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Review

Review

Molecular regulation of Nodal signaling during mesendoderm formation

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Abstract

One of the most important events during vertebrate embryogenesis is the formation or specification of the three germ layers, endoderm, mesoderm, and ectoderm. After a series of rapid cleavages, embryos form the mesendoderm and ectoderm during late blastulation and early gastrulation. The mesendoderm then further differentiates into the mesoderm and endoderm. Nodal, a member of the transforming growth factor β (TGF-β) superfamily, plays a pivotal role in mesendoderm formation by regulating the expression of a number of critical transcription factors, including Mix-like, GATA, Sox, and Fox. Because the Nodal signal transduction pathway is well-characterized, increasing effort has been made to delineate the spatiotemporal modulation of Nodal signaling during embryonic development. In this review, we summarize the recent progress delineating molecular regulation of Nodal signal intensity and duration during mesendoderm formation.

Key words: Nodal signal, Smad2, vertebrate embryo, mesendoderm

Introduction

Early development of vertebrates begins with a zygote that undergoes a series of rapid cleavages. Subsequently, these resulting embryos form three germ layers, the endoderm, mesoderm, and ectoderm during gastrulation. The endoderm, which is the innermost germ layer, differentiates into digestive and respiratory organs, including the liver, gallbladder, pancreas, thyroid, and lung. The mesoderm, which is the middle germ layer, gives rise to the heart, muscle, kidney, bone, vasculature, and hematopoietic system. The ectoderm, the outermost germ layer, develops into the skin and nervous system.

Mesendodermal cells are bipotent progenitors that differentiate into both mesoderm and endoderm. The induction of the mesendoderm is evolutionarily well organized and rigorously controlled by several signaling pathways, including Nodal, Wnt, and FGF, and a number of transcription factors, including Mix-like, GATA, Sox, and Fox [1,2]. Among these signaling pathways and transcription factors, Nodal signaling is the most important inducer of mesendoderm specification, where this function is performed by controlling the expression of Mix-like, GATA, Sox, and Fox transcription factors [2].

During Nodal signal transduction, mature ligands form dimers and bind to type I and II serine/threonine kinase receptors. These ligand–receptor interactions require an epidermal growth factor-Cripto-FRL1-Cryptic (EGF-CFC) protein, such as Cripto or Cryptic in mice, Oep in zebrafish, and FRL1/XCR in *Xenopus*, to serve as a co-receptor [3,4]. Constitutively active type II receptors then phosphorylate type I receptors, which, in turn, phosphorylate the C-terminal SSXS motif of receptor-regulated Smads (R-Smads), Smad2 and Smad3. Once activated, Smad2 and Smad3 complex with common Smad, Smad4, and then translocate into the nucleus to regulate transcription of target genes [5,6]. Deletion of Smad2 in mice disrupts primitive streak formation and mesoderm induction. By contrast, Smad3 mutant mice do not display defects related to mesendoderm formation, suggesting that Smad2 is the primary downstream effector of Nodal signaling for the induction of mesendoderm during embryonic development [7–9]. While the Nodal

signal transduction pathway is well characterized and seemingly straightforward, it is spatiotemporally modulated during embryonic development. In this review, we will focus on recent findings that provide significant insight into mechanisms of membrane receptor activation, phosphorylation and intracellular translocation of Smad proteins, and transcriptional regulation of target genes during mesendoderm formation.

