Nodal Morphogens

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Nodal signals belong to the TGF- β superfamily and are essential for the induction of mesoderm and endoderm and the determination of the left–right axis. Nodal signals can act as morphogens—they have concentration-dependent effects and can act at a distance from their source of production. Nodal and its feedback inhibitor Lefty form an activator/inhibitor pair that behaves similarly to postulated reaction–diffusion models of tissue patterning. Nodal morphogen activity is also regulated by microRNAs, convertases, TGF- β signals, coreceptors, and trafficking factors. This article describes how Nodal morphogens pattern embryonic fields and discusses how Nodal morphogen signaling is modulated.

In his 1901 book “Regeneration,” Thomas Hunt Morgan speculated that “*if we suppose the materials or structures that are characteristic of the vegetative half are gradually distributed from the vegetative to the animal half in decreasing amounts, then any piece of the egg will contain more of these things at one pole than the other*” and “*gastrulation depends on the relative amounts of the materials in the different parts of the blastula*” (Morgan 1901). Although Morgan’s speculations referred to the sea urchin embryo, they foretold our current understanding of morphogen gradients in frog and fish development. Morgan’s “*materials*,” “*structures*,” and “*things*” are the Nodal signals that create a vegetal-to-animal activity gradient to regulate germ layer formation and patterning. This article discusses how Nodal signaling provides positional information to fields of cells. I first portray the components of the signaling pathway and describe the role of Nodal

signals in mesendoderm induction and left–right axis specification. I then discuss how Nodal morphogen gradients are thought to be generated, modulated, and interpreted.

THE NODAL SIGNALING PATHWAY

Like most TGF- β signals, Nodal ligands activate serine/threonine kinase receptors that phosphorylate Smad proteins to regulate gene expression (reviewed in Schier 2003; Shen 2007; Wu and Hill 2009). In the case of Nodal, the signal is received by type I and II Activin receptors and EGF-CFC coreceptors. Receptor activation results in the phosphorylation of the transcription factors Smad2 and Smad3. This leads to their binding to Smad4, nuclear translocation, and association with additional transcription factors to regulate target genes. Several extracellular proteins, including processing enzymes and antagonists, regulate this

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Figure 1. The Nodal signaling pathway. After processing of the Nodal precursor by convertases, Nodal ligands signal through activin receptors and EGF-CFC coreceptors. Cerberus and Lefty act as extracellular inhibitors. MicroRNAs belonging to the miR-430 family target Nodal (zebrafish squint) and Lefty mRNAs for degradation and repression. MiR-15/16 represses the type II activin receptor. Rap2 is involved in the recycling of activin receptors whereas Dapper 2 targets activin receptor complexes for degradation in the lysosome. Pathway activation is mediated by phosphorylation of Smad2 and its association with Smad4 and transcription factors such as FoxH1, Mixer, and p53. Phospho-Smad2 is dephosphorylated by PPM1A and exported from the nucleus by RanBP3. Smad4 activity and stability is regulated by the ubiquitinase Ectodermin and the deubiquitinase FAM/Usp9x. See text for details.

core pathway. In addition, intracellular molecules such as transcriptional cofactors, proteins involved in receptor trafficking, and miRNAs regulate Nodal signaling. To understand how Nodal morphogen activity is regulated, one first needs to understand the molecular basis of Nodal signal transduction (Fig. 1).

Nodal Signals, Convertases, and Extracellular Antagonists

TGF- β signals belonging to the Nodal sub-family were initially found in chordates but were notably absent from Ecdysozoa such as

Drosophila or *Caenorhabditis elegans*. They are now also known to be present in deuterostomes (e.g., sea urchin) and in the protostome group of Lophotrochozoa (e.g., snails) where, similar to chordates, they control left–right asymmetry and chirality (Duboc et al. 2004; Chea et al. 2005; Grande and Patel 2009). The absence of Nodal in *Drosophila* or *C. elegans* might reflect the different, more derived modes of mesoderm and left–right specification in these systems. Although there is only one Nodal gene in mouse (Zhou et al. 1993), there are three in zebrafish (Cyclops, Squint, and Southpaw) (Erter et al. 1998; Feldman et al.

1998; Rebagliati et al. 1998a; Rebagliati et al. 1998b; Sampath et al. 1998; Long et al. 2003) and five in *Xenopus* (Xnr1, 2, 4, 5, and 6) (Jones et al. 1995; Joseph and Melton 1997; Takahashi et al. 2000).

Nodal proteins are translated as proproteins, consisting of a prodomain and a mature ligand domain. Studies in mouse have shown that the convertases Spc1 and Spc4 (also known as Furin and Pace4, respectively) cleave Nodal precursors at R-X-(K/R/X)-R consensus sequences (Beck et al. 2002). The Nodal precursor can be secreted and processed extracellularly by Spc1 and Spc4. Processing is essential for activation of the Nodal signaling pathway in zebrafish and mouse embryonic tissues (Beck et al. 2002; Le Good et al. 2005), but a non-processable Nodal precursor can activate the pathway in mouse extraembryonic ectoderm (Ben-Haim et al. 2006).

Additional signaling complexity is introduced by the finding that several related TGF- β ligands act through the same pathway as Nodal. For example, mouse GDF1 and GDF3, and the related frog and zebrafish Vg1, activate the pathway via Activin receptors and EGF-CFC coreceptors (Thomsen and Melton 1993; Cheng et al. 2003; Chen et al. 2006; Andersson et al. 2007; Karkera et al. 2007). Double mutant analysis has shown that Nodal can have both overlapping and nonredundant roles with GDF1. GDF1 and Nodal are both required for left–right specification, suggesting interdependent or synergistic roles (Rankin et al. 2000; Andersson et al. 2006). Indeed, GDF1 can heterodimerize with Nodal to generate a more active ligand than the corresponding homodimers (Tanaka et al. 2007).

