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Mesoderm induction and patterning: insights from neuromesodermal progenitors

Benjamin L. Martin*

Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794-5215

Abstract

The discovery of mesoderm inducing signals helped usher in the era of molecular developmental biology, and today the mechanisms of mesoderm induction and patterning are still intensely studied. Mesoderm induction begins during gastrulation, but recent evidence in vertebrates shows that this process continues after gastrulation in a group of posteriorly localized cells called neuromesodermal progenitors (NMPs). NMPs reside within the post-gastrulation embryonic structure called the tailbud, where they make a lineage decision between ectoderm (spinal cord) and mesoderm. The majority of NMP-derived mesoderm generates somites, but also contributes to lateral mesoderm fates such as endothelium. The discovery of NMPs provides a new paradigm in which to study vertebrate mesoderm induction. This review will discuss mechanisms of mesoderm induction within NMPs, and how they have informed our understanding of mesoderm induction more broadly within vertebrates as well as animal species outside of the vertebrate lineage. Special focus will be given to the signaling networks underlying NMP-derived mesoderm induction and patterning, as well as emerging work on the significance of partial epithelial to mesenchymal states in coordinating cell fate and morphogenesis.

Keywords

Neuromesodermal progenitors; mesoderm induction; mesoderm patterning; FGF; BMP; Wnt; Brachyury; epithelial to mesenchymal transition; EMT

1. Introduction

The vertebrate body axis forms in an anterior to posterior progression, with the head forming first (anterior) and the rest of the body plan forming sequentially away from the head [1]. A longstanding hypothesis, that this progressive mode of development relies on plastic multipotent progenitors at the posterior end of the embryo, was firmly supported in 2009 from retrospective clonal analysis in the mouse embryo [2]. This work showed that single cells in the mouse tailbud contribute daughter cells to both ectoderm (spinal cord) and mesoderm, and thus continue to make a germ layer decision after the end of gastrulation. These cells were given the name neuromesodermal progenitors (NMPs) based on their propensity to contribute to both neural and mesodermal lineages (Figure 1). Later work

*Correspondence: benjamin.martin@stonybrook.edu.

showed that cells in the tailbud of zebrafish embryos also have the potential to join the spinal cord or mesoderm, and that this decision is made continuously during axis elongation based on local canonical Wnt signaling cues [3]. High Wnt signaling induces mesoderm, while low Wnt signaling promotes spinal cord formation. The transition from NMP to mesoderm involves a developmental checkpoint that ensures neural specific genetic programs are repressed before cells can exit into mesodermal territories [4]. This checkpoint occurs during a partial epithelial to mesenchymal transition (EMT) as cells transition from epithelial NMPs to mesenchymal mesoderm [4]. More recent lineage tracing in chick embryos showed single NMPs contributing to both neural and mesodermal lineages [5]. The tailbud NMPs are identified as cells expressing both the neural associated transcription factor Sox2 and the mesoderm promoting transcription factor Brachyury [6], also referred to as Tbxt or T. In zebrafish there are two partially redundant *brachyury* genes *tbxta* and *tbxtb*, also referred to as *ntla* and *ntlb*, with *tbxta* playing the predominant role during development (based on loss of function phenotypes) [7]. This review will simply refer to "*brachyury"* to represent the combined function of the two zebrafish genes. Tailbud cells co-expressing Sox2 and Brachyury have been identified in mice, chick, zebrafish, and humans [3, 8–10], and thus NMPs appear to be a common feature of vertebrate embryonic development. NMPs have also recently been proposed to exist in invertebrate chordates [11].

The discovery of NMPs altered our understanding of lineage relationships within and between what are traditionally considered the three primary germ layers formed during gastrulation. While the spinal cord, brain, and epidermis are all considered to be tissues derived from the ectodermal germ layer, the normal biology of NMPs indicates that cells within the spinal cord are more closely related from a cell lineage perspective to paraxial mesoderm than they are to cells in the brain and epidermis [2]. This subsequently caused a broad shift in our understanding of neural induction and patterning. Instead of a model where all neural tissue is initially induced and then subsequently patterned into anterior (brain) and posterior (spinal cord) character, we now understand that there are separate cellular origins of brain and spinal cord [12–14]. Likewise, our view of mesodermal lineage relationships has changed, with certain anterior and ventral mesodermal types induced during gastrulation during primary germ layer segregation, while posterior mesoderm is generated from NMPs that share a common lineage with the spinal cord [2, 3]. The fact that mesoderm is continuously induced from NMPs during post-gastrulation axis extension provides a new context in which to study mechanisms of vertebrate mesoderm induction. This review will discuss aspects of NMP mesoderm induction that have revealed core conserved features of mesoderm induction in general, as well as mechanisms that appear to be NMP specific. Our consideration of mesoderm induction in this new context will further our understanding of the mechanisms of vertebrate body plan development, and how the mesodermal germ layer evolved within the animal lineage.

2. Signaling pathway usage during NMP and NMP-derived mesoderm induction

The mechanisms of vertebrate mesoderm induction have historically focused on gastrula stages of development and have revealed several signaling pathways critical for mesoderm

induction during this period. These include the Nodal, Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), and canonical Wnt pathways [15]. These pathways play variable roles in the induction of NMPs and the subsequent induction and patterning of NMP-derived mesoderm (Table 1). The Nodal and FGF signaling pathways exhibit the most difference between their gastrula and post-gastrula stage roles in mesoderm induction and will serve as the basis of discussion in this section. The function of canonical Wnt and BMP signaling during NMP mesoderm induction and patterning will be discussed later (see sections 3 and 4 for Wnt and 5 for BMP).