Functions of Nodal Signaling in Mesendoderm Induction

The essentiality of Nodal signaling in mesendoderm induction was first discovered in *Xenopus*. Scientists found cultured naive blastula ectoderm differentiates into mesodermal and endodermal tissues upon treatment with exogenous TGF-β2 and Activin [10,11]. In addition, injection of *Vg1* mRNA, which encodes a TGF-β factor, induces the formation of mesoderm and endoderm [12,13]. Other TGF-β/Nodal members with the ability to induce mesendoderm specification, including Nodal-related genes *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5*, *Xnr6*, and *Derriere*, were later identified in *Xenopus* [14–22]. Furthermore, mesoderm and endoderm formation are severely inhibited by injection of mRNAs encoding dominant-negative Nodal ligands, secreted Nodal antagonists, dominant-negative receptors, and dominant-negative Smad2 [16,23–25]. Therefore, Nodal signaling plays crucial roles in *Xenopus* mesendoderm specification. In *Xenopus* embryos, endoderm originates from the yolky vegetal cells and the marginal equatorial region becomes mesoderm and mesendoderm [2,26]. At the late blastula stage, maternal vegetally localized VegT, a T-box transcription factor, activates expression of Nodal members, which induces mesendoderm formation [14–17]. Overexpression of *Xnr1*, *Xnr2*, *Xnr4*, and *Derriere* in VegT-depleted embryos restores the expression of endodermal marker genes in the vegetal region and induction of mesoderm in the equatorial zone [14,15]. Furthermore, when blocking Nodal signaling through overexpression of the inhibitory forms of *Xnr2* and *Derriere*, injection of *VegT* mRNA fails to prevent mesoderm and endoderm defects in VegT-depleted embryos [14,15]. Furthermore, maternal β-catenin induces *Xnr* expression in the dorsal region, which is critical for the induction of the dorsal mesoderm [1,6]. Therefore, VegT cooperates with maternal β-catenin to activate Nodal gene expression in the vegetal region, which creates a dorsal–ventral Nodal signal gradient that induces and patterns mesendoderm formation [27].

In zebrafish embryos, three Nodal-related genes, *cyclops* (*cyc*), *squint* (*sqt*), and *southpaw* (*spaw*), have been characterized [28,29]. Zygotic expression of *sqt* initiates the perspective dorsal organizer at the mid-blastula transition stage and then both *sqt* and *cyc* are expressed across the entire margin from which mesendoderm originates [28]. There are several mechanisms of regulation employed for zygotic expression of *sqt* and *cyc*. First, maternal Wnt/β-catenin signaling promotes transcription of these genes in the future dorsal region [30,31]. In the lateral marginal zone, these genes are induced by factors from the yolk syncytial layer [32]. Furthermore, expression of these genes is positively self-regulated by Nodal signaling [31,33]. For a long period, scientists believed that zebrafish embryos did not express any maternal T-box transcription factors equivalent to *Xenopus* VegT that could initiate Nodal gene expression. However, Xu *et al.* [34] recently discovered Eomesa, a zebrafish T-box factor that binds to the *sqt* and *cyc* promoters and activates their transcription. Importantly, loss of maternal Eomesa disturbs the expression of mesendodermal genes during zebrafish embryogenesis [34].

It is interesting that mesendoderm specification is only mildly affected in *sqt* or *cyc* mutant embryos [35,36]. By contrast, *sqt/cyc* double-mutant embryos contain almost no mesodermal or endodermal tissue [35,37]. Therefore, to an extent, *sqt* and *cyc* have functionally redundant roles in mesendodermal induction. However, another Nodal gene, *spaw*, initiates in the two bilateral domains flanking Kupffer's vesicle and is responsible for establishing left-right asymmetry, although not for mesendoderm formation [29,38]. MZ*oep* mutants, which lack the maternal and zygotic EGF-CFC Nodal co-receptor one-eyed pinhead, phenocopy the mesendodermal defects observed in *sqt/cyc* double mutants [3,39]. Similar mesendodermal defects occur when Nodal-antagonist Lefty/Antivin mRNA or dominant-negative Nodal receptor mRNA are injected into embryos [40–43]. In addition, zebrafish embryos treated with SB-431542, a specific inhibitor of type I receptor ALK4/5/7, display notable loss of mesoderm- and endoderm-derived tissues [44]. Conversely, injection of mRNA for constitutively active Taram, a zebrafish Nodal type I receptor, activates mesendoderm formation and protects against mesendodermal defects in MZ*oep* mutants [45]. Furthermore, David *et al.* [46] demonstrated that activating the Nodal signaling pathway by overexpressing constitutively active Taram induces mesendoderm formation through bypassing the required signaling from the yolk syncytial layer.

