Direct and indirect control of oral ectoderm regulatory gene expression by Nodal signaling in the sea urchin embryo

Enhui Li1, Stefan C. Materna2, and Eric H. Davidson1,*
1Division of Biology, California Institute of Technology, Pasadena, CA 91125

Abstract

The Nodal signaling pathway is known from earlier work to be an essential mediator of oral ectoderm specification in the sea urchin embryo, and indirectly, of aboral ectoderm specification as well. Following expression of the Nodal ligand in the future oral ectoderm during cleavage, a sequence of regulatory gene activations occurs within this territory which depends directly or indirectly on nodal gene expression. Here we describe additional regulatory genes that contribute to the oral ectoderm regulatory state during specification in Strongylocentrotus purpuratus, and show how their spatial expression changes dynamically during development. By means of system wide perturbation analyses we have significantly improved current knowledge of the epistatic relations amongst the regulatory genes of the oral ectoderm. From these studies there emerge diverse circuitries relating downstream regulatory genes directly and indirectly to Nodal signaling. A key intermediary regulator, the role of which had not previously been discerned, is the not gene. In addition to activating several genes earlier described as targets of Nodal signaling, the not gene product acts to repress other oral ectoderm genes, contributing crucially to the bilateral spatial organization of the embryonic oral ectoderm.

Keywords

nodal signaling; gene regulatory network; oral ectoderm specification

Introduction

The oral ectoderm of the S. purpuratus embryo derives partly from animal pole cells that arise in early cleavage (mesomeres), and partly from veg1 lineage cells (Cameron et al., 1990, 1991; Ransick and Davidson, 1998). The embryo is initially polarized across its second axis (the oral-aboral axis) by means of a redox gradient that follows from an asymmetric distribution of maternal mitochondria (Coffman et al., 2009; Coffman and Davidson, 2001; Coffman et al., 2004; Coluccio et al., 2011). The earliest known transcriptional consequence of this polarization is activation of the nodal gene on the future oral side of the embryo, which in S. purpuratus occurs at 8 hpf, 6th cleavage (Fig.1) (Nam et al., 2007). Cis-regulatory analysis of nodal gene expression demonstrates inputs from putatively redox-sensitive transcription factors (Coffman et al., 2009; Nam et al., 2007;
Range et al., 2007), providing a causal link between the initial cytoplasmic anisotropy and the transcriptional response of *nodal*. Following its initial activation the *nodal* gene responds actively to its own signal transduction system, so that all cells of the oral ectoderm both produce and respond to the *nodal* signal, a community effect feedback mechanism (Bolouri and Davidson, 2010). This has two results: the feedback accounts for most of the amplitude of *nodal* gene expression, and it also ensures homogeneity of expression of downstream genes across the oral ectoderm. Much prior work demonstrates that *nodal* gene expression is required for specification of oral ectoderm to take place, and many downstream genes are known, expression of which fails if *nodal* translation is blocked (Duboc et al., 2004; Flowers et al., 2004; Lapraz et al., 2009; Saudemont et al., 2010). An initial draft gene regulatory network (GRN) for ectoderm specification (Su et al., 2009) proposed direct *nodal* signaling inputs into several oral ectoderm regulatory genes, but other genes were shown as indirectly affected, and some genes were indicated to be activated independently of *nodal* signaling. However, it was evident that the repertoire of genes included in this network was incomplete and that at least some linkages would change as new nodes were added in. More recently, Saudemont et al. (2010) published a GRN for the oral ectoderm containing a network model in which direct *nodal* signaling inputs are depicted in into all oral ectoderm genes that require *nodal* expression for their own normal expression. An immediate difficulty that arises from this topology is that it is inconsistent with current high resolution time course data (Materna et al., 2010) for genes downstream of *nodal* expression. As illustrated in Fig.1, where relevant timecourse data are reproduced, these genes are activated in sequence, some not until many hours after the most rapidly responding *nodal* target genes begin to be transcribed. This suggests that activation of these genes in consequence of *nodal* signaling is in fact not a direct, one-step process, and that additional network connections remain to be identified.

Here we identify a major intermediary mediator of *nodal* inputs into the oral ectoderm GRN, the *not* homeodomain gene, and focusing on the 8 genes included in Fig.1, we use perturbation analysis to distinguish between direct and indirect *nodal* signaling targets. We consider separately genes expressed in the animal oral ectoderm and genes expressed in the veg1 oral ectoderm. Unexpectedly, we discovered that the *not* gene performs a key role in establishing the bilateral organization of the oral ectoderm. A comprehensive revision of the oral ectoderm GRN model of Su et al. (2009) which contains many additional linkages will appear in a following publication.

