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Review

Nodal signals pattern vertebrate embryos

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Abstract. Vertebrate embryonic patterning requires several conserved inductive signals–including Nodal, Bmp, Wnt and Fgf signals. Nodal, which is a member of the transforming growth factor β (TGFβ) superfamily, activates a signal transduction pathway that is similar to that of other TGFβ members. Nodal genes, which have been identified in numerous vertebrate species, are expressed in specific cell types and tissues during embryonic development. Nodal signal transduction has been shown to play a pivotal role in inducing and patterning mesoderm and endoderm, and in regulating neurogenesis and left-right axis asymmetry. Antagonists, which act at different steps in the Nodal signal transduction pathway, have been shown to tightly modulate the inductive activity of Nodal.

Key words. Nodal; vertebrate; embryo; mesoderm; endoderm; neural; asymmetry.

Introduction

Vertebrate embryonic development starts upon fertilization of an oocyte, followed by rapid cell cleavage. As development proceeds, the embryo is patterned by regionalization to form three germ layers: ectoderm, mesoderm and endoderm. Subsequently, each germ layer gives rise to specific sets of differentiated cell types.

In 1924, Spemann and Mangold identified a region in early amphibian embryos that is capable of inducing a second embryonic axis during gastrulation. This region of the amphibian embryo, which is known as the organizer, acts as a signaling center for both anterior-posterior and dorsal-ventral patterning. Equivalents of the Spemann's organizer have now been identified in many vertebrate species; these equivalents include the node in mouse and chick, and the embryonic shield in fish. In the early 1990s, Nodal, a new member of transforming growth factor β (TGFβ) superfamily, was identified and shown to be highly expressed in the node of mouse embryos [1]. Its mutation by retroviral insertion causes loss of embryonic mesoderm [1–3]. Studies in several vertebrate models have indicated that the Nodal signal transduction pathway, including Nodal homologues and downstream signaling components, is highly conserved, and that Nodal genes are essential in evolutionarily divergent vertebrate embryos. The Nodal signaling pathway plays important roles in induction of mesoderm and endoderm, neural patterning along the anterior-posterior axis and specification of the left-right axis. Given its roles in germ layer formation and axes specification, it is not surprising that to assure normal development of vertebrate embryos the Nodal signaling pathway must be precisely regulated.

Nodal signal transduction

Except for choice of receptors, Nodal ligands use a mechanism similar to that of other members of the TGFβ superfamily to initiate signal transduction (fig. 1) [4]. In brief, a Nodal dimer binds to type I and type II serine/threonine kinase receptors, in the presence, or absence, of coreceptor epidermal growth factor-Cripto-FRL1-Cryptic (EGF-CFC) protein, resulting in formation of a lig-

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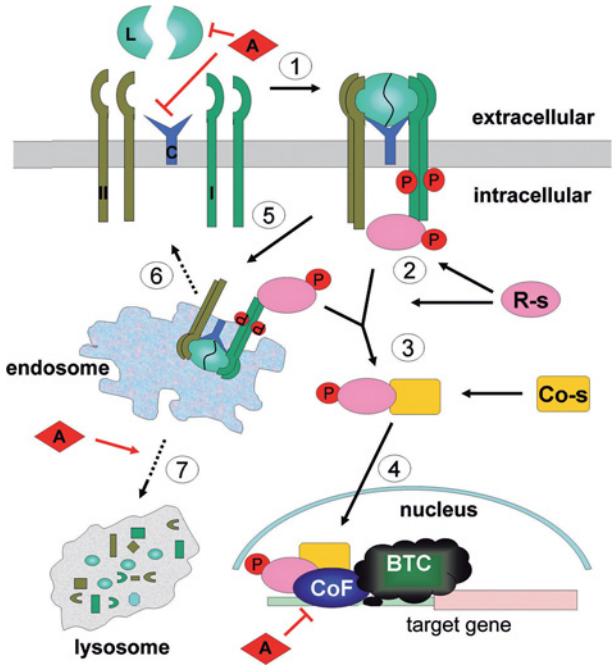

Figure 1. Nodal signal transduction. Step 1: Nodal dimer (L) binding to type II (II) and type I (I) receptors, facilitated by coreceptors (*C*), results in phosphorylation of the type I receptors. Step 2: The activated type I receptors phosphorylate R-Smads (R-s). Step 3: The phosphorylated R-Smads form a complex with Co-Smad (Co-s). Step 4: The R-Smad/Co-Smad complexes are translocated into the nucleus where they cooperate with cofactors (CoF) to activate target gene transcription; the R-Smad/Co-Smad/Co-F complex may associate with the basic transcription complex (BTC). Step 5: Ligand/receptor/coreceptor complexes are internalized while signal transduction continues. Step 6: Some receptors that have been internalized in endosomes may be recycled back to the membrane. Step 7: The remaining receptors and ligands are transported into lysosomes for degradation. Nodal antagonists (*A*) have been found to bind to the ligand, the receptor or the coreceptors, preventing the formation of ligand/receptor/coreceptor complexes. Antagonists may also inhibit signal transduction by binding nuclear cofactors to prevent target gene activation. One antagonist has been found to promote transportation of internalized receptors toward lysosomes. The mechanisms of these antagonists are discussed in a separate section.

and-receptor complex that is internalized by the cell. Within the receptor complex, the constitutively active type II receptor kinase activates type I receptors by phosphorylating their intracellular kinase domain. Activated type I receptors phosphorylate and activate downstream effectors Smad2 and Smad3 or both. These activated effectors form complexes with Smad4 that are translocated into the nucleus, where they interact with other transcription factors to regulate the expression of specific genes.

