Extracellular Modulation of BMP Activity in Patterning the Dorsoventral Axis

Shawn C. Little and Mary C. Mullins*

Signaling via bone morphogenetic proteins (BMPs) regulates a vast array of diverse biological processes in the developing embryo and in postembryonic life. Many insights into BMP signaling derive from studies of the BMP signaling gradients that pattern cell fates along the embryonic dorsal–ventral (DV) axis of both vertebrates and invertebrates. This review examines recent developments in the field of DV patterning by BMP signaling, focusing on extracellular modulation as a key mechanism in the formation of BMP signaling gradients in Drosophila, Xenopus, and zebrafish. Birth Defects Research (Part C) 78:224–242, 2006. © 2006 Wiley-Liss, Inc.

INTRODUCTION

The bone morphogenetic protein (BMP) family of secreted signaling molecules directs the development of multiple organs and tissue types at various stages in embryogenesis, and is implicated in congenital and adult diseases (Hogan, 1996; Massague, 1998; Chen et al., 2004). Ongoing investigations suggest the potential for modulating BMP signaling in the treatment of disorders as diverse as kidney disease, hypertension, and cancer, and in medical applications such as orthopedics, endodontics, and tissue engineering (Reddi, 2005; and references therein). Our understanding of development and disease requires thorough studies of the means by which BMP signaling generates diverse cellular responses in myriad biological contexts. Enormous insights into the physiologic roles of BMP signaling have been gained through the study of model organisms such as Drosophila, Xenopus, and zebrafish (Hammerschmidt and Mullins, 2002; De Robertis, 2006; O’Connor et al., 2006), which have vastly enhanced our understanding of human development.

BMP ligands are synthesized as pro-proteins, which are processed by proteases to form functional, signaling-competent, disulfide-linked dimers (Cui et al., 1998; Constam and Robertson, 1999). As members of the transforming growth factor (TGF)β superfamily, secreted BMP dimers signal by binding a heterotetrameric transmembrane receptor complex composed of two “type I” and two “type II” BMP receptors of the actin-vin receptor-like kinase (Alk) family (reviewed in Feng and Derynck, 2005). Ligand binding results in the formation of a stable receptor complex, which allows the kinase activity of the type II receptors to phosphorylate, and thereby activate, the type I receptors of the signaling complex. The active type I receptors phosphorylate the intracellular effectors of BMP signaling known as the receptor-associated (r-) Smads. Activation of the r-Smads leads to their oligomerization with the common (co-) Smad. The r-Smad-co-Smad complex then shuttles into the nucleus to effect BMP-dependent alterations in transcriptional programs.

The earliest requirement for BMP signaling in the developing organism is during the patterning of cell fates along the embryonic dorsal–ventral (DV) axis. Both vertebrates and invertebrates utilize BMP signaling in DV patterning, implying an ancient origin for the role of this conserved pathway in early development (De Robertis and Sasai, 1996; Holley and Ferguson, 1997). In addition to conservation of the ligands, receptors, and intracellular effectors that are required for signal propagation, several extracellular factors that regulate the availability of ligands in the extracellular space are also conserved. Increasing evidence gathered from studies of vertebrate and invertebrates shows that in the context of DV patterning, these extracellular modulators perform vital roles in the formation of differential BMP signaling levels that activate different programs of gene expression in a dose-dependent manner, thus specifying distinct cell fates across large fields of cells.

The best evidence for BMP signaling acting in a graded fashion to specify cell fate derives from studies done in Drosophila, Xenopus, and zebrafish. This review concentrates on recent studies, performed

Shawn C. Little and Mary C. Mullins are from the Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

Grant sponsor: NIH Training Grants; Grant numbers: T32 GM07229-28; T32 HD007516-05 (to S.C.L.); Grant sponsor: NIH; Grant number: GMS6326 (to M.C.M.).

*Correspondence to: Mary C. Mullins, Ph.D., Department of Cell & Developmental Biology, University of Pennsylvania School of Medicine, 1211 BRBII/III, Philadelphia, PA 19104-6058. E-mail: mullins@mail.med.upenn.edu

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bdrc.20079
in these organisms, that examine the roles of several factors that modulate BMP signaling extracellularly, and whose activities are vital for proper DV pattern formation. The first section focuses on Drosophila. We briefly review fly embryogenesis, introduce the molecules and activities of Drosophila BMP pathway factors, and assess in detail two studies on the formation of the signaling gradient via mechanisms of ligand redistribution (Shimmi et al., 2005; Wang and Ferguson, 2005). The second section follows a similar organization, but examines more thoroughly the modulation of BMP signals in DV patterning during early development of Xenopus and zebrafish.

**DROSOPHILA**

**The BMP Ligands**

The DV axis of the Drosophila embryo is patterned by a nuclear gradient of the transcription factor Dorsal (Dl) in ventral nuclei, and a BMP signaling gradient in dorsal regions (reviewed in Morisato and Anderson, 1995). Nuclei positioned from ventral to dorsal display progressively less nuclear Dl. This gradient of nuclear localized Dl sets up the early DV pattern of the embryo and regulates BMP signaling in two ways (Stathopoulos and Levine, 2002). First, it represses the expression of the BMP ligand gene **decapentaplegic** (**dpp**) ventrally, restricting its expression to the dorsal ~40% of the embryo (Fig. 1B). Second, low levels of nuclear localized Dl induce the expression of the BMP antagonist **short gastrulation** (**sog**) in ventral regions. Through the actions of Dpp and Sog, among other factors discussed below, differential BMP activity patterns tissues in dorsal regions of the embryo (Fig. 1A).

Dpp is required for the division of the dorsal nonneural ectoderm into dorsal epidermis and amnioserosa (Fig. 1A). The absence of Dpp by null mutation causes the entire ectoderm to be specified as the ventral neurogenic ectoderm (Fig. 1C) (Irish and Gelbart, 1987; Arora and Nusslein-Volhard, 1992). Hypomorphic mutations in **dpp** cause a loss of the amnioserosa, but leave intact some dorsal epidermis, whereas stronger mutations cause progressive expansion of neurectoderm at the expense of amnioserosa and epidermis. Ectopic expression of **dpp** causes the dose-dependent expansion of amnioserosa and dorsal epidermis (Ferguson and Anderson, 1992a; Wharton et al., 1993). These results support a role for BMP signaling acting as a morphogen in the early fly embryo, with high levels specifying amnioserosa and low levels the dorsal epidermis.

An additional BMP ligand, **Screw** (**Scw**), acts in conjunction with Dpp in DV patterning of the fly embryo (Arora et al., 1994). Unlike **dpp**, which shows expression confined to the dorsal nonneural ectoderm, **scw** shows expression ubiquitously throughout the embryo (Fig. 1B). **scw** null mutants, like **dpp** mutants, lack the amnioserosa and display an expansion of the neurectoderm, but unlike **dpp** mutants, the dorsal epidermis remains (Fig. 1C). Thus, **Scw** is only required for high levels of BMP signaling in DV patterning (Arora and Nusslein-Volhard, 1992). Although **scw** is broadly expressed throughout the embryo, its expression in ventral tissues is sufficient to rescue dorsal tissues in **scw** mutants (Neul and Ferguson, 1998; Nguyen et al., 1998a). Since **dpp** is not expressed in the ventral domain and BMP dimers form intracellularly, these studies provide support for Dpp and Scw functioning as homodimers, rather than as heterodimers (which would require their co-expression in the same cells), to establish differential BMP signaling activity in dorsal embryonic regions.

---

**Figure 1.** Drosophila DV cells fates and mutant phenotypes. **A:** Fate map of an embryo positioned with dorsal up and anterior to the left. **B:** Schematic of gene expression domains of BMP signaling components, represented in cross-section of an embryo with dorsal up. Scw and Sax are expressed by all cells of the embryo. **C:** Illustration of null mutant phenotypes. A and C: Adapted from Raftery and Sutherland (2003). B: Adapted from Eldar et al. (2002).
However, as discussed below, other recent studies suggest that Dpp-Scw heterodimers generate the highest BMP signaling level, required to specify the amnioserosa (Shimmi et al., 2005).