In mouse embryos, the primitive streak initiates pluripotent epiblast cell differentiation into germ layers. There is only one Nodal gene in mouse embryos, the expression of which is restricted to the primitive streak at the onset of gastrulation [47]. Mouse embryos with an insertional mutation in the Nodal locus fail to undergo gastrulation and do not form the primitive streak [48,49]. Similar mesendodermal defects are found in *ALK4*, *ActRIIA*, and *Cripto* mutants, which are deficient in Nodal receptors [50–52]. In addition, mouse embryos deficient in Smad2, the key mediator of Nodal signaling, exhibit developmental abnormalities soon after implantation and contain no mesodermal or endodermal tissues [8,9,53,54]. Furthermore, the use of genetic methods to decrease Nodal signaling in epiblasts revealed that Nodal induces specification of mesendoderm in a dose-dependent manner, where a higher level of signaling is required to induce definitive endoderm formation and a lower level is sufficient for mesoderm formation, which is corroborated by findings in *Xenopus* and zebrafish embryos [55–57].

Nodal signal also plays vital roles in mesendoderm differentiation of mammalian embryonic stem (ES) cells. Overexpression of Nodal in mouse ES cells enhances mesoderm and endoderm specification, but inhibits neuroectoderm formation [58]. In addition, mesendoderm differentiation is hindered by SB-431542, which dampens Nodal signaling in mouse and human ES cells [59–61]. Smad2-depleted human ES cells present with similar phenotypes [62]. These observations indicate that Nodal/Smad2 signaling mesendoderm formation-related functions are evolutionarily conserved across different animal species.

Regulation of TGF-β/Nodal Signaling in Mesendoderm Formation

As described above, moderate TGF-β/Nodal signaling is critical for mesendoderm induction. Signals are transduced from the cell membrane to the nucleus, which requires proper functioning of ligands, receptors, and Smads and their transcriptional cofactors. Each step is vital for signaling transduction and precisely regulated. In this section, we summarize the well-characterized aspects of post-translational regulation of Nodal signaling during induction of vertebrate mesendoderm (**Fig. 1**).

Regulation of ligand activity

Nodal ligands are synthesized as proproteins which have an N-terminal prodomain flanked by a mature ligand domain. Nodal precursors may be secreted, but have little Nodal signal-initiating activity [23,63]. Studies in mouse embryos have demonstrated that the convertases Furin (Spc1) and Pace4 (Spc4) extracellularly cleave