**Materials and Methods**

**Gene cloning and constructs**

Four genes investigated in this study were previously documented, *viz. nodal, bmp2/4*, and *nk2.2* (Su et al., 2009). The *not* gene was originally isolated by Peterson et al. (1999). Although studied earlier, for this work *gsc, nk1, and vegf3* genes were cloned by PCR, accorded to the predicted gene models (http://www.spbase.org/) (Cameron et al., 2009). A 24 hr cDNA library was used as the template for gene cloning. Primers for amplifying the *gsc* gene were 5′ CTGACATACAATCCACTGC; for the *vegf3* gene, 3′ TGTGACATACAATCCACTGC; and for the *nk1* gene were 5′ TCATTACCGAGGTAATCC and 3′ AATAACGTGTATGGCAAAGCGAAC. Amplified genes were inserted into the pGEM-T EZ vector.
Whole Mount In Situ Hybridization (WMISH)

The protocol for WMISH has been described previously (Ransick, 2004). The procedure used here were as follows: sea urchin embryos were fixed with glutaraldehyde fixative (1.3% glutaraldehyde, 32.5% filtered sea water, 32.5 mM MOPS (pH7), and 162.5 mM NaCl) at 4 °C overnight. After extensive washing with TBST (10 mM Tris · HCl, 150 mM NaCl, and 0.1% Tween-20), embryos were treated with proteinase K (5ug/ml in TBST) for 8 minutes, stopped with glycine solution (25 mM glycine in TBST), and post-fixed with paraformaldehyde fixative (4% paraformaldehyde, 32.5% filtered sea water, 32.5 mM maleic acid (pH7), and 32.5 mM NaCl). The hybridization reaction was performed at 60 °C for at least 18 hours in the presence of DIG labeled RNA probe, and DNP-labeled probe for double in situ hybridization. Post hybridization washes were 2× SSCT (300 mM NaCl, 30 mM sodium citrate (pH 7), and 0.1% Tween-20) for 15 minutes, 0.2× SSCT 20 minutes, and 0.1× SSCT for 10 minutes. Antibody incubation was performed at room temperature for 1 hour with 1:1000 diluted anti-DIG fab, or anti-DNP antibody. After extensive washes with MABT (100 mM maleic acid (pH7), 150mM NaCl, and 0.1% Tween-20), embryos were stained with BCIP/NBT, or INT/BCIP for the second color reaction. DIG labeled antisense RNA Probe was prepared using Roche DIG labeling kit. For double in situ, antisense RNA was first transcribed and then labeled with DNP using Label-IT kit. 0.5 to 1 ug labeled RNA probe was used for a 500 ul hybridization reaction.

MASO perturbation and mRNA quantification

Microinjections of morpholino antisense oligonucleotide (MASO) were performed to knock down expression of target genes. The MASO sequences used for bmp2/4, nodal, and lefty were described previously (Su et al., 2009). The sequence of the not MASO was GACATCAAGTTGGAACTCATCATAG. Concentrations of nodal, lefty, bmp2/4, or not MASOs in the injection solution were 100, 150, 300, and 300 µM respectively. Half of these concentrations were used in double MASO perturbation assays. Approximately 4 pL MASO solution was injected into fertilized sea urchin eggs. For QPCR analysis 200 embryos were harvested for RNA preparation using Qiagen RNAeasy Micro Prep kit. The isolated RNA was then reverse transcribed into cDNA using the BioRad iScript Kit. For Nanostring nCounter analysis, 300 embryos were collected, and total RNA was extracted using Qiagen RNAeasy Micro Prep kit. The tested gene code set, procedure, and data analysis of Nanostring nCounter system were described previously (Materna, et al. 2010).