**BMP Receptors and Ligand-Receptor Interactions**

The type II BMP receptor *punt* and the type I BMP receptor, Thickveins (Tkv), are required for all BMP signaling in the early embryo, displaying identical mutant phenotypes to *dpp* null mutants (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). Interestingly, a second type I BMP receptor, Saxophone (Sax), is only required for high BMP signaling activity, similar to Scw. Thus, *tkv* and *sax* exhibit remarkable similarities to *dpp* and *scw*, respectively. Similar to the behavior of *dpp* and *scw*, an excess of activated *tkv* receptor can rescue *sax* mutants, but activated sax has no effect in *tkv* mutants. These and other results suggest that Scw ligand preferentially signals via Sax, whereas Dpp signals via Tkv. Furthermore, Scw/Sax signaling synergizes with Dpp/Tkv signaling to promote high levels of BMP signaling required for the specification of amnioserosa (Neul and Ferguson, 1998; Nguyen et al., 1998a).

**Extracellular Modulators**

The differential BMP signaling levels that divide the dorsal ectoderm into two tissues are generated from ubiquitous BMP ligand expression dorsally via the combined activities of three secreted factors: Short gastrulation (Sog), Tolloid (Tolloid), and Twisted gastrulation (Tsg).

Sog, which is expressed in the presumptive ventrolateral neurulocorderm (Francois et al., 1994; Holley et al., 1995), can directly bind BMP ligands, interfering with ligand-receptor interactions and thus antagonizing signaling (Marques et al., 1997; Ross et al., 2001; Shimmi and O’Connor, 2003). *sog* mutants exhibit dorsalized defects consistent with an expansion of BMP activity ventrolaterally, including a partial loss of ventrolateral neurulocorderm and an expansion of dorsal epidermis (Fig. 1C) (Zusman et al., 1988; Ferguson and Anderson, 1992b; Francois et al., 1994; Holley et al., 1995; Biehs et al., 1996). In the embryo, Sog preferentially blocks the activity of Scw, while Sog alone cannot block the activity of Dpp (Neul and Ferguson, 1998; Nguyen et al., 1998a). A model of Sog activity suggests that Sog patterns dorsal ectoderm by diffusing from its ventrolateral domain dorsally, generating an inverse gradient of Scw antagonism, and thus forming a BMP signaling gradient. Consistent with this model, a graded distribution of Sog protein has been observed (Srinivasan et al., 2002), suggesting that dorsally-directed Sog movement might be sufficient to generate a gradient of BMP signaling to specify two dorsal cell fates. However, *sog* mutants also exhibit loss of the dorsal-most tissue, the amnioserosa, which requires the highest levels of BMP signaling. So, paradoxically, a factor that antagonizes BMP signaling is also required to generate the highest level of BMP signal.

The activity of Sog is counteracted by the metalloprotease Tolloid, which promotes BMP signaling by cleaving Sog (Marques et al., 1997; Shimmi and O’Connor, 2003). Tolloid is expressed in the dorsal domain (Fig. 1B), and *tolloid* null mutants display a phenotype similar to *scw* and *sax* mutants: a partial loss of dorsal epidermis, loss of the amnioserosa, and an expansion of the ventral epidermis (Fig. 1C) (Arora and Nusslein-Volhard, 1992; Ferguson and Anderson, 1992b). The mutant *tsg* also exhibits the loss of amnioserosa (Fig. 1C), consistent with a loss of BMP signal (Zusman and Wieschaus, 1985; Mason et al., 1994, 1997). However, unlike ligand mutants, *tsg* mutants do not display the expansion of neural ectoderm associated with other ventralized mutants. Although *tsg* is expressed with *tolloid* and *dpp* in the dorsal domain of the embryo, *tsg* expressed only ventrally is sufficient to rescue *tsg* mutant phenotype, suggesting it can promote BMP signal via a long-range mechanism (Mason et al., 1997). Biochemically, Tsg binds both to BMP ligands and to Sog (Ross et al., 2003; Shimmi and O’Connor, 2003). When present in a ternary complex of BMP-Sog-Tsg, Tsg strongly inhibits BMP signaling (Ross et al., 2001). This result seemingly contradicts the genetic finding that Tsg enhances BMP signaling.

The resolution of this contradiction arises from a model proposing that these three BMP modulators spatially redistribute secreted BMP ligands, resulting in highly concentrated BMP ligands dorsally, with lower levels dorsolaterally (Holley et al., 1996; Marques et al., 1997; Ashe and Levine, 1999; Decotto and Ferguson, 2001; Eldar et al., 2002; Shimmi and O’Connor, 2003). By this model, ventrolaterally produced Sog does not simply inhibit BMP signals. Instead, as Sog moves dorsally, it encounters and binds BMP ligands, subsequently transporting them dorsally in cooperation with Tsg. Continuous production of Sog ventrally ensures that Sog-BMP complexes undergo a net dorsal direction of movement. Upon reaching the dorsal side of the embryo, Tolloid activity, which is stimulated by BMP-bound Sog and Tsg (Yu et al., 2000; Shimmi and O’Connor, 2003), degrades Sog and destroys the inhibitory complex, releasing BMP ligand. Dorsal Tolloid activity serves as a “sink” for Sog, while the ventral domain acts as “source,” ensuring the graded distribution of a BMP inhibitor, and resulting in the redistribution of high BMP ligand levels into the dorsal-most region.

**Phospho-Mad Dynamics and the Direct Demonstration of Dorsal Ligand Transport**

The distribution of phospho-Mad (pMad; the Drosophila r-Smad) has been illuminating in understanding the extracellular modulation of BMP signaling and mechanism of ligand redistribution. A phospho-Smad1/5 specific antibody has revealed dynamic changes in pMad localization and levels in the early fly embryo (Dorfman and Shilo, 2001; Ross et al., 2001; Rushlow et al., 2001; Eldar et al., 2002; Shimmi and O’Connor, 2003; Sutherland et al., 2003; Mizutani et al., 2005; Shimmi et al., 2005; Wang and Ferguson,
2005). pMad is first detected at midcellularization (early stage 6) in a broad domain of low intensity spanning the dorsalmost ~20 cells. This broad, low signal is refined into undetectable pMad dorsolaterally and a high intensity stripe spanning the eight to 10 dorsalmost cells by the onset of gastrulation (late stage 6), which corresponds to the position of the amnioserosa. This rapid refinement of pMad levels suggests rapid turnover of pMad itself. As expected from the dorsal transport model, the refinement of pMad distribution is absent in tolloid and tsg mutants. sog mutants show pMad expanded into the ventrolateral ectoderm, but as expected from the absence of amnioserosa in sog mutants, there is also no dorsal region of higher pMad. Though indirect, the observation of a refinement of high levels of BMP signal with time in dorsal domains is consistent with the model of dorsal ligand transport. However, one difficulty in the interpretation of pMad data is the observation that in dorsolateral regions, which are clearly patterned by low levels of BMP signal, pMad is largely below the level of detection at the onset of gastrulation.

Direct visualization of long-range ligand transport was recently obtained by two groups working with tagged versions of Dpp (Shimmi et al., 2005; Wang and Ferguson, 2005). To monitor the position of Dpp during development, Shimmi et al. (2005) created transgenic embryos carrying functional hemagglutinin (HA)-tagged Dpp expressed in the endogenous dpp domain. Dpp-HA protein is initially found in the broad dorsal 40% of the embryo where the dpp gene is expressed. However, as development progresses, the Dpp-HA accumulates in a narrower dorsal stripe, with the same kinetics as pMad. This narrow Dpp stripe depends on the presence of Tsg and Sog, without which it remains broadly distributed across the entire dorsal domain. When Dpp-HA is expressed in only the anterior of the embryo, it accumulates in a dorsal stripe that extends even into the most posterior regions of the embryo. These observations provide direct evidence of Sog-Tsg dependent transport of a BMP ligand into the dorsal midline.