Nodal ligands

Nodal ligands, which initiate a signal transduction pathway, are targets for a variety of modulators. Nodal and nodal-related genes have been identified in several vertebrate species; the number of homologues found in each species varies. Humans, mice and chicks have a single nodal gene. However, zebrafish have at least three nodal genes: *squint* (*sqt*), *cyclops* (*cyc*) and *southpaw* (*spaw*), and *Xenopus laevis* has at least six nodal-related genes (*Xnr1–6*). It is likely that the roles played by the single *Nodal* gene in mouse and chick are apportioned among several nodal genes of frog and fish.

Newly synthesized Nodal protein precursors are secreted as homodimers with a hydrophobic leader and an N-terminal prodomain. Nodal protein precursors, which are secreted as homodimers, have a hydrophobic leader sequence and an N-terminal prodomain. Removal of the prodomain through proteolytic processing, involving subtilisin-like proprotein convertases Spc1/Furin and Spc4/Pace4, is required to produce the mature, functional form of the Nodal dimer [5–7]. Apparently, cleavage of the Nodal prodomain stimulates the ability of Nodal ligand to initiate signal transduction; however, it also promotes endocytic turnover of Nodal proteins [7]. In contrast, Nodal precursors have weak induction activity [5, 6], but are more stable than the mature forms [7]. The mature forms of different Nodal proteins share a higher degree of sequence identity than do their prodomains.

Nodal receptors and coreceptors

The receptors for Nodal ligands have been identified primarily based on null or gain-of-function phenotypes for the type I and type II serine/threonine kinase receptors. These phenotypes resemble those of Nodal null phenotypes, or phenotypes caused by overexpression of Nodal. Receptors identified in this way include two type II receptors, ActRIIA and ActRIIB, and two type I receptors, ALK4 (ActRIB) and ALK7 [8–14]; each of these receptors is also used for initiation of the Activin signal transduction pathway [15]. Mouse mutant embryos lacking *ALK4* or *ActRIIA* fail to form a primitive streak [9, 11]; this phenotype resembles that of *Nodal* mutants [3]. However, *ALK7* and *ActRIIB* mutant embryos are able to complete gastrulation [8, 16]. These genetic data imply that ALK4 and ActRIIA are the primary receptors for Nodal signaling during early development of vertebrate embryos.

Genetic studies in zebrafish have revealed that Nodal signal transduction requires the membrane-anchored protein Oep, which is an EGF-CFC protein first identified in the zebrafish mutant *one-eyed pinhead*, as a coreceptor [17, 18]. Biochemical analyses have also demonstrated that Nodal proteins can physically associate with ALK4 and type II receptors when these receptors bind to the CFC domain of the mouse EGF-CFC protein Cripto, supporting the notion that EGF-CFC proteins function as Nodal coreceptors [13, 14, 19]. Nodal can also directly bind the EGF-like domain of Cripto [19]. Although Nodal directly binds ALK7 in the absence of Cripto, the presence of

Cripto enhances ALK7-mediated Nodal signaling [13]. In contrast, Activin signal transduction does not require Cripto. In fact, Cripto can prevent Activin signaling; Activin bound to ActRIIA/IIB can form a complex with Cripto that prevents binding of the Activin–ActRIIA/IIB complex to ALK4 [20, 21].

Internalization of Nodal ligand-receptor complexes

The formation of ligand-receptor complexes on the plasma membrane are usually followed by internalization of the complexes. Internalization of a ligand-receptor complex can result in switching off receptor-mediated signaling or propagation of the signal [22]. TGFβ receptors are internalized through clathrin-coated pits, which facilitate signaling, or through lipid raft-caveolae, which promote proteasomal degradation of the receptors [23]. However, some reports claim that TGFβ and Activin can still transduce the signals in the absence of clathrin-dependent internalization of the receptors [24, 25]. As discussed below, Nodal signal transduction during embryonic development can be regulated by receptor internalization and degradation [26, 27].

Downstream effectors Smads

In the Nodal signaling pathway, type I receptors ALK4, or ALK7, associated with either ActRIIA, or ActRIIB, have been shown to activate the receptor-regulated Smads (R-Smads), Smad2 and Smad3 [13, 28]. The R-Smads are recruited to the activated type I receptors by SARA, and then phosphorylated on the last two serines of a conserved SSXS motif located at the extreme carboxyl terminus. The phosphorylated R-Smads are released from the receptor complex to form heteromeric complexes with a common Smad (Co-Smad), Smad4; these complexes enter the nucleus where they regulate transcription of downstream genes. Because of a difference in structure, Smad3 can bind directly to the conserved Smad-binding elements in the promoter of target genes; however, Smad2 must form complexes with other transcription factors to interact with DNA [29, 30]. Smad2 and Smad3 have been found to regulate different sets of genes in response to TGFβ signaling [31, 32]. Forkhead/winged helix transcription factors belonging to the FoxH1/Fast-1 family can interact with the Smad2/Smad4 complexes to form new transcriptional complexes that activate expression of target genes through Activin responsive elements [33–35]. FoxH1 proteins are not only activators of some Activin/Nodal/Vg1-responsive genes, they are also essential mediators of *Nodal* autoregulation during vertebrate embryogenesis [35–40]. Mixer, Milk and Bix3, which are members of the paired-like homeodomain Mix/Bix transcription factor family, are also binding partners of the Smad2/Smad4 complexes [41, 42], and play important roles in formation of endodermal derivatives in vertebrate embryos [41, 43–50]. In *Xenopus* embryos, Mixer may control the degree of mesoderm induction through repressing the expression of some genes and activating the expression of others [46].