Results

Dynamic expression patterns of regulatory genes in the animal and veg1 oral ectoderm

The high resolution time course data of Fig.1 illustrate a small cohort of Nodal-dependent genes which begin to accumulate within 2hr after activation of the nodal gene, viz. lefty, not, and vegf3, followed closely by bmp2/4. Expression of gsc trails the activation of nodal by 5hr, while other genes, here represented by nk2.2 and nk1, begin to function even later. As shown earlier using parameters measured for a number of regulatory and other genes (Bolouri and Davidson, 2003), in S. purpuratus the typical gene cascade step time, i.e., the interval between activation of a regulatory gene and the activation of its direct transcriptional targets, is for embryos at 15° C about 2–3 hrs. Thus a priori, the transcriptional network architecture controlling expression of all of the genes included in Fig.1 is not likely to consist of a simple set of direct, parallel inputs from Nodal signaling.

Furthermore, no two of these genes are expressed in exactly the same spatial pattern. Whole mount in situ hybridization (WMISH) observations at 3hr intervals were carried out on the genes of Fig.1 (excepting lefty), and the results are diagrammatically presented in Boolean form (Peter and Davidson, 2011) in Fig.2, along with WMISH images seen from the various
indicated directions for particularly interesting stages. Where useful, we have included double in situ hybridizations utilizing foxa as a marker for the veg2 endoderm domain, and for stomodeum at the center of oral ectoderm (Oliveri et al., 2006). In addition, Fig.3 provides diagrams of the progressively subdivided spatial subdomains of embryonic gene expression, as seen from the oral side. The subdomains shown represent diverse regulatory states as they are formed, from mid cleavage to beginning gastrulation, and in the legend are noted specifically expressed genes representing these spatial regulatory states.

Most of these genes had been studied earlier (op. cit.) though not always at this temporal resolution, but the not and vegf3 expression patterns deserve special comment as for these the observations summarized in Fig.2 qualitatively differ from prior descriptions. The not gene is the earliest gene encoding a transcription factor to be expressed in the oral ectoderm following nodal gene activation. Its expression pattern was incorrectly reported in an initial study (Peterson et al., 1999). From the time of their appearance, not transcripts are confined to the oral section of the embryo. However, not is expressed more extensively in the animal-vegetal dimension than is the nodal gene, as early as 15hr extending from the oral animal ectoderm down through the oral veg1 ectoderm, and the oral veg1 and veg2 endoderm, all the way into the non-skeletogenic mesodermal domain on the oral side (Fig.2). In contrast, nodal gene transcripts in S. purpuratus are confined to the animal and veg1 oral ectoderm. Furthermore, not expression is spatially dynamic: this gene ceases to be expressed in the oral mesoderm after 24hr, and then it is turned off in the oral veg2 and veg1 endoderm and ectoderm, so that after 30hr not expression can only be detected in the oral animal ectoderm (Fig.2). The vegf gene of Paracentrotus lividus, the orthologue of S.purpuratus vegf3, was reported to be expressed in the lateral ectoderm in mesenchyme blastula and gastrula stages where it has a role in patterning spicule formation and skeletogenesis (Duloquin et al., 2007). However, in S. purpuratus there is a much earlier phase of expression, as just discussed, during which its transcriptional expression in the whole oral ectoderm proper is very similar to that of nodal(Fig.2). But vegf3 expression is also dynamic (Fig.2), and in the late blastula the animal oral ectoderm expression fades out, to be replaced with a stable pattern of strong expression in two spots at the lateral corners of the oral veg1 ectoderm, and more weakly, in the aboral ectoderm as well (Figs. 2 and 3).

The nk1 gene appears throughout to mark an oral veg1 domain encompassing both veg1 ectoderm and veg1 endoderm on that side (Figs. 2 and 3). Neither its expression nor that of nk2.2 is spatially dynamic. They differ in that nk2.2 is also expressed in the aboral ectoderm (Chen et al., 2011), but on the oral side they are similar in that after mesenchyme blastula stage their expression is confined to the oral veg1 cells, excluding the lateral domains of expression of vegf3 (Fig.2) (Minokawa et al., 2004).

**Essential role of not in establishing the oral-aboral polarity of the ectodermal regulatory states**

An initial series of experiments showed that not transcription depends on synthesis of the Nodal ligand. Embryos bearing nodal/MASO were assayed by QPCR for nodal and not transcripts at 2hr intervals between 14 and 24hr, with the results shown in Fig 4A. Since the nodal gene is a direct target of Nodal signal transduction (Nam et al., 2007; Range et al., 2007), the quantitative kinetics of not response should match those of nodal if not is also a direct target as the time courses of Fig.1 might suggest. In the event this is exactly the result obtained (Fig.4A). Additional evidence is shown in similar experiments, with a similar rationale, in Fig.4B, where the effects of lefty MASO on nodal and not transcription are quantitatively compared. Lefty is a known antagonist of Nodal signal presentation, and given that the ectopic expression of nodal which follows application of lefty MASO results in up-regulation of nodal (Duboc et al., 2008), then, just as shown, the same result will obtain for not if its expression is indeed dependent on Nodal signaling. Taken together with
the time course data, these experiments indicate not to be the initial gene encoding a transcription factor that is activated by Nodal signaling on the oral side of the embryo.