Extracellular inhibitors such as Lefty and Cerberus antagonize Nodal signaling. Leftys are divergent members of the TGF- β family and block Nodal signaling by binding to Nodal itself and to EGF-CFC coreceptors (Meno et al. 1996; Bisgrove et al. 1999; Meno et al. 1999; Thisse and Thisse 1999; Thisse et al. 2000; Agathon et al. 2001; Chen and Schier 2002; Chen and Shen 2004; Cheng et al. 2004). Cerberus and Cerberus-like proteins like Charon bind directly to Nodal, inhibit its

binding to receptors, and thus regulate embryonic patterning (Bouwmeester et al. 1996; Piccolo et al. 1999; Bertocchini and Stern 2002; Silva et al. 2003; Hashimoto et al. 2004; Marques et al. 2004; Yamamoto et al. 2004; Tavares et al. 2007; Belo et al. 2008). Loss of Lefty antagonists results in ectopic and prolonged activity of Nodal signaling, leading to ectopic mesendoderm formation and abnormal left–right patterning (Meno et al. 1998; Meno et al. 1999; Agathon et al. 2001; Meno et al. 2001; Chen and Schier 2002; Feldman et al. 2002; Yamamoto et al. 2004).

Receptors and Signal Transducers

Nodal signals assemble receptor complexes consisting of type I and type II activin receptors (ActRIB; ActRIIA/B) that function as serine/threonine kinases (Reissmann et al. 2001; Yeo and Whitman 2001; Yan et al. 2002). Assembly results in the phosphorylation and activation of the type I receptor by the type II receptor. EGF-CFC proteins are extracellular GPI-linked factors that are required for Nodal signaling and embryogenesis (Schier et al. 1997; Shen et al. 1997; Ding et al. 1998; Zhang et al. 1998; Gritsman et al. 1999; Yan et al. 1999; Bamford et al. 2000; Shen and Schier 2000; de la Cruz et al. 2002; Dorey and Hill 2006; Onuma et al. 2006). For example, absence of the EGF-CFC protein one-eyed pinhead inactivates the pathway and renders embryos resistant to Nodal (Gritsman et al. 1999). It is thought that EGF-CFC proteins act as coreceptors by binding to Nodal and the type I activin receptor (Reissmann et al. 2001; Yeo and Whitman 2001; Yan et al. 2002; Cheng et al. 2003; Cheng et al. 2004; Chu et al. 2005; Minichiotti 2005).

Recent tissue culture studies have highlighted the importance of ligand and receptor trafficking in Nodal signaling. For example, the mammalian EGF-CFC protein Cripto can promote Nodal signaling by linking the processing and trafficking of Nodal (Constam 2009). Cripto forms a complex with Nodal precursors and convertases at the surface of responding cells and facilitates Nodal processing and translocation to early endosomes

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(Blanchet et al. 2008a; Blanchet et al. 2008b). Moreover, on internalization, Cripto facilitates the interaction with Activin receptors by attenuating the sorting of Nodal into intraluminal vesicles that are destined for lysosomal degradation (Blanchet et al. 2008b). Although the *in vivo* relevance of these findings remains to be tested, they suggest important roles of trafficking during Nodal signaling. Indeed, previous studies have shown that receptor trafficking plays an important role in TGF- β signaling. Receptors can either be recycled or targeted for degradation depending on the trafficking route (Constam 2009). Signaling is thought to occur in endosomes generated by clathrin-mediated internalization, whereas degradation is thought to be mediated by a lipid-raft/caveolar internalization pathway and trafficking to lysosomes (Di Guglielmo et al. 2003). It remains to be determined whether this model applies to Nodal signaling. For example, the degradative uptake of Nodal does not involve caveolin-positive carriers in tissue culture (Blanchet et al. 2008b), and signaling is mediated by interaction with EGF-CFC proteins localized to flotillin-positive lipid rafts (Blanchet et al. 2008b). It is clear, however, that the regulation of Activin receptor trafficking can modulate signaling (Jullien and Gurdon 2005). The Ras GTPase Rap2 promotes recycling of nonbound Activin Receptors and delays degradation of ligand-receptor complexes and thus up-regulates signaling (Choi et al. 2008). In contrast, Dapper 2 is induced by Nodal signaling and recruited to late endosomes where it binds the type I activin receptor and enhances the lysosomal degradation of Nodal receptors (Zhang et al. 2004). In addition, subunits of the PP2A phosphatase influence activin receptor levels and signaling (Batut et al. 2008). These studies indicate that ligand and receptor trafficking and stability have important roles in regulating the strength and duration of Nodal signaling.

Transcription Factors and Target Genes

Nodal receptor activation results in phosphorylation of Smad2/3 and association with

Smad4 and other transcription factors (Massague et al. 2005; Ross and Hill 2008). These activated Smad complexes accumulate in the nucleus as a result of decreased export rate and increased import rate compared with monomeric unphosphorylated Smads. Smads shuttle between nucleus and cytoplasm and thus can continuously monitor and respond to receptor activity (Bourillot et al. 2002; Inman et al. 2002; Xu et al. 2002; Nicolas et al. 2004; Schmierer and Hill 2005; Schmierer et al. 2008).

