The cessation of gastrulation
BMP signaling and EMT during and at the end of gastrulation

Sho Ohta,1,* Gary C. Schoenwolf1 and Gen Yamada2,*
1University of Utah School of Medicine; Department of Neurobiology and Anatomy; Salt Lake City, Utah USA; 2Division of Organ Formation IMEG (Institute of Molecular Embryology and Genetics); and Graduate School of Pharmaceutical Sciences Kumamoto University; Kumamoto, Japan

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*Correspondence to: Gen Yamada; Email: gensan@gpo.kumamoto-u.ac.jp and Sho Ohta; Email: shortail2006@yahoo.co.jp

**An integral component of gastrulation in all organisms is epithelial to mesenchymal transition (EMT), a fundamental morphogenetic event through which epithelial cells transform into mesenchymal cells. The mesenchymal cells that arise from epithelial cells during gastrulation contribute to various tissue rudiments during subsequent development, including the notochord, somites, heart, gut, kidney, body wall and lining of the coelom. The process of gastrulation has been the subject of several hundred scientific papers. Despite all that has been written, it is likely that what we currently know about gastrulation is still considerably less than what remains to be learned. One critical remaining question that we consider here is how does gastrulation cease at the right place along the body axis, and at the right time? In this commentary, we focus on the molecular mechanism for the cessation of gastrulation, using the chick embryo as a model system.**

**Avian Gastrulation**

It is appropriate that we first outline the process of gastrulation itself before describing its cessation. The avian embryo at the time the egg is laid consists of a disc containing two layers:1 the epiblast, facing the vitelline membranes and albumen, from which all of the embryonic tissues arise, and the hypoblast, facing the yolk, from which only extraembryonic tissues will arise. As the hypoblast spreads from the posterior to the anterior part of the blastodisc, progenitor cells appear between the epiblast and hypoblast. These cells constitute the primary mesoderm and are continuous with the first axial structure of embryo, the primitive streak. Primary mesoderm cells are first evident at the posterior margin of the area opaca, where it merges with the area pellucida 10 hours after the start of egg incubation.2 Later, epiblast cells (sometimes incorrectly called ectodermal cells) lateral to the primitive streak move into the groove of primitive streak and undergo an epithelial to mesenchymal transition (EMT) as the primitive streak elongates along the anterior-posterior axis of embryo (Fig. 1A and B). The combined movement of cells into the groove and their EMT to form mesenchymal cells is referred to in amniotes, including chick and mouse embryos, as the process of ingestion.

Anterior-posterior length of the primitive streak reaches a maximum by HH stage 4.3 Later, the primitive streak starts to regress, shortening along its anterior-posterior axis as the presumptive mesodermal cells ingress. Differentiation of rostral tissues, e.g., the neural tube, notochord, heart, and somites, occurs gradually and generally in an anterior-to-posterior sequence as the primitive streak regresses. By about HH stage 11–13, the remnants of the primitive streak consolidate at the posterior end of embryo into a bulb-like structure, “the tail bud” (Fig. 1C). The tail bud derives from part of the primitive streak and Hensen’s node and consists of a morphologically uniform mass of mesenchyme. The remnants of primitive streak lying at the level of the tail bud still can function to generate mesenchymal cells.
through a gastrulation-like ingressive cell movement, thereby contributing to caudal embryonic structures contained within the developing tail.4,5 As the tail bud extends caudally, the primitive streak remnant migrates to the ventral side of the tail bud and forms a thickened tissue called the ventral ectodermal ridge (VER) (Fig. 1D). The VER was first identified by Gruneberg and was suggested to be a signaling center that positively regulates tail bud outgrowth in a mechanism similar to that of the apical ectodermal ridge (AER) in regulation of the proximodistal outgrowth of the limb bud.6 However, evidence for a role for the VER is control - outgrowth of the limb bud.6,7 Whereas that of the apical ectodermal ridge tail bud outgrowth in a mechanism simi - by Gruneberg and was suggested to be a