Unexpectedly, Shimmi et al. (2005) found that Dpp transport does not occur in the absence of Scw. A straightforward explanation for this result is that Dpp and Scw are co-transported as a heterodimeric ligand. Shimmi et al. (2005) show that heterodimers of Dpp-Scw exist in the early fly embryo, and elicit approximately tenfold greater signal in cell culture than homodimers. Heterodimers are the preferred binding partners for Sog and Tsg, and the preferred Sog-BMP substrate for Tolloid, with homodimers exhibiting lower affinity to the extracellular modulators. These and other previous observations led Shimmi et al. (2005) to propose a model in which Dpp-Scw heterodimers produce the highest BMP signaling level in the early fly embryo, through their preferred dorsal transport and release by Sog, Tsg, and Tolloid, and their synergistic activation of the Sax and Tkv type I receptors.

However, Wang and Ferguson (2005) investigated again if Scw-Dpp heterodimers are required to obtain the highest BMP signaling levels by examining pMad levels. By localized injection of dpp and scw mRNA in nonoverlapping domains in the embryo, precluding the possibility of heterodimer formation, they could rescue dorsal pMad staining in scw mutants. These results led Wang and Ferguson (2005) to propose a model in which Scw homodimers associate with Dpp homodimers in a novel, higher order complex that elicits a synergistic signal in responding cells. Whether hetero- and/or homodimers are at work, the mechanism by which Scw and Dpp synergistically activate Sax and Tkv, respectively, leading to high pMad levels remains unknown.

Wang and Ferguson (2005) developed a novel method, termed perivitelline injection (PVI), to specifically detect secreted BMP ligand in the embryo. In this method, a functional transgenic Dpp-GFP fusion protein is detected by injection of anti-GFP antibody into the perivitelline space of live embryos. PVI specifically detects ligand that is either bound to the cell surface or has been internalized by endocytosis. In contrast to conventional antibody staining, PVI does not detect ligand at its site of production. Hence, this method allows evaluation of the distribution of secreted Dpp-GFP ligand. Wang and Ferguson (2005) reported several exciting and provocative results discussed below.

Dpp-GFP was expressed in a stripe of cells orthogonal to the domain of endogenous dpp. Using PVI, they observed dorsal accumulation of Dpp-GFP in a stripe of cells 8–12 diameters wide, extending by the beginning of gastrulation along the entire AP axis of the embryo, in a pattern similar to pMad localization. As Shimmi et al. (2005) determined, locally-produced Dpp is found at a substantial distance from its source all along the AP axis. A similar result was found with an epitope-tagged version of Scw. Dpp-GFP required the presence of Sog, without which Dpp accumulated near its site of production, confirming the requirement of an extracellular antagonist in dorsally-directed transport of BMP ligand.

Wang and Ferguson (2005) also asked how the absence of Tsg affected the distribution of Dpp-GFP. Surprisingly, Dpp-GFP could not be detected by PVI in tsg mutants, but was detected in sog mutants and at a lower level in tsg; sog double mutants. It should be noted that PVI, like pMad staining, is not sensitive enough to detect ligand-receptor interactions generating low BMP signaling dorsolaterally that specify epidermis, which can in part explain the absence of Dpp-GFP detected in tsg mutants. However, the reduced amount of Dpp-GFP in sog; tsg double mutants versus sog single mutants, suggests that Tsg stabilizes in some manner membrane-bound or internalized Dpp-GFP in sog mutants. The authors proposed that Tsg, in addition to its previously demonstrated role in dorsal transport, also promotes BMP signaling through the stabilization of ligand-receptor interactions and/or antagonizing currently unknown extracellular BMP inhibitors. This role for Tsg in promoting Dpp stability is independent of the presence of Sog, since sog; tsg double mutants display reduced broadly
distributed Dpp levels, compared to sog mutants. The proposal of Sog-dependent and -independent roles for Tsg in promoting BMP signaling is consistent with the behavior of Tsg in zebrafish embryos (see below) (Little and Mullins, 2004; Xie and Fisher, 2005).

Finally, Wang and Ferguson (2005) demonstrate that the onset of BMP signaling recruits additional ligand to sites of signaling, serving a positive feedback role that promotes high levels of pMad at the dorsal midline. This finding was based on the observation that the localized expression of an activated form of Tkv, but not the wild-type version, caused the accumulation of tagged Dpp at the site where activated receptor was found, and concomitantly depleted ligand from a large area of cells neighboring the region expressing activated Tkv. In addition, ligand recruitment did not occur if BMP-dependent transcription was inhibited. The authors proposed that this mechanism could rapidly increase levels of ligand in the dorsal domain while depleting ligand in lateral regions. This would create a “bistable” distribution of BMP ligand with high levels at most dorsal regions and low levels dorsolaterally, which is subsequently read by dorsal ectoderm cells to determine their fate.

Computational Approaches to Understanding Gradient Formation

The relatively simple geometry of the fly embryo, and well-characterized biochemical activities of the known components, have led to mathematical models of the shaping of the BMP signaling gradient. The earliest model (Eldar et al., 2002) successfully predicted the reliance of long-range Dpp transport on Sog and Tsg. However, a number of assumptions with little experimental basis are required for this model to function. A model proposed by Shimmi et al. (2005) explicitly examines the behavior of ligand heterodimers alongside both species of homodimer, explains the failure of long-range Dpp transport in the absence of Scw, and is consistent with synergistic signaling elicited by heterodimers. Additionally, the modeling performed by Mizutani et al. (2005) shows that many aspects of the formation of the signaling gradient, as read by pMad staining, can be explained by incorporating receptor-mediated ligand endocytosis and decay into the model of dorsal transport, removing the need to incorporate additional unsupported assumptions, and consistent with the inferred rate of ligand internalization and degradation.

Summary

BMP signaling occurs dynamically in the early Drosophila embryo (Fig. 2). BMP ligands expressed uniformly in the dorsolateral ectoderm of the blastoderm are spatially redistributed by midcellularization stage to the dorsal-most regions. This redistribution results from the production of Sog in the ventral ectoderm. Sog moves in a net dorsal direction, binds ligands and transports them dorsally in cooperation with Tsg. Tolloid releases BMP inhibition and Mad-dependent positive feedback reinforces signaling. The redistribution of BMP ligands can be visualized in vivo with tagged Dpp proteins, and directly reflects BMP signaling output, as measured by pMAD levels. Concomitant with BMP redistribution, pMad refines from a broad, low intensity dorsal domain into a narrow stripe of intense staining. Thus, by the onset of gastrulation, future amnioserosal cells encounter high levels of signal, while surrounding dorsolateral domains experience lowered levels. Substantial questions remain, foremost among them the identity of the predominant signaling species, relevant receptor complexes, and the mechanism of Mad-dependent positive feedback which recruits additional ligand to sites of signaling.

BMP SIGNALING IN VERTEBRATE DV PATTERNING

BMP signaling also patterns cell fates along the DV axis in vertebrates. This patterning has been studied most closely in Xenopus and zebrafish, which we will focus on here. Unlike Drosophila, BMP signaling in vertebrate embryos patterns ventral cell fates, not dorsal, and the appearance of dorsal fates in vertebrates requires BMP repression. Homologous factors to those in Drosophila transduce and modulate BMP signals in vertebrates (Holley et al., 1995; De Robertis and Sasai, 1996). We briefly review evidence implicating a gradient of BMP signaling in the patterning of ventral cell fates, and the mechanisms controlling BMP ligand gene expression. We then focus in detail on the recent characterization of factors that extracellularly modulate BMP signaling as the key means of setting up the signaling gradient.

The Requirement for a BMP Signaling Gradient in Vertebrate DV Patterning

Classic manipulations by Spemann and Mangold (1924), in which a dorsal blastopore lip (equivalent to the shield in zebrafish, node in mouse, and Henson’s node in chick) was transplanted ventrally into a host embryo, resulted in the formation of a “twinned” embryo with a second embryonic axis containing spinal cord and somites, cell fates normally of dorsal gastrula origin (see Fig 3A for a fate map) (Spemann and Mangold, 1924). The ectopic axis consisted largely of host-derived ventral tissue that would normally not contribute to dorsal structures. Instead of forming epidermis and ventral mesoderm, ventral host tissue was respecified to become neural and anterior somites. These experiments demonstrate that dorsal signals can override the program of ventral cell fate specification, and led to the question of the molecular nature of the dorsal organizing signals. Much research has demonstrated that the dorsal side of the embryo downregulates BMP activity through a Wnt/β-catenin signaling pathway.

