylase gene during sea urchin neurogenesis and role of serotonergic nervous system in larval behavior. J. Comp. Neurol. 466, 219–229.

Yaguchi, S., Yaguchi, J., Burke, R.D., 2006. Specification of ectoderm restricts the size of the animal plate and patterns neurogenesis in sea urchin embryos. Development 133, 2337–2346.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B.L., Kuehn, M.R., 1993. Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. Nature 361, 543–547.

Zhu, X., Mahairas, G., Illies, M., Cameron, R.A., Davidson, E.H., Ettensohn, C.A., 2001. A large-scale analysis of mRNAs expressed by primary mesenchyme cells of the sea urchin embryo. Development 128, 2615–2627.