Fig. 1D

The VER was first identified

Another major advance in understanding EMT was the discovery in 2002 of the Slug gene (now called Snail2) by Angela Nieto, working at the Cajal Institute, Madrid.23 Snail was first identified in Drosophila melanogaster as a transcription factor essential for the formation of mesoderm.23,24 Later, Snail2 loss-of-function experiments carried out in chick embryos showed that the Snail gene family has a role in triggering EMT.25 EMT is typically characterized by the loss of cell-cell adhesion and increased cell motility. Adhesion between neighboring cells depends on adherens junctions to which E-cadherin provides a structural support for cell-cell attachment. In the earlier steps in EMT, E-cadherin function is suppressed and Snail family genes expression is simultaneously upregulated in the epithelial cells committed to undergo EMT. Furthermore, Snail gain-of-function experiments demonstrated strong suppression of E-cadherin expression, and gel mobility assays provided evidence that Snail directly binds to the promoter region of E-cadherin.19 These experiments provided important insight into the molecular mechanism involved in the loss of cell-cell adhesion during EMT.

The members of TGFβ superfamily mentioned above can stimulate Snail family gene expression in different cellular contexts. For example, Bmp genes have been implicated in Snail family gene induction during EMT. In fact, BMP4/7 can induce Slug expression during neural crest formation in chick embryos.26 Recently, the binding site of SMAD1, which is a down-stream factor of BMP signaling, has been identified in the promoter region of Snail/Slug, suggesting that BMP signaling via SMAD elevates the transcription levels of Snail family genes.27 Although the role of BMP/SMAD signaling in regulating Snail/Slug expression during gastrulation is still unclear, the following evidence suggests that BMP signaling is involved

The members of TGFβ superfamily

in regulatory mechanisms that cease

Epithelial to Mesenchymal Transition (EMT)

In 1982, Elizabeth Hay and her graduate student, Gary Greenburg, working at Harvard University, first experimentally characterized EMT when they put a variety of epithelial tissue explants into collagen gels and noticed that mesenchymal-like cells spread into the gel from the grafted tissue.9 In the late 1980s, Jean-Paul Thiery, working at the Centre National de la Recherche Scientifique in France, found that rat bladder carcinoma cells in culture transformed into mesenchymal cells with a capacity for active migration.10 They were the first reports to link EMT as a central event in both embryonic tissue morphogenesis and cancer, but the mechanism underlying EMT remained unclear for many years.

It is now known that EMT is an orchestrated series of events involving alteration of cell-cell and cell-extracellular matrix (ECM) interactions. A typical epithelium is composed of a sheet of epithelial cells that are closely associated with one another. Individual epithelial cells interact with neighboring cells within the epithelium via adhesion molecules that inhibit the movement of individual cells out of the epithelial sheet (Fig. 2A and B).

To maintain the polarity and structure within the epithelium, the basal surface of epithelial cells is underlain by the basement membrane (Fig. 2C). In contrast to epithelial cells within an epithelial sheet, mesenchymal cells generally adhere to their neighboring cells less tightly, being loosely associated and completely surrounded by ECM. Thus, in comparison to epithelial cells, movement of mesenchymal cells within a tissue is considerably more dynamic. Once epithelial cells begin to undergo an EMT, the microenvironment surrounding these cells is dramatically altered, leading to destabilization of microtubules, disruption of cell-cell adhesion, and breakdown of the basement breakdown (Fig. 2D).

In the late 1980s, Hepatocyte growth factor (HGF) was thought to promote cancer cell movement and invasion. As an inducer of EMT, it was assumed that HGF was secreted from the surrounding connective tissues and resulted in EMT with the loss of cell-cell junctions and adhesion among neighboring epithelial cells.12,13 However recent studies have reported that HGF alone is not sufficient to induce the cascade of EMT-related genes in human kidney epithelial cells.14 In contrast, TGFβ is sufficient to induce such as cascade, including specific mesenchymal marker genes.15 In fact, TGFβ is essential for mesoderm and endoderm formation during gastrulation, and it is detected in the epiblast/ectodermal layer, as well as in the mesoderm underlying the primitive streak, in chick early development.16 In addition, recent studies have shown that several members of the TGFβ superfamily (e.g., Bone Morphogenic Protein: BMP, Activin, Nodal) play roles in mesoderm induction during gastrulation,17,19 and several TGFβ members have been implicated as major regulators of EMT in a number of systems (e.g., formation of the endocardial cushions, palatal shelf fusion).20,21