A Wnt/β-catenin pathway establishes the location of the dorsalmost aspect of the DV axis, the Spemann organizer, from which BMP antagonists emanate to restrict and modulate BMP signaling ventrolaterally. Nuclear translocation of β-catenin in cells of the future dorsal-most region
Figure 2. Model of dynamics of BMP signaling activity in the Drosophila embryo. Sog is produced from ventral neurogenic ectoderm (yellow) surrounding the dorsal domain of Dpp expression (violet). The blue line represents changes in BMP activity as the embryo proceeds from early blastoderm (top) to the onset of gastrulation (bottom). Sog moves into increasingly dorsal domains (red lines), while Sog-Tsg mediated ligand transport, Tolloid activity, and positive feedback events result in a dramatic increase in BMP activity centered about the dorsal midline. Circles underneath the black line represent BMP signal received by cells across the embryo. Cells receiving no signal (red) become neural ectoderm. Those receiving low levels of BMP (dark green) are specified as dorsal ectoderm, whereas those experiencing high levels become amnioserosa (AS) (bright green). Adapted from Srinivasan et al. (2002), Shimmi and O'Connor (2003), and Wang and Ferguson (2005).
precedes formation of the morphologically identifiable organizer. The ablation of nuclear β-catenin, either chemically or genetically, results in the failure to form the organizer and a loss of dorsal identity (Heasman et al., 1994, 2000; Wylie et al., 1996; Kelly et al., 2000; Bellipanni et al., 2006). β-Catenin induces the expression of BMP antagonist genes in dorsal regions, thus initiating the restriction of BMP signaling to ventrolateral regions of the blastoderm and future gastrula. Additionally, in zebrafish, β-catenin induces the expression of the transcriptional repressor Bozozok (Fekany et al., 1999; Ryu et al., 2001), which directly represses bmp2b gene expression dorsally (Leung et al., 2003).

When misexpressed at high levels, BMP activity can override the activity of the organizer and cause radial ventralization (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Harland, 1994; Hemmati-Bri vanlou and Thomsen, 1995; Schmidt et al., 1995; Jones et al., 1996; Dosch et al., 1997; Hemmati-Bri vanlou and Melton, 1997; Suzuki et al., 1997). Both ectoderm and mesoderm respond in a dose-dependent manner to overexpression of BMP ligands, and increasingly ventral cell fates are induced by higher amounts of BMP. For example, the overexpression of BMP4 on the dorsal side of a Xenopus embryo expands the ventral blood and epidermis at the expense of dorsal neural tissue, anterior somites, and notochord. In loss of function studies in Xenopus, knockout of BMP ligands by morpholino injection results in reduction or loss of ventral cell fates, and moderate expansion of neural markers, demonstrating the requirement for BMP signaling in ventral cell specification (Reversade et al., 2005).

Analysis of BMP signaling mutants in zebrafish provides genetic evidence for the existence of a BMP signaling gradient in the early embryo (reviewed in Hammerschmidt and Mullins, 2002; Schier and Talbot, 2005). Mutations in multiple components of a BMP signaling pathway cause a series of dorsalized phenotypes (Mullins et al., 1996). The strongest dorsalized mutants are seen in null bmp2b (swirl) and bmp7 (snailhouse) mutants (Kishimoto et al., 1997; Nguyen et al., 1998b; Dick et al., 2000; Schmid et al., 2000), which display radialized neural ectoderm and paraxial mesoderm, accompanied by complete loss of ventrolateral fates, indicating a severe loss of BMP signal (Fig. 3C). Similarly, strong dorsalizations are observed in embryos lacking all function of either the type I BMP receptor alk8 in maternal-zygotic lost-a-fin mutants (Mintzer et al., 2001), or of the r-Smad, smad5, in maternal somitaburtn169 mutants (Kramer et al., 2002). Morphologically, these strongly dorsalized embryos are severely impaired and do not survive beyond the 14-somite stage. A slightly less severe phenotype is seen in hypomorphic bmp7 mutants, in which the neur ectoderm and most anterior somites do not completely encircle the embryo (Dick et al., 2000; Schmid et al., 2000). Moderately dorsalized mutants, resulting from moderate reduction in BMP signaling, form normal anterior but weakly dorsalized trunk and severely dorsalized tail tissues. More mildly dorsalized phenotypes are restricted to tail tissues. The decreasing severity of these phenotypes is reflected in alterations during gastrulation of the extent of dorsal and ventral marker gene expression (Nguyen et al., 1998b; Dick et al., 2000; Schmid et al., 2000). A continuum of dorsalized phenotypes can also be induced via dose-dependent inhibition of BMP signaling in gain-of-function studies in both fish and frogs (Dosch et al., 1997; Knecht and Harland, 1997; Neave et al., 1997). These results, together with the analysis in Xenopus gain-of-function studies and in zebrafish mutants of lateral ectodermal cell fate specification of neural crest and sensory placodal tissues (Marchant et al., 1998; Nguyen et al., 1998b, 2000; Tribulo et al., 2003), reveal the graded function of BMP signaling, acting as a morphogen, to pattern tissues along the DV axis (Fig. 3).

The Expression of BMP Ligand Genes in Time and Space

The BMP ligand genes are expressed in a dynamic manner in the early embryo (Dale et al., 1992; Nishimatsu et al., 1992; Fainsod et al., 1994; Clement et al., 1995; Hemmati-Bri vanlou and Thomsen, 1995; Schmidt et al., 1995; Wang et al., 1997; Fritz and Sheets, 2001). In Xenopus, mRNA encoding bmp2, bmp4, and bmp7 are maternally contributed to the egg and are found ubiquitously throughout the early embryo. Maternal bmp2 transcript is degraded at the mid-blastula stage, and bmp2 is not transcribed zygotically until mid-gastrulation. Zygotic
transcription of \textit{bmp7} and \textit{bmp4} are activated at the midblastula transition and are expressed throughout the embryo, then degraded dorsally at the early gastrula stage. As gastrulation proceeds, the domain of maternal BMP expression becomes restricted zygotically, beginning with a clearing on the dorsal side at midblastula, and gradually moving into more lateral domains as gastrulation proceeds.

A similar pattern of BMP ligand expression is found in zebrafish (Kishimoto et al., 1997; Martinez-Barbera et al., 1997; Nikaido et al., 1997; Dick et al., 2000; Schmid et al., 2000; Furthauer et al., 2004). mRNA encoding \textit{bmp4} and \textit{bmp7} is found in immature oocytes (Kramer et al., 2002), but are either weakly detected or are absent at the two to four cell stage (Dick et al., 2000). Genetically, neither \textit{bmp2b} nor \textit{bmp7} are maternally required for proper patterning (Nguyen et al., 1998b; Kishimoto et al., 1997; Schmid et al., 2000), although the BMP-related ligand Radar/Gdf6a may function maternally in DV patterning (Goutel et al., 2000; Sidi et al., 2003). Zytotically, \textit{bmp2b} and \textit{bmp7} are expressed throughout the early blastula and are then cleared from

---

\textbf{Figure 4.} Schematic of BMP signaling in the zebrafish embryo. \textbf{A:} At early blastula, genes encoding BMP ligands are expressed throughout the blastoderm. \textbf{B:} Ligand gene expression is subsequently eliminated in the dorsal domain. \textbf{C:} By the onset of gastrulation, a BMP signaling gradient has been established. The dorsal and ventral regions of the embryo express a variety of pro- and anti-BMP factors. For more information on dorsally and ventrally expressed genes, see De Robertis (2006).

the dorsal side (Fig. 4A and B). Zygotic *bmp4* is expressed ventrally just prior to the onset of gastrulation, so that during gastrulation all three BMP ligands are broadly expressed in the ventral half of the embryo. Their expression is maintained in ventroposterior cells that contribute to the tail bud, from which tail structures will arise and which requires continued BMP signals.