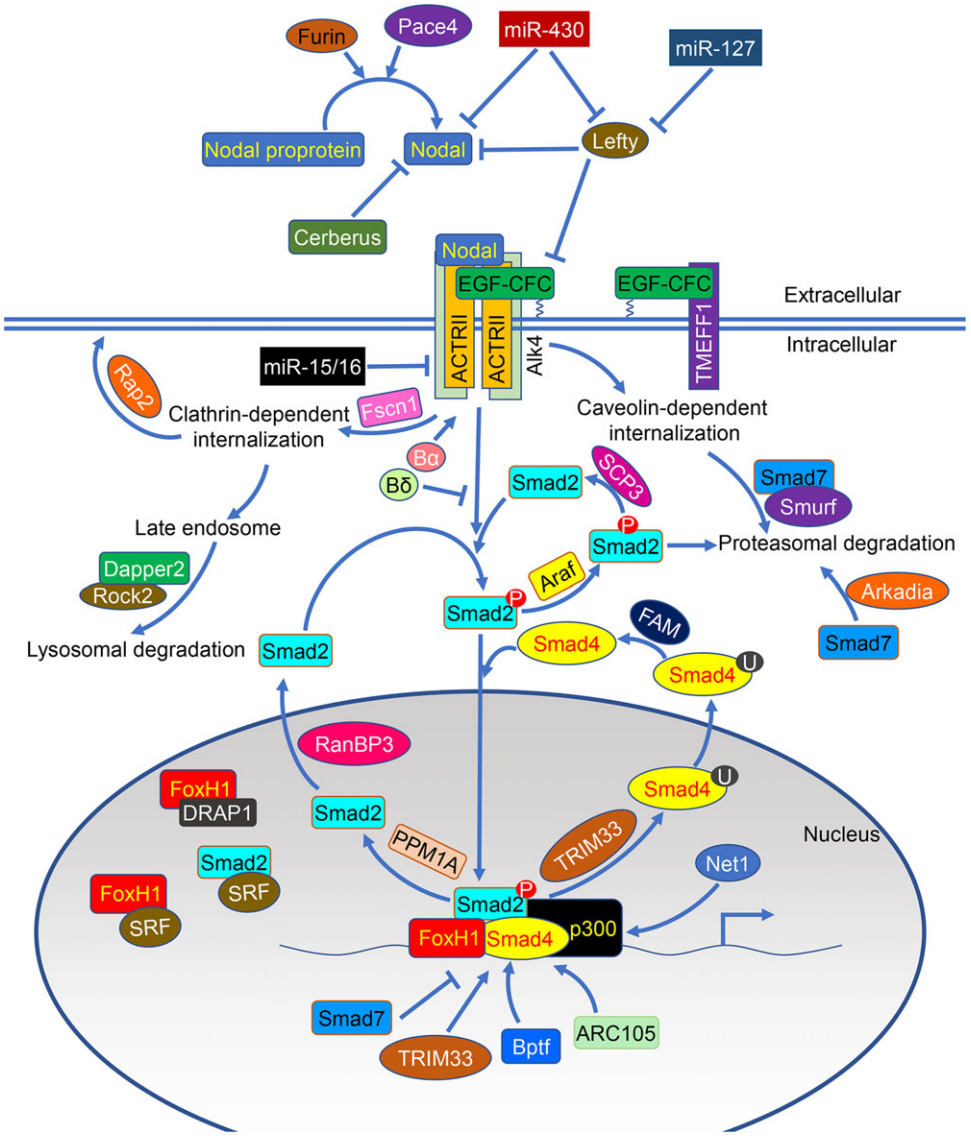

Figure 1. Regulation of Nodal signaling After transcription, Nodal mRNA is targeted by the miR-430 family. Nodal ligands are then translated as proproteins that will be processed into mature forms by Furin and Pace4. These mature ligands interact with type I and II receptors to initiate signaling. Ligand activity is limited by the extracellular antagonists Cerberus and Lefty. Lefty also inhibits receptor activity by binding to the EGF-CFC co-receptor and is negatively regulated by miR-430 and miR-127. At the receptor level, miR-15/16 inhibits the expression of type II receptor Actr2a. TMEFF1 interferes with receptor activity by competitively associating with EGF-CFC protein. Clathrin-dependent internalization is essential for signal transduction. Fscn1 promotes type I receptor endocytosis by facilitating the transport of ALK4/ALK5 from clathrin-coated vesicles to early endosomes. GTPase Rap2 directs recycling of internalized receptors back to the cell membrane, while Dapper2 and Rock2 interact with and accelerate lysosomal degradation of activated type I receptors. These receptors also undergo caveolin-dependent internalization, where Smad7 recruits Smurf1/2 to the type I receptor for proteasomal degradation. In addition, Smad7 is recognized by the RING-finger domain of the E3 ubiquitin-protein ligase Arkadia for ubiquitylation and degradation. The phosphatase PP2A subunit Bα enhances type I receptor stability, while another subunit, Bδ, suppresses the kinase activity of type I receptor. Upon ligand binding, the R-Smads are phosphorylated by the type I receptor at their C-terminal SSXS motif and, thus, activated. Regulation of phosphorylation is crucial for R-Smad activity. Araf phosphorylates the linker region of Smad2 to promote its degradation, while the phosphatase SCP3 dephosphorylates the linker regions of Smad2. Additionally, activated Smad2 is dephosphorylated by PPM1A in the nucleus, resulting in the nuclear export of Smad2 as directed by the Ran-binding protein RanBP3. The transcription of Nodal target genes requires the association of the Smad complex with FoxH1 or Mixer2. ARC105 acts as another transcriptional coactivator. DRAP1 interacts with FoxH1, while SRF interacts with both FoxH1 and Smad2. These interactions disrupt Smad–FoxH1 complex formation. Smad7 selectively associates with Smad-responsive elements to interfere with the formation of functional Smad–DNA complexes. Chromatin acetylation induced by p300, as well as NURF-catalyzed ATP-dependent nucleosome sliding, is crucial for the transcriptional activation of Nodal target genes. Net1 promotes Smad2 and p300 interactions. TRIM33 monoubiquitylates the MH2 domain of Smad4, thus restricting its nuclear localization, while FAM reverses this modification. However, TRIM33 also recruits Smad2/Smad3 to H3K9me3- and H3K18ac-marked promoters to enhance expression of Nodal target genes.