Nodal functions in embryonic development

As noted above, Nodal signals play a central role in the pattern formation of vertebrate embryos. As described in the previous section, the Nodal signaling pathway shares common mediators and modulators with other TGFβ signaling pathways. For example, Nodal, Activin, Vg1, GDF1 and inhibin all transduce signals through receptors ActRIIB and ALK4, and transcription factors Smad2 and Smad3 [4]. This complicates interpretation of their functional associations with specific upstream factors. Therefore, our review focuses primarily on Nodal ligands. The functions of these ligands are summarized in table 1.

Function in endoderm formation

During organogenesis, endodermal derivatives differentiate to form the gastrointestinal epithelium, which gives rise to the gastrointestinal tract, lungs, liver, pancreas, biliary system, thyroid gland and thymus. At the onset of gastrulation, progenitors of definitive mouse embryonic endoderm partially mix with mesoderm progenitors in the posterior region of the epiblast where primitive streak formation begins [51, 52]. As gastrulation proceeds, endoderm progenitors separate from the mesoderm, and migrate through the primitive streak to form the definitive endoderm. In zebrafish blastulas, endoderm progenitors arise predominantly from the dorsal and lateral blastoderm margin, which also contains mesodermal progenitors [53, 54]. In *Xenopus*, the entire vegetal region and the suprablastoporal region commit to the endoderm fate in the late blastula [55]. Certain *Nodal* genes are expressed in endoderm progenitors immediately before, or after, or before and after, the onset of gastrulation in mice [1, 3], zebrafish [56] and frogs [57], implying a role for *Nodal* in endoderm formation.

In mouse, *Nodal* insertional mutants [1, 3], and *ALK4* [11], *ActRIIA* [9] and *Cripto* [58] null mutants, fail to form an elongated primitive streak, have excessive ectoderm, and are deficient in endoderm and mesoderm. Analyses of hypomorphic mutant mice, which can undergo gastrulation because loss of Nodal activity is incomplete, reveal that the degree of endoderm loss is associated with the amount of residual Nodal activity [59]. Recent studies indicate that, while Nodal is involved in maintaining pluripotency of human and mouse embryonic stem (ES) cells [60, 61], excess Nodal promotes ES cells to differentiate into endodermal and mesodermal

Table 1. Phenotypes resulting from mutation/knockdown or misexpression of Nodal members[1].

Species	Genes	Genetic manipulations	Major phenotypes
Mouse	*Nodal*	mutation	no mesoderm, more embryonic ectodermal cells with reduced size [1, 2]
Zebrafish	*squint* (*sqt*)	mutation	cyclopia, defects in the prechordal plate and ventral nervous system [69]
	cyclops (*cyc*)	mutation	cyclopia, no floor plate, reduced neuron number in ventral midbrain, axonal disturbance in brain and spinal cord [87, 171, 172]; left-right axis defects (only in cyc^{b229} mutants) [173]
	sqt, cyc	double mutation	lack of endoderm and mesoderm, cyclopia, no anterior trunk spinal cord [69, 109]
	southpaw (*spaw*)	knockdown	abnormal positioning of heart and pancreas, randomized gut looping [118, 132]
Xenopus	*Xnr1*	overexpression	induce mesoderm and endoderm [76, 83, 174]; cause laterality defects [117]
	Xnr2	overexpression	induce mesoderm and endoderm [74, 76, 83]
	Xnr3	overexpression	induce neural tissues [84, 100]
	Xnr4	overexpression	induce mesoderm and endoderm [76, 85, 174]
	Xnr5	overexpression	induce mesoderm and endoderm [75]
	Xnr6	overexpression	induce mesoderm and endoderm [75]
Chick	*cNR-1*	ectopic expression	randomization of heart laterality [131]

[1] In the case that data resulting from mutation or knockdown are available for a gene, its overexpression phenotypes were not described. Knockdown means morpholino-mediated inhibition of mRNA translation [175].

lineages while inhibiting production of neuroectoderm [62–64].

In zebrafish, zygotic expression of nodal gene *sqt* starts at the mid-blastula transition (MBT) in dorsal blastomeres, and shortly thereafter in the dorsal extra-embryonic yolk syncytial layer (YSL) [56]. During late blastulation, both *sqt* and *cyc* are expressed throughout the entire blastodermal margin [56], a region where mesodermal and endodermal precursors are found. Zygotic expression of these *nodal* genes is regulated in a variety of ways: in dorsal blastoderm it depends on factors downstream in the genetic pathway regulated by maternal β-catenin [65, 66]; in the ventrolateral margin it depends on factors in the YSL [67]; the expression is also subject to positive autoregulation [66, 68]. Although *sqt*;*cyc* double mutant embryos can develop through gastrulation, they are unable to express early endodermal markers and lack endodermal derivatives, such as gut [69]. In zygotic *oep* mutants, endoderm fails to form, despite the relatively normal development of mesoderm [70]. It appears that maternally supplied Oep confers enough activity to specify mesoderm, but endoderm specification apparently requires a greater level of Nodal signaling [71]. Overexpression of *lefty1*/*antivin*, an antagonist of Nodal, can cause a complete loss of endoderm [72]. On the contrary, expression of a constitutively active form of *acvr1b*/*taram-a* [73], a zebrafish orthologue of mammalian ALK4, is sufficient to activate endodermal gene expression [73]. Therefore, as in mouse, Nodal signals are required for endoderm induction in zebrafish.