BMP activity itself positively regulates BMP ligand gene expression, thus ensuring continued BMP activity (Metz et al., 1998). Maternal signaling via Smad5 likely initiates embryo-wide zygotic expression of *bmp2b* and *bmp7* in zebrafish, and *bmp4/7* in Xenopus (Kramer et al., 2002). Disruption of BMP-mediated positive feedback is essential for the loss of BMP expression from the dorsal domain. The initial dorsal clearance of BMP expression is mediated by at least two overlapping mechanisms. In zebrafish, β-catenin-dependent activation of the transcriptional repressor Bozozok/Dharma/Nieuwkoid extinguishes expression of *bmp2b* on the dorsal side (Koos and Ho, 1999; Fekany-Lee et al., 2000; Leung et al., 2003). A similar mechanism involving Xiro1 and Siamois may operate to repress *bmp4* in Xenopus (Lemaire et al., 1995; Baker et al., 1999; Gomez-Skarmeta et al., 2001). Additionally, β-catenin-induced FGF signaling on the dorsal side locally represses BMP activity (Furthauer et al., 1997, 2001, 2004; Tsang et al., 2004; Stern, 2005), at least in part through the reduction of BMP-dependent Smad activity by mitogen activated protein kinase (MAPK)-dependent inhibitory phosphorylation (Kretzschmar et al., 1997; Pera et al., 2003; Kuroda et al., 2005). The ventrolaterally expressed Vox, Vent, and Ved transcription factors repress dorsal gene expression in ventral regions (Kawahara et al., 2000a, 2000b; Melby et al., 2000; Imai et al., 2001; Shimiizu et al., 2002; Gilardelle et al., 2004). They are repressed dorsally by Bozozok and their expression is maintained ventrally by BMP and Wnt8 signaling (Fekany-Lee et al., 2000; Lekven et al., 2001; Ramel and Lekven, 2004; Ramel et al., 2005). These early mechanisms establish broad BMP-positive and BMP-negative regions demarcating initial dorsal and ventral domains in the late blastula stage embryo.

**Extracellular Antagonism of BMP Signaling**

These mechanisms of dorsal repression of BMP signaling do not explain the classic observations of Mangold and Spemann (Spemann and Mangold, 1924) that transplanted dorsal tissue can redirect the development of ventral host tissue to take on dorsal identity, a manipulation indicating the existence of signals emanating from the organizer into surrounding tissues. Additionally, supplying BMP ligand mRNA ubiquitously throughout a zebrafish embryo can rescue *bmp2b* and *bmp7* mutants to wild type, indicating that additional mechanisms must regulate DV pattern (Kishimoto et al., 1997; Nguyen et al., 1999b). Furthermore, the broad ventral expression of BMP ligand genes does not explain how BMP signaling acts in a graded fashion along the DV axis.

These activities are mediated by the production from the organizer of extracellular antagonists of BMP signaling. Two such molecules are Noggin (Nog) and Chordin (Chd), secreted polypeptides produced by the organizer by the start of gastrulation (Smith and Harland, 1992; Sasai et al., 1994). These factors can induce dorsal fates in ectoderm and mesoderm in a dose-dependent manner and produce a secondary axis when ectopically expressed ventrally, thus mimicking the activity of organizer tissue (Dosch et al., 1997; Wilson et al., 1997; Bauer et al., 1998; Eimon and Harland, 1999; Furthauer et al., 1999). Nog and Chd themselves do not directly signal cell fate, but instead block BMP signaling by directly binding BMP ligands extracellularly (Smith and Harland, 1992; Sasai et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996). Chd, like its ortholog Sog, binds BMP ligands via four cysteine-rich (CR) domains. Chd and Sog can function interchangeably in frog and fly embryos (Holley et al., 1995; Schmidt et al., 1995). BMP ligands bound to these antagonists cannot interact with receptors and thus cannot signal. The crystal structure of the BMP-Nog complex reveals that this antagonist binds BMP at residues required for ligand-receptor interaction (Groppe et al., 2002). Noggin, as well as the BMP antagonists Follicostatin (Hemmati-Brivanlou et al., 1994) and Cerberus (Bouwmeester et al., 1996), are not found in invertebrate genomes and appear to be a more recent development in the evolution of BMP modulation.

Unlike other antagonists, Chordin expression extends outside the organizer into future neural tissue in both Xenopus and zebrafish, as well as into marginal dorsolateral regions well outside the organizer in zebrafish (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997; Kuroda et al., 2004). The zebrafish *chordin* mutant displays reduced neural plate, reduced paraxial tissue, and slight reduction of the dorsal-most axial mesoderm, with expansion of ventral fates, indicating excess BMP signaling (Hammerschmidt et al., 1996). A similar phenotype is seen when Chordin is depleted by antisense oligonucleotides or morpholinos in Xenopus (Oelgeschlager et al., 2003a). The removal of Chd also attenuates the ability of the organizer to induce a secondary axis in transplant experiments (Oelgeschlager et al., 2003a). Chordin overexpression induces dorsal fates in a dose-dependent manner, and this can occur at a distance from the site of Chordin expression (Jones and Smith, 1998; Blitz et al., 2000).

The restricted expression of Chd and Nog on the dorsal side, combined with the broad expression of BMP ligands ventrally, has led to a model in which BMP antagonists diffuse laterally from their sites of expression, conceptually similar to the dorsal diffusion of Sog in Drosophila (Holley and Ferguson, 1997; Thomsen, 1997). The ventrally-directed movement of Chd/Nog causes a DV gradient of anti-BMP activity, resulting in an inverse gradient of BMP activity with high levels ventrally and decreasing levels in progressively more lateral regions (Fig. 4C). Thus, in this model, the movement of antagonists from dorsal to ventral sets up a BMP activ-
Extracellular antagonism is indispensable for DV patterning. The triple depletion of Chd, Nog, and Fol in Xenopus tropicalis causes the complete loss of neural tissue and dorsal mesoderm (Khokha et al., 2005), demonstrating the redundant activity of extracellular antagonists in blocking BMP signaling. The establishment of the organizer is not affected by triple antagonist knockdown, yet the dorsalizing activities of early β-catenin signal are not sufficient to maintain any dorsal identity if BMP antagonists are absent. In zebrafish, mutations in both chd and the transcriptional repressor bozozok massively derepress BMP expression and lead to radialized ventral fates, an effect which is masked when bmp2b is also mutated (Gonzalez et al., 2000). Thus, both transcriptional repression and extracellular antagonists are required to repress ventralizing BMP signals in dorsal regions to allow dorsal development.

**Additional Extracellular Modulators of BMP Activity**

Many additional factors influence BMP signaling at the extracellular level. In the next section, we discuss recent findings regarding several of these factors.

**The Tolloid Family**

Multiple homologs of the Drosophila metalloprotease Tolloid are found in vertebrates (Ge and Greenspan, 2006). The first vertebrate Tolloid-related protease identified, BMP1, copurified with BMP ligands as a bone-inducing activity from demineralized bone extracts (Wozney et al., 1988). BMP1 homologs are found in fish, frog, mouse, and human. Additional related genes are, in Xenopus, Xolloid (Xld) and Xolloid-related (Xlr) (Goodman et al., 1998; Dale et al., 2002), and in fish, tololoid (tl1), bmp1a, and bmp1b (Blader et al., 1997; Muraoka et al., 2006). Of these factors, those studied most intensely for their roles in DV patterning have been, in frog, Xld and Xlr, and in fish, tll1 (the gene ablated in the fish mutant mini fin) (Connors et al., 1999) and bmp1a. These factors are members of the “BMP synexpression group” of genes that are expressed ventrally and are positively regulated by BMP signaling (Karaulanov et al., 2004). Tolloids cleave Chordin, thus promoting BMP signaling and ventral cell fate, and function analogously to Drosophila Tolloid degradation of Sog, again illustrating the conservation of this signaling pathway.

In Xenopus, the overexpression of Xld, Bmp1, and Xlr promotes ventral cell fates (Piccolo et al., 1997; Goodman et al., 1998; Dale et al., 2002), and in zebrafish, TII overexpression phenocopies the chd mutant (Connors et al., 2006). In vitro, Tolloid does not degrade other BMP antagonists such as Noggin, nor does Tolloid counteract their dorsalizing effects in the embryo (Piccolo et al., 1997). This is consistent with the observations that TII overexpression does not increase the severity of the zebrafish chd mutant phenotype, and that the strongest phenotype of Tolloid overexpression is equivalent to a chd mutant. Moreover, the phenotype of a tll1;chordin double mutant fish embryo is also equivalent to a chordin mutant, indicating that toolloid acts upstream of chordin and plays no additional role than regulating Chordin activity in the early zebrafish embryo (Wagner and Mullins, 2002). It should be noted that Tolloid-related factors target many extracellular matrix components (Ge and Greenspan, 2006). However, all evidence to date indicates that, during DV patterning, the Tolloid family promotes BMP signaling via degradation and inactivation of Chordin, and not other BMP antagonists.

