Published in final edited form as:

Dev Dyn. 2008 August ; 237(8): 2177–2186. doi:10.1002/dvdy.21631.

Bmp signaling is necessary and sufficient for ventrolateral endoderm specification in *Xenopus*

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Abstract

Here we show that Bmp signaling is necessary and sufficient for the specification of ventral endoderm in *Xenopus* embryos. Overexpression of *Bmp4* in ectoderm induces markers of endoderm, including *Sox17* β , *Mixer* and *VegT*, but cannot induce the expression of the dorsoanterior markers, *Xhex* and *Cerberus*. Furthermore, knockdown approaches using overexpression of Bmp antagonists and morpholinos designed against *Bmp4*, *Bmp2* and *Bmp7* demonstrate that Bmp signaling is critical for ventral, but not dorsoanterior endoderm formation. This activity is not simply a result of embryonic dorsalization as markers for dorsal endoderm are not expanded. We further show that no endodermal cells of either ventral or dorsal character form when both Wnt and Bmp signals are abolished. Overall, this report strongly suggests that Bmp plays an essential role in ventral endoderm specification.

Keywords

Bmp; endoderm; *Xenopus*; ventrolateral; TGF β

Introduction

Endoderm is one of the first cell types to emerge in the embryo and gives rise to a host of critical tissues, including the pancreas, liver and lung. In the frog, endoderm is initially specified by cytoplasmic determinants localized vegetally within the oocyte. The T-box transcription factor VegT plays an essential role in endoderm formation in *Xenopus* (Zhang et al., 1998; Xanthos et al., 2001). During blastula stages, a gradient of VegT induces different cell fates. High levels of VegT in the vegetal blastomeres induce endodermal cell fates while lower levels of VegT in the overlying cells induce mesodermal cell fates equatorially. Although VegT is thought to trigger these inductive events, zygotically transcribed TGF β molecules, primarily Nodal-related factors, maintain the expression of VegT, forming a positive feedback loop that ensures both mesodermal and endodermal specification (Kofron et al., 1999; Xanthos et al., 2001). These Nodal signals are necessary

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and sufficient for both the formation of endoderm and mesoderm and therefore must be interpreted selectively in each tissue to form correct lineage specific cell fates. Sox17 β and Mixer are transcription factors expressed within the endoderm and represent such downstream cell fate modulators (Hudson et al., 1997; Henry and Melton, 1998). These factors are involved in defining the boundary between endoderm and mesoderm (Engleka et al., 2001; Kofron et al., 2004). In fact, expression of Sox17 inhibits the ability of Nodal-like molecules to induce mesoderm, thus suggesting that it helps to distinguish the two tissue types (Hudson et al., 1997; Engleka et al., 2001). Mixer, also an endoderm inducer, is thought to antagonize the formation of mesoderm and thus controls widespread mesoderm formation within the embryo (Henry and Melton, 1998; Kofron et al., 2004).

The endoderm is comprised of several different cellular subtypes, which create a dorsal-ventral polarity. Endoderm formed on the dorsal side is not equivalent to that of the ventral side and, in fact, the dorsal endoderm provides a substantial and powerful effect on the formation of the Spemann's Organizer. Recently, it has been shown that - in addition to its role in dorsal ventral patterning - functional β -catenin is critical for the formation of the dorsal anterior endoderm, and is necessary for the correct expression levels of Sox17 β target genes, including *Edd* and *FoxA2* (Jones et al., 1999; Zorn et al., 1999; Sinner et al., 2004). Other molecular signals that specify distinct endodermal domains have yet to be elucidated.

Here we show that Bmp signaling plays an essential role in endoderm formation during *Xenopus* development; overexpression of Bmp4 induces endodermal fates and a triple knockdown of Bmp2, 4 and 7 selectively inhibit ventral, but not dorsoanterior, endoderm formation. This provides strong evidence that Bmp signaling is involved in the specification of the ventral aspect of this critical germ layer.