Embryos treated with an effective not MASO displayed normal hatching and blastular development, but gastrulation and skeletogenesis were delayed. The time-course of not MASO perturbations showed that no effect was seen at 12hr or 15hr, i.e., before any Not protein could have accumulated. The effects of not MASO on all known regulatory genes expressed at 18 and 24hr were assessed by Nanostring nCounter (183 gene codeset). This experiment revealed a sharply limited set of responsive ectodermal genes. Nanostring data to be published elsewhere show that this MASO had no side effects, since no genes are affected outside the oral field, and the number of genes affected was reproducible and small. Since the bmp2/4 gene is not a not target, there is no global disturbance of the O/A axis, and therefore not MASO affects fewer genes than does nodal MASO.

Ectodermal genes affected by not MASO were studied in detail by QPCR (Fig.5). Here we see that the direct and early Nodal target genes, viz. nodal, chordin, lefty, and bmp2/4, are unaffected at blastula stages by arrest of Not translation. However, in contrast, expression of the gsc gene, a previously considered a direct Nodal signaling target (Duboc et al., 2010; Saudemont et al., 2010; Su et al., 2009), is also reduced by ~75% by not MASO at 18hr. In addition nk1 expression is sharply repressed by not MASO, though no effects were seen on expression of the other veg1 oral ectoderm regulatory genes nk2.2 and lim1 (Fig.5B). Fig. 5A also shows clearly that not negatively controls its own expression. Previously a high resolution profiling of not gene expression showed that not transcript level undergoes a significant drop from its peak at 25hr, after being activated by the Nodal signaling (Materna et al., 2010). Self-repression of not accounts for the peak-like form of its expression time course. Another gene mildly up regulated by not MASO is vegf3 (Fig.5B).

Nodal (activated Smad) and not inputs to downstream genes of course need not be either exclusive or if dual, synergistic. To disentangle the regulatory relationships, a series of experiments was undertaken in which the effects on downstream gene transcription of lefty MASO or not MASO administered singly was compared in the same batch of embryos with the effects of lefty plus not MASO’s administered together. The principle is that if the up-regulation of Nodal signaling targets resulting from lefty MASO were not dependent as well as Nodal-dependent, this up-regulation would be abolished by the simultaneous presence of not MASO. On the other hand, for genes activated by Nodal signaling independently of any not input, the results of lefty plus not MASO would be the same as for lefty MASO alone. Results for gsc; not itself, and vegf3 are shown in Fig.6. Here we see evidence that gsc has a positive OR logic response to both not input and to Nodal signaling. Because not is itself a primary target of Nodal (Figs. 4 and 6), nodal MASO removes both feeds and virtually abolishes gsc expression (>98%; Fig.6; Fig.S1), and we can conclude there are no other required positive inputs to gsc. not MASO alone modestly depresses gsc expression (75%) while about a 6 fold boost of gsc expression is obtained with lefty MASO alone. This drops to about 3-fold when not MASO is also present. Thus response to the Nodal signal activates not, and both Nodal and not activate gsc. As we saw earlier, not represses itself, so its expression is boosted by not MASO, as it is also by lefty MASO, and since they are independent these two effects should be additive, as in fact they are (Fig.6). Finally, Fig.6 shows that vegf3 is regulated oppositely by not and Nodal (see also Fig.5B, where not MASO appears to de-repress vegf3). Alone, lefty MASO fails to up-regulate vegf3 as it does to all simple Nodal targets, but if not MASO is also present up-regulation occurs. The implication is that Nodal signaling is able to turn on vegf3 expression provided that not repression of vegf3 is blocked. This might explain why nodal/MASO alone does not significantly depress vegf3 expression: absence of the positive Nodal input would be offset by absence of the repressive not input.
Spatial control of signal ligand gene expression by not

To examine the spatial consequences of the apparent repression of vegf3 by not, WMISH observations were made on embryos bearing not MASO (Fig. 7A). As reported earlier (Duloquin et al., 2007), in control embryos vegf3 is expressed bilaterally at the lateral edges of the oral ectoderm, but in not MASO expression spreads across the whole of the oral ectoderm. Thus, just as implied by the QPCR experiment of Fig. 5, not expression is antagonistic to vegf3 expression. Spatially, this repressive function of the not gene is responsible for removing vegf3 expression from the oral ectoderm where it is initially transcribed (Fig. 2) and also for confining this expression to the flanking edges of the veg1 oral ectoderm. not gene function in vegf3 clearance was also supported by the observation that overexpression of not mRNA significantly reduced the transcript level of vegf3 (unpublished data).