Recent analysis has focused on the relative contributions of the Tolloid genes to DV patterning. The tll1 (mini fin) mutant in zebrafish exhibits a weakly dorsalized phenotype in which the ventral fin fold is absent (Connors et al., 1999). tll1 is expressed at low levels broadly at the onset of gastrulation, and is progressively restricted to the ventral half of the embryo at mid gastrulation and to the tail bud at the end of gastrulation, consistent with a role in patterning ventral cell fates. The absence of tll1 activity causes the expansion of chd expressing tissue and concomitant reduction in BMP expression in the tail bud, indicating the importance of tll1 in maintaining BMP activity after gastrulation. Transgenic embryos carrying a heat shock promoter driving tll1 expression demonstrate that tll1 activation as late as somitogenesis stages is sufficient to modulate Chd activity, allowing BMP signaling to pattern tail tissues (Connors et al., 2006). This, together with the spatial requirement of TII in ventral marginal domains (Connors et al., 2006), demonstrates a localized requirement for TII activity in the ventral tail bud. However, the contribution of Tolloid-related factors to zebrafish DV patterning is larger than suggested from the analysis of tll1. bmp1a is expressed maternally and zygotically and is distributed uniformly throughout the early embryo (Muraoka et al., 2006). Combined knock down of both tll1 and bmp1a results in strongly dorsalized phenotypes (Muraoka et al., 2006). In Xenopus, similar early expression and ubiquitous distribution are seen with Xenopus bmp1 and Xolloid (Goodman et al., 1998), although knockdown of these factors has not yet been performed.

The morphogen gradient hypothesis includes a requirement for a “sink” located opposite the site of morphogen production that provides a means of degrading the morphogen to prevent its accumulation, which could over time build up to high levels across the entire field of cells, eliminating a concentration gradient. The BMP antagonists provide this sink dorsally, but what prevents the antagonists from accumulating ventrally and blocking all BMP activity? The discovery of the anti-Chordin activity of Tolloid provides a plausible sink by which the diffusing BMP antagonist Chordin could be destroyed, thus
maintaining a gradient of BMP activity. On the one hand, ventral expression of tll1 in zebrafish seems consistent with this proposal. Indeed, in the context of the tail bud, which is a relatively small collection of cells that undergo further patterning and specification events, localized tll1 is required for its proper patterning. However, the ubiquitous, early expression of bmp1 and other Tolloids leaves unclear if a ventrally-localized sink is required. Given that their absence results in strong dors-alization, Tolloids may be required broadly in space to promote BMP signaling ventrally; however, it is unknown if protein expression or activity is restricted to the ventral region.

The CR-domain family: Twisted Gastrulation and Crossveinless-2

Tsg and Crossveinless-2 (Cv-2) are two related factors that modulate BMP signaling in the early embryo. Both factors contain CR domains reminiscent of the four CR domains contained in Chordin (Garcia Abreu et al., 2002). Tsg and Cv-2 bind BMPs in vitro, suggesting the possibility that they behave as antagonists. However, recent work has uncovered complexity in the behavior of these related molecules, and suggests that they may both promote and antagonize BMP signaling, depending on the vertebrate species and their molecular environment.

The Cv-2 gene was originally identified in Drosophila as a factor promoting high levels of BMP signaling required for specification of the posterior cross-vein in the wing (Conley et al., 2000). This gene is not expressed during DV patterning of the fly embryo. In contrast, vertebrate Cv-2 is expressed in regions of BMP expression in the developing mouse, chick, and fish, as part of the BMP synexpression group (Coffinier et al., 2002; Moser et al., 2003; Coles et al., 2004; Kamimura et al., 2004; Karaulanov et al., 2004; Rentzsch et al., 2006). In cell culture, Cv-2 exhibits both pro- and anti-BMP effects, depending on the cell line. For example, Cv-2 inhibits BMP signals in 293T cells and osteoblast culture (Moser et al., 2003; Binnerts et al., 2004), but increases phospho-Smad1 levels in COS cells (Kamimura et al., 2004). In Xenopus, overexpression dorsally has no effect, while ventral expression can induce secondary axes (Moser et al., 2003). In zebrafish, Cv-2 misexpression induces a mixture of weakly dors-alized and ventralized phenotypes (Rentzsch et al., 2006). However, like Tsg, loss of function in zebrafish with morpholinos indicates that the primary role of Cv-2 is to promote BMP signaling. This is accomplished largely by competing with Chordin for binding to BMP ligands. Interestingly, the switch in Cv-2 behavior is mediated by proteolytic cleavage of Cv-2 itself (Rentzsch et al., 2006). An uncleavable mutant form of Cv-2 is strongly dorsalizing, whereas the N-terminal half of the protein, which contains the five CR domains, is strongly ventralizing. Full length, but not the N-terminal segment of Cv-2 binds to heparin beads, suggesting that the anti-BMP effect of Cv-2 may be mediated by retention of BMP ligands in the extracellular space through binding to heparan sulfate proteoglycans. As with Tsg (see below), it remains to be shown how binding of BMPs via CR domains can result in an increase in BMP signaling.

Several reports have endeavored to elucidate the function of vertebrate Tsg, combining biochemical analysis with misexpression in the embryo. Biochemically, Tsg can bind to Chd and BMP ligand independently or in a BMP-Chd-Tsg complex (Oelgeschlager et al., 2000; Chang et al., 2001; Larrain et al., 2001; Scott et al., 2001). Together, Chd and Tsg strongly repress ligand-receptor interaction. However, Tsg stimulates the activity of Tolloid, and inhibits the remaining anti-BMP activity of Chd fragments produced by Tolloid, promoting BMP signaling (Oelgeschlager et al., 2000; Yu et al., 2000; Larrain et al., 2001; Scott et al., 2001; Shimmi and O’Connor, 2003; Xie and Fisher, 2005). In gain-of-function experiments, overexpression of Tsg causes dorsialized phenotypes at low levels in Xenopus and at any level in zebrafish (Ross et al., 2001; Oelgeschlager et al., 2003b; Little and Mullins, 2004; Xie and Fisher, 2005). At high levels in Xenopus, or in combination with Tolloid, Tsg overexpression switches to ventralizing activity (Oelgeschlager et al., 2000, 2003b; Larrain et al., 2001; Ross et al., 2001). When combined with Chd overexpression, low levels of Chd and Tsg synergize, causing dorsalized phenotypes at levels that neither can do alone (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). The results from these biochemical and overexpression experiments led to a model in which Tsg could either promote or antagonize BMP signaling, with its behavior relying on molecular context, specifically the presence or absence of Tolloid (Larrain et al., 2001). By this model, the function of Tsg depends on the status of Chd: full length Chd causes antagonistic activity, while Chd fragments or the presence of Tolloid stimulate pro-BMP activity. But which of these activities predominates in the embryo?

Loss-of-function approaches by morpholino knock down of Tsg have led to seemingly contradictory findings. In X. laevis, high levels of morpholino induce expansion of the ventral markers sizzled and Bambi, and combined with low level knockdown of Chd (amounts that have no effect on their own), reduce the extent of neural markers sox2 and otx2 (Blitz et al., 2003). These results are consistent with a ventralized phenotype, indicating increased BMP signaling when Tsg is reduced. These effects are seen after midgastrulation, with markers at earlier stages appearing normal, leading to the suggestion that Tsg maintains dorsal identity, presumably after BMP-mediated DV patterning. In X. tropicalis, Tsg knockdown causes more profound ventralization, with high levels of morpholino expanding sizzled and reducing sox2 during gastrulation, demonstrating an early ventralizing effect of Tsg depletion (Wills et al., 2006). At neurula stages, Tsg knockdown causes both X. tropicalis and X. laevis to exhibit reduction of neural tissue and expansion of ventral fates, and co-knockdown of Chd with Tsg enhances the ventralized phenotypes. These results indicate that Tsg inhibits specification of ventral identity.