2.1 Nodal signaling

Nodal signaling plays a key conserved role during both gastrula-stage mesoderm and endoderm induction in vertebrate embryos [15, 16]. Genetic analysis has indicated in both zebrafish and mouse that Nodal signaling plays a critical role during mesoderm and endoderm induction [15, 16]. However, this role appears to be restricted specifically to anterior mesoderm. A loss of function mutation in the single mouse Nodal gene disrupts primitive streak formation, but in 25% of embryos there are posteriorly localized mesodermal cells, indicating that Nodal signaling is not absolutely required for all mesoderm to form [25]. Likewise, loss of function of the two early acting Nodal genes in zebrafish, or of the essential co-receptor *tdgf1* (also referred to as *one-eyed pinhead* or *crypto*) results in an absence of anterior mesoderm, but posterior mesoderm, including somitic tissue, is formed [26–28]. Posterior somites are generated from NMPs, indicating that Nodal signaling is not required for NMP or NMP-derived mesoderm induction. Supporting this, timed inactivation of Nodal signaling in zebrafish using small molecule inhibitors showed that once gastrulation begins, inhibition of Nodal signaling does not impact mesoderm induction in any region of the embryo [17].

2.2 FGF signaling

FGF signaling was first identified as being important for mesoderm induction based on its ability to induce mesoderm in frog embryos when over-expressed [29]. Later work showed that inhibition of FGF signaling in frogs and zebrafish causes a specific loss of posterior mesoderm, with anterior mesoderm forming [30–32]. Subsequent work in a number of other vertebrate model systems revealed a conserved role of FGF signaling in inducing posterior mesoderm [18]. In addition to vertebrate mesoderm induction, FGF signaling also plays key roles in mesoderm migration and patterning [18, 33]. As development progresses, the role of FGF signaling in mesoderm induction changes over time. During gastrula stages, FGF induces posterior mesoderm at least in part through transcriptional activation of *brachyury*. In zebrafish and *Xenopus*, loss of FGF signaling results in a loss of *brachyury* expression, and *brachyury* is in turn itself required for posterior mesoderm formation [32, 34–36]. In zebrafish, inhibition of FGF signaling after gastrulation also disrupts mesoderm formation, as NMP-derived cells fail to transition into committed mesodermal progenitors. However, the mechanism is distinct, as loss of FGF signaling after gastrulation results in an expansion of *brachyury* expression as opposed to a loss [19]. In this context, FGF signaling activates the expression of transcription factors *msgn1* and *tbx16*, which in turn are required to repress NMP markers *brachyury* and *sox2* [19, 37–39]. In the absence of FGF signaling, prospective NMP-derived mesoderm cells become trapped in the partial EMT state and

remain in the tailbud, unable to exit and commit to mesodermal differentiation (discussed in further detail in section 4) [19]. In the chick model, FGF signaling was shown to play an additional role in cells once they join the paraxial mesoderm. Here, FGF signaling promotes the random motility of paraxial mesoderm cells which is essential for the proper axial extension of the embryo [40]. In zebrafish, convergence and extension of the paraxial mesoderm is important for axial elongation and is also associated with non-directional rearrangement of cells [41].

Given that much of the posterior mesoderm in vertebrate embryos is derived from NMPs, the data suggests that FGF signaling induces NMPs during gastrulation, and then FGF is required within NMPs to induce mesoderm through regulation of transcription factors that promote EMT and mesoderm differentiation. In addition to gastrula stage FGF signaling being required for posterior mesoderm formation, it is also required for posterior neural (spinal cord) formation. This activity includes activation of soxB1 transcription factor expression and is independent of the neural inducing activity of BMP signaling inhibition [14, 42–45]. This activity adds further support for a role of FGF signaling in inducing the NMP population during gastrulation. Furthermore, protocols for the in vitro derivation of NMPs from pluripotent stem cells require the addition of FGF [6, 10, 46, 47].

2.3 Integrating new views of neural and mesoderm induction during development

The discovery of NMPs created a shift in our understanding of nervous system development by showing that spinal cord cells originate from a population of cells that is unique from those that give rise to the brain. This is opposed to a historical model of neural development called the activation and transformation model, which posits that all neural tissue is first induced (activation) with an anterior brain character and then patterned (transformed) into posterior tissues of the hindbrain and spinal cord by morphogen gradients [48]. Our current updated understanding is that the cells that generate the brain are induced first through local inhibition of BMP signaling, whereas the spinal cord forms from induction by FGF signaling, independent of BMP inhibition [12, 14, 42]. The cells that form spinal cord also come from a distinct source (the NMPs) compared to cells that will generate the brain, and do not pass through an anterior neural intermediate before becoming spinal cord [13]. It is not clear whether all, or just part of the spinal cord is generated from NMPs in all vertebrates. For instance, fate mapping in zebrafish indicates that portions of the spinal cord exhibit clonal restriction to that fate without contributions to paraxial mesoderm [49]. However, Wnt signaling manipulations suggest that these spinal cord cells come from a neuromesodermal competent population, despite not realizing both fates during development [3]. The role of Nodal and FGF signaling in mesoderm induction point towards a similar division within the mesodermal germ layer with respect to distinct signaling mechanisms and cellular origins of anterior vs. posterior tissues. In the mesoderm, Nodal signaling induces anterior tissues, while FGF signaling is required for generating posterior mesoderm. Most of the posterior mesoderm also comes from a distinct cellular origin (NMPs) compared to the anterior tissue. Thus, the generation of neural and mesodermal tissues in vertebrate embryos appear to occur through distinct anterior and posterior development modules. However, these modules interact with each other based on positive feedback between FGF and Nodal signaling during early development [50].

3. The role of Brachyury (T) during mesoderm induction

NMPs are defined as cells that co-express the transcription factors *sox2* and *brachyury* [3]. *Brachyury*, which is the founding member of the T-box transcription factor family, has long been associated with mesoderm development since the *Brachyury* mouse mutant was first described in 1927 [51]. Mice heterozygous for loss of *Brachyury* function have short tails, hence the name *Brachyury* which means “short tail” in Greek. Homozygous mutant loss of function results in embryonic lethality and a loss of posterior mesoderm, with only the anterior-most 8–12 somites forming of the approximately 60 that normally form [52, 53]. Mutant embryos also lack axial mesoderm that will form the notochord. The essential role of *Brachyury* in notochord formation is conserved within the chordates [54–60]. Additionally, work in many different animal species have revealed that *Brachyury* plays an essential role in posterior mesoderm induction in many of them, including all vertebrates that have been examined [61].