Materials and Methods

Transcription Reactions

DNA plasmids were linearized at the appropriate restriction sites and mRNA was transcribed with mMessage machine (Ambion, Austin, TX). The plasmids used are as follows:

RNA	Vector	Linearization	Reference
<i>Noggin</i>	pCS2+	NotI	(Smith and Harland, 1992)
<i>Bmp4</i>	pSP64T	EcoRI	(Nishimatsu et al., 1992)
<i>mBmp4</i>	CS107	AscI	(Marc Dionne, unpublished)
<i>Alk3 Q233D</i>	pCS2+	NotI	(Hsu et al., 1998; Wieser et al., 1995)
<i>DN-Alk3</i>	p64T	EcoRI	(Graff et al., 1994)
<i>Chordin</i>	pCS2+	NotI	(Sasai et al., 1994)
β -galactosidase	pCS2	NotI	(Turner and Weintraub 1994)

Microinjections

Female frogs were primed for ovulation with human chorionic gonadotropin (Condie and Harland, 1987). *Xenopus laevis* eggs were collected and fertilized *in vitro* according to the standard protocol (Sive et al., 2000). We used 1× modified ringers (MR) solution (instead of MBS) for egg collection and 1/3× MR for fertilization. Embryos were transferred into dishes containing 1/3× MR with 2.5% ficoll and injected at the one-cell stage into the animal pole (presumptive ectoderm) for animal cap assays or the marginal zone (presumptive mesoderm) and vegetal pole (presumptive endoderm) for whole mount *in situ* hybridization assays.

For *X. tropicalis*, ovulation of adult *X. tropicalis* (Nasco) and *in vitro* fertilization of eggs were conducted as described (<http://tropicalis.yale.edu>). Fertilized eggs were de-jellied in 3% cysteine (pH 8.0) in water for 10–15 minutes before transferring to agarose-coated dishes containing 3% ficoll 400 in 1/9X modified frog ringer's solution (MR) prior to injection. Miniruby fluorescent dextran (Molecular Probes, Lane and Smith 1999) was used as a tracer for microinjection of MOs, and nuclear-localized beta-galactosidase mRNA (Turner and Weintraub 1994) was used as a lineage tracer for mRNA microinjection. Embryos were raised at 28°C and collected at gastrula stages.

Morpholino sequences

Bmp2: GCCCCTTTCCTTACCTTCATGGTGA

Bmp4: CCAGGAATCATGGTGTCTTGACAGA

Bmp7.1: TGCAGAACATTTCATTTCCCTTGGAT

β-catenin: TTTCAACAGTTTCCAAAGAACCAGG

In situ hybridizations

Anti-sense probes were generated from the following plasmids:

Probe	Vector	Reference
<i>Xbra</i>	pGEM7	(Smith et al., 1991)
<i>Sox17β</i>	pBSSK	(Hudson et al., 1997)
<i>Mixer</i>	pBSKS	(Henry and Melton, 1998)
<i>xtMixer</i>	CS107	Tgas105b05
<i>xtSox17α</i>	pCMVSPORT6	Image: 6994003
<i>xtCerberus</i>	CS107	Tgas016c17
<i>xtHex</i>	CS107	Tgas075h17
<i>xtMyf5</i>	CS107	Tgas04013
<i>xtVent2</i>	CS107	Image:4462078
<i>xtBra</i>	CS107	Tgas023E12
<i>xtDerriere</i>	CS107	Tgas141f11

Xenopus embryos were developed to stage 10.5, fixed in MEMFA and stored in methanol. In situ hybridization analysis was performed as described (Harland, 1991; Sive et al., 2000, Khokha 2002) on whole or bisected embryos.

Ectoderm and Vegetal pole explants

Synthetic mRNA was injected into the animal poles of one-cell embryos, developed to stage 8 and transferred to 1% agarose coated dishes containing 3/4× NAM (Peng, 1991). Ectodermal tissue was excised using an eyebrow knife and hair loop and cultured in 3/4× NAM until intact sibling embryos reached stage 10.5 or 18. Uninjected embryos were developed to stage 8 and transferred to 1% agarose coated dishes containing 3/4× NAM. For vegetal explants, endodermal tissue was excised and cultured as described for ectodermal explants above.

