RESEARCH ARTICLE

Cell-cycle-dependent TGFβ–BMP antagonism regulates neural tube closure by modulating tight junctions

Smita Amarnath1 and Seema Agarwala1,2,3,*

ABSTRACT

Many organs form by invaginating and rolling flat epithelial cell sheets into tubes. Invagination of the ventral midline of the neural plate forms the median hinge point (MHP), an event that elevates the neural folds and is essential for neural tube closure (NTC). MHP formation involves dynamic spatiotemporal modulations of cell shape, but how these are achieved is not understood. Here, we show that cell-cycle-dependent BMP and TGFβ antagonism elicits MHP formation by dynamically regulating interactions between apical (PAR complex) and basolateral (LGL) polarity proteins. TGFβ and BMP-activated receptor (r)-SMADs [phosphorylated SMAD2 or SMAD3 (pSMAD2,3), or phosphorylated SMAD1, SMAD5 or SMAD8 (pSMAD1,5,8)] undergo cell-cycle-dependent modulations and nucleo-cytosolic shuttling along the apicobasal axis of the neural plate. Non-canonical TGFβ and BMP activity in the cytosol determines whether pSMAD2,3 or pSMAD1,5,8 associates with the tight junction (PAR complex) or with LGL, and whether cell shape changes can occur at the MHP. Thus, the interactions of BMP and TGFβ with polarity proteins dynamically modulate MHP formation by regulating r-SMAD competition for tight junctions and r-SMAD sequestration by LGL.

KEYWORDS: Apicobasal polarity, Hinge point, LGL, Midbrain, Neural tube closure defects, Organogenesis, PAR

INTRODUCTION

Many tubular organs develop from flat epithelial sheets that are invaginated and rolled to form closed tubes (Andrew and Ewald, 2010; Martin-Belmonte et al., 2008; Pilot and Lecuit, 2005). Invagination of the ventral midline of the neural plate leads to the formation of the median hinge point (MHP), a dynamic event which elevates the neural folds, ultimately facilitating their apposition and fusion at the dorsal midline (Colas and Schoenwolf, 2001; Eom et al., 2013; Ybot-Gonzalez et al., 2007). Neural tube closure (NTC) defects (e.g. exencephaly, spina bifida) result in the brain and spinal cord when hinge points do not form correctly (Copp and Greene, 2013; Eom et al., 2013; Ybot-Gonzalez et al., 2007).

Although invagination events are fundamental to the generation of tubular organs, the cellular and molecular mechanisms underlying their formation are not well understood (Lecuit and Lenne, 2007). As with other invagination events, MHP formation requires dynamic and polarized changes in cell shape, which can be induced by cytoskeletal reorganization, polarized endocytosis, cell cycle kinetics or a combination of these behaviors (Colas and Schoenwolf, 2001; Eom et al., 2013; Lee and Harland, 2010; Nishimura et al., 2012; Suzuki et al., 2012). Recent studies suggest that cell-shape changes at the MHP can also result from junctional remodeling, which occurs in the planar axis during convergent extension, as it narrows and elongates the neural plate (Eom et al., 2013; Nishimura et al., 2012). However, many polarized cell behaviors involved in MHP formation (apical constriction, basal nuclear translocation, changes in apicobasal length), occur along the apicobasal axis and play a role for the apicobasal polarity pathway in MHP formation and NTC has recently begun to emerge (Eom et al., 2013; Klezovitch et al., 2004; Lee and Harland, 2010).

The apicobasal polarity pathway establishes tight junctions and segregates the apical and basolateral compartments of epithelial cells (Margolis and Borg, 2005). This segregation is mediated in part by apical polarity proteins such as the PAR [PAR3-atypical protein kinase C (aPKC)–PAR6] and the Crumbs (Crumbs–PAR6–Stardust) complexes. These associate with, and maintain tight junction integrity by excluding basolateral proteins such as Lethal giant larva (LGL), Disks large and Scribble from the apical compartment (Bilder, 2004; Yamanaka et al., 2006, 2003). Although this segregation is important for the maintenance of a stable epithelium, tissues undergoing three-dimensional shape changes require modulations of the apicobasal polarity pathway to create a flexible and dynamic epithelium (Andrew and Ewald, 2010; Eom et al., 2012, 2011; Martin-Belmonte et al., 2008; Mostov et al., 2003).

An interesting and unresolved question is how dynamic modulations of the apicobasal polarity pathway are achieved in vivo, and how this pathway interacts with the signaling cascades (e.g. SHH, BMP, WNT) that govern early tissue specification and morphogenesis. In this study, we have explored the interactions between the apicobasal polarity pathway and the TGFβ and BMP signaling cascades in the amniote midbrain. Mutant mice with defective BMP or TGFβ signaling (Bmpr2−/−, Bmp5−/−, Bmp7−/−, Smad5−/−, Nog−/−, Rgsna−/−, Tgf1−/−, Skir+) frequently display cranial NTC defects, which cannot be attributed to defects in cell fate specification alone (Castrano and Mishina, 2009; Chang et al., 1999; McMahon et al., 1998; Solloway and Robertson, 1999; Berk et al., 1997; Harris and Juriloff, 2010; Kuang et al., 2006; Niederkolfer et al., 2004). Instead, evidence from diverse systems has begun to accumulate suggesting that TGFβ and BMP signaling directs tissue shape, size and three-dimensional morphogenesis by regulating epithelial organization (Eom et al., 2012, 2011; Gibson and Perrimon, 2005; von der Hardt et al., 2007; Shen and Dahnmann, 2005; Zavadil and Böttger, 2005).