Smad4 mutant phenotypes are less severe than Smad2/3 mutant phenotypes, indicating Smad4-independent gene regulation by Nodal (Chu et al. 2005). Moreover, Smad3/4 have relatively poor affinity for DNA, whereas Smad2 has no DNA binding activity. Thus, Smad proteins must form complexes with specific transcription factors to recognize and regulate Nodal-responsive *cis*-elements (Massague et al. 2005; Ross and Hill 2008). Proteins such as FoxH1, Mixer, and p53 form complexes with Smad proteins and contribute to the specific recognition and regulation of subsets of Nodal target genes (Chen et al. 1996; Germain et al. 2000; Hoodless et al. 2001; Yamamoto et al. 2001; Hart et al. 2002; Cordenonsi et al. 2003; Takebayashi-Suzuki et al. 2003). For example, loss of FoxH1 or Mixer leads to distinct phenotypes in zebrafish (Kikuchi et al. 2000; Pogoda et al. 2000; Sirotkin et al. 2000), whereas FoxH1; Mixer double mutants show more than additive phenotypes (Kunwar et al. 2003). These results have led to the model that Smad2/3/4 cofactors regulate distinct but partially overlapping sets of Nodal downstream genes (Ross and Hill 2008).

The activity of Smad complexes is regulated post-translationally (Lin et al. 2006; Episkopou et al. 2001; Niederlander et al. 2001; Iratni et al. 2002; Dupont et al. 2005; Cordenonsi et al. 2007; Levy et al. 2007; Mavrikakis et al. 2007; Nagano et al. 2007; Yun et al. 2007; Sasai et al. 2008; Dai et al. 2009; Dupont et al. 2009). Smad2/3 is activated by receptor-mediated phosphorylation and inhibited by phosphatases such as PPM1A (Lin et al. 2006). De-phosphorylated Smad2/3 is then recognized

by RanBP3 and exported from the nucleus (Dai et al. 2009). Interference with PPM1A or RanBP3 activity leads to an increase of TGF- β signaling. Additionally, ubiquitination and de-ubiquitination of Smad4 contribute to Nodal signaling. Smad4 monoubiquitination by Ectodermind lowers pathway activity, whereas de-ubiquitination by FAM/Uspx allows interaction with P-Smad2 and pathway activation (Dupont et al. 2005; Dupont et al. 2009). In contrast, the ubiquitin ligase Arkadia increases pathway activity, but its exact mechanism of action is controversial (Episkopou et al. 2001; Niederlander et al. 2001; Koinuma et al. 2003; Levy et al. 2007; Mavrikakis et al. 2007; Nagano et al. 2007). Cofactor activity is also regulated. For example, Drap1 can bind to FoxH1 and attenuate Nodal signaling, and Serum response factor interacts with Smad2 and FoxH1 to inhibit the formation of Smad2-FoxH1 complexes (Iratni et al. 2002; Yun et al. 2007). The Zn-finger factor XFDL156 binds p53, blocking its interaction with P-Smad2 (Sasai et al. 2008). Conversely, Ras/MAPK signaling promotes p53 phosphorylation and enhances its interaction with P-Smad2 (Cordenonsi et al. 2007).

A plethora of Nodal target genes have been identified (Dickmeis et al. 2001; Bennett et al. 2007a; Guzman-Ayala et al. 2009). For example, genomic analyses in zebrafish show that Nodal initiates a cascade of regulatory events by inducing the expression of transcription factors and additional signaling molecules (Dickmeis et al. 2001; Bennett et al. 2007a). In addition, Nodal also has direct effects on cell behavior by regulating cytoskeletal components and other differentiation genes. With the exception of Nodal itself, the feedback inhibitor Lefty and the transcription factor pitx2, no downstream genes have been found that are shared between Nodal signaling in mesendoderm and left-right specification in all vertebrates (Adachi et al. 1999; Bisgrove et al. 1999; Norris and Robertson 1999; Osada et al. 2000; Saijoh et al. 2000; Faucourt et al. 2001; Shiratori et al. 2001; Norris et al. 2002; Vincent et al. 2004; Saijoh et al. 2005; Shiratori et al. 2006; Guzman-Ayala et al. 2009). This suggests

that although the core Nodal signaling pathway has been conserved across evolution, the downstream targets are divergent between different tissues.

THE ROLES OF NODAL SIGNALS IN DEVELOPMENT

To discuss how Nodal proteins provide positional information, one first needs to understand how the pathway is used during mesendoderm induction and left-right patterning. For a discussion of Nodal signals in other developmental contexts, see Shen 2007.

Mesendoderm Induction

The formation of the three germ layers—endoderm, mesoderm, and ectoderm—is a prerequisite for the formation of vertebrate organs (Kimelman and Schier 2002; Stern 2004; Schier and Talbot 2005; Solnica-Krezel 2005; Heasman 2006; Tam and Loebel 2007; Arnold and Robertson 2009). Nodal signals are essential for the induction and patterning of mesoderm and endoderm (Conlon et al. 1991; Conlon et al. 1994; Feldman et al. 1998; Gritsman et al. 1999; Agius et al. 2000; Schier and Shen 2000; Brennan et al. 2001; Schier 2003; Bennett et al. 2007b; Shen 2007). For example, in the absence of Nodal signaling, mouse embryos do not form the primitive streak and zebrafish embryos lack all endoderm and head and trunk mesoderm, including notochord, heart, kidney, blood, liver, pancreas, and gut (Conlon et al. 1991; Conlon et al. 1994; Feldman et al. 1998; Gritsman et al. 1999; Bennett et al. 2007b). Nodal signaling is active during blastula stages, when cells are pluripotent. During normal embryogenesis, spatially localized activity of the Nodal pathway induces and patterns mesendoderm at the appropriate position (Fig. 2). Conversely, ectopic activation of the pathway induces ectopic mesendoderm (Jones et al. 1995; Toyama et al. 1995; Schier 2003; Shen 2007). The in vitro differentiation of embryonic stem cells uses this inducing property of Nodal in which activation of Nodal signaling helps to

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Figure 2. Mesoderm and endoderm induction in zebrafish. Ectoderm, mesoderm, and mesendoderm precursors are arranged from animal to vegetal positions at the blastula stage. The Nodal signals Squint and Cyclops and the inhibitor Lefty are expressed at the blastula margin. Genetic studies suggest that Cyclops has short-range activity whereas Squint and Lefty have long-range activity. See text for details.

maintain the pluripotent state and drive development along the mesendodermal pathway (Parisi et al. 2003; Brandenberger et al. 2004; Kubo et al. 2004; Vallier et al. 2004; D'Amour et al. 2005; James et al. 2005; Pfendler et al. 2005; Vallier et al. 2005; Camus et al. 2006; Takenaga et al. 2007; Smith et al. 2008; Vallier et al. 2009).