Investigating the role that Brachyury plays during continuous mesoderm production from NMPs has revealed molecular insights into its function as a mesoderm inducing factor. While Brachyury is an essential positive transcriptional regulator required for mesoderm induction from NMPs at the whole embryo level, its function is not absolutely required at the cell autonomous level. Mosaic analysis of Brachyury function in both zebrafish and mouse has revealed that even in the complete absence of Brachyury function, NMPs can still contribute to posterior somites when surrounded by wild-type cells [7, 62, 63]. This indicates that an essential function of Brachyury is the transcriptional regulation of genes that function in a cell non-autonomous fashion, such that wild-type cells that surround cells lacking Brachyury function can rescue the mutant phenotype. In zebrafish, critical direct targets of Brachyury are the canonical Wnt ligands *wnt3a* and *wnt8a* (Figure 2A) [7, 64]. Wnt signaling in turn activates *brachyury* expression, which creates an autoregulatory loop to sustain posterior Wnt signaling during the course of axis extension [7]. The posteriorly localized Wnt signal is necessary for both NMP maintenance and for mesoderm induction from the NMPs (as further discussed in section 4) [3, 10, 22]. After the discovery that zebrafish Brachyury directly activates Wnt ligands, it was subsequently discovered that this is also true in mouse, sea urchin, and sea anemone [65–67]. Additionally, a Brachyury/canonical Wnt signaling autoregulatory loop that was found to be critical for NMP derived mesoderm formation has also been observed in a diverse set of animals, including mouse, sea urchin, acorn worm, and sea anemone, revealing the deep evolutionary ancestry of this regulatory relationship (Figure 2B) [22, 65–70]. Thus, a central evolutionarily conserved role of Brachyury (predating the Cambrian explosion over 500 million years ago) during mesoderm induction is the maintenance of canonical Wnt signaling [61].

In addition to the activation of canonical Wnt ligands, zebrafish Brachyury also directly activates the expression of the retinoic acid metabolizing enzyme *cyp26a1* [62], and this positive regulation by Brachyury is conserved in mouse (Figure 2C) [63, 71]. During mouse gastrulation, retinoic acid signaling plays an important role in inducing the NMP population [72], a function that exhibits species specific differences between mouse and zebrafish [73]. However, in both species, as well as in chick, low levels of retinoic acid signaling is required for post-gastrulation NMP maintenance [8, 62, 74, 75]. Retinoic acid is normally

produced in the most recently formed somites of vertebrate embryos, in cells that express the enzyme *aldh1a2*, which catalyzes the formation of retinoic acid from retinaldehyde [76]. The most recently formed somites are in close proximity to the tailbud and the NMP population. Retinoic acid is a potent inhibitor of *brachyury* expression, which in turn causes a loss of Wnt ligand expression [62]. Thus, in order for NMPs to be sustained in the undifferentiated state, they must be protected from the neighboring retinoic acid source (Figure 2C). Indeed, loss of Cyp26a1 function in zebrafish and mouse causes a failure to sustain NMPs and results in posterior truncations and a loss of the posterior-most somites [62, 74, 75]. In zebrafish, *cyp26a1* mutant cells transplanted into wild-type host embryos are able to contribute to the posterior-most somites of the host embryos, which would normally be missing in whole embryos *cyp26a1* mutants [62]. This result suggests that neighboring wild-type cells that express Cyp26a1 can act as a retinoic acid sink and degrade enough retinoic acid to protect transplanted cells lacking Cyp26a1. This non-autonomous role of Cyp26a1 is also supported by other work in zebrafish [77]. Thus Brachyury, by direct activation of canonical Wnt ligands and *cyp26a1*, creates a molecular niche that supports the maintenance of NMPs and their subsequent induction into mesoderm [62].

There is much still to be learned about the role of Brachyury in both NMP maintenance and differentiation into mesoderm. Many other direct transcriptional target genes regulated by Brachyury that have been identified, suggesting that Brachyury is also regulating NMPs and mesoderm induction in other ways. A report using mice indicated that Brachyury plays a direct role in the neural/mesodermal fate decision by antagonizing the function of the Sox2 transcription factor, which promotes neural fate in NMPs [71]. However more recent work has suggested that Brachyury does not play such a role, as mosaic analysis in mouse embryos of Brachyury mutant cells shows there is not an increased propensity for these cells to become neural instead of mesoderm [63]. Some of the Brachyury mutant cells in mosaic embryos are able to contribute to posterior somites (as mentioned above), but many tend to stay in the tailbud in the region of the NMPs, suggesting a role in promoting exit of NMPs into the mesodermal compartment [63]. Recent work using quail embryos also showed that the NMPs with a higher ratio of Brachyury compared to Sox2 exhibit increased motility and exit into paraxial mesoderm [78]. Whether this activity is related to the regulation of Wnt and/or retinoic acid signaling in the mouse remains to be seen.

Some of the other Brachyury direct targets are required for the proper segmentation of the NMP-derived paraxial mesoderm into somites, thereby coordinating the generation of new mesoderm with the continuous segmentation process that occurs in vertebrate axial elongation [79, 80]. The Brachyury/Wnt autoregulatory loop also intersects with Hox gene regulation. Hox genes are transcription factors that activate genes important for specifying cell fate at specific axial levels of the body [81]. Wnt signaling activates Hox gene expression through regulation of the Caudal homeobox (Cdx) transcription factors, which in turn activate Hox gene expression [61]. The regulation of Hox genes by Wnt signaling adds an additional layer of coordination to the induction of NMP-derived mesoderm and the acquisition of axial identity. This topic was extensively covered in a recent review [6], however, even more recent work has shown that the *brachyury* (*tbxta*) promoter in zebrafish is directly activated by a posterior Hox gene, which helps drive *brachyury* expression in

the NMP region [82]. Thus, there appears to be a positive feedback loop between the Brachyury/Wnt loop and Hox genes.