However, apparently contradictory results were found when low
levels of Tsg morpholino were co-injected with BMP7 morpholinos into X. laevis (Zakin et al., 2005). Either morpholino alone elicits only a weak effect on the domain of otx2 and sizzled expression, whereas the combined treatment caused expansion of otx2, severe decrease or loss of sizzled, loss of ventral fin, and tail truncation, i.e., dorsalized phenotypes. This was reminiscent of the phenotype seen in tsg−/−;bmp7−/− compound mouse mutant embryos (Zakin et al., 2005). It appears that in Xenopus species, different levels of Tsg knockdown can result in opposing phenotypes.

In zebrafish, different levels of morpholino against Tsg elicit qualitatively different phenotypes. Low level knockdown does not cause alterations in early DV patterning. Instead, the ventrally positioned cardinal vein exhibits an edema and pooling of blood cells by 36 to 48 hr postfertilization (hpf) (Ross et al., 2001; Little and Mullins, 2004). This effect is specific to Tsg knockdown, as it can be rescued by coinjection of tsg mRNA, and it is consistent with the observation that tsg transcripts are found in the region of the developing vasculature at 24 hpf (Little and Mullins, unpublished observation). Interestingly, til1 is also expressed in this region (Connors et al., 1999). Recent work has shown that BMP activity after gastrulation limits the extent of lateral mesoderm that gives rise to vasculature and hematopoietic lineages (Gupta et al., 2006), and it is possible that Tollloid and Tsg play a role in regulating BMP signaling in this process.

However, high levels of Tsg morpholino cause moderately dorsalized phenotypes in wild-type zebrafish embryos (Little and Mullins, 2004; Xie and Fisher, 2005). Alterations in dorsal and ventral gene expression domains are seen at midgastrulation, consistent with an early effect of Tsg on BMP signaling. Dorsalization is also seen when low levels of morpholino, which do not alter DV patterning, are injected into bmp2b heterozygotes, a result similar to that seen in the combined knockdown of bmp7 and tsg in X. laevis (Zakin et al., 2005). These results indicate that in zebrafish, Tsg promotes ventral cell fates. This occurs at least in part in cooperation with til1, and involves the inactivation or removal of Chd fragments generated by Tollloid-related factors (Xie and Fisher, 2005). In addition, Tsg must promote BMP signaling as least partially independently of Chd, since Tsg knockdown can partially suppress the phenotype of chordin null mutants. It is currently unclear whether this occurs via the inhibition of additional anti-BMP factors, or involves novel mechanism(s) such as stabilization of ligand-receptor interactions, as proposed in Drosophila (Wang and Ferguson, 2005), or interaction of Tsg-BMP complexes with hypothetical additional factors that could promote high levels of BMP signal.

How can we resolve the apparently contradictory effects of Tsg knockdown on DV pattern and BMP signaling? It seems possible that Tsg could promote BMP during DV patterning in zebrafish, antagonize BMP in X. tropicalis, and in X. laevis Tsg might promote BMP ventrally while antagonizing dorsally, as predicted by the model of Larrain et al. (2001). There may be differences in the biochemical properties of Tsg from different species or additional factors regulating Tsg activity. Alternatively, Tsg's dual activities to promote and antagonize BMP signaling in DV patterning may be conserved in these vertebrates. Considering these opposite activities, it is expected that one activity is epistatic to the other, i.e., that the BMP-promoting activity of Tsg predominates over the BMP-antagonistic activity of Tsg. Thus, in the complete loss of Tsg function, the BMP promoting defect of Tsg would predominate, yielding a dorsalization of the embryonic axis. In this circumstance, the loss of BMP-antagonistic function has no consequence because BMP signaling is also defective. Partial loss of function of Tsg could yield weaker dorsalizations or ventralizations, depending on dosage requirements for Tsg's opposing activities in each of these organisms. Genetic analysis of null and hypomorphic alleles of tsg in zebrafish and X. tropicalis would be valuable to further elucidate the Tsg function in DV patterning. Analysis of the spatial requirements for these opposing activities may also provide important insights. Interestingly, there is evidence in Drosophila that tsg can both promote and antagonize BMP signaling in spatially distinct DV domains of the embryo (Ross et al., 2001).

Sizzled

The positional cloning of the ventralized zebrafish mutant gene, ogon, revealed it to be a mutation in the sizzled gene (Collavin and Kirschner, 2003; Martyn and Schulte-Merker, 2003; Yabe et al., 2003). ogon mutants exhibit expanded blood progenitors and multiple fin folds, similar to chd mutants, but largely normal anterior structures (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996; Miller-Bertoglio et al., 1999).

Named for its sequence homology to the Wnt receptor Frizzled, Sizzled is a member of the secreted Frizzled-related proteins (sFRPs), which are putative antagonists of Wnt signaling (Rattner et al., 1997; Pera and De Robertis, 2000). However, Sizzled does not inhibit Wnt signals, but instead antagonizes BMP signaling (Bradley et al., 2000; Collavin and Kirschner, 2003; Yabe et al., 2003). Sizzled function relies entirely on the presence of Chordin. sizzled; chordin double mutants are not more strongly ventralized than chordin single mutants, and overexpression of Sizzled has no effect in chordin mutants or Xenopus embryos injected with chordin morpholinos (Yabe et al., 2003; Lee et al., 2006).

Unlike other BMP antagonists, sizzled is expressed broadly on the ventral side of the embryo as part of the BMP synexpression group (Salic et al., 1997; Yabe et al., 2003).

Sizzled acts uniquely among known BMP antagonists: it represses BMP signaling by inhibiting Tollloid-mediated degradation of Chordin (Lee et al., 2006; Muraoka et al., 2006). Sizzled directly binds Tollloid-related enzymes in biochemical assays, blocking Tollloid’s ability to cleave Chordin. The kinetics of Sizzled-Xlr binding show that Sizzled competes with Chordin for binding to Tollloid, thus acting as a competitive inhibitor, although Sizzled itself is not cleaved by Tollloid. This binding is mediated...
by CR domains found in Sizzled and other sFRPs. Xenopus Sizzled inhibits both Bmp1 and Xir in vitro, while zebrafish Szl more strongly interacts with Bmp1a and does not strongly inhibit Tll1. This result is supported by the phenotype of the zebrafish double mutant tll1; sizzled, which has a wild-type phenotype (Wagner and Mullins, 2002). From this result, we know that Sizzled retains activity through inhibition of Bmp1a or other Tolloids, even in the absence of Tll1.

sizzled and chordin are expressed in non-overlapping domains. Where then are these two proteins required in the embryo? In overexpression, Sizzled can exert effects on BMP signaling outside of its domain of expression, suggesting long-range trans-signaling outside of its domain of expression (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996; Miller-Bertoglio et al., 1999; Yabe et al., 2003), indicating that Sizzled normally prevents BMP signaling from extending into dorsal regions. The mechanism by which Sizzled achieves this may involve the local inhibition of BMP signaling and prevention of high levels of BMP that otherwise would diffuse dorsally. Alternatively, or in addition, Sizzled may diffuse dorsally, protecting Chd from Tolloid cleavage, and allowing Chordin to restrict BMP signaling to a ventral domain. However, localized ventral expression of sizzled is not required for proper patterning, since injection of sizzled mRNA at the single cell stage rescues ogon to a wild type phenotype (Yabe et al., 2003). Finally, the sizzled mutant phenotype, which shows only mild defects in dorsal-anterior patterning compared to chordin mutants, suggests that Chd does not require protection from Tolloid activity throughout its functional domain. However, multiple sFRPs are present in the early embryo, which may deter Tolloid function on the dorsal side.

