Coordinate involvement of Nodal-dependent inhibition and Wnt-dependent activation in the maintenance of organizer-specific bmp2b in zebrafish

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ABSTRACT A vertebrate signaling center, known in zebrafish as the organizer, is essential for axis patterning and formation and is regulated by multiple cell signaling pathways, including Wnt, Nodal, and Bmp. Organizer-specific Bmp2b plays important roles in the maintenance of the Bmp activity gradient and dorsal-ventral patterning. However, it is unknown how transcription of bmp2b in the organizer is regulated. In this study, we generated a bmp2b transgenic line Tsg(-2.272bmp2b:gfp) that reproduced organizer-specific bmp2b expression. Dissection analysis revealed that a 0.273-kb minimal promoter was indispensable for bmp2b expression in the dorsal organizer. Reporter assays showed that organizer-specific bmp2b is negatively regulated by the Nodal signal and positively regulated by the Wnt signal in both embryos and cell lines. Promoter analysis and chromatin-immunoprecipitation (ChIP) indicated that one consensus Smad-binding element (SBE) (CAGAC) and one Lef/Tcf-binding element (LBE) (AGATAA) were present in the 0.273-kb promoter, and could be directly bound by Smad2 and β-catenin proteins. Together, these results suggest that maintenance of organizer-specific bmp2b expression involves opposite and concerted regulation by Nodal and Wnt signaling.

KEY WORDS: zebrafish, organizer-specific bmp2b, transcriptional regulation, Nodal, Wnt

Introduction

A signaling center known as the organizer in zebrafish plays an essential role in germ layer formation and body axis patterning during early embryogenesis. Embryological manipulations have confirmed that analogous signaling centers are ubiquitously present in vertebrate embryos, and are known as “Spemann Organizer” in Xenopus, “Hensen’s node” in chicken, and “node” in mouse. These signaling centers are responsible for controlling cell fate, and studies using transplant experiments have demonstrated that their activity can induce a second body axis (Harland and Gerhart 1997; Kelly et al., 2000; Medina et al., 1997; Schier 2001; Yasuo and Lemaire 2001).

In zebrafish, the organizer forms during the shield stage at 6 hours postfertilization (hpf) in a process that involves regulation by members of multiple cell signaling pathways. Among these, Wnt signal plays an important role during dorsal organizer generation. Recent study reported that, the maternal Wnt8a which functions as the dorsal determinant, is required for the primary dorsal center localization (Lu et al., 2011), while the zygotic Wnt signal restricts...
organizer size after gastrulation (Ramel and Lekven 2004; Schier 2001). In addition, Nodal, Fibroblast growth factors (Fgf), Bone morphogenetic proteins (Bmps) and other factors are required for the formation of the dorsal organizer and correct axis patterning (Belo et al., 2009; Hikasa and Sokol 2013; Langdon and Mullins 2011; Maegawa et al., 2006; Schier 2001; Schier and Talbot 2001; Schier and Talbot 2005).

Bmps, which are members of the TGF-β superfamily, exhibit a broad spectrum of biological activities and function as morphogens during embryonic and organ development, contributing to the formation of bone, blood vessels, heart, kidney, neurons, and liver (Langdon and Mullins 2011; Miyazono et al., 2010; Yamamoto and Oelgeschlager 2004). In Xenopus and zebrafish gastrulas, Bmps are distributed in a gradient along the dorsal-ventral axis, where they play an important role in germ layer induction and dorsoventral patterning. In the ectoderm, high levels of Bmp activity specify epidermal fates, intermediate levels lead to the formation of the central nervous system (CNS), and low levels are required for induction of the neural crest. In the mesoderm, high levels of Bmp facilitate formation of the lateral plate mesoderm (LPM), blood tissues, and kidneys, whereas lower Bmp activity is essential for notochord formation (De Robertis and Kuroda 2004; Heasman 2006; Langdon and Mullins 2011; Plouhinec et al., 2013).