As mentioned above, Brachyury is also required for the formation of the notochord in chordate embryos. Both the notochord and floor-plate are generated from midline progenitors that reside within the tailbud, and which have NMP-like characteristics including co-expression of *brachyury* and *sox2*, as well as the ability to continuously generate both mesoderm (notochord) and neural tissue (floor plate) [83]. However, unlike the cell non-autonomous role of Brachyury in NMP-derived mesoderm, Brachyury is required in a cell-autonomous fashion for notochord development [84]. Mosaic analysis of *brachyury* mutant cells in both zebrafish and mouse indicates that they are completely unable to join the notochord, and in the case of zebrafish instead join the floor plate [84, 85]. The distinct activity of Brachyury between these two populations of cells is likely due at least in part to differences in cofactors present. Brachyury binds to the BMP effector Smad1, which can cause differential target gene regulation compared to Brachyury not bound to Smad1 [86]. The NMPs are a region of high BMP activity, while the midline and notochord progenitors have low BMP activity, and thus an absence of activated Smad1 [20, 87]. In *Xenopus* embryos, the absence of Smad1 binding to Brachyury promotes activation of notochord marker *goosecoid* expression [86]. The difference in Brachyury function within NMPs and midline progenitors may also be the result of differential regulation of signaling pathways in these populations. FGF signaling plays an essential role in promoting notochord fate, which is conserved across chordates [30–32, 88–90]. Brachyury functions in an autoregulatory loop with FGF signaling [35]. Brachyury can induce FGF ligand expression, and FGF signaling is in turn required for *brachyury* expression [35]. Loss of Brachyury functional analysis in zebrafish revealed that the regulation of FGF signaling activation by Brachyury appears restricted to the axial mesoderm [7], which may play a role in the differential activity of Brachyury revealed by mosaic analysis.

4. Insights into the epithelial to mesenchymal transition that generates mesoderm

Epithelial to mesenchymal transition (EMT) describes the cellular state change that occurs in tightly adhering epithelial cells as they lose their adhesions and become migratory and invasive mesenchymal cells [91, 92]. The term EMT was coined by Elizabeth Hay, who first described the process after observing mesoderm formation in the chick embryo [93]. Since then, EMT has been recognized to be a critical event during mesoderm induction across animal species, where cells undergo EMT to internalize and form the mesodermal germ layer [91, 94]. Here I will discuss the process of EMT in NMP-derived mesoderm induction and how it has informed our understanding of the molecular regulation of this process, as well as the biological importance of intermediate transitional states between full epithelial and mesenchymal characteristics.

4.1 NMPs undergo a two-step EMT during mesoderm induction

Several lines of evidence suggest that NMPs are an epithelial cell type, and subsequently undergo EMT as they form mesoderm [6]. Much of our understanding of the NMP to

mesoderm EMT comes from zebrafish, based on their amenability for pairing live imaging with genetic manipulations. Live-imaging of zebrafish tailbud cell movements revealed that cells within the region corresponding to the location of NMPs have collective epithelial-like migration and then transition to rapid individual cell migration as they transition to mesoderm, consistent with these cells being in a mesenchymal state [95]. Live-imaging of zebrafish mesoderm formation during gastrulation and in the tailbud at post-gastrulation stages showed that the EMT process occurs in a two-step fashion [19, 96, 97]. In the first step, cells transition from epithelium to mesenchyme that migrates in a non-directional fashion. In the second step the cells migrating in a non-directional manner switch to directional migration as measured by individual cell tracking, which allows them to join the mesodermal cell population [96]. The cells in the transitional, or partial EMT state, are more adhesive than the fully mesenchymal mesoderm formed after the completion of the second step [97]. The T-box transcription factor Tbx16 is essential for the completion of the second EMT step in zebrafish [96, 98]. In the absence of Tbx16 function, cells complete the first EMT step but are unable to complete the second, and thus remain trapped in the partial EMT state indefinitely. This causes the cells to remain in the tailbud until the end of axis extension, unable to join the mesoderm [99, 100]. Mouse embryos with a loss of function in the related transcription factor TBX6 have a similar phenotype, where embryos have enlarged tailbuds due to cells being unable to leave and join the mesoderm [101]. However, unlike in zebrafish *tbx16* mutants, a portion of cells in the mouse *Tbx6* mutants exit the tailbud to join the region normally occupied by the paraxial mesoderm, but instead give rise to ectopic spinal cords [101]. The nature of this phenotype, and the difference with zebrafish, is discussed in section 4.2.

The cellular transition and molecular regulation of the two-step EMT during NMP-derived mesoderm induction was further characterized in zebrafish embryos. The initiation of the 1st EMT step is regulated by canonical Wnt signaling, with Wnt signaling inducing an apical constriction and delamination of cells from the NMP epithelium, a process which, with respect to cell morphology and Wnt signaling dependence, looks remarkably like the mesodermal EMT during mouse gastrulation (Figure 3) [19, 102]. In the absence of Wnt signaling NMPs downregulate expression of the direct Wnt target gene *brachyury* and do not leave the NMP epithelium. These low Wnt cells will eventually contribute to the spinal cord [3, 7, 19]. Once cells enter the partial EMT state, FGF signaling is required to promote the 2nd EMT step, causing them to obtain directional migration out of the tailbud to join the mesodermal population. The activity of FGF signaling is due at least in part to its positive regulation of *tbx16* and *msgn1* expression, which promote exit from the tailbud in both zebrafish and mouse (*Tbx6* in the case of mouse) [37, 38, 100, 101, 103]. The FGF and Wnt signaling pathways activate each other in the zebrafish tailbud, which may help ensure the continuous and balanced allocation of cells to the mesoderm by positive reinforcement between the 1st and 2nd EMT steps [104]. In the mouse, a partial EMT occurs in the epiblast, and the cells that enter the partial EMT state are incorporated into the tailbud as NMPs. This process is dependent on the activities of the Tgf-beta signaling pathway and the SNAI1 transcription factor [105]. Thus, partial EMT appears to be a common aspect of NMP development, although with species specific differences in molecular regulation and timing.