We have recently shown that BMP signaling maintains the stability of the neural epithelium by regulating biochemical interactions between phosphorylated SMAD1, SMAD5 or SMAD8 (pSMAD1,5,8) and the PAR polarity protein complex. BMP attenuation reduces these interactions and is required for the induction of cell-shape changes at the MHP (Eom et al., 2011).
Sustained BMP blockade can disrupt epithelial organization and induce epithelial-to-mesenchymal transitions (EMTs) in diverse systems (Gibson and Perrimon, 2003; Shen and Dahmann, 2005). However, BMP induction by low BMP signaling at the ventral midline of the neural plate is not accompanied by overt epithelial disorganization. Instead, polarity is modulated along the apicobasal axis by graded pSMAD1,5,8 activity, with neural progenitors cycling between high and low BMP states as they progress through the cell cycle while undergoing interkinetic nuclear migration (Eom et al., 2011, 2013; Sauer, 1935). Such a cyclic BMP signal could potentially mediate the cell-shape changes required for NTC without disrupting epithelial organization, but how is such a complex and cell-cycle-dependent BMP profile established?

In this study, we show that a cell-cycle-dependent TGFβ signal modulates BMP activity along the apicobasal axis of the neural plate. BMP–TGFβ antagonism determines whether pSMAD1,5,8 or TGFβ-activated receptor (r)-SMADs (phosphorylated SMAD2 or SMAD3, pSMAD2,3) associate with the PAR complex at apical junctions. We report for the first time that BMP and TGFβ can modulate BMP activity along the apicobasal axis by graded pSMAD1,5,8 activity, with neural progenitors cycling between high and low BMP states as they progress through the cell cycle while undergoing interkinetic nuclear migration (Eom et al., 2011, 2013; Sauer, 1935). Such a cyclic BMP signal could potentially mediate the cell-shape changes required for NTC without disrupting epithelial organization, but how is such a complex and cell-cycle-dependent BMP profile established?

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To determine their subcellular localization, we colabeled pSMAD proteins with markers of chromatin (DAPI), mitotic chromatin (pHH3) and the cytosol (acetylated tubulin). Given that acetylated-tubulin was predominantly distributed along the cell cortex, we additionally used the absence of DAPI or pHH3 staining to determine the cytosolic distribution of pSMAD proteins. These data demonstrate that, during interphase, pSMAD expression was mainly nuclear, with 1.44× higher levels of pSMAD2,3, relative to pSMAD1,5,8 (pSMAD1,5,8 versus pSMAD2,3: 1.45×10⁵±1.02×10⁴ versus 1.01×10⁵±5.26×10³; P≤0.001; yellow arrowheads, Fig. 1B–H⁠; interphase insets labeled i, Fig. 1F"; H", F"; IJ, Fig. S1C–F). By contrast, significant pools of pSMAD proteins were detected in the cytosol during mitosis, although there was 3.7× as much pSMAD1,5,8 as pSMAD2,3 (pSMAD1,5,8 versus pSMAD2,3: 1.81×10³±6.29×10² versus 4.83×10³±1.15×10³; P≤0.001; white arrowheads, Fig. 1A–H⁠; insets labeled m, Fig. 1F"; H", F"; IJ, Fig. S1C–F).

It should be noted that relatively low levels of pSMAD2,3 and high levels of pSMAD1,5,8 also colocalized with the pHH3+ mitotic chromatin (white arrowheads, Fig. 1A–H⁠; insets labeled m, Fig. 1F"; H"; F"; IJ, Fig. S1C–F) (Eom et al., 2011). However, given that mitotic cells are thought to be transcriptionally silent, the functionally relevant pools of pSMAD during this cell cycle phase are likely to be cytosolic (chromatin-independent) as we show below (Caravaca et al., 2013; Michelotti et al., 1997). Taken together, these results suggest that although pSMAD1,5,8 and pSMAD2,3 are modulated in opposite directions in tandem with the cell cycle, pools of pSMAD2,3 and pSMAD1,5,8 are detected in the cytosol during mitosis and are shuttled into the nucleus during interphase.

The opposite effects of BMP and TGFβ signaling in hinge point induction

TGFβ signaling is necessary and sufficient for inducing the MHP

Cell cycle progression in the neural plate is asynchronous and results in a mosaic pattern of pSMAD1,5,8 expression. Together with the results presented in Fig. 1, this suggests that BMP activity at the MHP is low, cyclic and mosaic, features which are essential for MHP formation (Eom et al., 2011, 2012). But how is such a complex BMP profile established during NTC? Given the complementary modulation of pSMAD1,5,8 and pSMAD2,3 in the neural plate, we asked whether TGFβ and BMP signaling might cross-repress each other and affect MHP formation in opposite directions (Fig. 1; Fig. S1).

Unlike controls, constitutively active (ca) Smad2 misexpression at neural plate stages [Hamburger–Hamilton (HH) stage 4–5] induced ectopic hinge points in lateral neural plate when examined at 3 h to 2 days [embryonic day (E)1–E3] after electroporation (Fig. 2A–B⁠; data not shown). Conversely, TGFβ blockade achieved by Lefty2 misexpression at the ventral midline flattened the endogenous MHP, suggesting that TGFβ signaling is both necessary and sufficient for MHP formation (Fig. 2C–D⁠) (Schier, 2003; Shen, 2007). Together with previous work, these observations demonstrate that TGFβ and BMP signals regulate MHP formation in opposite directions (summarized in Fig. 2E–G).
Interestingly, ventral midline TGFβ manipulations, which disrupted MHP formation, also failed to elevate and/or fuse the neural folds and led to NTC defects in 24 out of 26 cases (Fig. S2A–D). These results stress the importance of TGFβ signaling in MHP formation, and the role of MHP formation in NTC.
Cross-repression between TGFβ and BMP signaling cascades

The opposite effects of BMP and TGFβ signaling on MHP formation led us to ask whether the two pathways cross-repressed each other. Western blot analyses of electroporated whole-cell lysates showed that caSmad2 misexpression reduced pSMAD1,5,8 levels, whereas Lefty2 and BMP4 misexpression increased it (Fig. 2H). Conversely, caSmad2 and Noggin misexpression increased pSMAD2,3 levels, whereas Lefty2 and BMP4 misexpression did the opposite (Fig. 2I). Given that these changes were not accompanied by reductions in total (phosphorylated and non-phosphorylated) SMAD levels, they are likely to represent a reduction in SMAD phosphorylation (Fig. 2J). Thus, BMP and TGFβ induce the phosphorylation of their own r-SMADs, while blocking the ability of the other pathway to do so (summarized in Fig. 2K) (Miyazono et al., 2010).