Several mechanisms spatially restrict activation of Nodal signaling. In zebrafish and frog, Nodal genes are transcribed in the vegetal region, which overlaps with endoderm precursors and is adjacent to presumptive mesoderm. Thus, local transcription generates a local source of Nodal signals. It appears that high levels of Nodal signaling in and close to the source induce endoderm, whereas lower levels induce mesoderm in neighboring cells (Schier 2003; Schier and Talbot 2005; Shen 2007).

In contrast to the transcriptional restriction of Nodal gene expression in zebrafish and frog, the local activation of mouse Nodal signaling is initially achieved post-transcriptionally. Nodal transcription is initiated throughout

the epiblast. Nodal precursor protein produced by the epiblast induces the transcription of the convertases Furin and Pace4 in extraembryonic ectoderm (Ben-Haim et al. 2006). These convertases are secreted and process Nodal in the adjacent epiblast, initiating an autoregulatory feedback that enhances Nodal transcription in a subset of epiblast cells that include mesendoderm precursors (Beck et al. 2002). In addition to the local generation of Nodal mRNA and protein, inhibition of the pathway by extracellular antagonists further restricts mesendoderm formation. For example, loss of the inhibitor Lefty leads to the transformation of presumptive ectoderm into mesendoderm (Meno et al. 1999; Agathon et al. 2001; Chen and Schier 2002; Feldman et al. 2002; Perea-Gomez et al. 2002).

Left-Right Patterning

Vertebrate organs are positioned asymmetrically not only along the anterior-posterior and dorsal-ventral axes but also the left-right axis (Shiratori and Hamada 2006). *Nodal* genes are expressed in the left lateral plate mesoderm and required for left-right axis specification (Levin et al. 1995; Collignon et al. 1996; Lowe et al. 1996; Yan et al. 1999; Lowe et al. 2001; Long et al. 2003; Kumar et al. 2008). Asymmetric activation of the pathway induces asymmetric organ morphogenesis (Yan et al. 1999; Bamford et al. 2000; Concha et al. 2000; Concha et al. 2003; Halpern et al. 2003; Yashiro et al. 2007; Davis et al. 2008; de Campos-Baptista et al. 2008; Kurpios et al. 2008; Bakkers et al. 2009; Roussigne et al. 2009). For example, activation of Nodal signaling in left heart progenitors allows leftward movement and enhances the speed of cardiomyocytes (Baker et al. 2008; de Campos-Baptista et al. 2008; Bakkers et al. 2009). In the zebrafish diencephalon, left-sided Nodal expression promotes neurogenesis in the left habenula and the movement of the parapineal organ to the left (Concha et al. 2000; Concha et al. 2003; Halpern et al. 2003; Roussigne et al. 2009). During mouse development,

Nodal signaling is required for asymmetric organogenesis, ranging from lung lobe formation (Yan et al. 1999) to gut morphogenesis (Davis et al. 2008; Kurpios et al. 2008). In the absence of Nodal signaling, organ asymmetry is lost, randomized, or isomeric.

Nodal expression in the left lateral plate mesoderm is dependent on Nodal expression in cells in or next to the embryonic midline (Fig. 3). For example, mouse Nodal is expressed in the node and induces Nodal in the left lateral plate mesoderm. This process requires intact cilia and fluid flow in the node (Nonaka et al. 1998; Nonaka et al. 2002) and appears to be mediated by the movement of Nodal protein to the lateral plate mesoderm (Brennan et al. 2002; Nonaka et al. 2002; Saijoh et al. 2003; Nakamura et al. 2006; Oki et al. 2007). In contrast to mesendodermal patterning, graded Nodal signaling does not appear to be required for left–right patterning. Thus, Nodal signaling might simply control the binary decision of

left versus right. However, similar to the restriction of Nodal signaling in mesendoderm development, extracellular antagonists belonging to the Lefty family restrict Nodal signaling to only the left side (Meno et al. 1998; Feldman et al. 2002; Nakamura et al. 2006).

NODAL SIGNALS AS MORPHOGENS

Nodal signals can act as morphogens—they directly act at a distance from their site of production and induce concentration-dependent responses in target cells.

Direct Long-Range Effects of Nodal Signals

The range of vertebrate TGF- β signals was a contentious issue in the 1990s. It was unclear whether such signals can act directly at a distance or depend on relay signals that indirectly mediate their effects (Gurdon et al. 1994; Jones et al. 1996; Reilly and Melton 1996; McDowell et al. 1997). Studies of Activin revealed long-range effects, whereas studies of TGF- β 1 suggested short-range activity; however, because the tested ligands are neither expressed in the blastula nor required for normal mesendoderm induction, the *in vivo* relevance of these experiments was unclear (Schier and Shen 2000). The isolation of Nodal genes allowed the analyses of endogenously expressed and functionally essential TGF- β s. This revealed both short- and long-range activity of Nodal signals during mesendoderm induction (Chen and Schier 2001).