4.2 The partial EMT transitional state acts as a developmental checkpoint

Work from the cancer field has indicated that cells in the partial or intermediate EMT state have unique properties, such as increased stemness, invasiveness, and drug resistance, making the study of partial EMT states in cancer progression particularly important [106]. However, much less is known about the biological properties of cells in the partial EMT state in normal developmental processes, and whether there is significance to maintenance of metastable partial EMT states. As mentioned previously, zebrafish cells fated to become NMP-derived mesoderm that lack *tbx16* function become trapped in the partial EMT state, a phenotype similar to mouse *Tbx6* mutants. Both zebrafish Tbx16 and mouse TBX6 play an important role in transcriptional repression of the NMP marker *sox2* [107, 108]. It was recently shown in zebrafish that ectopically maintaining *sox2* in mesoderm fated NMPs is sufficient to phenocopy the Tbx16 loss of function phenotype, and eliminating Sox2 function in *tbx16* mutants allows NMP fated mesoderm to exit the tailbud and differentiate into mesoderm [4]. The activity of Sox2 in preventing NMP fated mesoderm from exiting the tailbud and holding them in a metastable partial EMT state was found to be dependent upon interactions with the mesoderm inducing canonical Wnt signal. Inhibiting Wnt signaling in Sox2 gain of function or Tbx16 loss of function mesoderm fated cells (which maintain *sox2* expression) allows them to exit the tailbud, however they differentiate into ectopic spinal cord rather than mesoderm [4]. Thus, the partial EMT state, which is promoted by a unique interaction of Sox2 and Wnt signaling, acts as a developmental checkpoint to ensure mesoderm fated cells that express *sox2* do not exit the tailbud, making certain these cells adopt the appropriate mesodermal fate instead of neural fate (Figure 3) [4]. Recent work using quail embryos also showed that higher levels of Sox2 relative to Brachyury limits NMP cell migration and prevents incorporation into mesoderm [78].

In zebrafish, lowering Wnt signaling in Tbx16 loss of function cells causes them to differentiate into ectopic spinal cord [4]. This phenotype is similar to *Tbx6* mutant mouse embryos, which form ectopic spinal cords [101], suggesting that differences in Wnt signaling levels between the two species may account for the normal differences in phenotypes. Based on the phenotypes, the differences imply that zebrafish have a higher relative level of Wnt signaling in the NMP region than mouse, and lowering the level in *tbx16* loss of function phenocopies the mouse *Tbx6* mutant. Wnt signal activation was previously shown to accelerate the tailbud exit and differentiation of NMP-fated mesoderm [3, 108]. Since zebrafish development is extremely rapid relative to mouse, which facilitates their need to swim and evade predators early in development, the hypothesized higher levels of Wnt signaling may play a role in part to promote rapid mesoderm induction and differentiation.

5. Mesoderm patterning by BMP and FGF signaling

Newly induced NMP-derived mesoderm primarily gives rise to paraxial mesoderm, which forms the somites. Although there are distinct non-NMP sources of lateral mesoderm [9, 109], a minority of NMP-derived mesoderm adopts lateral mesodermal fates [2, 3, 24]. The primary lateral fates identified thus far are vascular endothelium that will form posterior blood vessels and nephric mesenchyme [3, 20, 24]. This medial-lateral (also referred to as

dorsal-ventral) mesodermal patterning is much simpler than what occurs during gastrulation-stage mesodermal patterning, where there are many more types of mesoderm being specified at the same time that mesodermal cells are undergoing very broad cell movements [15]. The simplified patterning event in NMP-derived mesoderm provides an opportunity to better understand mechanisms of patterning, without the complicated pleiotropies caused by gastrula-stage manipulations.

The first indication that NMP-derived mesoderm is continuously patterned by local signaling cues after it is induced came from experiments manipulating canonical Wnt signaling [3]. The role of Wnt signaling in patterning gastrula-stage mesoderm is well studied and is critical for promoting dorsal/medial cell fates such as axial and paraxial mesoderm [110]. During NMP-derived mesoderm patterning, this role appears to be conserved. After mesoderm is induced, Wnt signaling is required for paraxial mesoderm fate, and in its absence cells adopt the lateral endothelial fate [3]. Wnt signaling directly activates the expression of key paraxial mesoderm genes *msgn1* and *tbx16* (in zebrafish) and the patterning role of Wnt signaling in NMP-derived mesoderm is likely due at least in part to the regulation of these genes [108, 111–114]. The knowledge that NMP-derived mesoderm is patterned into multiple cell fates set the stage to examine other signaling pathways known to pattern mesoderm, and to use the relative simplicity of the system to tease out the molecular mechanism of patterning downstream of signal activation.

The BMP and FGF signaling pathways play key roles in mesodermal patterning, in addition to their roles in mesoderm induction. BMP induces ventral/lateral mesoderm, whereas FGF induces medial/dorsal fates [18, 23]. However, the downstream targets of these pathways that mediate the patterning during gastrulation were unknown. Like their gastrulation stage roles, BMP and FGF signaling also pattern NMP-derived mesoderm, with BMP inducing the lateral/ventral endothelial and nephric mesenchyme fates, and FGF inducing the medial/dorsal paraxial mesoderm fate [20, 24]. With respect to the paraxial/endothelial fate decision, the absence of BMP signaling causes endothelial fated cells to become paraxial mesoderm, and form ectopic somites at the ventral midline where axial blood vessels would normally form. On the other hand, activation of BMP induces endothelial fate in the paraxial mesoderm fated cells, and after signaling activation a large functional network of blood vessels form where somites normally occur (Figure 4A). Loss of FGF signaling gives the same patterning phenotype as gain of BMP signaling (Figure 4A) [20]. Although FGF signaling functions to repress BMP signaling during gastrulation [115, 116], the FGF loss of function phenotype in NMP derived mesodermal patterning is independent of any direct repressive role on BMP signaling. Instead, BMP and FGF signaling interact at the level of bHLH transcription factor activity, with the medial FGF patterning the result of FGF induced *msgn1*, *myf5*, and *myod* [20]. BMP signaling inhibits their activity by activation of Id proteins (*id1*, *id3*), which are HLH proteins that bind to and inhibit the activity of bHLH transcription factors [20]. This mechanism mediated by FGF and BMP signaling also functions during gastrula stage mesoderm patterning in zebrafish and in mouse NMP-derived mesoderm (Figure 4B) [20].