In frog, overexpression of *Xnr2* can induce dorsoanterior endodermal markers in animal cap ectoderm, and a dominant negative cleavage mutant form of *Xnr2* prevents endoderm formation by inhibiting the Nodal signal transduction [74]. Another two nodal-related genes, *Xnr5* and *Xnr6*, also have the ability to induce formation of endoderm and mesoderm [75]. Maternally supplied transcription factor VegT is believed to be a key initiator of endoderm formation in *Xenopus* [76]. Overexpression of *Xnr1*, *Xnr2* or *Xnr4* can rescue the expression of various endoderm genes in VegT-depleted embryos [76]. This suggests that maternal VegT induces endoderm, at least in part, by activating zygotic expression of nodal-related genes in *Xenopus*.

Function in mesoderm induction

As described in the previous section, mesoderm progenitors intermingle partially with endoderm progenitors in mouse and zebrafish in the late blastula. Mesoderm progenitors in the *Xenopus* late blastula are situated in the equatorial region. Mesoderm progenitors that are located in different regions of the dorsovental axis in amphibians and fish have different fates in the late blastula [53, 77–

79]: cells in the dorsal organizer primarily contribute to the dorsal mesoderm, giving rise to axial midline structures such as the prechordal plate and the notochord as well as head mesoderm; cells in the lateral mesoderm region primarily give rise to somites, adult blood precursors, heart and kidney; and cells in the most ventral region form ventral tissues such as embryonic blood precursors. Different mesoderm fates are induced by specific factors in a dose-dependent manner.

Studies in amphibian species in the 1960s revealed that the mesoderm is induced by signals released from vegetal endoderm cells [80]. Since then developmental biologists have made vigorous efforts to identify endogenous mesoderm inducers in the vegetal endoderm. Although VegT has been proved to be a key maternal factor for mesoderm induction in amphibians [81], orthologue in mammals and fish have not been found. In the early 1990s, Activin and Vg1, also members of TGFβ family, and fibroblast growth factors (Fgfs), were shown to induce mesoderm upon overexpression [82]. The first evidence for a pivotal role of Nodal in mesoderm induction arose from a retroviral insertion in the *Nodal* locus of a mouse mutant that lacked a primitive streak and most mesoderm [1, 3]. Soon after that, three nodal-related genes, *Xnr1*, *Xnr2* and *Xnr3*, were identified in *Xenopus* [83, 84]. *Xnr1* and *Xnr2* are initially expressed throughout the vegetal region, but become restricted to the dorsal marginal zone during late blastulation [83]; *Xnr3* is exclusively expressed in the superficial layer of the Spemann organizer immediately preceding, and during gastrulation [84]. These *Xnrs,* along with *Xnr4*, *Xnr5* and *Xnr6*, which were identified later [75, 85], dorsalize ventral mesoderm in a dose-dependent manner, rescue dorsal mesoderm in ventralized embryos, and induce mesoderm in animal cap explants [83, 84], supporting a role for Nodal-related proteins in mesoderm induction. An absolute requirement for endogenous Nodal signal during mesoderm induction has been confirmed in zebrafish Nodal-related mutants. Zebrafish *sqt;cyc* double mutant embryos lack mesoderm derivatives, including the notochord, heart, pronephros, blood and most somites; these mutants also lack endoderm derivatives [69]. Zebrafish *MZoep* mutant embryos, which lack both maternal and zygotic Oep activity, have a phenotype that is identical to that of *sqt;cyc* double mutants [18]. In addition, mouse *Nodal* and zebrafish *sqt* and *cyc* are able to induce expression of mesoderm markers in *Xenopus* animal cap explants, indicating a conserved activity of Nodal proteins in vertebrates [83, 86, 87].

Nodal proteins have been proved to be morphogens for dorsoventral and anterioposterior patterning of mesoderm. The zebrafish Nodal morphogens have been most thoroughly studied. In *Xenopus* animal cap assay, differing levels of *sqt* or *cyc* expression can induce different mesendodermal markers [86–88]. By comparing $sqt^{-/-}$; $cyc^{+/+}$and $sqt^{-/-}$; $cyc^{+/-}$ mutant embryos, Dougan et al. demonstrated that ventrolateral mesendodermal fates can be induced by a lower level of *nodal*-related gene activity that is required to induce formation of dorsal mesendoderm and that Nodal signals act in the marginal region to pattern the animal-vegetal axis [68]. That patterning of the zebrafish organizer requires a gradient of Nodal activity is supported by the findings that show specification of prechordal plate progenitors in the dorsal margin requires sustained high levels of Nodal signaling, while the specification of notochord progenitors, which are located closer to the animal region, requires lower levels of Nodal activity [71]. When the Nodal antagonist *lefty1*/*antivin* is used to attenuate the Nodal signaling level, a lower level of *lefty1* results in loss of the cephalic mesoderm, an intermediate level leads to loss of both head mesoderm and notochord, and a high level causes loss of all head and trunk mesoderm-derived structures [72]. Nodal proteins may regulate mesoderm patterning partly by regulating expression, directly or indirectly, of other important organizer-specific signaling molecules, including *Fgfs*, *goosecoid* and *noggin* [18, 66, 89–91]. Morphogens, such as Nodal proteins, diffuse across the embryo some distance from where they are produced. Indeed, Chen and Schier (2001) have shown that Sqt can induce expression of some mesodermal markers over a long distance, while Cyc can induce the same markers over a much smaller range, indicating that Sqt is a long-range morphogen and Cyc is a short-range morphogen [88]. Using GFP-Nodal and GFP-Lefty2 fusion constructs, Sakuma et al. (2002) demonstrated in chick embryos that both fusion proteins are able to diffuse over a long distance and that GFP-Lefty2 diffuses faster than GFP-Nodal [14]. Lefty proteins, which require Nodal signaling for expression, are long-range inhibitors of Squint activity as well as antagonists of Squint autoregulation [92]. Lefty proteins may play a pivotal role in amplifying small differences in the Nodal signaling level and in generating spatial concentration patterns of Nodal signals. Thus, the Nodal-Lefty system supports the pattern-generating reaction-diffusion model [93]. As we shall discuss below, there are other Nodal inhibitors in vertebrate embryos. It will be interesting to determine the impact of these inhibitors, alone or in combination, in the formation of the spatial gradient of Nodal morphogenic activity.