It is interesting that vegf3 is not the only gene encoding a signaling ligand transcription of which is cleared from the oral quadrant of veg1 ectoderm in consequence of not expression. The wnt5 gene is expressed in veg1 ectoderm and endoderm from 18–27hr, thereafter only in veg1 ectoderm, in a horseshoe-like pattern, extending around the aboral circumference and into the lateral veg1 ectoderm on either side (for details of the wnt5 expression pattern through time see Fig. S2). Exclusion of wnt5 expression from the oral circumference of the veg1 territory is also due to not repression. This is shown clearly in the not MASO experiment of Fig. 7B. The veg1 activators of wnt5 remain to be established.

Given the observations of Duloquin et al (2007) that the Vegf3 signal is used by skeletogenic mesenchyme cells in the blastocoel as a patterning cue, we monitored the disposition of these cells and their spicule forming activity in embryos bearing not MASO. Ingression and initial formation of the ring formed by the newly ingressed skeletogenic cells around the base of the blastocoel were as in control embryos, but thereafter they failed to behave in a normal way. In untreated embryos, 3–4 ingressed skeletogenic cells occur in bilateral clusters, and these are sites of spiculogenesis; only occasional stragglers linger on the oral side, and the remainder of the skeletogenic cells maintain an aboral circular array (Fig. 7C control, 36hr). In the presence of not MASO, however, all treated embryos showed the altered pattern of skeletogenic cell disposition, and abnormal spicule formation. More PMC cells were positioned orally during the early to mid-gastrula stages (Fig. 7C). In the later stage, a number of supernumerary and underdeveloped spicules were seen on the oral side, with a loss of preferential formation of lateral spicules.

Spatial inputs into nk genes by not and Nodal

As shown in Fig. 5B expression of the veg1 nk1 gene is about 80% abolished by not MASO. From the transcript accumulation kinetics in Fig. 1, as pointed out above, it is most unlikely that nk1 is a direct Nodal signaling target. Indeed it is to be predicted that the delayed onset of nk1 expression, even with respect to not, indicates that an additional unknown, later appearing positive input in veg1 lineage cells is required for nk1 expression (or alternatively, the eventual removal of a negative clamp on nk1 expression). In any case the epistatic relation of nodal to nk1 is mediated by not. In contrast, nk2.2 expression is impervious to not MASO (Fig. 5), nor is spatial expression of this gene in any way subject to not control (Fig. 8A). But nk2.2 is likely a Nodal signaling target: Fig. 8B demonstrates a significant 75% decrease in nk2.2 expression in the presence of nodal MASO, and an increase in expression caused by lefty MASO typical of direct Nodal targets. The magnitude of nk2.2 expression changes upon nodal or lefty MASO treatment indicated that expression of the nk2.2 gene in both oral and aboral subdomains is Nodal dependent. The Nodal signaling pathway is able to activate oral nk2.2 expression directly; on the other hand, aboral nk2.2 expression is indirectly driven by nodal through another signaling gene, bmp2/4.
downstream of the nodal pathway. Still, the 5hr delay between activation of the nodal gene and of the nk2.2 gene, plus the incomplete decrease in nk2.2 transcript level induced by nodal MASO, suggests an additional possible OR input. One potential player could be BMP2/4, which is also up-regulated by lefty MASO, but the experiment in Fig. 8B in which bmp2/4 MASO was also introduced excludes this.

Discussion

We here distinguish between direct and indirect targets of Nodal signaling, and explore the role of a heretofore unstudied sea urchin homeobox gene, not. A variety of regulatory relationships emerge: The not gene is a direct target of Nodal signaling; the gsc gene is both a direct and an indirect target of Nodal signaling; the nk1 gene is not a target of Nodal signaling but is driven by not input; the nk2.2 gene is a direct target of Nodal signaling but has no input from not. The not gene turns out to be a major mediator of Nodal signaling effects in the oral ectoderm, and in addition is responsible for the bilateral expression of two other signaling ligand genes, wnt5 and vegf3, by repressing both orally, permitting their expression in the lateral ectoderm.