An early response to either FGF loss of function or BMP gain of function in NMP-derived mesoderm is the expanded expression of the endothelial inducing gene *etv2* [20]. Etv2 is

both necessary and sufficient for inducing endothelial fate [117–122], and recent single cell sequencing of mutant zebrafish *etv2* embryos revealed that in the absence of Etv2 function presumptive endothelial cells become somite derived skeletal muscle [123]. Gain of Etv2 function can also transfate cells in the somite into endothelial cells [118]. Etv2 labels both endothelial and hematopoietic progenitors in mouse and zebrafish. In the mouse, loss of ETV2 function results in the complete loss of blood and vessels [119]. In zebrafish, loss of Etv2 function results in the loss of vessels and primitive myeloid cells. Further research is needed to determine whether hematopoietic lineages are also derived in part from NMPs, and if BMP and FGF signaling play a similar patterning role.

7. Conclusions and future directions

The study of NMP-derived mesoderm induction is in its infancy, and while it so far has revealed critical insights into mesoderm induction, there are still many unanswered questions. Key molecular aspects of molecular pathways involved in NMP-derived mesoderm induction are yet to be described, such as the direct molecular targets of the canonical Wnt pathway that induce apical constriction in NMPs and the initiation of the EMT process. There are other basic questions that remain, such as why NMPs exhibit enrichment in the G2 phase of the cell cycle [124–126], and whether chemo-attractants or repellents play a role in directed migration out of the tailbud into the mesodermal territory, among many others. Furthermore, differences exist between the growth dynamics and lineage contribution of NMPs between vertebrate species, and further work is needed to determine how these differences may impact body plan variations [127, 128].

Additional important evolutionary questions remain as well. It is not clear to what extent partial EMT states observed in mouse and zebrafish NMPs occur during mesoderm formation in other animals. Recent evidence shows that in the diploblastic cnidarian *Nematostella vectensis*, the endomesodermal germ layer is established through cells entering into a partial EMT state, without becoming fully mesenchymal [129]. However, these cells can be artificially induced to become fully mesenchymal causing individual cell delamination [129]. Additionally, the mesodermal patterning mechanism involving regulation of bHLH transcription factor activity first observed in NMP-derived mesoderm may also have ancient origins, as bHLH transcription factors of the type found in vertebrate mesoderm as well as their HLH inhibitors are found in the earliest branching metazoans [130]. Finally, evidence is building that NMP-like cells or the regulatory circuits that govern their development exist outside of the vertebrate lineage [11, 66], and it will be fascinating to see how broadly they are represented across the animal kingdom.

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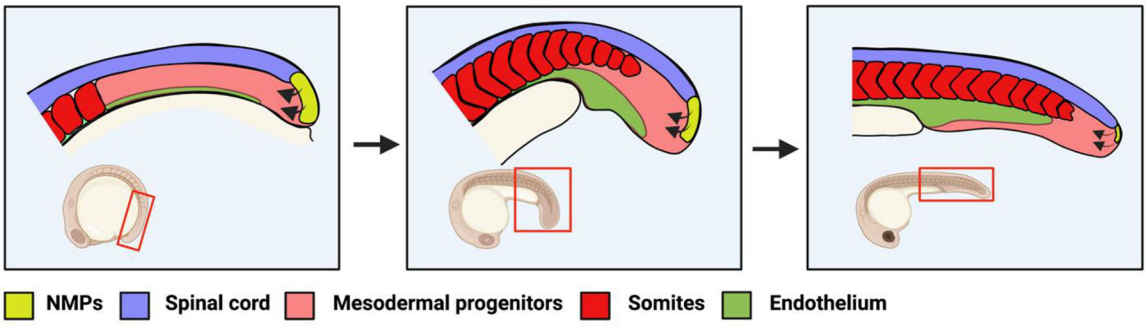

Figure 1 –. Neuromesodermal progenitors generate spinal cord and mesoderm after gastrulation.

Vertebrate embryos (here is shown a zebrafish embryo) contain NMPs (yellow) in the tailbud which generate spinal cord (purple) and mesodermal progenitors (pink). The mesoderm will differentiate into somites (red) and vascular endothelium (green). The process of mesoderm induction from NMPs occurs continuously (arrows) over the course of axial extension until the NMPs are depleted. The red boxed region of the inset embryo represents the area depicted in the schematics.