RT-PCR Analysis

RNA was isolated and RT-PCR was performed as previously described (Wilson and Melton, 1994). For new primers, PCR products were analyzed after 30 cycles of amplification. The following primer sequences were used:

Gene	Primer Sequence	Reference
<i>Sox17β</i>	F-AACTCCCACCAGCAGGCTACTTTG R-TGTCAATGTCACTCTCCAGATGTCC	(Myers et al., 2004)
<i>Mixer</i>	F-CACCAGCCCAGCACTTAACC R-CAATGTCACTCACTGAAG	(Henry and Melton, 1998)
<i>VegT</i>	F-CAAGTAAATGTGAGAAACCGTG R-CAAATACACACATTTCCCGA	(Zhang et al., 1998)
<i>Xbra</i>	F-GGATCGTTATCACCTCTG R-GTGTAAGTCTGTAGCAGCA	(Wilson and Melton, 1994)
<i>ODC</i>	F-CAGCTAGCTGTGGTGTGG R-CAACATGGAACTCACACC	(Agius et al., 2000)
<i>Cerberus</i>	F-GCTTGCAAAACCTTGCCCTT R-GTGATGGAACAGAGATCTTG	(Bouwmeester et al., 1996)
<i>Xhex</i>	F-AACAGCGCATCTAATGGGAC R-CCTTTCCGCTGTGTCAGAGG	(Newman et al., 1997)
<i>Mix.1</i>	F-GCAGATGCCAGTTCAGCCAAT R-TTTGTCCATAGGTTCCGCCCTG	(Wilson and Melton, 1994)
<i>Bmp2</i>	F-AGCCAGCCGAGCAAATACAGTG R-GGTGTCCAATAGTCTCACACAGGG	
<i>Bmp4</i>	F-AGCCCCGTTGTCATAGAACTTC R-AGTGTGCGTGTGGTGATTCCAG	
<i>Bmp7</i>	F-TTTATCCAGAGGAGGTTGCGAGGC R-TGTCAGCGTCATTGAGGAAGTTG	
<i>Alk3</i>	F-TTCTCCAAAAGCCCTGTCACGG R-CAATAATGGTAGCCCTGAGCCAC	

Gene	Primer Sequence	Reference
<i>Xhox-3</i>	F-ATATGATGAGCCACGCAGCAG	(Suzuki et al., 1997)
	R-CAGATGCTGCAGCTCTTTGGC	
<i>Msx-1</i>	F-GCTAAAAATGGCTGCTAA	
	R-AGG TGG GCT GTG TAA AGT	

Results

Bmp4 induces ventral endodermal fates

The Bmp family plays critical roles in the establishment of dorsal/ventral patterning and induction of neural tissue in the vertebrate embryo. We hypothesized that Bmp4 may contribute to the specification of endodermal sub-types. To test this theory, we overexpressed Bmp4 and assayed for its ability to induce endoderm in ectodermal explants. Indeed, Bmp4 can induce *Sox17 β* , *VegT*, *Mixer*, and *Mix.1* (Fig. 1A–B), as well as inducing its known downstream targets *Xhox3* and *Msx1* (Fig. 1C; Hemmati-Brivanlou and Thomsen, 1995; Suzuki et al., 1997). Explants exposed to Bmp4 protein also display *Sox17 β* induction (data not shown). Although Bmp4 can induce markers of a general endodermal character, it cannot induce the dorsoanterior markers, *Cerberus* and *Xhex*, suggesting that Bmp4-mediated endoderm induction specifies more ventrolateral endodermal fates and thus discriminates this Bmp-like activity from that of Nodal, which can induce the broad range of fates (Jones et al., 1995; Osada and Wright, 1999) (Fig. 1B). To verify that the observed induction of *Sox17 β* is truly a result of Bmp signaling and not the consequence of a promiscuous ligand activating the Nodal pathway, we overexpressed the activated Bmp receptor, *Alk3 Q233D* (Wieser et al., 1995). Like *Bmp4*, *Alk3 Q233D* can induce *Sox17 β* and *Xbra* in ectoderm (Fig. 1D). This result confirms that Bmp signaling is sufficient to induce endodermal gene expression.

If Bmp signaling is of physiological importance for endoderm formation, then at least the mediating receptor and perhaps the cognate ligand should be expressed within this domain. Therefore, to deduce whether components of Bmp signaling are present within the endoderm, we performed RT-PCR analysis on vegetal explants for *Bmp2*, *Bmp4*, *Bmp7* and *Alk3*. This analysis revealed that all four molecules are expressed vegetally as well as in the animal pole, suggesting that they could be involved in the formation of endoderm (Fig. 2). This evidence supports the work of others who detected robust Bmp activity in the ventral vegetal endodermal cells at stage 10 using an antibody against phosphorylated Smad1 (Faure et al., 2000; Kurata et al., 2001).