Function in neural induction and anteroposterior patterning

The first step in neurogenesis is the adoption of a neural fate by dorsal ectoderm during late blastulation, giving rise to the neuroectoderm. Transplanted *Xenopus* Spe-

mann organizer, zebrafish embryonic shield or chick node have each been shown to induce a second embryonic axis in which epidermis is transformed into the neural tube, indicating that the organizer secretes neural inducers [94, 95]. Several studies have indicated that organizer-derived Bmp antagonists, including Chordin, Noggin and Follistatin, and Fgfs play critical roles in neural induction [96, 97]. Following neural induction, the neuroectoderm differentiates into two regions along the anteroposterior axis, the anterior neuroectoderm, which gives rise to the forebrain and anterior midbrain, and the posterior neuroectoderm, which gives rise to posterior midbrain, hindbrain and the spinal cord. It is believed that cells that acquire a neuroectodermal fate earliest have a default anterior neural identity and that posterior signals are required for commitment to posterior neural fates [98, 99].

Nodal genes are expressed in the organizer or its equivalents in several species, suggesting their potential involvements in neural induction. For instance, *Xnr3* is specifically expressed in the epithelial layer of the Spemann organizer [84]. Overexpression of *Xnr3* has been shown to directly induce neural tissue that expresses the anterior neural marker *Cp11* and the pan-neural markers *NCAM* and *Nrp1* in *Xenopus* animal cap explants [100]. It may be that Xnr3 antagonizes epidermal induction by Bmp signaling [101]. In mouse, *Nodal* mutants fail to express *Otx2*; *Otx2* expression in the visceral endoderm of early gastrula is required to establish anterior and posterior patterns. At later stages, these mutants express *Otx2* weakly in the epiblast; *Otx2* expression in the epiblast is required for continued anterior development [102]. Mosaic early gastrula, composed of *Nodal*-mutant primitive endoderm and wild-type embryonic epiblast cells, form an embryonic axis that lacks forebrain and anterior midbrain, indicating that Nodal in the primitive endoderm is required for development of the anterior neuroectoderm [103]. Although zebrafish *MZoep* mutants develop a well-patterned central nervous system [18], blocking Fgf signaling in these mutants results in a dramatic reduction of neural tissues; the same treatment has little impact on neural tissue development in wild-type embryos, implying that coordination of Nodal signals with Fgf signals plays an important role in neural induction [104]. Studies in several vertebrate species have shown that, during late blastulation and early gastrulation, Nodal signaling is required for maintaining expression of some neural inducers in the organizer [74, 90, 105, 106], indicating that Nodal signals play an indirect role in neural induction.

Nodal signaling is also involved in anteroposterior patterning of the neuroectoderm, showing that determining its role in neural development is not straightforward. In *Xenopus*, local overexpression of *Xnr1* in the organizer endoderm causes loss of anterior neural tissues [107]. Simultaneous inhibition of Nodal and Bmp signaling in *Xenopus* explants of ventral marginal zone can induce headlike structures [108]. In zebrafish, Nodal-deficient *MZoep* mutants have an expanded expression domain for markers of the anterior neural markers [18], and *sqt;cyc* double mutants lack anterior trunk spinal cord [109]. In the absence of mesoderm, overexpression of *antivin*/*lefty1* at high doses causes expansion of the anterior neuroectoderm, such that posterior neuroectoderm is completely lost [110], supporting a role for Nodal in posterierizing the neuroectoderm. Reduced *Nodal* expression in mice results in varied degrees of defective anterior patterning that correlates with anterior midline mesodermal and endodermal defects [59]. A gradient of Nodal activity in the distal visceral endoderm (DVE) appears to play an important role in establishment of the anterior-posterior axis in mice [111]. On the whole, these results indicate that Nodal signaling suppresses anterior neural development and promotes posterior neural development, which contrasts the role of Nodal signaling in neural induction. Since Nodal signal transduction regulates expression of neural inducers, factors that induce formation of posterior neuroectoderm and also their antagonists, fine-tuning the multiple roles played by Nodal in embryonic development may be crucial for proper neural induction, and anteroposterior patterning.

Function in establishment of left-right asymmetry

The left-right (LR) axis is one of the three major body axes in chordates. The generation of LR asymmetry can be partitioned into three sequential phases [112]. During the first phase, bilateral symmetry in the embryo is broken, which, depending on species, occurs just prior to, or soon after, gastrulation. During the second phase, transfer of LR-biased signals from the organizer to the lateral plate mesoderm activates asymmetric gene expression. During the third phase, the asymmetry of LR signals causes local changes in cell behavior that lead to formation of asymmetric tissues and organs.