TGFβ signaling regulates cell shape and is a partial regulator of cell fates at the MHP

We next explored the cell behaviors regulated by TGFβ signaling during hinge point formation. Hinge point formation in the neural plate is known to involve apical constriction and the basal localization of cell nuclei (defined in Materials and Methods, Table 1) (Eom et al., 2011; Lee et al., 2007). Ectopic hinge points induced by caSmad2 misexpression displayed non-autonomous, basal nuclear localization (arrowhead, Fig. 3A–B′) either alone, or in conjunction with the apical constriction of mitotic cells (Figs 3A–D, 3A–B″, Table 1). Both phenotypes were identical to those displayed by the endogenous MHP and by ectopic hinges induced by BMP blockade (Eom et al., 2011).

To determine whether caSmad2 misexpression specifically induced the MHP or generic invaginations of the neural plate, we...
Table 1. Identification and quantification of ectopic hinge points

<table>
<thead>
<tr>
<th>DNA electroporated</th>
<th>Age of</th>
<th>Total no. of</th>
<th>Average ratio of apicalotbasal</th>
<th>Average apical</th>
<th>No. of cells per site displaying</th>
<th>Total no. of</th>
<th>Number of cells/site displaying</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>harvest</td>
<td>brains</td>
<td>edge to vertex in site</td>
<td>constriction</td>
<td>PAR3+ puncta</td>
<td>cells</td>
<td>apical LGL – GFP</td>
</tr>
<tr>
<td>GFP</td>
<td>HH7–HH8</td>
<td>17</td>
<td>0.89±0.019</td>
<td>85</td>
<td>0.43±0.957</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Hh12–HH14</td>
<td>5</td>
<td>0.97±0.004</td>
<td>30</td>
<td>0.51±0.017</td>
<td>20</td>
<td>10.4±0.789</td>
<td>30</td>
</tr>
<tr>
<td>Lgl1–GFP</td>
<td>HH7–HH8</td>
<td>8</td>
<td>0.87±0.043</td>
<td>40</td>
<td>0.32±0.021</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>Hh12–HH14</td>
<td>4</td>
<td>0.96±0.002</td>
<td>24</td>
<td>0.52±0.008</td>
<td>15</td>
<td>10.9±0.379</td>
<td>32</td>
</tr>
<tr>
<td>caSmad2</td>
<td>HH7–HH8</td>
<td>17</td>
<td>0.57±0.019</td>
<td>68</td>
<td>0.3±0.955</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td>Hh12–HH14</td>
<td>4</td>
<td>0.7±0.033</td>
<td>20</td>
<td>0.33±0.025</td>
<td>20</td>
<td>18.4±0.307</td>
<td>40</td>
</tr>
<tr>
<td>caSmad2</td>
<td>HH7–HH8</td>
<td>6</td>
<td>0.59±0.019</td>
<td>30</td>
<td>0.24±0.022</td>
<td>32</td>
<td>50</td>
</tr>
<tr>
<td>Lgl1–GFP</td>
<td>HH6</td>
<td>0.7±0.028</td>
<td>36</td>
<td>0.28±0.029</td>
<td>25</td>
<td>17.8±0.552</td>
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<tr>
<td>caSmad2</td>
<td>HH7–HH8</td>
<td>6</td>
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<td>18.4±0.307</td>
<td>40</td>
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Embryos were electroporated at HH4–HH5, and harvested at either HH7–HH8 or HH12–HH14. The criteria for distinguishing between normal topological variations of the apical surface and ectopic hinge points are detailed in the Materials and Methods. Graphs for apical constriction and nuclear localization at HH7 are presented in Fig. 3C–D. The average nuclear location per site is measured relative to the the apical surface. NA, not applicable. Results are presented as mean±s.e.m.

Next determined whether caSmad2 manipulations also induced ventral midline cell fates at ectopic hinge points. Unlike controls, early (HH4–HH5) caSmad2 electroporations induced robust expression of FOA2, an exclusive marker of the MHP and the future floor plate (Fig. 3E–F′) (Bayly et al., 2012; Sasaki and Hogan, 1993). By contrast, late (HH8–HH9) caSmad2 electroporations failed to induce FOA2 expression (data not shown). Finally, early or late caSmad2 electroporations did not induce ventral midline markers such as LMX1A or LMX1B, or SHH (Fig. S2E–H′). Although TGFβ signaling is a partial and transient regulator of ventral midline cell fates, the robust induction of FOA2 suggests that it specifically induces the MHP (Bayly et al., 2012). Interestingly, early or late stage BMP manipulations do not induce ventral midline cell fates (Eom et al., 2012, 2011). Thus, although BMP and TGFβ signaling regulate shape changes at the MHP in opposite directions, they do not affect ventral midline cell fate specification in a complementary manner.

TGFβ signaling directs MHP formation by regulating tight junction integrity

Given the apicolateral nature of TGFβ-induced cell behaviors (apical constriction, basolateral nuclear localization) at ectopic hinge points, we next asked whether TGFβ signaling interacted with apical (PAR3) and basolateral (LGL) polarity proteins during MHP formation. Because suitable LGL antibodies are unavailable, we visualized the apical and basolateral compartments in neural cells by combining PAR3 immunostaining with non-phenotypic electroporations of Lgl1–GFP (1 µg/µl) (Eom et al., 2011). Such non-phenotypic Lgl1–GFP-electroporated controls displayed smooth apical contours and the complete segregation of apical (PAR3+) and basolateral (LGL1–GFP+) compartments (Fig. 4A–A′). By contrast, caSmad2 and Lgl1–GFP co-electroporations induced ectopic hinges accompanied by the loss of apical PAR3, the ectopic incursion of Lgl1–GFP into the apical compartment, and PAR3 and LGL1–GFP overlap (arrowheads, Fig. 4C–C′; Table 1). We noted that when present, endosomes in control brains were not PAR3+ (yellow arrowheads, Fig. 4A–B′). By contrast, caSmad2 electroporations resulted in ectopic endosomes in the cytosol that were both PAR3+ and EAA1+ (Fig. 4C–D′). The removal of PAR3+ apical membranes into the cytosol through endocytosis might partially explain how apical constriction is accomplished by TGFβ signaling (Eom et al., 2011; Lee and Harland, 2010).