Bmp antagonists disrupt early endoderm formation

We have shown that Bmp signaling is sufficient for endoderm induction and that components of this pathway are present within endogenous endoderm. Therefore, we wanted to examine whether Bmp signaling is necessary for endoderm formation in *Xenopus*. Toward this goal, we overexpressed the Bmp antagonists, *Chordin* and *Noggin*, and dominant negative *Alk3* (DN-*Alk3*) in the vegetal hemisphere and assayed for effects on *Sox17 β* expression during gastrulation. Embryos containing any sign of cell death were

discarded and not used in the analysis. As shown in Fig. 3, we observed disrupted *Sox17* β expression in 67% of embryos injected with *Chordin* (n=36) and 48% of embryos injected with *Noggin* (n=27) versus 11% of water injected control embryos (n=27). In a duplicate experiment, Chordin affected 52% (n=50; Fig. 3C), Noggin affected 68% (n=62; Fig. 3D), compared to 15% in controls (n=61; Fig. 3A). In a separate experiment, we overexpressed *DN-Alk3* in the endoderm and observed disrupted *Sox17* β expression in 75% of injected embryos (n=55) compared to 15% of control embryos (n=42) (Fig. 3B). The more lineage-specific inhibition of *Sox17* β observed in this sample may reflect the difference between cell-autonomous (*DN-Alk3*) and secreted (*Chordin* and *Noggin*) Bmp antagonists. To verify that the reduction of *Sox17* β expression observed in antagonist-injected embryos was not due to cell death, we repeated these experiments using a range of mRNA doses, and using nuclear *lacZ* as a tracer for microinjection (Figure 3E–J). While only 5.9% of embryos injected vegetally with *lacZ* alone showed reduced *Sox17* β expression (n=17, Figure 3H), embryos coinjected with *lacZ* and low or high doses of Bmp antagonists had broadly reduced *Sox17* β expression while retaining clear nuclear β -gal staining, confirming a non cell-autonomous effect on *Sox17* β . Specifically, we found that *Sox17* β expression was reduced in 77.8% of embryos injected with 10pg of *Noggin* mRNA (n=27, Fig 3F), 85.2% of embryos injected with 100pg of *Noggin* (n=27, Fig 3G), 82.1% of embryos injected with 100pg *Chordin* mRNA (n=28, Fig 3I), and 84% of embryos injected with 500pg of *Chordin* mRNA (n=25, Fig 3J). The degree of expression lost was variable with slightly different targeting, as might be expected for the effects of secreted antagonists. These data strongly suggest that Bmp signaling has an endogenous function during early endoderm formation.

Bmps are necessary for endoderm formation in *Xenopus*

Although Bmp antagonists are thought to specifically affect Bmp signaling during development, it remains plausible that they may also inhibit other pathways. Therefore, to define the specific role of Bmp signaling in *Xenopus* endoderm specification, we used morpholino oligos (MO) to knock down Bmp2, Bmp4, and Bmp7, together or in various combinations (Fig. 4A, B). We injected *X. tropicalis* embryos with 10ng of each MO per blastomere at the 2-cell stage. While we observed little to no reduction in the expression of *Sox17* α in either the single or double knockdowns, a triple knock down of Bmp2, Bmp4 and Bmp7 dramatically decreased the expression of *Sox17* α (92.1%, n=38, sum of four experiments) and *Mixer* (86.8%, n=38 sum of four experiments) (Fig. 4A,B). Loss of endodermal cell fates could be rescued by co-injection of mouse *Bmp4* mRNA (Fig. 4B). Interestingly, embryos injected with all three Bmp MOs retain the most dorsal expression of *Sox17* α (65.8% n=38, sum of four experiments) and *Mixer* (71.1%, n=38 sum of four experiments) despite the substantial reduction of their ventral endoderm expression (Fig. 4B, Fig. 5). This was especially clear when Bmp morphants were bisected along the dorsal-ventral axis, which revealed strong staining in the dorsal lip but not ventrolaterally. Consistent with this, *Cerberus* and *XHex*, two markers of dorsoanterior endoderm, retained strong expression in embryos depleted of all three Bmps (*Cerberus*: 100%, n=30; *Xhex*: 89.5%, n=19; Fig. 5), further suggesting that Bmp signaling is necessary for ventrolateral, but not dorsoanterior, endoderm specification. This result is consistent with our previous findings that expression of Bmp4 itself is not sufficient to induce dorsoanterior endoderm. Furthermore, embryos lacking Bmp2, 4 and 7 still contain mesoderm as judged by staining