Revisions of the oral ectoderm GRN

An updated model of the whole oral ectoderm GRN to about 30hrs (gastrulation) is to be presented elsewhere, and here we focus only on the shallow layer of interactions immediately downstream of nodal gene expression. Fig.9 summarizes our current view of the architecture in this region of the GRN, as presented in BioTapestry (Longabaugh et al., 2009). A prominent new feature in the upper or animal oral ectoderm is the feed forward circuitry by which gsc expression is controlled; i.e., Nodal signaling activates not, and not in turn activates gsc, while gsc also receives a direct input from Nodal signaling. Fig.1 shows that the kinetics with which gsc is activated depend ultimately on the not input. Together the Smad and Not inputs account for virtually all expression of the gsc gene, but once its transcription gets going the dual input reliably promotes its expression. As we show elsewhere, gsc is an essential gene in oral ectoderm specification, due to its required participation in a specific subcircuit responsible for activation of downstream genes. A second prominent new feature uncovered in this work and also involving a not gene function, is repression, after some hrs, of vegf3 gene expression in the animal oral ectoderm (Figs.2, 7A). The early transcriptional driver of vegf3 expression is probably Nodal as well (Fig.6). These relations constitute an incoherent feed forward loop (Fig.9), and nicely account for the transience of oral vegf3 expression. However, the developmental function, if any, of the transient expression of vegf3 in both animal and veg1 oral ectoderm remains unknown.

Additional new regulatory relations are portrayed in Fig.9 for veg1 oral ectoderm. The nk1 gene is expressed in all veg1 cells on the oral side, under positive not control with respect to the oral-aboral dimension. But its confinement to veg1 as well as its transcript accumulation kinetics, as discussed above, suggest an additional input which may, as indicated in Fig 9, be confined to the veg1 cell lineage. The nk2.2 gene is controlled by Nodal signaling but there is no not input, and it is expressed in animal and veg1 aboral ectoderm as well (Fig.2), obviously under control of some other input. An unexpected finding is that not repression prohibits both vegf3 and wnt5 from transcription in the oral circumference of the veg1 ectoderm. These genes could share a general veg1 activator, in which case vegf3 would also have to respond to an aboral repressor. The effect of not MASO on skeletogenic patterning in the gastrula (Fig.7C) suggests that while vegf3 expression is responsible for the lateral skeletogenic cell clusters there could be a role for wnt5 expression in organizing the aboral skeletogenic chain that these cells normally construct overlying veg1 on the aboral side of...
the embryo. In any case it is clear that not repression contributes an essential aspect of the bilateral spatial organization of regulatory state in this embryo.