The PAR complex is known to associate with, and stabilize, tight junctions by excluding Lgl from the apical compartment (Bilder, 2004; Dollar et al., 2005; Plant et al., 2003; Yamanaka et al., 2003; Zavadil and Böttiger, 2005). The paralateral transfer of LGL–GFP into the apical compartment, and the removal of apical PAR3 into endosomes thus suggests that TGFβ misexpression disrupts tight junction integrity in the neural epithelium by regulating the subcellular localization of apicolateral polarity proteins (Plant et al., 2003; Yamanaka et al., 2003).

TGFβ pathway members biochemically interact with the PAR complex at apical junctions

In previous work, we demonstrated that BMP signaling stabilized tight junctions through biochemical interactions between pSMAD1,5,8 and the PAR complex (Eom et al., 2011). Given the opposite effect of caSmad2 on tight junctions, we next asked whether pSMAD2,3 might also biochemically associate with the PAR complex. Co-immunoprecipitation and western blotting of whole-cell lysates from cells that had been electroporated with EGFP revealed that pSMAD2,3 associated with all members of the PAR complex (Bayly et al., 2012; Sasaki and Hogan, 1993). To further confirm that pSMAD2,3 associates with the PAR complex at apical junctions, we combined pSMAD2,3 immunohistochemistry with low (1 µg/µl) non-phenotypic Par3 (Eom et al., 2011). Such non-phenotypic Par3−GFP co-electroporations induced robust expression of FOXA2, an exclusive marker of the MHP and the future floor plate (Fig. 3E–F′) (Bayly et al., 2012; Sasaki and Hogan, 1993). By contrast, late (HH8–HH9) caSmad2 electroporations failed to induce FOA2 expression (data not shown). Finally, early or late caSmad2 electroporations did not induce ventral midline markers such as LMX1A or LMX1B, or SHH (Fig. S2E–H′). Although TGFβ signaling is a partial and transient regulator of ventral midline cell fates, the robust induction of FOA2 suggests that it specifically induces the MHP (Bayly et al., 2012). Interestingly, early or late stage BMP manipulations do not induce ventral midline cell fates (Eom et al., 2012, 2011). Thus, although BMP and TGFβ signaling regulate shape changes at the MHP in opposite directions, they do not affect ventral midline cell fate specification in a complementary manner.

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proteins displayed variable expression in the mitotic cytosol (see Fig. S3A–A‴). However, 80% of mitotic cells (n=20 out of 25) unexpectedly displayed a partial overlap between pSMAD2,3 and the PAR3–GFP domains, frequently forming a PAR3–GFP-negative ‘sub-apical cap’ immediately basal to the apical domain (Fig. 4I–I‴; see also, Fig. 1B–B‴, right, white arrowhead). By contrast, pSMAD1,5,8 fully encompassed apical PAR3–GFP expression and additionally extended throughout the cytosol (Fig. S3D–D‴; Fig. 1D–D‴) (Eom et al., 2011). These data confirm the co-immunoprecipitation and western blotting observations that suggest greater association of pSMAD1,5,8 with apical junctions by regulating their phosphorylation in opposite (Fig. 5A–A‴). BMP4 and Noggin misexpression mimicked the effects of Lefty2 and caSmad2, respectively, and suppressed PAR complex and the r-SMADs for the other pathway. This was demonstrated by caSmad2 misexpression, which increased PAR3–pSMAD2,3 interactions, and reduced PAR3–pSMAD1,5,8 interactions, whereas Lefty2 misexpression did the opposite (Fig. 5A–B‴). BMP4 and Noggin misexpression mimicked the effects of Lefty2 and caSmad2, respectively, and suppressed PAR3–PAR-complex interactions in the cytosol while increasing pSMAD1,5,8–PAR-complex interactions (Fig. 5C–F‴).

We next electroporated non-phenotypic levels of Par3–GFP either alone (controls) or with caSmad2 or Noggin and combined these with the immunodetection of pSMAD proteins in mitotic cells as described above (Fig. S3A–F‴; Fig. 4I–I‴). Owing to the cell-cycle-dependent modulation of pSMAD proteins, control and experimental brains displayed variable overlap between PAR3–GFP and pSMAD proteins in the cytosol. However, compared to controls, caSmad2 and Noggin-induced ectopic hinge points displayed increased overlap of PAR3–GFP with pSMAD2,3, but not with pSMAD1,5,8, thus confirming the results obtained by co-immunoprecipitation and western blotting (Fig. S3A–F‴; Fig. 5). Interestingly, neither ligand affected PAR3 or total SMAD protein levels, although both altered pSMAD levels (Figs 2J and 5G).
form of BMP and TGFβ function in the cytosol (summarized in Fig. 5I).

pSMADs biochemically interact with LGL

We noted that pools of cytosolic pSMAD proteins were not associated with apical polarity proteins (Fig. 4I–I‴; Fig. S3A–A‴, D–D‴) (Eom et al., 2011). We therefore asked whether the non-junctional cytosolic pSMAD pools might also play a role in modulating epithelial organization. We focused on potential pSMAD interactions with LGL because LGL can induce ectopic hinge points in lateral neural plate, compete with PAR3 for PAR6 binding, and shuttle between the apical compartment and the cytosol in a BMP- and TGFβ-dependent manner (Fig. 4A–D′) (Betschinger et al., 2003; Dollar et al., 2005; Eom et al., 2011; Plant et al., 2003; Yamanaka et al., 2003).