R-X-(K/R/X)-R consensus sequences of Nodal precursors, thereby generating mature Nodal ligands [64]. This proteolytic processing is critical for Nodal signal transduction and mesendoderm induction in mouse and zebrafish embryos [63,64].

Lefty protein, a divergent TGF-β member, acts as an extracellular Nodal antagonist by inhibiting the formation of receptor complexes by binding to Nodal ligands and EGF-CFC co-receptors. When Lefty is absent, Nodal signaling and mesendoderm formation are augmented [40,43,65–73]. Moreover, Lefty proteins are important negative-feedback regulators of Nodal signaling because the *lefty* genes are directly regulated by Nodal [40,72,74,75]. Aside from Lefty proteins, DAN/Cerberus members are also classic Nodal antagonists. DAN proteins are cysteine-rich extracellular proteins and include *Xenopus* Cerberus and Coco and mouse Cerberus-like (Cerl), which can interact with Nodal ligands and suppress signal transduction [76–79].

The levels of Nodal ligands and antagonist proteins are also important for signaling activity. The miR-430/427/302 family plays a crucial role in Nodal signaling and mesendoderm formation [80,81]. In zebrafish embryos, in addition to its many other functions [82–84], miR-430 targets the Nodal ligand *sqt* and its antagonists *lefty1* and *lefty2* [81]. However, inhibition of *lefty* gene expression overshadows the inhibition of *sqt* expression. Loss of miR-430 causes an imbalance and reduction in Nodal signaling and mesendoderm specification [81]. Similar phenomena have been observed in *Xenopus* embryos, where the miR-430 orthologue miR-427 targets two Nodal ligands, *Xnr5* and *Xnr6b*, and both *lefties*. Knockdown of miR-427 inhibits Nodal signaling and mesendoderm induction [80]. A similar phenomenon occurs in human ES cells. The miR-430 orthologue miR-302 targets only *lefties* and does not affect *nodal* expression. Loss- and gain-of-function studies revealed that miR-302 promotes Nodal signal transduction and mesendoderm formation [80]. In addition, miR-127 strengthens Nodal signaling by targeting *lefty2* mRNA for degradation [85]. Overexpression of miR-127 enhances, while knockdown of miR-127 prevents, mesendoderm differentiation in mouse ES cells [85].

Regulation of receptor activity

The transmembrane serine/threonine kinase type I and type II receptors, as well as the EGF-CFC co-receptors, transduce Nodal signals from the cell membrane to the cytosol. MicroRNAs play essential roles in modulating Nodal type II receptor Acrt2a expression. In *Xenopus* embryos, miR-15 and miR-16 play crucial roles in organizer formation by targeting type II receptor Actr2a [86]. Expression of miR-15/16 is negatively regulated by dorsal Wnt/β-catenin signaling, and thus is restricted ventrally. In ventral blastomeres, miR-15/16 targets and inhibits the expression of Actr2a, leading to the expression of relatively more type II receptors and a higher responsiveness to Nodal signals in the dorsal region [86].