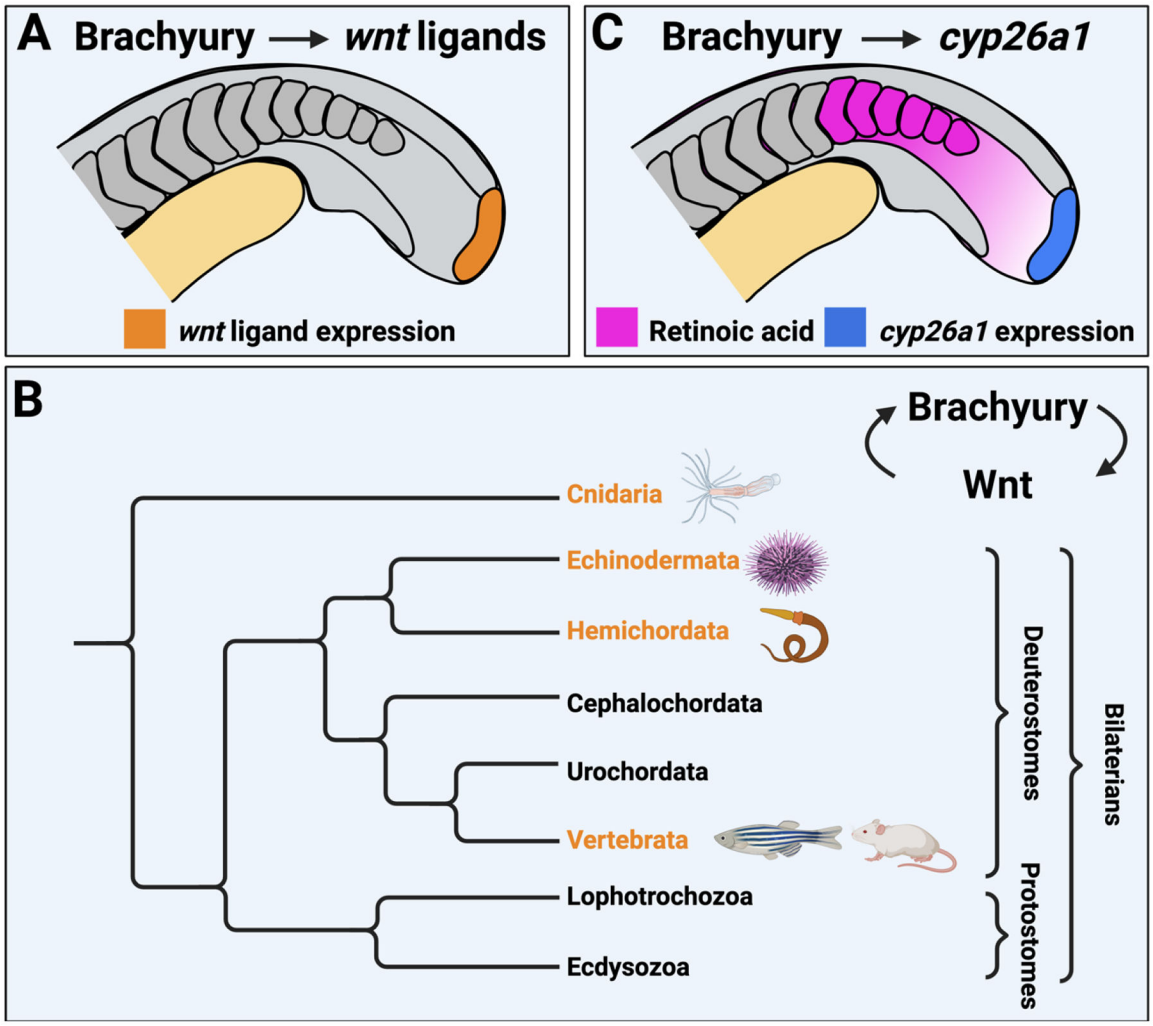

Figure 2 –. Conserved direct Brachyury targets involved in NMP maintenance and NMP-derived mesoderm induction.

(A) Brachyury directly activates the expression of canonical Wnt ligands in the NMP region (orange). Wnt signaling in turn activates *brachyury* expression. (B) A simplified animal phylogeny illustrating the groups in which there is experimental evidence for a Brachyury/Wnt autoregulatory loop (orange). The specific animals in which this has been observed are shown as icons next to the phylogenetic tree. (C) Brachyury target gene *cyp26a1* is expressed in the NMPs (blue). Cyp26a1 degrades retinoic acid to protect NMPs from the nearby posterior somite retinoic acid source (magenta).

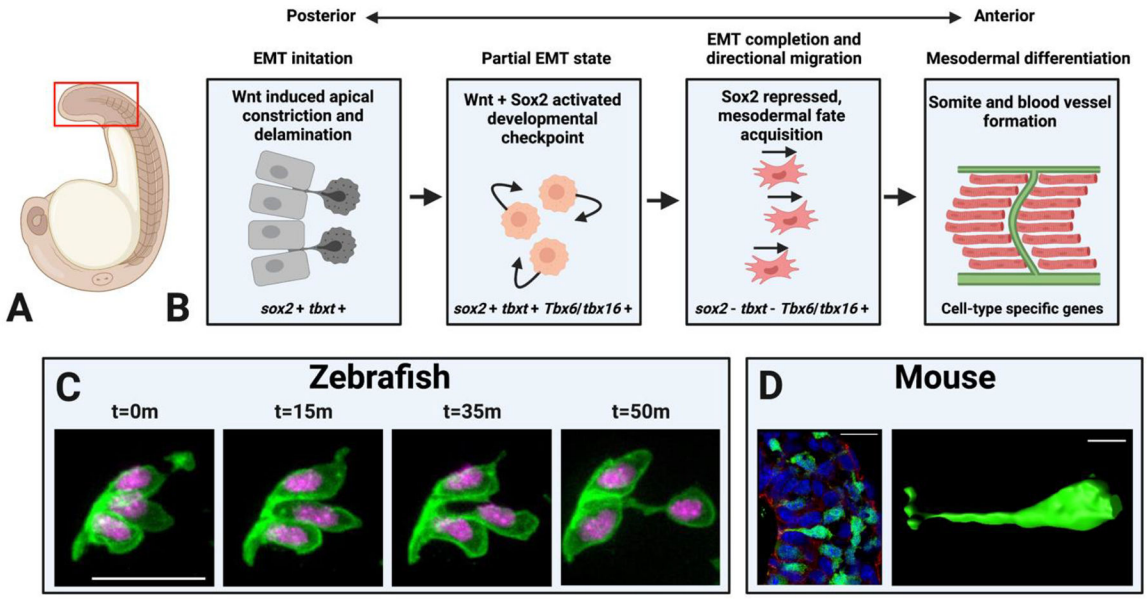

Figure 3 –. Insights into the mesodermal epithelial to mesenchymal transition.

(A) A zebrafish embryo depicting (red box) the region of interest shown in (B). (B) The stages of the NMP-derived mesodermal EMT are depicted as schematics with events occurring beginning in the posterior wall of the tailbud (left) and progressing towards the anterior (right). The marker genes expressed at each step are indicated under each schematic. (C) Micrographs from a time-lapse of an individual cell undergoing EMT during zebrafish NMP-derived mesoderm induction, adapted from [16] with permission. Scale bar = 20μm. (D) Micrograph (left) and surface rendering (right) of an individual cell undergoing EMT during gastrula stage mesoderm induction in a mouse embryo, adapted from [94] with permission. Scale bar in left image = 25μm, in right image = 10μm). Both the mouse and zebrafish cells exhibit apical constriction and eventual delamination from the epithelium.