We confirmed that low, non-phenotypic levels of electroporated Lg1–GFP remained confined to the cytosol of control brains (Fig. 6A) (Eom et al., 2011). Cytosolic lysates prepared from such control electroporations unexpectedly revealed that LGL–GFP can biochemically interact with pSMAD2,3 and pSMAD1,5,8 in the neural plate (Figs 5H and 6B,C). Low levels of electroporated Lg1–GFP combined with pSMAD immunohistochemistry also demonstrated a greater degree of LGL1–GFP overlap with pSMAD2,3 than with pSMAD1,5,8 in the mitotic cytosol, confirming the co-immunoprecipitation and western blotting results (Fig. 6B,C).

Interestingly, compared to controls, caSmad2 misexpression reduced LGL1–GFP and pSMAD2,3 interaction, whereas Lefty2 misexpression did the opposite (Fig. 6D,E). By contrast, caSmad2 misexpression increased pSMAD1,5,8–LGL1 interactions, whereas
Lefty2 misexpression produced the opposite results (Fig. 6F,G). BMP4 misexpression mimicked the effects of Lefty2 misexpression by increasing pSMAD2,3–LGL1–GFP interactions and reducing pSMAD1,5,8–LGL1–GFP interactions (Fig. 6H,I). Noggin misexpression did the opposite and mimicked the effects of caSmad2 misexpression by reducing pSMAD2,3–LGL1–GFP interactions and increasing pSMAD1,5,8–LGL1–GFP interactions (Fig. 6J,K). We next examined the colocalization of pSMAD proteins in mitotic cells following non-phenotypic Lgl1–GFP electroporations either alone, or combined with caSmad2, Lefty2, BMP4 or Noggin manipulations (Fig. 6D–K). Unlike controls, caSmad2 and Noggin induced ectopic hinges, and displayed reduced pSMAD2,3–LGL1–GFP sequences overlapping in the cytosol and increased LGL1–GFP–pSMAD1,5,8 overlap, whereas BMP4 and Lefty2 misexpression produced the opposite results (Fig. 6B–K). These observations corroborate the co-immunoprecipitation and western blotting results and show for the first time that BMP and TGFβ signaling in regulating epithelial organization during NTC. Interestingly, caSmad2 and Noggin induced ectopic hinges, and caused a substantial and non-cell-autonomous upregulation of SLUG (also known as SNAI2), a transcription factor that can induce the EMT transcriptional cascade (Thiery and Sleeman, 2006). SLUG upregulation by caSmad2 and Noggin misexpression was accompanied by the downregulation and/or mislocalization of NCAD (CDH2), an adherens junction constituent involved in maintaining epithelial organization (Fig. 5F). Taken together, these results suggest that BMP and TGFβ signaling might regulate epithelial organization during NTC through canonical, as well as non-canonical mechanisms.

**DISCUSSION**

In this study, we describe a novel mechanism by which spatiotemporally dynamic morphogen gradients can be established during embryonic development. We show that cross-regulation between BMP and TGFβ pathways results in complementary, cell-cycle-dependent pSMAD1,5,8 and pSMAD2,3 modulation along the apicobasal axis of the neural plate (Fig. 7). Functionally discrete pSMAD pools are seen in the mitotic cytosol, which differentially interact with apical and basolateral polarity proteins in a ligand-dependent manner. These
interactions help establish a dynamic epithelium where polarized cell behaviors (apical constriction, basal nuclear migration or retention, polarized endocytosis) can mediate cell-shape changes along the apicobasal axis. Mediolateral modulation of pSMAD1,5,8 regulates the spatial specificity of such changes, ensuring that the MHP forms at the ventral midline where BMP activity is at its lowest and TGFβ activity is high.

Non-canonical BMP–TGFβ interactions modulate apicobasal polarity during MHP formation

A role of TGFβ signaling in the disruption of epithelial organization is well established (Zavadil and Böttinger, 2005). Evidence for a role for BMP signaling in regulating epithelial organization in a variety of tissues and morphogenetic events, including NTC, has also recently begun to emerge (Eom et al., 2013; von der Hardt et al., 2007; Khalsa et al., 1998; Ray and Wharton, 2001; Sela-Donenfeld and Kalcheim, 1999; Shoval et al., 2007). In this study, we show that although BMP and TGFβ regulate epithelial organization in opposite directions, they converge on common downstream signaling mechanisms, which involve regulating the subcellular localization of LGL and PAR complex proteins and modulating their interactions.

TGFβ signaling can disrupt epithelial organization and induce EMT in many tissues using the transcription-dependent upregulation of EMT genes such as Snail, Slug, Zeb1 and Zeb2 (Nieto, 2002; Thiery and Sleeman, 2006; Zavadil and Böttinger, 2005). The current study suggests that similar TGFβ-mediated
Cell-cycle-dependent BMP and TGFβ apicobasal polarity interactions establish a dynamic epithelium during NTC

Sustained TGFβ misexpression or BMP blockade results in EMT and abnormal epithelial reorganization, including the formations of ectopic cysts or rosettes (Eom et al., 2012; Gibson and Perrimon, 2005; Shen and Dahmann, 2005). By contrast, increased BMP or reduced TGFβ signaling flattens the neural epithelium presumably because increased pSMAD1,5,8 at apical junctions make the epithelium inflexible and incapable of executing morphogenetic bending. However, the wild-type neural plate occupies neither end of this spectrum and forms a dynamic epithelium capable of undergoing shape changes without undergoing EMT.