for *Xbra*, and still have intact Smad2 signaling as judged by staining for the *Vg-1* related gene *Derriere*. However, patterning within the mesoderm appears to be affected since *Myf5* and *Vent2* are missing or largely reduced (Fig. 4C). The reduction of expression of these markers is consistent with our expectation for embryos depleted of Bmps, since *Vent2* is a direct target of Bmp signaling and *Myf5* is known to be sensitive to manipulation of Bmp levels (Dosch et al. 1997; Sinner et al. 2004) and supports the established role of Bmp in dorsal ventral mesodermal patterning.

Since Wnt signaling is a critical component of dorsal ventral patterning, which is known to set up the anterior endodermal domain (Zorn et al., 1999; Jones et al., 1999; Sinner et al., 2004), we sought to determine whether knocking down all Bmp and Wnt signaling would eliminate the formation of all endodermal tissues. Knockdown of Wnt signaling with a β -catenin MO (Heasman, 2000; Khokha, 2002) reduces the expression of dorsoanterior endoderm marked by loss of *Cerberus* expression (12/12=100%) but did not appear to affect general endoderm specification (Fig. 5). However, a quadruple knockdown of Bmp2, 4, 7 and β -catenin abolishes all endoderm (Fig. 5). In embryos depleted of all four gene products, expression of *Mixer* and *Sox17a* was either absent (*Mixer*: 42.9%, n=14; *Sox17a*: 10%, n=20), or very weakly expressed in only a few cells (*Mixer*: 57.1%; *Sox17a*: 90%), while *Cerberus* expression was absent in all injected embryos (n=12). Thus the combined effect of Bmp and Wnt signaling is essential for all aspects of endoderm in *Xenopus* embryos.

Discussion

In this report we show that Bmp signaling is critical for the specification of ventrolateral endoderm in *Xenopus*. While Bmp signaling has been implicated in having many critical roles in developmental processes, including neural induction and dorsal ventral patterning, its affect on endoderm has not been widely studied (Jones et al., 1992; Suzuki et al., 1994; Hawley et al., 1995; Schmidt et al., 1995; Harland and Gerhart, 1997; Dale and Jones, 1999; Shivdasani, 2002). It has been known for many years that Bmp4 is expressed within the ectodermal domain and that high concentrations of Bmp4 or the correct formation of heterodimers between Bmp4 and Bmp7 can induce mesoderm (Suzuki et al., 1997; Dale and Jones, 1999). We now show that this is also true for endoderm. Although Bmp4 is expressed within the ectoderm during gastrulation stages, this does not mean that it is capable of inducing mesoendodermal derivatives within these cells. It is possible that the complete signaling network is not normally activated within these cells in the endogenous stage, which occurs only with addition of high levels of exogenous protein. The main focus of this paper however is to provide genetic evidence for the involvement of endogenous bmp signaling within the embryonic milieu and thus here we show both that bmp signaling is necessary and sufficient for endodermal specification.

Several lines of evidence over the past ten years lend support to our finding that Bmp signaling plays a role in endoderm formation. Perhaps the most striking of these is fact that Bmp activated phosphorylated Smad1 is present throughout the vegetal cells in *Xenopus* after MBT as early as stage 9 (Kurata et al., 2001). Phosphorylated Smad2, the downstream modulator of Nodal-like TGF β signals, is not detected throughout the vegetal cells until stage 9.5, although some expression is observed in the dorsal marginal region at stage 9