In addition to secreted inhibitors of the Nodal pathway, several transmembrane proteins serve as antagonists and block signal transduction. During *Xenopus* embryogenesis, the transmembrane protein TMEFF1, a follistatin module-containing protein, selectively inhibits Nodal, but not Activin [87]. TMEFF1 interacts with the CFC domain of the Nodal co-receptor Cripto, which is responsible for Cripto binding to ALK4. The consequence of this competition for binding is ALK4 is excluded from the Cripto complex, thereby restricting Nodal signaling and mesendoderm specification [88]. In zebrafish embryos, the transmembrane protein Nicalin and its binding partner Nomo antagonize Nodal signaling [89]. Overexpression of both genes leads to cyclopia, a phenotype related to Nodal deficiency. Conversely, Nomo inhibitors can cause an increase in anterior axial mesendoderm [89].

Receptor trafficking is very important for regulation of TGF-β signal transduction. TGF-β receptors can be internalized in a clathrin-dependent manner into EEA1-positive early endosomes, where the Smad2 anchor for receptor activation (SARA) facilitates TGF-β signaling [90,91]. Conversely, lipid-raft caveolae-mediated internalization of receptors is responsible for receptor degradation [90,91]. In zebrafish embryos, the actin-bundling protein Fscn1 controls receptor trafficking and promotes Nodal signaling [92]. Fscn1 specifically interacts with TGF-β type I receptor ALK5 and Activin/Nodal type I receptor ALK4, where it facilitates the trafficking of internalized receptors from clathrin-coated vesicles to early endosomes by acting as a molecular linker between these type I receptors and the actin cytoskeleton. Furthermore, *fscn1a* is a direct target gene of Nodal signaling. Thus, *fscn1a* and Nodal signaling form a positive-feedback loop that controls endoderm induction [92]. In *Xenopus* embryos, the Ras GTPase family member Rap2 enhances Activin/Nodal signaling by controlling receptor trafficking [93]. In the absence of Activin/Nodal activation, Rap2 directs receptors into a recycling pathway that maintains the receptors on the cell membrane. Upon ligand stimulation, Rap2 prevents receptor turnover rather than enhances receptor recycling. Overall, Rap2 plays a positive role in Nodal signal transduction and mesoderm induction [93].

Dapper2 acts as a negative modulator of Nodal signaling by promoting the degradation of type I receptor ALK4/5 [94]. Zebrafish *Dapper2* is a Nodal-regulated gene that is expressed in mesoderm precursors during the shield stage. Dapper2 interacts with activated ALK4/ALK5 in late endosomes and facilitates their degradation in lysosomes [94]. The serine/threonine kinase Rock2 is a binding partner of Dapper2 that binds to and accelerates lysosomal degradation of TGF-β type I receptors internalized from the cell surface, thereby serving as a negative modulator of Nodal signaling during zebrafish mesendoderm induction [95]. In this study, the authors hypothesized that Dapper2 presents TGF-β type I receptors to Rock2 for lysosomal degradation. Interestingly, Rock2 is required for Dapper2-induced degradation of TGF-β type I receptors, while Dapper2 is dispensable for Rock2-mediated inhibition of TGF-β/Nodal signaling [95]. It is likely that other unidentified adaptor proteins compensate for the loss of Dapper2 presentation of type I receptors to Rock2.