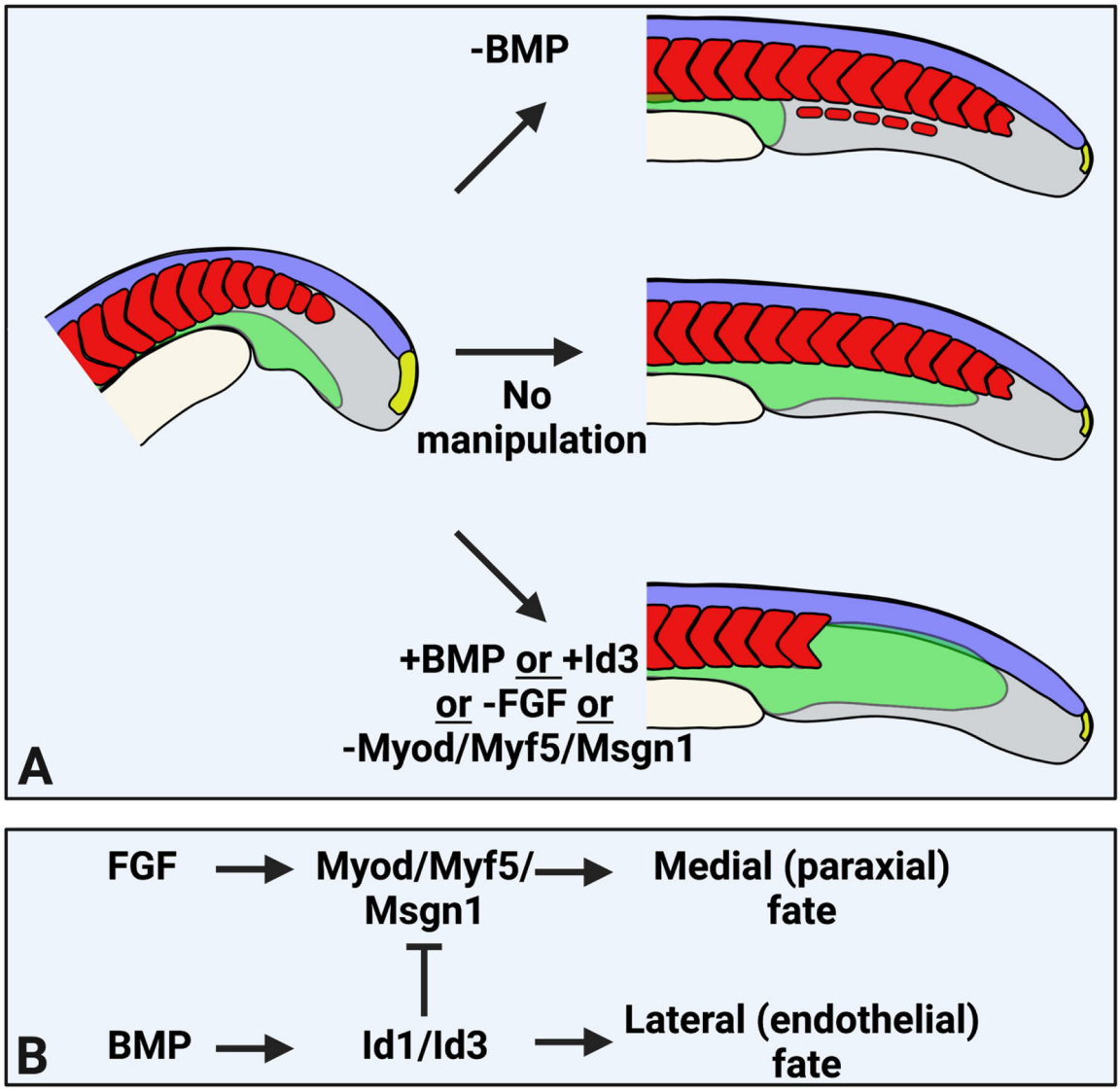

Figure 4 –. Patterning of mesoderm by modulation of bHLH transcription factor activity.

(A) Experimental manipulations that change the patterning of NMP-derived mesoderm in vivo, resulting in loss of endothelium and formation of ectopic somite tissue (top), or loss of somites and expansion of endothelium (bottom). (B) A model depicting the molecular mechanism of patterning downstream of BMP and FGF signaling. FGF signaling activates bHLH transcription factor expression, while BMP signaling activates the Id genes which inhibit bHLH proteins. This mechanism has been shown in zebrafish and in cultured mouse NMP-derived mesoderm.

Table 1 –

Mesoderm inducing signaling pathways and their role in NMPs.

Signaling Pathway	Role in gastrula stage mesoderm induction and patterning	Role in NMP-derived mesoderm induction	Role in NMP-derived mesoderm patterning
Nodal	Essential for mesoderm (and endoderm) induction and promotes dorsal/medial patterning [15, 16].	Not essential as loss of function during NMP-derived mesoderm induction has no impact on mesoderm formation [17].	No role currently identified.
FGF	Essential for induction of posterior mesoderm and patterning into dorsal/medial fates [15, 18].	Essential for EMT completion during NMP-derived mesoderm induction. Loss of FGF signaling in mesoderm fated NMPs causes them to be trapped in the partial EMT state and prevents mesodermal differentiation [19].	Essential for patterning NMP-derived mesoderm into the paraxial mesoderm fate [20].
Canonical Wnt	Essential for mesoderm induction and patterning, with maternal signaling promoting dorsal/medial fates and zygotic signaling promoting ventral/lateral fates [15, 21].	Essential for both the maintenance of NMPs and the induction of NMP-derived mesoderm. This pathway is required for EMT initiation during NMP-derived mesoderm induction [3, 10, 22].	Essential for patterning NMP-derived mesoderm into the paraxial mesoderm fate [3].
BMP	Plays a role in mesoderm induction and patterning. Best known for pattering mesoderm into ventral/lateral fates [15, 23].	No direct role currently identified.	Essential for patterning NMP-derived mesoderm into the lateral mesoderm fate [20, 24].