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in the regulation of EMT during gastrulation. (1) Bmp genes, such as Bmp2/4/7, are expressed in the primitive streak along its A-P axis.28 (2) pSmad, a downstream factor of BMP signaling, is also strongly expressed in the pre-migratory cells adjacent to the primitive streak, as well as in the lateral neural plate, a region that give rises to neural crest cells.17 (3) Bmpr1a-null mutant mice are unable to initiate gastrulation.29 (4) Bmp4 mutant mice display gastrulation defects and a failure to form sufficient mesoderm.30 (5) Similarly, Bmp2 mutant mice show abnormalities in the formation of both extraembryonic and embryonic mesodermal derivatives.31 (6) Smad1+/-:Smad5+/- double heterozygous mutant embryos also display decreased mesoderm.32,33 These findings suggest that EMT could be mediated by BMP signaling during gastrulation. Assuming such a possibility, attenuation of BMP signaling-dependent EMT at the time of cessation of gastrulation could function to arrest mesoderm formation. In the following paragraphs, we will describe the expression pattern of Bmps and their antagonist,
Figure 2. (A) General structure of epithelial cells. (B) Schematic of an adherens junction. E-cadherin mediates epithelial cell-epithelial cell binding at the adherens junction. (C) Schematic of epithelial cell and ECM binding. Integrin mediates the binding of epithelial cells to the ECM. (D) Schematic of the process of EMT. Typical epithelial cells tightly bind to neighboring cell through cell-cell junctions and cell-ECM binding. Once epithelial cells commit to undergo EMT, they lose cell-cell adhesion and the basement membrane becomes disrupted. Eventually, the committed cell acquires a migratory behavior. (E) Notably, Noggin expression is observed in the ventrolateral region of the tail bud mesoderm at HH stage 17. Basement membrane breakdown is also observed adjacent to the site where Noggin expression is faint (arrowhead). By HH stage 24, Noggin expression extends across the entire tail bud ventral mesoderm, and the basement membrane is formed beneath the entire ventral ectoderm.

Noggin, surrounding the primitive streak at the stage when gastrulation begins to cease.

Expression Patterns of Bmps and Noggin in Late Gastrulation

As described above, the remnants of the primitive streak persist at the ventral side of the tail bud, during the initial stages of tail bud elongation, as a thickened midline tissue called the VER. Furthermore, we mentioned that the epithelial cells in the VER continue to ingress and migrate to the ventral tail region.\(^5\) Examination of the expression of Bmp genes and their antagonists in the ventral tail bud, revealed intriguing dynamic patterns. Bmp4 is expressed in the ventral tail bud mesoderm underlying the VER,\(^5\) whereas Bmp7 is expressed predominantly in the ventral ectoderm and VER.\(^5\) In addition, Bmp2 is expressed at low levels in the ventral tail bud mesoderm, partially overlapping the domain of Bmp4 expression. Conservation and variation in the Bmp gene expression pattern among
species has been reported. In mouse embryos, Bmp2 expression is restricted to the VER in the developing tail, whereas Bmp4 is expressed in the ventral tail bud mesoderm. Although the expression patterns of these Bmp genes are essentially constant, the expression pattern of Noggin changes characteristically during the cessation process. Additionally, other BMP antagonists including Chordin, Gremlin and Follistatin are not detected in or adjacent to the VER. Noggin is predominantly expressed in the ventrolateral region of the tail bud at the stage when the ingressive cell movement is continuing to occur (Fig. 2E). However, cell ingression gradually attenuates as the Noggin expression domain starts to expand toward the midline ventral tail bud mesoderm. Finally, the ingressive movement ceases when Noggin expression extends across the entire ventral mesoderm (Fig. 2E). These dynamic gene expression patterns led us to hypothesize that the gastrulation-like ingressive cell movements from the VER arise via EMT induced by BMP signaling, in a manner similar to that occurring in gastrula stage embryos, and raised the notion that temporal/spatial Noggin expression is a key to suppress EMT in the VER at the right time and right place. This hypothesis is supported by changes in proteins expressed in the vicinity of the VER. Basement membrane breakdown occurs adjacent to the VER at the time when Noggin is faintly expressed at the midline of the ventral tail bud mesoderm, which indicates that epithelial cells in the VER continuously undergo EMT. However, as Noggin expression expands across the entire ventral mesoderm, basement membrane breakdown can no longer be observed.