The first evidence for Nodal involvement in generating LR asymmetry arose from the identification of asymmetric expression of *cNR-1*, a chick orthologue of Nodal; *cNR-1* expression is restricted to the left lateral plate mesoderm [113]. Mouse *Nodal* is expressed symmetrically before and during gastrulation [3], but during early segmentation its expression is restricted to the left side of the node and lateral plate mesoderm [114, 115]. The Notch signaling pathway directly regulates expression of the *Nodal* gene in mouse [116]. Expression of the *Xenopus* Nodal-related gene, *Xnr1*, is also restricted to the left side after the onset of somitogenesis [117]. Two of the zebrafish *nodal* genes, *cyclops* (*cyc*) and *southpaw* (*spaw*), are also expressed asymmetrically. Around the 20-somite stage, expression of *cyc* is confined to the left lateral plate

and the presumptive epiphysis, which lies on the left side of the dorsal diencephalons [56]. *spaw* is initially expressed in two bilateral domains flanking Kupffer's vesicle, an equivalent of the mouse definitive node, at the 4–6 somite stages [118]. At the 10–12 somite stage, *spaw* is expressed asymmetrically in the left lateral plate mesoderm, preceding asymmetric *cyc* expression, while its expression pattern flanking Kupffer's vesicle remains symmetric. Moreover, some components and antagonists of the Nodal signaling pathway, including *oep* [17], *Lefty* [72, 119, 120] and *Cerl-2* [121], have asymmetric expression domains during embryogenesis.

How is asymmetric Nodal expression initiated? Studies over the past few years have indicated that its asymmetric expression immediately follows the disruption of LR symmetry in the node or equivalents. In mice, leftward rotation of monocilia on the ventral surface of the embryonic node cause a leftward fluid flow, which would result in leftward diffusion of determinants that activate the Nodal signaling cascade [122–126]; rotational monocillia in chick and rabbit ventral node cells, dorsal blastopore cells of *Xenopus* early neurula and epithelial cells of fish Kupffer's vesicle may play a similar role in LR symmetry breaking [127–129]. Recently, Tanaka et al. discovered that Fgf signaling triggers secretion of nodal vesicular parcels (NVPs) from the floor of the mouse node, and that these NVPs are transported to the left by the nodal flow, where their contents, including Sonic hedgehog and retinoic acids, are released locally [130]. However, it is not clear whether NVPs contain Nodal proteins or how NVP contents might regulate *Nodal* expression at the node. Many studies have suggested that left-sided Nodal signals induce left-sided expression of the *paired-like homeodomain transcription factor* 2 (*pitx2*) in the left plate mesoderm, where Pitx2 in turn activates expression of downstream morphogenetic factors at later stages [112] (also see below).

Several lines of evidence have shown that genetic interference with the Nodal signal transduction disrupts normal LR asymmetry in vertebrate embryos. For instance, ectopic expression of *nodal*-related genes in chick, *Xenopus* or zebrafish can cause defects in the LR asymmetry of the heart and gut [117, 118, 131]. When zebrafish *southpaw* is knocked down with antisense morpholinos, the embryos show displacement of the pancreas to the left-sided or midline position and severe disruption of cardiac LR asymmetry, accompanied by reduction or loss of the left-sided expression domains of *cyc*, *pitx2*, *lefty1* and *lefty2* in the lateral plate mesoderm [118]. Horne-Badovinac et al. (2003) showed that reducing left-sided Nodal activity randomizes the migration pattern of the lateral plate mesoderm cells, and gut looping [132]. Hypomorphic mouse mutants lacking *nodal* expression in the left lateral plate mesoderm exhibit random heart looping [59]. Null mutations in EGF-CFC genes cause irregular LR patterning of internal organs in zebrafish and mice [133–135]. Ectopic *lefty* expression, or, alternatively, null of Lefty, an antagonist of Nodal signaling, can alter the unilateral expression pattern of *nodal* and *pitx2*, and affect tissue and organ laterality [136–139]. Lefty proteins expressed either in the midline or on the right side of the embryo may act as barriers that prevent left-sided Nodal/Activin signals from acting on the right side [136]. Several members of the Cerberus/Dan family of secreted factors, including mouse Cerl-2 [121], zebrafish Charon [140], and chick Cer [141] and Charonte [142, 143], are involved in establishing LR asymmetry. Cer1–2 and Charon antagonize Nodal signaling [121, 140]; however, chick Cer and Charonte stimulate *Nodal* expression by antagonizing Bmp signaling in the left side [141–143].

Several null mutations in the human *CFC1* locus have been identified in patients having randomized organ positioning [144] or transposition of the great arteries and double-outlet right ventricle [145]. A study conducted on patients with LR axis malformations by Kosaki et al. (1999) led to the identification of one patient having a nonsense mutation and another having a missense mutation (S342K) in the *LEFTY A* locus [146]. These findings support the proposition that the Nodal signal transduction pathway plays an evolutionarily conserved role in LR axis formation.

Negative regulators of the nodal signaling pathway

Since Nodal signals play critical roles in various aspects of vertebrate embryonic patterning, Nodal signaling activity must be precisely controlled in a temporal and spatial fashion. The Nodal signal transduction pathway has multiple steps; each step affords the opportunity to modulate signal transduction. Antagonists of the pathways allow gradients of Nodal activity to be precisely determined. We should emphasize that many Nodal antagonists can also inhibit other related signaling pathways. Unlike Bmp signaling, which has many antagonists [147, 148], only a few Nodal antagonists have been identified (table 2).