We suggest that such a dynamic epithelium is created by cyclic TGFβ and BMP activity, which allows neural cells to shunt between full to partially polarized states as they progress through the cell cycle. When partially polarized, tight junctions are ‘floppy’ and permit the incursion of LGL into the apical compartment, and the removal of apical PAR3 into the cytosol by endocytosis. This type of junctional remodeling results in the removal of apical membranes into endosomes and might partially explain apical constriction, as it does in bottle cells during Xenopus gastrulation (Lee and Harland, 2010). The compromised polarity might also explain the basal retention and/or migration of nuclei because LGL misexpression, which induces PAR3 endocytosis and apical constriction, also induces basal nuclear migration or retention at ectopic hinge points, possibly through the regulation of cell cycle kinetics or the cellular cytoskeleton (Eom et al., 2011).

In the type of dynamic neural epithelium envisaged above, cells would undergo repeated cycles of shape changes, but return to a stable epithelial state in the interim. Given that cell cycle progression in the neural plate is asynchronous, adjacent cells would experience different levels of BMP signaling, as demonstrated by the mosaic expression of pSMAD1,5,8 when viewed apically (Eom et al., 2011). This would permit sets of MHP cells to change shape over time, while adjacent cells would remain integrated within the neural epithelium. Such a neural plate could be bent and shaped by BMP–TGFβ interactions without generalized epithelial disruption.

Despite their reciprocal modulation along the apicobasal axis, both types of pSMAD proteins undergo nucleocytosolic shuttling along the apicobasal axis and are mainly present in the cytosol during mitosis. Thus, shifts in polarity, which facilitate shape changes might occur during mitosis and involve specific mitotic phases. But how polarity shifts might affect events during subsequent phases of the cell cycle is not understood, although novel mechanisms that correlate junctional remodeling with cytokinesis have been recently proposed (Denes et al., 2015).

In vitro studies also demonstrate that when exposed to appropriate signals, epithelial cells grown in three-dimensional cultures can organize into complex repeated structures, such as branched tubules, by combining polarity modulations with cell cycle kinetics (Ewald et al., 2008; Mostov et al., 2003; Zegers et al., 2003). The current study provides mechanistic insight into how such signals might be established and modulated in a cyclic manner.
in vivo. Such dynamic modulation of morphogen levels might underlie not just three-dimensional tissue morphogenesis, but also the production of repeated tissue patterns (e.g. rhombomeres, somites). Given that such morphogen gradients could also simultaneously regulate other aspects of pattern formation (e.g. cell fate specification, cell proliferation, differentiation) their broad range of actions might ensure that tissue patterning occurs in a coordinated manner in three dimensions.

**MATERIALS AND METHODS**

**Chick embryos**

All experiments were conducted in accordance with the guidelines provided by the institutional IACUC. Fertilized Leghorn eggs (Ideal Poultry, Texas) were incubated at 38°C in a humidified forced-draft incubator. Embryos were staged according to Hamburger and Hamilton (1951).

**Expression vectors**

In vivo gene expression was driven by the pXex, pEFX, pMes or pCS2 expression vectors (Agarwala et al., 2001; Johnson and Krieg, 1994; Swartz et al., 2001). The construction of EGFP, membrane targeted EGFP (mEGFP, LgI–GFP, Par3–GFP, BMP4, Noggin, Lefty2 and caSmad2 expression vectors has been described previously (Bayly et al., 2007; Bisgrove et al., 1999; Eom et al., 2011). All experiments were conducted in accordance with biosafety and recombinant DNA protocols approved by the Institutional Biosafety Committee.

**In ovo electroporation**

1-4 μg/μl DNA was electroporated into HH4–HH9 midbrains according to previously established protocols (Bayly et al., 2007; Brown et al., 2012a,b). Electroporations were classified as early (HH4–HH5) or late (HH8–HH9) based on whether they were conducted prior to, or after midbrain neural tube closure at HH8 (Colas and Schoenwolf, 2001). Embryos were harvested between HH7 and E3.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Eom et al., 2011). Embryos were stained with antibodies against pH3 (1:500; Upstate, NY, # 07-424), PAR3 (1:500; Upstate, #07-330), pSMAD1,5,8 (1:1000; Cell Signaling Technology, MA, # 9511S), pSMAD2,3 (1:100; Cell Signaling Technology, # 9743S), EEA1 (1:30; BD Biosciences, NJ, # 610456), FOXA2 (1:200; DSHB, IA, #4E7), SHH (1:200; DSHB, #5E1), LMX (1:200; DSHB, # 50.5A5), acetylated tubulin (1:500; Life Technologies, # 32-2700), N-CAD (1:100; DSHB, IA, # 6B3), SLUG (1:50; DSHB, IA, #62.1E6) and GFP (1:1000; Molecular Probes, CA, # A1120). Alexa-Fluor-conjugated secondary antibodies were used for fluorescence detection (Afonso and Henrique, 2006). All antibodies used in the current study were validated for use in chicks in previous studies (Eom et al., 2011, 2012; Afonso and Henrique, 2006; Liem et al., 1995; Nagy et al., 2012; San Miguel-Ruiz and Letourneau, 2014).

**Imaging**

Confocal images were obtained with an Olympus IX51 spinning disc microscope and data analyses were carried out with Slidebook Pro (3I, CO). Images are presented as single 0.5–0.8-μm thick optical sections.

**pSMAD fluorescence intensity measurements**

pSMAD2,3 and pSMAD1,5,8 immunohistochemistry was combined with nuclear (DAPI) or cytosolic (acetylated-tubulin-positive) markers in HH6–HH7 midbrains, a period which overlapped with MHP formation (see Fig. 1A–‘F’). Confocal midbrain images were collected as described above, and a sampling box subtending 90 μm×90 μm was placed across the MHP. pSMAD fluorescence was measured in 35 cells taken from six brains according to previously established protocols (McCloy et al., 2014; Burgess et al., 2010). Briefly, the strong cortical expression of acetylated tubulin staining along the cell periphery was used to draw cell outlines and DAPI staining was used to draw nuclear or chromatin outlines. Whole-cell and nuclear (chromatin in mitotic cells) pSMAD fluorescence was measured using Fiji software. Cytosolic pSMAD fluorescence was determined by computing the difference between the two measurements. The corrected cell fluorescence for each cellular compartment was obtained by applying a correction for background fluorescence as described in previous studies (McCloy et al., 2014; Burgess et al., 2010). Pair-wise comparisons of pSMAD fluorescence were made as follows: total (cytosolic+nuclear) pSMAD1,5,8 for mitotic versus interphase cells; total pSMAD2,3 for mitotic versus interphase cells; cytosolic pSMAD1,5,8 versus pSMAD2,3 in mitotic cells; nuclear pSMAD1,5,8 versus pSMAD2,3 in interphase cells. The statistical significance of each pair-wise comparison was determined by the Mann–Whitney test (see Fig. 1J for details).