The Molecular Basis of Our Understanding of the Cessation of Gastrulation

To test the hypothesis stated above, we performed a Noggin overexpression experiment asking whether BMP signaling is necessary for continuous ingressive cell movement from the VER. We also overexpressed Bmp2, asking whether expansion of Noggin expression across the entire ventral tail bud mesoderm is required for arresting ingressive cell movement. Noggin overexpression leads to a decreased tail bud ventral cell mass, which suggests attenuation of the ingressive cell movement from the VER. Indeed, epithelial cells abnormally accumulated in the VER of Noggin overexpressing embryos, and a basement membrane was formed beneath the accumulated epithelium. Furthermore, Slug (Snail2) expression adjacent to the VER was considerably decreased in Noggin overexpressing embryos. Decreased number of cells expressing phosphorylated Smad (pSmad1/5/8) were also observed in the VER after Noggin overexpression, suggesting that Smad-dependent signaling could be involved in EMT from the VER. This is consistent with the results of Noggin mutant mice. Noggin mutant mice have elevated pSmad signaling at the region corresponding to the VER and display an increased tail bud ventral cell mass with aberrant EMT. Furthermore, ablation of the VER in the mouse developing tail bud results in downregulation of Noggin level in the ventral tail mesoderm and leads to hyperexpansion of the ventral mesodermal region without direct evidence of increased cell proliferation. This result suggests the possibility that the VER secretes some factor(s) to regulate the level of Noggin expression and results in formation of the proper cell mass in the ventral tail mesoderm. In contrast, Bmp2 overexpression led to persistence of cell ingression after the time when gastrulation has normally ceased. Furthermore, upregulation of Slug expression was also observed in Bmp2 overexpressing embryos. Taken together these results suggest that BMP signaling via pSmad1/5/8 is necessary to maintain the ingressive cell movement from the VER, and that Noggin is required for downregulation of BMP signaling to induce cessation of gastrulation through attenuation of EMT (Fig. 3).

Recent studies have demonstrated that pSmad1/5/8 regulates EMT in different cellular contexts, including transcriptional regulation and proteolysis. As mentioned above, Smad binding sites are found in the promoter region of Snail, and binding of Smad to these sites can facilitate transcription of Snail. Subsequently,
transcriptional repression of E-cadherin is mediated through Snail binding to the E-cadherin promoter region. This down-regulation of E-cadherin expression leads to a mechanical disruption of the adherens junctions. Besides transcriptional regulation of E-cadherin, expression of several proteinase genes is also regulated by BMP signaling. Matrix metalloproteinase has been implicated as one of the major proteinases to induce basement membrane disruption during EMT. In fact, BMP signaling through Smad1 has been reported to upregulate MMP2 expression in pancreatic cancer cell invasion.34

Another recent study has shown that cross talk between Snail signaling and Wnt/β-catenin signaling is involved in transcriptional repression of E-cadherin. β-catenin has a dual role in EMT. It enhances cell-cell adhesion when bound to cadherin complexes in adherens junctions and also functions as a transcriptional coactivator upon entry into the nucleus.35 Dissociation of β-catenin from adherens junctions becomes a trigger for the loss of cadherin-mediated cell adhesion. Subsequently, released β-catenin itself interacts with TCF/LEF and acts as transcriptional coactivator to regulate EMT-related genes in the nucleus, including Snail, Slug and Twist.36 Intriguingly, Smad-dependent signaling during EMT has been shown to upregulate LEF1 expression.37 Moreover, Smad3 seems to directly interact with β-catenin and facilitate nuclear translocation of β-catenin.38 These reports support a potential link between Smad1/5/8 and Wnt/β-catenin signaling during EMT.

**Outlook**

In this commentary, we have summarized the molecular mechanisms underlying the cessation of gastrulation. In tail bud stage embryos, remnants of the primitive streak are incorporated into the ventral tip of the tail bud as the VER. Epithelial cells of the VER show a gastrulation-like ingressive cell movement, and BMP signaling is required to maintain this ingressive cell movement. In subsequent embryonic development, expansion of *Noggin* expression throughout the entire ventral tail bud mesoderm inhibits BMP signaling and attenuates ingressive cell movement. Phosphorylated Smad1/5/8, a downstream factor of BMP signaling, plays a critical role in the cellular processes of EMT such as the transcriptional repression of E-cadherin and basement membrane breakdown via MMP2. Therefore, inhibition of BMP signaling by *Noggin* likely affects these processes in its role in the cessation of gastrulation.

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