In zebrafish, several lines of evidence suggest that the Nodal signals Cyclops and Squint have short- and long-range effects, respectively (Chen and Schier 2001). First, misexpression of Squint from a localized source induces long-range activation of Nodal downstream genes in surrounding tissue, whereas Cyclops only has short-range activity (Fig. 2). Second, *in vivo* target gene induction in Squint mutants is only short-range and originates from Cyclops. Conversely, Squint-mediated gene induction is still long-range in Cyclops mutants. Third, the long-range effect of Squint appears to be

Figure 3. Model for left–right patterning by Nodal and Lefty. Nodal/GDF1 heterodimers form in the region of the node and activate Nodal signaling in the lateral plate mesoderm (LPM). Cilia-induced fluid flow biases Nodal activation toward the left. Subsequent Nodal autoregulation, induction of Lefty expression, and long-range inhibition by Lefty lead to robust pathway activation on the left and inhibition on the right. See text for details.

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direct, because Squint generated in coreceptor mutant (i.e., nonresponding) cells can traverse a field of nonresponsive, coreceptor mutant cells and activate Nodal target genes in distant wild-type cells. These experiments rule out a Squint-induced relay signal and provided evidence that Squint can act directly at a long range. Further support for direct long-range Nodal action during mesendoderm formation has been provided by the distribution of *Xenopus* Nodal protein Xnr2 (Williams et al. 2004). Local expression of Xnr2-GFP results in the extracellular movement of the fluorescent protein from the source into nonexpressing tissue.

Nodal signals also have long-range activity during left–right specification. For example, mouse Nodal can be generated in nonresponsive coreceptor mutant node cells, traverse nonresponsive mesoderm, and activate Nodal target genes in distant wild-type cells in the LPM (Oki et al. 2007). Similarly, Nodal expression in the LPM is required for expression of *lefty1* in the midline (Yamamoto et al. 2003). Taken together, these studies clearly establish that Nodal ligands can have direct long-range effects.

Concentration-dependent Effects of Nodal Signals

Nodal signals can induce dose-dependent effects in responding cells as seen by varying the concentration of Nodal ligands and determining downstream gene expression. Such experiments have revealed at least two thresholds for Nodal-dependent gene activation. For example, low levels of Nodal are sufficient to induce targets such as *ntl/T/Brachyury* and *floating head/Xnot*, whereas the targets *goosecoid* and *casanova/sox32* are activated only by high levels of Nodal (Gurdon and Bourillot 2001; Schier and Talbot 2005). Further evidence for dose-dependent effects of Nodal signaling comes from mutants that partially decrease Nodal activity. For example, partial reduction of Nodal, Smad2/3, or EGF-CFC coreceptor activity leads to loss of high threshold target gene expression and absence of endoderm and

prechordal plate mesoderm (Schier et al. 1997; Gritsman et al. 2000; Thisse et al. 2000; Dougan et al. 2003; Vincent et al. 2003).

Together with the local expression and long-range activity of Nodal ligands, these findings have suggested a model wherein Nodal ligands such as Squint form a concentration gradient from the vegetal to the animal pole, much as T.H. Morgan had predicted (Schier 2003). This gradient provides positional information so that cells acquire fates according to their location in the gradient. A gradient of Nodal signals has not yet been visualized, but an activity gradient can be visualized by the domains of target gene expression, P-smad2 levels, and nuclear accumulation of Smad2-fluorescent protein fusions (Faure et al. 2000; Gritsman et al. 2000; Chen and Schier 2001; Lee et al. 2001; Harvey and Smith 2009). For example, zebrafish Smad2 accumulates in a vegetal-to-animal gradient in zebrafish blastula nuclei (Harvey and Smith 2009). These observations support the idea that an activity gradient of Nodal signaling specifies different cell types along the vegetal-animal axis.

Time-dependent Effects of Nodal Signals

The spatial concentration gradient model is consistent with the gene expression domains of Nodal downstream genes and the blastula fate map, but it has several limitations. First, zebrafish mesendoderm can eventually form normally even in the absence of the long-range signal Squint, i.e., solely by the action of the short-range ligand Cyclops (Feldman et al. 1998; Chen and Schier 2001; Dougan et al. 2003). Second, downstream responses are not only determined by the concentration of the signal at a given time but also the duration of pathway activity. For example, expression of high-threshold targets is absent on premature block of the pathway by receptor inhibitors or loss of coreceptors (Gritsman et al. 2000; Aoki et al. 2002; Hagos and Dougan 2007). A similar loss of high-threshold target gene expression is observed on delayed pathway activation caused by late expression of coreceptors (Gritsman et al. 2000). Moreover, cells

exposed to a uniform dose of Nodal mRNA progressively move from low- to high-threshold fates with increasing time of exposure (Hagos and Dougan 2007). Analogously, nuclear accumulation of Smad2 increases within a one-hour time window in the late zebrafish blastula (Harvey and Smith 2009). These experiments reveal a clear role for prolonged exposure to Nodal ligands and suggest that the cumulative dose (concentration and time) of Nodal determines the fate of responding cells. The cumulative dose model might also explain why short-range Cyclops can induce all mesodermal cell types in the absence of long-range Squint. Different times of exposure of blastomeres to Cyclops might allow the differential activation of Nodal signaling and the generation of different cell types.