When type I receptors are internalized via caveolin-positive lipid rafts, they are targeted by Smad7, an inhibitory Smad protein [96–98]. Smad7 recruits the HECT-domain E3 ubiquitin ligases Smurf1/2 to receptors, leading to proteasomal degradation of the receptors and suppression of TGF-β/Nodal signaling [96–98]. Consistent with this, overexpression of Smad7 or Smurf2 in *Xenopus* inhibits Nodal signaling-induced mesoderm formation [99,100]. Furthermore, Arkadia augments Nodal signaling by recognizing Smad7 and inducing its ubiquitylation and degradation [101]. *In vivo* data indicate that Arkadia is required for Nodal signaling-induced mesendoderm specification in *Xenopus* and formation of the node and establishment of left-right asymmetry in mouse embryos [102,103]. In addition, the regulatory subunits of phosphatase PP2A regulate Nodal signaling through modulation of receptor activity [104]. However, Bα and Bδ subunits have opposing functions in Nodal signaling and mesendoderm induction. Bα acts as a positive regulator of Nodal signaling by enhancing type I receptor stability, while Bδ inhibits Nodal signaling by suppressing type I receptor kinase activity [104].

Regulation of Smad phosphorylation

Upon ligand binding and subsequent receptor activation, Smad2 and Smad3 are phosphorylated by type I receptors at their

C-terminal SSXS sequence, form a complex with Smad4, and then translocate into the nucleus. Regulation of Smad2/3 phosphorylation plays an important role in Nodal signal transduction and mesendoderm formation. Except for when the MH2 domain is phosphorylated, which activates Smad2/3, phosphorylation of the linker region represses TGF-β/Nodal signaling. Liu *et al.* [105] demonstrated that Araf, a Raf kinase family member, functions as a negative regulator of Nodal signaling during zebrafish mesendoderm specification. Mechanistically, Araf physically interacts with and phosphorylates Smad2 in the linker region at S253, which promotes the degradation of activated Smad2 and inhibits Nodal signaling and mesendoderm induction [105]. Additionally, *Xenopus* SCP3, a small C-terminal domain-containing phosphatase, is essential for the full activation of Nodal/Activin signaling and acts by dephosphorylating Smad2 linker regions [106]. SCP3 knockdown reduces mesendoderm formation and expression of Nodal target genes during *Xenopus* embryogenesis [106]. Lin *et al.* [107] found that the nuclear phosphatase PPM1A dephosphorylates activated Smad2/3 and promotes Smad2/3 nuclear export. PPM1A knockdown enhances TGF-β signal transduction in mammalian cells and, conversely, *ppm1a* overexpression abolishes Nodal signaling during zebrafish mesoderm induction [107]. Furthermore, dephosphorylated Smad2/3 is selectively recognized by RanBP3, a nuclear Ran-binding protein. RanBP3 and its Ran-binding activity are essential for Smad2/3 nuclear export [108]. In *Xenopus* ectodermal explants, injection of *RanBP3* mRNA represses Activin-induced mesendodermal gene expression [108].

Regulation of Smad transcriptional activity

Smad3/4 binds relatively poorly to DNA, while Smad2 has no affinity for DNA. Therefore, Smad2/4 and Smad3/4 complexes need to interact with transcription cofactors, such as FoxH1/Fast1 or Mixer, to activate expression of target genes [109–112]. FoxH1 and Mixer are very important transcriptional coactivators of Smad2/3, but are not involved in transcription of all Nodal target genes [113], indicating there are additional transcription factors serving as coactivators during Nodal signal transduction. In *Xenopus* embryos, ARC105, a component of the Mediator complex, acts as a coactivator of Nodal signaling by interacting with Smad proteins to form a transcriptional complex. In support of this, ARC105 knockdown was found to inhibit mesendoderm formation [114]. Several years ago in a previous study, we globally identified Smad2 targets in early zebrafish gastrulas using the ChIP-chip assays. Importantly, by identifying DNA-binding sites for transcription factors besides Smad2 in the Smad2-bound regions, we confirmed well-known Smad2-binding partners, such as FoxH1 and Lef1/β-catenin. In addition, many previously unknown partners of Smad2 during zebrafish embryogenesis, including Oct1 and Gata6, have been revealed [115], which will aid the characterization of the regulatory cascades involved in mesendoderm formation.