(Faure et al., 2000; Kurata et al., 2001). Furthermore, during gastrulation phospho-Smad1 is detected strongly in the ventral vegetal cells and excluded from the dorsal zone, consistent with the proposed role of Bmp4 signaling in ventral but not dorsoanterior endoderm specification (Faure et al., 2000; Kurata et al., 2001). Genetics in the mouse and Zebrafish also support the involvement of Bmp signaling in the formation and patterning of endoderm. Mouse embryos having mutations in Bmp4, its type I receptor, BmpRIA, and its type II receptor, BmpRII, all arrest at gastrulation, failing to form mesoderm and subsequent tissues (Mishina et al., 1995; Winnier et al., 1995; Beppu et al., 2000). This inhibits studying the role of these molecules in the direct formation of endoderm, although it does demonstrate the importance of a role for Bmp signaling during gastrulation. Studies on BmpRIA mutant chimeric mice demonstrate that embryos develop defects in endoderm morphogenesis with an improperly formed and patterned endoderm (Davis et al., 2004). Similar effects have been shown with Bmp2b embryonic mutations in Zebrafish (Tiso et al., 2002). In these mutant embryos, endoderm is formed, but patterning is severely affected. Multiple Bmps and signaling components exist in all vertebrates, making single mutations difficult to interpret in light of the complexity of signaling cascades. While there are clearly endodermal abnormalities in these examples, genetic redundancy may account for the varying degrees of disruption.

Additional evidence for the involvement of Bmp signaling in endoderm formation emerges from the collection of known transcriptional targets for Bmp. *Vent1* and *Vent2*, both known Bmp targets, are expressed within the ventral posterior endoderm in *Xenopus*, consistent with a role in mediating Bmp signaling within this domain (Sinner et al., 2004). *Vent2* was also localized to the endoderm of Zebrafish embryos at tailbud stage (Costa et al., 2003). Furthermore, Mix.1 is a transcription factor known to be involved in early endoderm formation activation and is dependent upon Bmp4 signaling (Mead et al., 1996). We are currently investigating the roles these molecules may play to mediate Bmp role in the formation of ventrolateral endoderm.

While the bulk of literature supports our finding, several reports contradict our results (Sasai et al., 1996; Poulain et al., 2006). Sasai et al., showed that ectoderm expressing Noggin and Chordin led to the induction of the endoderm marker, *Edd*, and the pancreas marker, *Xlhbox8*, during late neurula stages (Sasai et al., 1996). This would suggest that inhibition, not activation, of Bmp signaling leads to endoderm formation. While this appears contradictory to our results, this may be attributable to the significantly different stages analyzed in the two reports and thus may reflect the induction of distinct tissue types by Bmp antagonists during embryogenesis. Poulain et al., further suggest that Bmp signaling inhibits endoderm specification in Zebrafish. This result is in direct contrast to ours as they overexpress Bmp2, 4 and 7 vegetally and observe local reduction in *Sox17* β expression during gastrulation (Poulain et al., 2006). Further work is required to understand the basis of these discrepancies.

In this paper, we propose a model for endoderm specification which appears to be consistent with our results and others. Initially, VegT plays an essential role in specifying endodermal and mesodermal cell fates as depletion leads to a substantial reduction in these two germ layers (Zhang et al., 1998). These signals are actively maintained by Nodal signaling after

MBT (Kofron et al., 1999; Xanthos et al., 2001). Cells receiving the VegT signal are then acted upon either by Bmp or β -catenin signals, which are ventrally or dorsally located, respectively. Both Bmp and β -catenin signals are essential for further endodermal specification. β -catenin signaling is activated on the dorsal side of the embryo and results in dorsoanterior endoderm expression marked most clearly by *Cerberus* expression. Knockdown of β -catenin results in a dramatic reduction in *Cerberus* expression (Jones et al., 1999; Zorn et al., 1999; Sinner et al., 2004). On the ventral side, Bmp4 overexpression can induce *Mixer* and *Sox17 β* in naïve ectoderm but not *Cerberus*. Consistent with this observation, depletion of Bmps strongly affects ventrolateral endodermal fates, but preserves the dorsal endodermal cells, which express *Mixer*, *Sox17 α* and *Cerberus*. We propose that this maintenance of dorsal expression is due to the remaining β -catenin mediated activation of dorsal Wnt signaling. The autonomy of these two endoderm-inducing pathways, the Bmp-mediated and the Wnt-mediated, account for our observation that dorsal endoderm is preserved in Bmp morphants, but not necessarily expanded. This is in contrast to mesodermal patterning, where manipulation of Bmp signaling directly changes the proportion of dorsal versus ventral mesodermal fates but not the induction of mesoderm, as we see from the preservation of *Xbra* but reduction in *Vent2* and *Myf5* expression in Bmp morphants. Overall, our results, coupled with many reports in the literature, lend a tremendous amount of support for the involvement of Bmp signaling in endoderm formation and point to a mechanism by which Bmp and Wnt signaling act to specify distinct regions within the endoderm.