**Complexity of the oral ectoderm patterning GRN in both time and space**

Careful perusal of the expression matrices in Fig. 2 show that there are multiple, dynamically evolving patterns of gene expression to be accounted for, even within the very limited sample of early oral ectoderm genes included in this study. Many additional genes have now been discovered to participate in the oral ectoderm GRN, as will be discussed in detail in a forthcoming work, and it is apparent that oral ectoderm specification is a progressive process directed by a hierarchical GRN. Although the importance of nodal expression as an initial input in oral ectoderm specification is rightly emphasized (Duboc et al., 2004), it is simplistic to summarize this process or the underlying GRN as a parallel one-step function of direct Nodal signaling, as was done in the model published by Saudemont et al. (2010). Even in its earliest transitions, as summarized in Fig. 9, the actual regulatory circuitry is diverse and anything but parallel in design. In the overall process of oral ectoderm specification many different spatial regulatory state domains arise. These include in the oral-aboral dimension the central vs. lateral oral regulatory domains of the veg1 ectoderm discussed in this paper. Further toward the upper oral ectoderm boundary with the apical neurogenic domain, there appears on either side of the animal oral ectoderm the bilaterally organized ciliated band, again the outcome of a separate regulatory state marked initially by onecut (hnf6) expression; and more centrally there arises the stomodeal region, the separate regulatory state of which is marked early on by foxa and brachyury expression (Fig. 3). In the animal-vegetal dimension sequential regulatory state boundaries are formed from the start. The nodal target gene we have focused on here, not, is expressed all the way down through veg1 and veg2 endomesoderm descendents on the oral side, while at the same blastula stages in this species nodal transcription itself is confined to veg1 and animal ectoderm (Fig. 2). The more extensive not expression is due to vegetal diffusion of Nodal over several cell diameters, and indeed phosphorylated Smad, the immediate early consequence of Nodal signal transduction, covers the whole oral hemisphere, from apical to vegetal pole (Bergeron et al., 2011; Yaguchi et al., 2007). As discussed elsewhere, we recently discovered that not executes a critical function in the initial specification of the veg2 oral mesoderm, as well as performing the ectodermal regulatory roles considered here. Above the veg2 endoderm (as shown by the double WMISHs with foxa in Fig. 2) specification in the oral veg1 domain ultimately results in separation of veg1 endoderm from veg1 ectoderm fates; some genes such as vegf3 are initially expressed throughout veg1 but by gastrulation retreat to veg1 ectoderm, while others such as nk1 continue to be expressed in both oral veg1 endoderm and oral veg1 ectoderm (Figs. 2 and 3). Above the veg1 ectoderm, which becomes the vegetal strip of the ciliated band, are installed the animal oral ectoderm and stomodeal regulatory states. In short, oral ectoderm regulatory state specification is a temporally progressive process of continuing spatial subdivision, resulting in multiple confined domains. This process is carried out in a single cell thick, two dimensional epithelial grid composed of several hundred contiguous cells. The nodal gene executes diverse roles in initiating the earliest underlying GRN. As the hierarchical circuitry of this GRN comes clearly and completely into view, it promises to provide a canonical example of the genomic control of spatial regulatory state patterning.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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References


**Research Highlights**

A homeobox gene not is identified as a direct target of Nodal signaling during early sea urchin embryogenesis. The not gene is essential in establishing the oral-aboral polarity of the ectodermal regulatory states. Nodal signaling through Smad, and indirectly through not, drives progressive expression of oral ectodermal genes.
Fig. 1. Temporal expression of early oral ectodermal genes including nodal, bmp2/4, lefty, not, gsc, nk1, nk2.2, and vegf3. Expressions of these genes were quantified hourly from fertilization to 27 hpf using Nanostring nCounter (Materna et al., 2010). Oral ectodermal genes are activated sequentially throughout early to mid-blastular stages of sea urchin embryos. Expression of nodal and lefty genes starts between 8 and 9 hpf, and is closely followed by not, vegf3, gsc, and nk1.
Fig. 2. Expression pattern of *nodal*, *bmp2/4*, *not*, *gsc*, *nk1*, *nk2.2*, and *vegf3* in animal and veg1 oral ectoderm during blastular to early gastrula stages. WMISH was used to monitor the spatial patterns of ectodermal genes every three hours from 15 hpf to 33 hpf. An expression matrix summarizing the gene expression pattern and dynamics is presented on the left panel of each subfigure. Representative WHISH images of gene expression are shown on the right. Color codes in the expression matrix indicate gene expression level as specified in the legend at the bottom of the figure. Weak aboral expression of *vegf3* is marked by arrow heads; expression of *foxa* in the stomodeum is marked by “*” in the double in situ pictures. lv, lateral view; vv, vegetal view; oev, oral ectodermal view.
Fig. 3.