Smad transcriptional complexes also act as negative regulators during mesendoderm induction. During mouse embryogenesis, the transcriptional corepressor DRAP1 physically interacts with FoxH1 and blocks the binding of FoxH1–Smad2/Smad4 transcriptional complex to the Nodal-response elements [116]. Loss of Drap1 causes severe gastrulation defects that are consistent with increased Nodal expression in mouse embryos [116]. In addition, serum response factor (SRF) precludes Activin/Nodal signaling and mesendoderm induction in *Xenopus* embryos [117]. This MADS box-containing transcription factor disrupts Smad2–FAST1 complex formation, thereby impeding Nodal signal transduction [117]. Apart from targeting internalized type I receptors for degradation, Smad7 also inhibits TGF-β signaling in the nucleus [118]. In addition, Smad7 selectively associates with Smad-responsive elements through its MH2 domain and, thus, interferes with the formation of functional TGF-β1-induced Smad–DNA complexes [118]. Consistently, nuclear Smad7 has inhibitory functions in zebrafish mesendoderm formation [118].

Epigenetic mechanisms are also involved in the regulation of Nodal-mediated mesendoderm formation. The founding member of the ISWI family of chromatin remodeling complexes, NURF (nucleosome remodeling factor), promotes gene transcription by catalyzing ATP-dependent nucleosome sliding [119,120]. BPTF, the largest subunit in the NURF complex, associates with Smad2/3 and is required for the development of mesodermal, endodermal, and ectodermal tissue lineages in mouse embryos and ES cells [121]. Physical links between BPTF and Smad proteins facilitate the recruitment of other components of the NURF complex to change nucleosome density around DNA-binding sites [122]. The histone acetyl transferase p300 is able to loosen chromatin and make it accessible to be bound by transcription factors [123]. The interactions of Smad complex with p300 promote transcriptional activation of target genes [124–126]. By contrast, histone deacetylase (HDAC) inhibits the transcription of TGF-β/Nodal target genes [127]. Recently, Wei *et al.* [128] found that the RhoA-specific guanine nucleotide exchange factor Net1, a new Smad2 partner, enhances TGF-β/Nodal signaling by promoting the association between Smad2 and p300 and decreasing the interaction of Smad2 with HDAC1 in the nucleus. Loss- and gain-of-function experiments revealed that, independent of its guanine nucleotide exchange factor activity, nuclear Net1 is vital for Nodal-induced mesendoderm specification [128]. TRIM33 (tripartite motif containing 33; i.e. TIF1γ or Ectodermin), is a multi-domain regulator of transcription. It contains a RING domain with E3 ubiquitin ligase activity, two B-boxes, a coiled-coil domain, a PHD domain, and a bromodomain. TRIM33 binds to the promoter regions of H3K9me3- and H3K18ac-marked Nodal target genes through its PHD finger and bromodomain, and then recruits Smad2/3 to displace the chromatin-compacting factor HP1γ [129]. This is a key process for the transcriptional activation of Nodal target genes during mesendodermal differentiation of mouse ES cells [129].

Conclusion and Perspectives

The Nodal/Smad2 pathway is considered to be a major regulator of mesendoderm induction during vertebrate embryogenesis. Over the past decade, significant progress has been made in characterizing how Nodal signaling is regulated to achieve proper formation of the mesendoderm. We summarized the latest advances in differential regulation of TGF-β/Nodal signaling from the cell membrane to the nucleus. These studies provide us with novel insights into the spatiotemporal regulation of TGF-β/Nodal signaling during mesendoderm development, as well as contribute to other studies related to TGF-β signaling and functions. Despite this substantial progress, many questions still remain to be answered. For example, endoderm and mesoderm are derived, at least in part, from a bipotent mesendodermal population. It remains unknown whether Nodal signaling is asymmetrically activated during mesendodermal precursor division and differentiation. Furthermore, the precise functions and regulatory mechanisms of Nodal signaling during this process have yet to be characterized. These should be among the future areas of research in Nodal signaling, and additional studies will undoubtedly yield exciting new findings in this field.

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