The duration of Nodal signaling might be measured at several points in the pathway. For example, to elicit a particular response, sufficient ligand has to accumulate over time. Thus, in Squint mutants, induction of cell fates is delayed, whereas overexpression of Nodal ligands can accelerate fate specification (Hagos and Dougan 2007). Timing is also likely to influence events at the receptor level. For example, blocking receptor activity after exposure to high doses of Nodal attenuates the response and leads to absence of high-threshold target gene expression (Hagos and Dougan 2007). This result argues against the idea that a given concentration of Nodal is sufficient to induce a long-lasting response and suggests that cumulative dose is measured at the receptor level or downstream. Indeed, studies on Activin suggest that one mechanism of memory of previous exposure to ligand might be the high stability and residence of signal-receptor complexes in intracellular vesicles after endocytosis and before lysosomal degradation (Dyson and Gurdon 1998; Gurdon and Bourillot 2001; Jullien and Gurdon 2005). Taken together, these studies suggest that the cumulative dose of Nodal signaling determines cells fates. It is unclear, however, how concentration and duration are translated into positional identities (Ashe and Briscoe 2006).

A Reaction–Diffusion System: Mid-range Activation by Nodal and Long-range Inhibition by Lefty

The noncanonical TGF- β signal Lefty is a potent feedback inhibitor of Nodal signaling (Meno et al. 1999). Several studies have suggested that Nodal and Lefty constitute an activator/inhibitor pair as postulated in reaction–diffusion models of pattern formation (Saijoh et al. 2000; Chen and Schier 2002; Hamada et al. 2002; Schier 2003; Nakamura et al. 2006). In such models, a locally acting activator induces both its own synthesis and the synthesis of a long-range inhibitor (Fig. 4) (Turing 1952; Meinhardt and Gierer 2000). In the classic reaction–diffusion system, such interactions can result in self-organization that generates patterns in an initially homogenous field of cells. Nodal/Lefty share the activator/inhibitor and self-enhancement features of this system. For example, in the zebrafish blastula, Nodal activates Nodal and Lefty transcription at the margin, and Lefty is required to restrict the range of Nodal signaling by blocking both the generation of Nodal locally and the response to Nodal at a distance (Meno et al. 1999; Chen and Schier 2002). The Nodal/Lefty interaction occurs in three steps: (1) Nodal expression is activated, resulting in pathway activation. (2) Pathway activation results in Lefty expression. (3) Lefty inhibits the pathway.

Figure 4. Reaction/Diffusion Model for Patterning by Nodal and Lefty. Nodal induces its own expression and forms a concentration gradient (c , concentrations of Nodal and Lefty; x , distance). Lefty is induced by Nodal and blocks Nodal signaling. The model postulates that Lefty has a longer range and more shallow distribution than Nodal. Thus, Nodal signaling is active close to the source but inhibited at a distance. See text for details.

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The exact pattern of pathway activation depends on the local concentrations of Nodal and Lefty. In the blastula, genetic experiments indicate that Squint and Cyclops are in excess close to the source, whereas Lefty is present at higher levels at a distance from the source (Chen and Schier 2002). In the absence of Lefty, more Nodal is generated, and cells at a distance are no longer inhibited by Lefty. On excess of Lefty, Nodal production and signaling are inhibited. Thus, Nodal/Lefty constitute a reaction–diffusion activator/inhibitor pair but they do not generate pattern *de novo* in an initially homogenous field of cells (Fig. 2). Rather, localized maternal determinants activate Nodal expression, which then induces Lefty. The Nodal/Lefty interaction is also an example of how a morphogen, Nodal, can modulate its effects by inducing a secondary signal, Lefty. Thus, target cells are not merely passive responders but change the activity gradient by their response.

The Nodal/Lefty activator/inhibitor pair also plays a role during left–right specification (Hamada et al. 2002; Nakamura et al. 2006). In this system, Nodal/Lefty interactions appear to amplify small differences between left and right lateral plate mesoderm (Fig. 3). Both left and right can initially express low levels of Nodal but by an unknown mechanism, cilia-induced flow in the node is thought to generate a slightly higher accumulation of Nodal in left lateral plate mesoderm. This initial asymmetry is amplified by Nodal autoregulation and the induction of Lefty on the left and in the midline. The long-range activity of left-sided and midline Lefty then suppresses Nodal amplification on the right. Mathematical modeling supports this self-enhancement lateral inhibition model (Nakamura et al. 2006). As in the blastula, this system is not entirely self-organizing but biased by a prepattern. In contrast to the blastula, however, the output is not graded but discrete: On (left) versus off (right).

Taken together, these studies have provided strong genetic evidence that Nodal/Lefty are part of a two-component reaction–diffusion system. However, the presumptive long-range distributions of Nodal and Lefty proteins have

not been visualized *in vivo*, and it remains unclear how exactly their interaction provides positional information.

Modulation of Nodal Morphogen Activity

The distribution and activity of morphogens are controlled by multiple factors. Prominent roles are played by the rate and level of morphogen production at the source, the rate of morphogen movement from the source into surrounding tissues, and the availability and stability of the morphogen. For example, the higher the production, diffusion, and stability of a morphogen, the longer is its range. Conversely, the effects of morphogens are determined by the responsiveness of target cells. For example, receptor levels and inhibitors can influence cellular responses. In the following section, some of the molecular mechanisms that modulate the range and activity of Nodal morphogens is discussed. It is worth emphasizing, however, that this analysis is still in its infancy. For example, when experimental manipulations change the Nodal signaling range or response, it is often unclear whether this change is caused by alterations in morphogen production, mobility, trafficking or stability, changes in regulatory feedback interactions, or differences in target cell responsiveness.