**Western blotting and immunoprecipitation**

Western blotting and immunoprecipitations were carried out using previously established protocols (Eom et al., 2011).

**Whole-cell lysates**

Midbrain lysates were electroporated at HH4–HH5 with EGFP, caSmad2, Lefty2, Noggin, BMP4, and non-phenotypic concentrations of LgI–GFP (1 μg/ml), either alone or in combination with caSmad2 (Eom et al., 2011). Whole-cell lysates were prepared in triplicate at HH12–H14 (late E2 to early E3) from electroporated regions identified in whole embryos under a fluorescent stereomicroscope. The fluorescent region was micro-dissected under visual control and contained the entire apicobasal thickness of the neural plate. The late harvesting times were required for obtaining sufficient quantities of lysates from electroporated regions of the midbrain. The choice of this time point is validated in Table 1, which shows that hinge points induced at HH4–H5 and examined between HH7 and E3 are morphologically identical and resemble the endogenous MHP based on morphological, molecular and cellular criteria outlined in the ‗criteria for defining hinges‘ section below.

**Cytosolic extracts**

Cytosolic extracts were prepared from 40–50 HH12–HH14 midbrains collected as described above and processed according to the manufacturer’s instructions (Thermo Scientific, IL). The protein concentration was estimated using the BCA method (Thermo Scientific) (Smith et al., 1985). The purity of cytosolic extracts was checked by western blotting for α-tubulin (1:1000; Sigma, MO, #T6074) and histone H1b (1:500; Millipore, CA, #05.457) antibodies (Fig. 5H).

**Co-immunoprecipitation and western blotting**

Co-immunoprecipitations were performed in electroporated HH12–HH14 lysates (three biological replicates) incubated with 20 μg/ml of antibodies against PAR3, aPKC, PAR6, pSMAD1,5,8 or pSMAD2,3 (details as above) (Eom et al., 2011). Protein A/G agarose beads were used to precipitate the protein complexes, which were separated by SDS-PAGE. Western blotting was performed with antibodies against aPKC, PAR3, PAR6, pSMAD1,5,8, pSMAD2,3 (1:100 each, details as above), GFP (1:100; Molecular Probes, CA, #A1120), SMAD1 (1:50; Millipore, CA, #051459) or SMAD2 (1:100; Abcam, UK, #ab47083) antibodies, with the signal detected by ECL chemiluminescence (Thermo Scientific, IL). The anti-GFP antibody could not be used for immunoprecipitating LgI–GFP. As a result, co-immunoprecipitation and western blotting were not performed in reverse in Fig. 6.

**Cell measurements**

Differences in cell behaviors were quantified in midbrains electroporated at HH4–HH5 and harvested at HH7. To validate our tissue collection strategy for biochemical experiments, identical measurements were also made at HH12–HH14 (Table 1). Quantitative measurements were made with ImageJ according to established protocols using 15–100 cells from 4–17 brains for each quantitative measurement (Eom et al., 2011). Sample sizes for individual measurements are reported in Table 1. Data are presented as mean±s.e.m. Given that the data displayed non-normal distributions, the statistical significance of pair-wise comparisons between control and experimental groups was made using the Mann–Whitney test (Table 1).
Criteria for defining hinges
Apical constriction

Given that large caSmad2 electroporations produced tissue-level dysmorphologies, we confined all quantitative analyses to focal electroporations of two to nine cells in the lateral neural plate. A sampling box subtending 90 µm × 90 µm was centered over a mosaic cluster of electroporated cells in control or caSmad2-electroporated midbrains. A measure of apical constriction was obtained according to previously established protocols from midbrains stained with DAPI and PAR3 (Eom et al., 2011; Lee et al., 2007). Briefly, we measured the ratio of the apical width (aw) to the widest point or width (ww) of all electroporated mitotic cells with a clearly identifiable outline within the sampling box (e.g. Fig. 3A–B’; Table 1).

Basal nuclear localization

The nuclear location of midbrain cells with respect to the apical surface was determined in DAPI- and PAR3-stained sections according to previously established protocols (Table 1) (Eom et al., 2011). Given that basal nuclear localization was a non-autonomous event, we measured the location of all nuclei (electroporated and un-electroporated) within the sampling box as outlined above.

Vertex to edge ratio at hinge points

To distinguish between normal topological variances along the apical surface and tissue invagination due to hinge point induction, we centered a sampling box over a mosaic cluster of electroporated cells as described above. We measured the apico basal span of EGFP electroporated cells at the center and at the edge of the sampling box. For caSmad2 electroporations, we centered the sampling box at the vertex of a hinge point where the apico basal span was at its shortest. Given that the average vertex: edge apico basal span ratio was 0.89 for controls, we considered an invagination of the apical surface to be a hinge if the vertex: edge apico basal span ratio was ≤ 0.65 (Table 1).

PAR3+ puncta and apical LGL1

The number of cells displaying PAR3+ puncta per sampling box were counted in control and experimental embryos using the sampling and statistical paradigms described above (Table 1). In brains electroporated with low levels of Lgl1–GFP, or caSmad2 and Lgl1–GFP, we also counted the vertex to edge ratio at hinge points due to deregulation of the cyclin B-Cdc2/PP2A balance.