Acknowledgments

We thank Eric Chiao, Dale Frank and Elena Casey for valuable discussions and comments. We also thank Heather McCullough and Jeff Leonard for assisting with experiments. Special thanks to Eddy DeRobertis, Richard Harland, Janet Heasman, Hugh Woodland, Kunxin Luo, Doug Melton, Joan Massague and Jim Smith for contributing plasmids. CS107 mBMP4 construct was generated by Marc Dionne. This manuscript was funded by the generosity of the Donald E and Delia B. Baxter Foundation and from the National Institutes of Health (R01 HD41557).

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Figure 1. Bmp4 and Alk3Q233D induce endodermal markers

(A) Embryos were injected with doses of *Bmp4* mRNA ranging from 0.1 ng to 1.0 ng. Ectoderm was explanted from these embryos, harvested at stage 10.5 and analyzed by RTPCR for the expression of *Sox17β* and *Xbra*. (B) Embryos were injected with 0.5 or 1.0 ng *Bmp4*. Ectoderm was explanted and harvested at stage 10.5. RT-PCR was performed using primers for *Sox17β*, *VegT*, *Mixer*, *Mix.1*, *Cerberus* (*Cer*). (C) Embryos were injected with 0.5ng of *Bmp4* and explants were performed as in (A), but with primers for *Xhex* or *Xhox3*, *Msx1*, and *Sox17β*. (D) Embryos were injected with 0.5 ng *Alk3 Q233D* and explants

were performed as in (A). *ODC* was used as a loading control for all experiments (A – D). WE, whole embryo; -RT, minus reverse transcriptase.

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Figure 2. *Bmp2*, *Bmp4*, *Bmp7* and *Alk3* are expressed in vegetal cells

Animal and vegetal hemispheres were explanted at stage 8.0, cultured and harvested at stage 10.5. RT-PCR analysis was performed using primers for *Bmp2*, *Bmp4*, *Bmp7*, *Alk3*, *Sox17β* and *Xbra*. cDNA amounts were normalized. *ODC* was used as a loading control. WE, whole embryo; -RT, minus reverse transcriptase.

Figure 3. Bmp antagonists disrupt early endoderm formation

One-cell embryos were injected vegetally with mRNA encoding the following Bmp Antagonists: (B) *DN-Alk3* (0.5 ng), (C) *Chordin* (0.5 ng), and (D) *Noggin* (0.5 ng) and assayed by *in situ* hybridization with a probe for *Sox17β* at stage 10.5. (A) is a control injected with water. In F-J, 2-cell stage embryos were injected vegetally with 200pg of *lacZ* mRNA, and mRNA for *Chordin* or *Noggin* as indicated. Embryos were fixed at stage 11, stained for *lacZ* using red gal substrate and analyzed for *Sox17β* expression.

Figure 4. Bmp signaling is essential for endoderm formation in *Xenopus*

X. tropicalis embryos were injected at the two-cell stage with 10ng per blastomere of each MO indicated, then analyzed for expression of *Sox17 α* (A, B) or *Mixer* (B) at stage 10.5. Miniruby fluorescent dextran was used as a tracer for MO injection. In (B), a subset of MO-injected embryos were re-injected in one ventral blastomere at the 4-cell stage with 0.5ng of mouse *Bmp4*. Nuclear β -galactosidase (red) was used as a tracer. (C) Triple Bmp morphant embryos were also analyzed for expression of *Xbra*, *Derriere*, *Myf5* and *Vent2*.

Figure 5. Combination of Wnt and Bmp signaling is required for endoderm formation
X. tropicalis embryos were injected with 10ng per blastomere of each MO indicated at the 2-cell stage, then analyzed at stage 10.5 for expression of the general endoderm markers *Sox17 α* and *Mixer*, or the dorsoanterior endoderm markers *Cerberus* and *Xhex*.