Antidorsalizing Morphogenetic Protein (ADMP)

A recent provocative report suggests that a dorsally produced BMP plays a role in the specification of ventral fates (De Robertis, 2006). The origin of this hypothesis was the observation in Xenopus that triple knockdown of BMP2, 4, and 7 expands radially rhombomere 5 of the hindbrain but not more anterior neural tissue, indicating the presence in the anterior of a low level of BMP activity (Reversade et al., 2005). The phenotype of triple knockdown Xenopus embryos resembles that of zebrafish maternal-zygotic alk8 mutants, which exhibit radialization of posterior, but not the most anterior neural tissue (Mintzer et al., 2001). The Xenopus triple BMP knockdown is weaker than zebrafish embryos with mutations in either bmp2b or bmp7 (Kishimoto et al., 1997; Nguyen et al., 1998b; Dick et al., 2000; Schmid et al., 2000). In these mutants, all neural markers are radialized, including anterior ones, indicating that the complete loss of either ligand eliminates BMP signaling. This is not observed in Xenopus, even with triple BMP knockdown, indicating a low level of BMP activity remaining.

Additionally, although ultraviolet (UV)-treated Xenopus embryos that fail to establish an organizer develop only ventral structures, when BMP2, 4, and 7 are reduced in these embryos, neural tissue becomes radially expanded (Reversade et al., 2005). In other words, the reduction of three BMPs induced radially dorsalized ectoderm only in the absence of organizer and/or dorsal tissues, suggesting that when BMP2/4/7 are reduced in normal embryos, a dorsal signal allows specification of some ventral identity.

A candidate for such a signal is the BMP family member ADMP (Moos et al., 1995; Jouin and Stern, 1999; Dosch and Niehrs, 2000; Lele et al., 2001; Willott et al., 2002). ADMP is expressed in the organizer and its vicinity at the onset of gastrulation in both fish and frog, and is found in dorsal axial structures as gastrulation proceeds. Like other BMPs, ADMP binds Chordin and Tsg, and overexpression of ADMP causes ventralization (Dosch and Niehrs, 2000; Lele et al., 2001; Willott et al., 2002; Reversade and De Robertis, 2005). Knockdown of ADMP alone mildly dorsalizes the embryo (Willott et al., 2002; Reversade and De Robertis, 2005). The quadruple knockdown of BMP2, 4, 7, and ADMP leads to complete loss of ventral ectoderm and radial expansion of neural identity, revealing an effect of ADMP at a striking distance from its dorsal source of synthesis. Most impressively, some ventral identity is restored in quadruple knockdown embryos by transplanting dorsal organizer tissue from a normal, unmanipulated embryo (Reversade and De Robertis, 2005). This demonstrates that dorsal tissue produces a ventralizing signal that partially patterns ventral cell fates on the opposite side of the embryo, and indicates that BMP activity mediates the remaining BMP activity in triple BMP2/4/7 knockdown embryos. Interestingly, triple knockdown of BMP2, 4, and 7, together with inhibition of Tolloid activity, also causes radial expansion of neural tissue, suggesting that Tolloid normally proteolyzes Chordin, causing release of ADMP ventrally, which then can pattern ventral tissues.

How could ADMP pattern cell fates at such a substantial distance? An intriguing model is that, like in Drosophila, Chordin may transport ADMP from its dorsal region of synthesis distantly into ventral regions. Cleavage of Chordin ventrally by Tolloid-related proteases would result in the delivery of ADMP into ventral regions, where it specifies ventral tissues. This is a satisfying model and would indicate conservation in function of Chordin and its ortholog Sog in the transport of BMP ligands across substantial distances to pattern the embryo.

But does transport of ADMP by Chordin normally function in the embryo when BMP2, 4, and 7 are present? Unlike the Drosophila sog mutant, which exhibits aspects of both dorsalization and ventralization reflecting its dual roles as an antagonist and agonist of BMP signaling, the chordin loss-of-function phenotype is a ventralization, with the possible exception of a mild, incompletely penetrant tail dorsalization (Wagner and Mullins, 2002; Hammerschmidt and Mullins, 2002). Loss of ADMP causes mild dorsalization, indicating that it plays a limited role in generating ventral BMP activ-
ity. Remarkably, double knockdown of ADMP and Chordin results in a completely phenotypically wild-type embryo (Reversade and De Robertis, 2005). In this biological context, ADMP clearly exerts a ventralizing function independent of Chordin, since epistasis is not observed. Thus ADMP ventralizing activity does not rely entirely on Chordin or Chordin-mediated transport.

ADMP may also function as a negative feedback regulator of dorsally-expressed BMP antagonists. Like other dorsally-expressed genes, ADMP expression requires dorsal signals, which it paradoxically antagonizes. ADMP may function in all or part by binding dorsally-produced BMP antagonists and sequestering them in the dorsal domain, thereby allowing ventrally produced BMPs to function. In zebrafish, where single null alleles of either bmp2b or bmp7 cause radial expansion of neural tissue, this may be the primary function of ADMP. This function of ADMP as a dorsal antagonist could also account for the observed effects of triple and quadruple BMP knockdown in Xenopus, but implies that the BMP morpholino knockdown is not a complete one, a property often difficult to assess with morpholinos. In this case, in BMP2/4/7 triple knockdown embryos, ADMP limits the activity of dorsally produced BMP antagonists, thereby allowing a region of ventral identity to be specified by the remnant of low, ventrally produced BMP activity. This could also explain the ability of transplanted, normal dorsal tissue to rescue BMP2/4/7/ADMP depleted embryos: the return of dorsal ADMP sequesters some portion of BMP antagonists, allowing a remaining low level of ventral BMP to function.

However, ADMP may also impart ventralizing signals in its own right, as it interacts with the divergent BMP receptor Alk2 (Reversade and De Robertis, 2005). Interestingly, ventral BMPs induce their own set of negative feedback regulators (e.g., Sizzled, BAMBI), which limit BMP activity in time and/or space. Understanding the interaction between dorsal and ventral signaling centers, each acting as sources of negative and positive feedback regulators, will be the source of exciting future studies.

**Perspective and Future Directions**

We have reviewed the impressive gains recently made in understanding BMP signal modulation in DV patterning. Extracellular modulation has been revealed as the key means of setting up a gradient of BMP activity, and we now appreciate the many layers of extracellular control. Yet many exciting and fundamental questions remain regarding the interactions in time and space between BMP promoting and antagonizing activities arising from dorsal and ventral signaling centers, and the manner in which the resulting BMP signals are translated into distinct transcriptional readouts.

Although the model of a BMP activity gradient is well established, this has not yet been direct demonstration of a gradient of any factor in vertebrates. How far can BMP antagonists travel from their sites of production? Is the putative movement of antagonists mediated by diffusion or an active process? The extracellular matrix performs critical functions in the shaping and signaling of BMP and other gradients in the Drosophila wing imaginal discs. Does the extracellular matrix play a role in mediating, potentiating, or attenuating antagonist movement and/or BMP signaling in the embryo? Are there additional regulators of BMP signaling in these contexts?

Which asymmetry, or combinations thereof, are essential to form a robust self-regulating BMP morphogen field? Although chordin, bmp2b, bmp7, tolloid, and sizzled are expressed asymmetrically along the DV axis, broad misexpression of each singly can rescue its respective mutant to a completely wild-type phenotype. Multiple mechanisms likely compensate for perturbations in asymmetric expression of these factors to maintain the BMP morphogen field, but how much change can be tolerated? Broad overexpression of any one of these factors can tip the balance, causing either ventralization or dorsalization. However, sufficient self-regulation of the field can compensate for ectopic expression of physiological levels of at least one factor at any given time. The self-regulatory ability of the BMP morphogen field through multiple layers of extracellular and intracellular control likely ensures robustness of the BMP morphogen gradient to normal embryonic perturbations in expression domains and levels. As in Drosophila (Eldar et al., 2002; Mizutani et al., 2005; Shimmi et al., 2005), it is highly likely that computational simulations of the dorsal and ventral signaling centers will aid our understanding of their interactions and assist us in generating new predictions. In the years ahead, we are certain to see exciting developments which will vastly improve our understanding of these and many other important questions regarding the formation and interpretation of BMP gradients in DV patterning.

**REFERENCES**


Blader P, Rastegar S, Fischer N, Strahle U. 1997. Cleavage of the BMP-4 an-
EXTRACELLULAR MODULATION OF BMP ACTIVITY 239

Goodman SA, Albano R, Wardle FC, et al. 1998. BMP1-related metalloproteinas promote the development of