Cerberus/Dan family members

Members of the Cerberus/Dan gene family encode secreted factors, including Cerberus (Cer), Cerberus-like 2 (Cerl-2), Charon, Caronte, Coco, Dan, Gremlin and others. Each of these factors contains a 'can-domain' that is structurally related to the cysteine-knot motif found in members of the TGFβ superfamily [147]. The Bmp signal transduction pathway was the first shown to be inhibited by members of the Cerberus/Dan family; these factors inhibit Bmp signaling by binding to Bmp ligands [149]. Re-

Table 2. Summary of Nodal antagonists.

Factors	Types	Antagonistic mechanisms
Cerberus/Dan family members	secreted proteins	binds to the Nodal proteins to prevent their signal transduction [108–121, 140]
Bmp3/Bmp7	secreted proteins	form heterodimers with Nodal proteins and result in mutual inhibition [19]
Lefty proteins	secreted proteins	inhibit the Nodal signal transduction by binding to the Nodal coreceptors EGF-CFCs as well as to the Nodal ligands [154, 155]
Tomoregulin-1	transmembrane protein	binds to egf-cfcs to prevent the Nodal signal transduction [159]
Nicalin/Nomo	endoplasmic reticulum membrane proteins	probably modifying or trapping the Nodal signaling components [160]
Dapper2	endosomal protein	promotes degradation of nodal receptors [27]
Drap1	transcriptional repressor	binds to the DNA binding domain of FoxH1 [167]

cently, it was shown that some of these factors can also inhibit Nodal and/or Wnt signals by directly binding the ligands [108, 121, 140, 150]. Two isoforms of *Xenopus* Cer have been identified; the larger isoform is known as Cer-L, and shorter one as Cer-S [108]. Cer-L can physically interact with Xnr1, Bmp-4 and Xwnt-8, indicating that it antagonizes Nodal, Bmp and Wnt signaling. By contrast, Cer-S binds to Xnr1, but not to Bmp-4, Xwnt-8, Activin or Vg1, suggesting that Cer-S is a Nodal-specific antagonist. Overexpression of *Cer-S* messenger RNA (mRNA) in frog embryos prevents development of anterior endoderm and trunk mesoderm, phenocopying loss-of-function of Nodal signals by other means. The mechanisms that regulate the spatial and temporal distribution of endogenous Cer-L and Cer-S isoforms have not been determined. Although *Cerberus-like* (*Cerl*) null mutant mice have no defects in embryonic patterning and growth [151], *Cerl*$^{-/-}$*; Lefty1*$^{-/-}$ compound mutants develop an ectopic primitive streak and die before birth; the defects in these double mutants are rescued in *Nodal* hemizygous mice, suggesting that *Cerl* and *Lefy1* have overlapping functions [152]. The mouse Cerberus-like gene *Cerl-2* is expressed in the perinodal region during the early headfold stage and specifically in the right side of the node during early somitogenesis [121]. *Cerl-2* null mice have multiple laterality defects, including randomization of the L/R axis; the defects in these mice are partially rescued in hemizygous *Nodal* mutants [121]. Zebrafish *charon*, which is probably a mouse *Cerl-2* orthologue, is specifically expressed in regions surrounding Kupffer's vesicle, the zebrafish equivalent of the mouse definitive node [140]. Knockdown of the expression of Charon, which can bind Spaw, leads to bilateral expression of left side-specific genes in the lateral plate mesoderm and diencephalons, and defects in early and late asymmetric heart development [140]. These results indicate that Cerl-2/Charon plays an important role in left-right patterning by antagonizing Nodal activity.

Bmps

Bmp proteins constitute a subfamily of the TGFβ superfamily. During early vertebrate embryos, Bmp signals are required for ventral mesoderm formation, contrasting with the required role of Nodal in dorsal and lateral mesoderm induction. Interestingly, Yeo et al. (2001) found that Bmp3 and Bmp7 formed heterodimers with Nodal when they were overexpressed in *Xenopus* embryos, and this interaction led to mutual antagonism of Nodal and Bmp signals [19]. This implies that Bmp antagonism may play a role in the establishment of the Nodal dorsoventral gradient; however, it remains unknown whether direct interactions between Bmps and Nodals occur *in vivo* and play a role in embryonic development.

Lefty proteins

Two *Lefty* genes, *Lefty1*/*antivin*/*LEFTY B* and *Lefty2*/*EBAF*/*LEFTY A,* have been identified in vertebrates; their expression domains partially overlap Nodal expression domains [72, 119, 120, 137, 146]. Although they belong to the TGFβ superfamily, they lack a cysteine that is required to form stable dimers, and, therefore, cannot induce receptor-mediated, Smad-dependent signal transduction. Instead, Lefty proteins antagonize Nodal signaling by preventing formation of Nodal/EGF-CFC/receptor complexes [153]. EGF-CFC proteins, which include mammalian Cripto and Cryptic, zebrafish Oep and frog FRL-1, are required for Nodal binding to receptors [19]. Lefty can bind to EGF-CFC, preventing their access to ligands or receptors [154, 155]. Lefty proteins can also physically interact with Nodal ligands to block the Nodal signal transduction [154]. In addition to the Nodal pathway, Lefty can inhibit EGF-CFCs-dependent Vg1/GDF signal transduction pathways [155]. Biochemical and genetic data indicate that Lefty may also antagonize Bmp signal transduction [138, 156], given the contrasting roles

that Nodal and Bmp play in embryonic development, these results are surprising.