A diagram highlighting ectodermal gene expression domains of early sea urchin embryos, viewed from the oral ectoderm. Developmental stages include the late cleavage stage (A, 10h), blastula stage (B, 15h), mesenchyme blastula stage (C, 20h; D, 24h), and early gastrula stage (E, 30h; F, 33h). Domains and subdomains are color-coded and labels are shown in a color-matching font. Genes expressed in the oral animal ectoderm include gsc (see Fig. 2) and foxg (Tu et al. 2006). The stomodeal ectoderm is formed at mid-mesenchyme blastula stage and is located in the center of the oral animal ectoderm. Genes expressed in this subdomain include foxa and bra (Oliveri et al., 2006; Croce et al., 2001; Peterson et al., 1999). The expression patterns of these genes overlap those of animal oral ectoderm. The apical domain resides at the animal pole of the embryo above the oral ectoderm. Veg1 derived oral ectoderm is located just below the animal oral ectoderm and both nk1 and nk2.2 are expressed in this territory (see Fig. 2). Ciliated band genes, such as hnf6 (Otim et al. 2004), become expressed at late mesenchyme blastula stage. Their expression surrounds the oral animal ectoderm, and includes the veg1 oral ectoderm. The veg1 lateral domain, marked by vegf3 expression, neighbors that of oral veg1, and is formed during late mesenchyme blastula stage. Animal—animal ectoderm; Apical—apical plate; Ec—ectoderm; En—endoderm; Meso—mesoderm; Skeletogenic—skeletogenic mesenchyme.
Perturbation analysis reveals *not* to be a direct target of Nodal signaling. Expression of *nodal* and *not* was analyzed by QPCR in A) *nodal* or B) *lefty* knockdown embryos. Gene expression levels were quantified at two hour intervals from 14 hpf to 24 hpf. Changes of mRNA levels are shown as ddCt (1 ddCt = 1.9 fold difference). *nodal* abundance is significantly lower in *nodal* MASO injected embryos, but higher in *lefty* morphants. Changes in transcript abundance of *not* in response to *lefty* MASO is opposite to the effects observed in *nodal* perturbations, indicating that *not* is directly activated by Nodal signaling. Perturbation assays were repeated at least three times, and standard deviation was shown by error bars in the chart.
Fig. 5.

Sp-not is a key regulator of oral ectoderm formation. Expression of A) oral animal ectodermal genes and B) veg1 ectodermal genes was quantified by QPCR after injection of not MASO. At least three batches of sea urchin embryos were assayed at 18 hpf and 24 hpf. Changes of transcript levels are shown as ddCt (1 ddCt = 1.9 fold difference). gsc and nk1 genes exhibit significantly lower transcript levels in not MASO injected embryos compared to controls. Perturbation assays were repeated at least three times, and standard deviation was shown by error bars in the chart.
Fig. 6.
Nodal signaling and not drive oral ectodermal gene expression cooperatively or antagonistically. Single or double perturbation assays were performed to investigate how the nodal pathway and the not gene regulate the expression of the gsc, not, and vegf3 genes. Transcript levels were measured by QPCR at 16 hpf. Magnitude of gene expression changes was compared among nodal-, lefty-, not-knockdown, and lefty/not- double knockdown assays, to infer the functions of nodal and not in oral ectodermal gene expression. Perturbation assays were repeated at least three times, and standard deviation was shown by error bars in the chart.
Fig. 7.
Changes of gene expression pattern and skeleton formation in not MASO treated embryos. A, B) Gene expression patterns of wnt5 and vegf3 were analyzed and compared between not morphant and untreated embryos at mesenchyme blastula stages (24 hpf or 27 hpf). Loss of Not function caused ectopic expression of vegf3 and wnt5 in the veg1 oral ectoderm. C) Additionally not MASO injection altered the patterning of PMC cells and delayed the formation of skeleton.
Expression of *nk2.2* depends on Nodal signaling but not function of Not. A) Injection of *not* MASO does not affect spatial expression of *nk2.2* and leaves oral ectodermal expression unaltered B) Transcript levels of *nk2.2* are significantly affected by *nodal* MASO, *lefty* MASO, and *lefty/bmp2/4* double MASO injections. *nodal* knockdown results in lower transcript levels of *nk2.2* while *lefty* knockdown causes an increase demonstrating that Nodal signaling activates *nk2.2* expression. Activation of *nk2.2* by Nodal does not necessarily involve Bmp2/4 as *lefty/bmp2/4* double MASO injection caused a similar upregulation as *lefty* MASO alone. Perturbation assays were repeated at least three times, and standard deviation was shown by error bars in the chart.

*Fig. 8.*
Fig. 9.
Animal oral and veg1 ectodermal GRN for sea urchin embryogenesis. Linkages identified in this study are incorporated into the current GRN model. Eight genes (except vegfr) were included in the network. The figure was created using the Biotapestry program. The not gene plays an essential role in replaying nodal signaling pathway and patterning of ectodermal gene expression. Not can function both as an activator and repressor. It drives the expression of the nk1 and gsc in the veg1 and animal ectoderm, restricting target genes in the oral ectoderm. On the other hand, not represses the wnt5 and vegf3 genes, clearing their expression from the oral territory. nodal and not inputs to downstream genes can be synergistic, as both are required for the positive regulation of gsc expression. Additionally,
the Nodal signaling pathway (through Smad) and *not* function antagonistically in regulating
*not* and *vegf* expression.