Expression

The concentrations and ratio of Nodal and Lefty signals determine the range of Nodal signaling—the more Nodal and the less Lefty, the higher the activation of the pathway. The expression levels of these genes must therefore be exquisitely regulated. For instance, in frog and zebrafish, Nodal genes are first activated on the future dorsal side and then are expressed in the vegetal and marginal region of the blastula (Schier 2003). This expression pattern is reflected in the activation of the pathway. In frogs, P-Smad2 is first detected on the dorsal side, and in zebrafish, nuclear Smad2 levels are highest in dorsal blastomeres (Faure et al. 2000; Lee et al. 2001; Harvey and Smith 2009). Correspondingly, genetic studies have shown

that the induction of dorsal mesodermal cell fates requires higher levels of Nodal signaling than ventral and lateral mesoderm, and misexpression studies have revealed that the induction of dorsal mesoderm markers such as *gsc* requires higher levels of Nodal signaling than the induction of pan-mesodermal genes such as *ntl* (Schier et al. 1997; Gritsman et al. 2000; Dougan et al. 2003). Moreover, fate mapping studies have shown that partial reduction of Nodal signaling leads to the loss of dorsal mesodermal cell fates (Gritsman et al. 2000; Dougan et al. 2003). Instead, these cells acquire more animal cell fates and form neural structures. Importantly, no transformation into more ventral mesodermal cell fates is observed on inhibition of Nodal signaling. Thus, despite higher nuclear accumulation of Smad2 on the dorsal side and the suggestion that Nodal signaling patterns mesoderm along the dorsal–ventral axis (Harvey and Smith 2009), there is no evidence for a requirement of graded Nodal signaling in specifying dorsal versus ventral mesodermal fates. Rather, graded Nodal signaling patterns the vegetal–animal axis and blocks the formation of neural structures at the dorsal margin (Feldman et al. 2000; Carmany-Rampey and Schier 2001; Dougan et al. 2003).

Following the induction of Nodal gene expression, regulatory interactions between Nodal and Lefty generate an intricate system to modulate Nodal signaling. Nodal activates its own transcription, which if unchecked results in a positive feedback that dramatically increases Nodal gene expression. However, because Nodal also activates Lefty expression and Lefty inhibits pathway activity, a negative feedback dampens, spatially restricts, and temporally attenuates Nodal signaling (Meno et al. 1999; Chen and Schier 2002; Feldman et al. 2002; Dougan et al. 2003). It is still unclear how exactly these auto- and cross-regulatory interactions contribute to the robustness and precision of patterning, but recent evidence suggests that the balance of Nodal/Lefty is carefully regulated not only at the level of transcription but also post-transcriptionally by microRNAs. In particular,

zebrafish miR-430 dampens the mRNA levels and translation of Squint, *lefty1*, and *lefty2* (Choi et al. 2007), one of the many roles of this microRNA (Giraldez et al. 2005; Giraldez et al. 2006; Mishima et al. 2006). In the absence of miR-430, both agonist and antagonist are up-regulated. Intriguingly, this up-regulation leads to an imbalance of Nodal versus Lefty inputs, so that Lefty activity prevails and Nodal signaling is reduced. It is unclear why Lefty derepression dominates Squint derepression. It is conceivable that Lefty is misexpressed at higher levels than Squint on loss of miR-430 repression. Alternatively, it is possible that nonlinear steps in Nodal/Lefty regulation favor inhibition of the pathway in the absence of miR-430. A similar inhibition of Nodal antagonism is observed in human ES cells, where the miR-430 orthologue miR-302 is required to repress Lefty and promote mesendoderm development (Rosa et al. 2009).

MicroRNAs have also been implicated in the regulation of type II activin receptor expression. *Xenopus* miR-15/16 is expressed in ventral–lateral regions and inhibits type II Activin receptor expression (Martello et al. 2007). This results in higher activin receptor levels in dorsal compared with ventral blastomeres, resulting in higher responsiveness to Nodal signals on the dorsal side. This effect is further augmented by Rap2, which is involved in recycling of Activin receptors (Choi et al. 2008). Rap2 is initially enriched dorsally and thus also contributes to higher receptor levels and thus the earlier and higher induction of Nodal signaling in dorsal blastomeres. These studies demonstrate that microRNAs are important modulators of Nodal morphogen signaling.

Stability and Movement

Nodal pathway activation in a field of cells is not only dependent on the levels of Nodal and Lefty at the source, but also on the levels that reach target cells. These levels are determined by the movement and stability of Nodal and Lefty—the more stable and mobile, the longer the range of the signal.

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The movement of Nodal proteins is poorly understood. It has been shown in *Xenopus* explants that the Nodal protein Xnr2 is distributed through extracellular routes and not through uptake and release from cells, a process called transcytosis (Williams et al. 2004). It is unclear, however, if this extracellular movement is purely diffusive or if active transport is involved. Studies of the TGF- β signals Activin and Dpp favor this possibility, but detailed biophysical studies are required to directly test Nodal diffusion (McDowell et al. 1997; Kinoshita et al. 2006; Kicheva et al. 2007; Kicheva and Gonzalez-Gaitan 2008). Such studies are also crucial to determine whether the different activity ranges of Squint, Cyclops, and Lefty are because of different diffusibilities. For example, studies of Dpp have measured a diffusion coefficient of $0.1 \mu\text{m}^2/\text{s}$ in imaginal discs (Kicheva et al. 2007; Kicheva and Gonzalez-Gaitan 2008). Modeling studies indicate that this value would only allow short-range signaling within the 2–3 hour time frame during which the zebrafish blastula is patterned by Nodal signals (Lee, Robson, and Schier, unpublished results). Hence, it is unclear how diffusibility contributes to the range of Nodal signaling. This is a particularly important issue because one of the tenets of reaction–diffusion models is that the inhibitor (Lefty) has a longer range than the activator (Nodal).