Tomoregulin-1

Tomoregulin-1 (TMEFF-1) is a transmembrane protein that was first identified in *Xenopus*. It contains two Follistatin modules and one EGF motif in its extracellular domain, and has a short intracellular tail [157]. TMEFF-1 inhibits mesoderm induction by *Xnr1* and *Vg1* by associating with the CFC domain of Cripto or FRL-1 [158, 159]. Although TMEFF-1 does not inhibit Activin signal transduction, it can block mesodermal, but not epidermal, induction by Bmp2; TMEFF-1 inhibition of Bmp2 mesodermal induction requires the TMEFF-1 intracellular tail [159]. Overexpression of *TMEFF-1* on the dorsal side of *Xenopus* embryos causes a reduction, or absence, of head structures, resembling phenotypes induced by overexpression of *cmXnr2*, which encodes a dominant-negative form of Xnr2 [159].

Nicalin and Nomo

Nicalin is a transmembrane protein that belongs to the aminopeptidase/transferring receptor superfamily and Nomo is a Nicalin-binding protein [160]. In cultured mammalian cells, overexpression of either *Nicalin* or *Nomo* inhibits Nodal- or Activin-stimulated reporter gene expression. Knockdown of *Nomo* in zebrafish embryos results in an increase of anterior mesodermal and endodermal derivatives at the expense of more posterior ones; the effect of *Nomo* can be prevented by overexpression of *lefty1*. Since Nicalin and Nomo are located in the membrane of the endoplasmic reticulum (ER), they are believed to inhibit Nodal signal transduction by modifying or trapping, possibly both, Nodal signaling components that are routed through the ER [160].

Dapper2

Dapper is a Disheveled-binding protein that was first identified in *Xenopus* [161]. Mammals and fish have two orthologues of frog Dapper, named Dapper1 and Dapper2 [162–165]. Xenopus dapper can inhibit Disheveled-mediated Wnt signaling and JNK activation. In zebrafish late blastula, the expression domains of both *dapper1* and *dapper2* overlap those of *sqt* and *cyc* [27, 164]. Zhang et al. (2004) have demonstrated that, in *in vitro* cell culture, zebrafish Dapper2 inhibits TGFβ signaling, but not Wnt signaling; this contrasts with the effect of human Dapper1, which inhibits Wnt, but not TGFβ, signaling [27]. Knockdown of zebrafish *dapper2* causes an increase of mesoderm tissues; *lefty1* overexpression neutralizes this effect. In contrast, *dapper2* overexpression leads to loss of axial mesoderm and induces cyclopia; *lefty1* knockdown alleviates these effects. Furthermore, *dapper2* knockdown rescues *sonic hedgehog* expression in the ventral brain and the floorplate in *Zoep* mutants. These observations all indicate that *dapper2* is an antagonist of Nodal signaling *in vivo* [27]. Biochemical studies indicate that Dapper2 binds either ALK4 or ALK5, promoting degradation of these receptors via the lysosomal pathway. Since Dapper2 is primarily located in late endosomes, it may facilitate transportation of internalized receptors from late endosomes to lysosomes. Another study revealed that zebrafish *dapper2* is required for normal convergent extension and may also promote Wnt/Ca^{2+}-PCP pathway activity [164].

Drap1

Drap1 interacts with DR1, which can bind to the TATA-binding protein (TBP) to repress transcription [166]. Surprisingly, Iratini et al. (2002) discovered a specific role of Drap1 in regulating Nodal signal transduction during mouse embryonic development [167]. They demonstrated that *Drap1* null mutants have an expanded primitive streak and arrested mesoderm cell movement; this phenotype is partially suppressed in *Nodal* hemizygous mutants. Their biochemical analyses indicated that Drap1 could bind to the DNA binding domain of FoxH1, preventing FoxH1 from binding to DNA and, therefore, from forming FoxH1-Smad2-Smad4 transcription regulatory complexes. Drap1 provides a mechanism for cells to modulate the response to Nodal signaling at the level of transcription.

Conclusion

Nodal signals play important roles in germ layer formation and patterning in vertebrate embryos. It is now widely accepted that Nodal proteins are morphogens for embryonic patterning. However, because available detection methods are not sufficiently sensitive, the spatial and temporal distribution of endogenous Nodal proteins is unknown. The embryonic Nodal gradient results in the formation of concentration gradients of the activated components of the Nodal signal transduction pathway, as is evidenced by the expression pattern of phosphorylated Smad2 in *Xenopus* blastula and gastrula [168–170]. Regional responses of Nodal-activated target genes may be dependent on Nodal concentration, lesser concentrations of Nodal resulting in fewer activated *cis*-acting Nodal response elements.

As demonstrated by Beck et al. (2002) [5] and Le Good et al. (2005) [7], mature Nodal proteins are degraded rapidly; the mechanism causing the rapid degradation of mature Nodal dimers is unclear. Like other TGFβ signals, active Nodal signals can associate with receptor-corecep-

tor complexes; the cell internalizes Nodal-receptor-coreceptor complexes. One can imagine that internalization of these complexes as well as turnover or recycling of the components must involve dozens of intracellular factors. Identifying these factors could help elucidate the mechanisms underlying specific developmental processes. In addition, it will be interesting to elucidate the mechanisms and developmental specificity of cross-talk between Nodal signaling and other signal transduction pathways.

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