Deposited in PMC for release after 12 months.

This work was supported by the National Institutes of Health (NIH) (National Institute of Neurological Disorders and Stroke) [grant number R01 NS049091 to S.A.]. Deposited in PMC for release after 12 months.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S. Amarnath conducted and analyzed the experiments. S. Agarwala directed the research and wrote the manuscript.

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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.179192.supplemental

References


Science


Supplementary Figures

Fig. S1. pSMAD modulation and nucleo-cytosolic shuttling in the neural plate.
A-B': pSMAD2,3 (A, A') and pSMAD1,5,8 (B, B') expression along the mediolateral (m-l) axis of the neural plate. A and B show low-power views of the neural plate. Boxed areas in A, B circumscribe the MHP and are magnified in A' and B'. Arrowheads in A and B indicate the midline. a-b, apicobasal axis. Scale bars for A and B are shown in B, and for A' and B' in B'.
A, A': pSMAD2,3 expression is high throughout the mediolateral axis of the neural plate and the MHP.
B, B': pSMAD1,5,8 expression is modulated along the mediolateral axis and is low at the MHP.
C-F': Cell cycle dependent modulation of pSMAD2,3 (C-D') and pSMAD1,5,8 (E-F') in lateral neural plate at HH7. C and E show high power views of Figs. 1A and C (blue bracket). m: apical
location of pH3+ mitotic cells. i: location of interphase cells. Boxed areas in C and E are
magnified in D, D’ and F, F’ respectively. Scale bars for C, C’, and E, E’ are shown in C’, and
for D, D’ and F, F’ in D.
C-C’: pH3 (C) and DAPI (C’) co-labeling with pSMAD2,3 in lateral neural plate shows high
and nuclear expression of pSMAD2,3 during interphase.
D, D’: Low, and predominantly cytosolic (pHH3 and DAPI-negative) pSMAD2,3 expression in
mitotic cells in lateral neural plate.
E, E’: DAPI co-labeling shows low and nuclear expression of pSMAD1,5,8 during interphase.
F, F’: High and ubiquitous (chromatin-associated and chromatin-independent) expression of
pSMAD1,5,8 during mitosis (F, F’).
**Supplementary information**

**Table D**

<table>
<thead>
<tr>
<th>Treatment</th>
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</tr>
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<tr>
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**Figures E, E’, E”, E’’’**

- E: DAPI stained, GFP expression shown.
- E’: GFP expression.
- E”: LMX expression.
- E’’’: GFP and LMX co-expression.

**Figures F, F’, F”, F’’’**

- F: DAPI stained, caSmad2 expression shown.
- F’: GFP expression.
- F”: LMX expression.
- F’’’: GFP and LMX co-expression.

**Figures G, G’, G”, G’’’**

- G: GFP expression.
- G’: SHH expression.
- G”: GFP and SHH co-expression.

**Figures H, H’, H”, H’’’**

- H: DAPI stained, caSmad2 expression shown.
- H’: GFP expression.
- H”: SHH expression.
- H’’’: GFP and SHH co-expression.
Fig. S2A-C. TGFβ signaling affects neural tube closure
HH4-5 midline electroporations (yellow arrowheads) of EGFP (A), caSmad2 (B) and Lefty2 (C), examined at E2 after NTC has occurred. Unlike controls, caSmad2 and Lefty2 manipulations prevent dorsal midline fusion (white arrowheads), and induce neural tube defects. Note that sustained caSmad2 misexpression (B) also results in cell delamination (*) into the lumen of the neural tube, a hallmark of EMT (see also Fig. 3F).

D: Quantitation of neural tube closure defects (NTDs) following EGFP, caSmad2 and Lefty2 misexpression.

Fig. S2E-H**: caSmad2 misexpression is a partial regulator of ventral midline cells fates in the midbrain.
Ventral midline expression of LMX1A/B (E-F**) and SHH (G-H**) does not differ between controls (E-E'', G-G'') and midbrains electroporated with caSmad2 (F-F'', H-H'') at early (HH4-5) or late (HH8-9) stages (data not shown). This is in contrast to robust FOXA2 expression induced by early caSmad2 electroporations (Fig 3E-F**). Yellow arrowheads: ventral midline. White arrowheads: fused dorsal midline (E, G), or unfused (F, H) neural folds.
Fig. S3. TGFβ and BMP signaling affects PAR3-pSMAD interactions in opposite directions

A-A’’: Non-phenotypic electroporations of Par3-GFP display apically restricted localization of PAR3-GFP and co-localization with pSMAD2,3 (but not DAPI) in the mitotic cytosol (arrowheads). Inset (A’’): magnified image of the cell indicated by the left arrowhead.

B-C’’: Compared to controls (A-A’’), co-electroporations of Par3-GFP with caSmad2 (B-B’’) or Noggin (C-C’’) result in ectopic hinges (yellow arrowheads) which display cells with increased overlap between PAR3-GFP and pSMAD2,3 (white arrowheads). Inset (C’’): magnified image of the cell indicated by the left white arrowhead.

D-D’’ Non-phenotypic electroporations of Par3-GFP display apical overlap with pSMAD 1,5,8 (white arrowheads).

E-F’’’: Compared to controls (D-D’’), ectopic hinges (yellow arrowheads) induced by caSmad2 (E-E’’) or Noggin (F-F’’) electroporations display reduced overlap between Par3-GFP and pSMAD1,5,8 (white arrowheads).
Fig. S4. TGFβ and BMP signaling regulate EMT genes

A-C''': Compared to the controls (A-A''''), caSmad2 (B-B'''') and noggin (C-C'''')
misexpression induces ectopic hinges (arrowheads), which express SLUG, a regulator of the
EMT cascade. The white dashed lines demarcate the neural plate from head mesenchyme.

D-F''': Compared to controls (D-D''''), NCAD expression is downregulated and/or mislocalized
in caSmad2 (E-E'''') and Noggin (F-F'''') induced ectopic hinges (arrowheads).