It is also unclear how processing, stability, post-translational modifications, and trafficking contribute to the range of Nodal signals. Different Nodal signals can have different signaling ranges. For instance, Cyclops has a shorter signaling range than Squint (Chen and Schier 2001). The differences in range are thought to be caused by differences in both the prodomains and mature domains (Chen and Schier 2001; Jing et al. 2006; Tian et al. 2008). For example, a chimeric protein consisting of the Cyclops prodomain and Squint mature domain has a longer range than Cyclops protein but a shorter range than Squint. An acidic region in the amino terminus of the Squint mature ligand is required for the longer range of the Cyclops-Squint chimera (Jing et al. 2006). Conversely, a presumptive lysosomal targeting

region in the Cyclops prodomain restricts its range (Tian et al. 2008). Expression of the mature domain of Squint or mouse Nodal results in an unstable signal that only acts at a short range, whereas the mature domain of Cyclops does not have any activity by itself and requires the prodomain (Le Good et al. 2005; Tian et al. 2008). In contrast, insertion of an N-glycosylation site increases the stability of mouse Nodal in cell culture and extends its range in zebrafish embryos (Le Good et al. 2005). These results have suggested that increased proteolytic maturation of Nodal potentiates local signaling, whereas increased Nodal stability extends long-range signaling. In tissue culture, Nodal precursor is more stable than processed Nodal, indicating that the site and timing of processing might influence the range of signaling (Blanchet et al. 2008a; Constam 2009). Interaction with EGF-CFC proteins can target mouse Nodal into specific endocytic compartments and might thus also contribute to the stability and intracellular movement of Nodal ligands (Le Good et al. 2005; Blanchet et al. 2008a; Blanchet et al. 2008b). These studies suggest that the location and regulation of Nodal processing plays a major role in determining stability and range.

The range of Nodal signaling is modulated by additional extracellular proteins. For example, Nodal secreted in the node can activate signaling in the distant LPM only in the presence of the glycosaminoglycan chondroitin sulfate (Oki et al. 2007). Because ectopic Nodal can still activate the pathway on chondroitin sulfate perturbation, it is likely that the interaction of Nodal with sulfated glycosaminoglycans increases ligand movement or stability and not receptor binding or activation.

Further complexity arises from the potential for TGF- β heterodimer formation. For example, the related TGF- β signal GDF1 is also involved in left–right specification (Rankin et al. 2000). Similar to Nodal, it is expressed in the node and LPM. Node expression is required for left-sided activation of Nodal target genes, and expression in the LPM is required for the expansion of Nodal signaling in the LPM. Native GDF1 homodimers appear

largely inactive but GDF1/Nodal heterodimers are active and even more potent than Nodal homodimers in activating Nodal reporter genes (Tanaka et al. 2007). These heterodimers also have a longer activity range but it is unclear if this is only because of increased specific activity in pathway activation or also caused by increased diffusion or stability.

Interpretation

The mechanisms underlying the interpretation of Nodal signals in target cells are poorly understood. Studies of Activin in *Xenopus* have revealed a linear relationship of TGF- β concentration, receptor activation, Smad complex formation, and downstream gene activation (Gurdon and Bourillot 2001). Moreover, Activin can act in a ratchet mechanism, i.e., cells have a memory of the highest levels of Activin they have been exposed to and respond accordingly. This memory resides at the level of receptor-ligand complexes and is maintained by the intracellular localization of signaling complexes in endosomes (Jullien and Gurdon 2005). It is unclear whether Nodal signals behave similarly to Activin. For example, Activin is not dependent on EGF-CFC proteins for pathway activation and cannot be inhibited by Leftys (Gritsman et al. 1999; Cheng et al. 2004). Thus, it is possible that Nodal-receptor complexes are disrupted by Lefty, undermining potential ratchet mechanisms and pathway linearity.

Finally, it is not clear how Nodal target genes respond to different levels of Nodal signaling. Based on studies of other morphogens, it is assumed that the differential affinity for *cis*-regulatory elements determines the threshold at which a downstream gene is activated. The responses of known downstream genes to different concentrations of Nodal are consistent with this model, but direct evidence is lacking. The situation is further complicated by the existence of maternally generated prepatterning in early fish and frog embryos. The localization of pathway antagonists in the presumptive ectoderm changes the response properties of target tissues and complicates simple models

wherein a naïve group of cells homogeneously responds to different morphogen concentrations. For example, ectoderm is maternally expressed and localized to the animal pole in *Xenopus*, thus creating a zone that is less responsive to Nodal and BMP signals (Dupont et al. 2005; Dupont et al. 2009). Additional transcription factors such as Sox3, Xema/FoxI1e, Serum response factor, and XFDL156, and the Cerberus-like protein Coco are also expressed in presumptive ectoderm and protect ectoderm from Nodal inducers (Bell et al. 2003; Suri et al. 2005; Mir et al. 2007; Yun et al. 2007; Zhang and Klymkowsky 2007; Sasai et al. 2008). Thus, pre-patterning will modulate the generation of and response to Nodal signaling gradients.

CONCLUDING REMARKS

Studies of Nodal signaling led to the discovery of the bona fide mesendoderm inducers and left-right determinants, identified the first endogenous vertebrate morphogen, established the existence of an activator/inhibitor pair as postulated in reaction-diffusion models, revealed roles for microRNAs in morphogen signaling and highlighted the complex roles of processing, trafficking, and post-translational modifications during morphogen signaling. Despite this progress, a quantitative or biophysical understanding of Nodal morphogens is largely elusive. We do not know the shape of the putative Nodal morphogen gradient, the *in vivo* diffusion, processing, trafficking, and degradation properties of Nodal and Lefty, the binding and reaction kinetics of the pathway components, the mechanisms by which signal concentration and duration are translated into positional information, or the robustness and precision encoded by the Nodal morphogen gradient. Filling these gaps will be necessary to truly understand how the Nodal morphogen patterns developing tissues.

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