Bmp activity gradients are maintained by a large network of molecular regulators that act at the transcriptional and translational levels. The Bmp antagonist Chordin (Chd) contributes to low Bmp activity on the dorsal side of early Xenopus and zebrafish embryos, where it is secreted and binds to Bmp protein, preventing its intracellular signal transduction. In contrast, in Drosophila, the Chordin homolog Sog, is produced on the ventral side of the embryo, where it binds and inhibits diffusion of the Bmp homolog Dpp from the dorsal side of the embryo. The complex is then transported back to the dorsal side of the embryo for degradation (Ben-Zvi et al., 2011; Ben-Zvi et al., 2008; O’Connor et al., 2006; Plouhinec et al., 2011). Other known regulators, such as the metalloproteinase Tolloid, can bind and digest Chd. In addition, twisted Gastrulation (Tsg) functions extracellularly to facilitate formation of a Chd-Tsg-Bmp trimolecular complex. When this complex flows to the ventral side of the embryo, the high levels of Crossveinless-2 (CV2) there contribute to degradation of Bmp (Plouhinec et al., 2011; Umulis et al., 2009; Zakin and De Robertis 2010).

In early zebrafish gastrulas, different from other bmp genes with the expression in ventral mesoderm, bmp2b is also enriched at organizer region exclusively. Its asymmetric expression pattern is initiated by transcriptional repression by bozozok (boz) during the late blastula stage at approximately 4 hpf, the expression of boz in the organizer ceases from 50% epiboly stage onward (Leung et al., 2003; Solnica-Krezel and Driever 2001; Yamanaka et al., 1998). We recently showed that organizer-derived Bmp2b is required for maintenance of the Bmp activity gradient during embryonic development (Xue et al., 2014). However, the elements that mediate transcriptional regulation of organizer-specific bmp2b remain unknown.

In this study, we aimed to determine the functional elements that regulate organizer-specific bmp2b expression. We generated the novel transgenic line Tsg(-2.272bmp2b:gfp), in which the 2.27-kb bmp2b promoter drives organizer-specific bmp2b expression. To determine the role of bmp2b promoter elements on its transcriptional regulation, we isolated a 0.273-kb minimal promoter from the main 2.272-kb sequence. The minimal promoter, which harbored one consensus Smad-binding element (SBE) and one Lef/Tcf binding element (LBE), responded to Nodal/Smad2 and Wnt/β-catenin signal through direct binding of these elements by Smad2 and β-catenin protein, respectively. Our results indicate that maintenance of organizer-specific bmp2b involves its direct repression by Nodal signal and activation by Wnt signal.

Results

Zygotic GFP expression of Tsg(-2.272bmp2b:gfp) transgenic embryos is specifically restricted to dorsal organizer region

We recently reported that a 2,620-base pair (bp) bmp2b pro-
Molecular dissection identifies a functional sequence of bmp2b promoter for dorsal organizer expression

In order to determine which region of the 2.272-kb bmp2b promoter is required for organizer-specific bmp2b localization, we created a pGL3(-2.272bmp2b:luc) construct in which the 2.272-kb bmp2b promoter drives expression of luciferase. A series of truncated forms of the constructs Tg(-2.272bmp2b:gfp) and pGL3(-2.272bmp2b:luc) were generated to dissect the 2.272-kb bmp2b promoter (Fig. 2). Deletion analysis identified a 0.273-kb proximal promoter region essential for GFP expression in embryos. Injection of the Tg(-0.273bmp2b:gfp) plasmid into embryos at the one-cell stage gave rise to transient GFP expression specifically (usually >80%) in the organizer region at the shield stage (Fig. 2B). This truncated promoter also drove transcription of the luciferase reporter in HEK293 cells transfected with the pGL3(-0.273bmp2b:luc) plasmid (Fig. 2A). Taken together, these data suggest that the 0.273-kb minimal promoter is sufficient to mediate expression of the reporter gene in the organizer region.

Nodal and Wnt signals contribute differently to regulation of organizer-specific bmp2b

A question of interest to us is how organizer-specific bmp2b expression is controlled. We used the pGL3(-0.273bmp2b:luc) construct to perform a luciferase reporter assay in different cell lines. Luciferase expression was repressed by co-transfection of zebrafish Smad3b (Jia et al., 2008) in Hep3B cells, suggesting that Nodal signaling inhibits bmp2b expression. While in HEK293 cells, luciferase expression was enhanced by co-transfection of human Lef1 which recruits β-catenin to Wnt target genes (Roel et al., 2009) (Fig. 3A), suggesting that canonical Wnt signaling promotes bmp2b expression.

To substantiate the regulatory roles of these two signaling pathways on bmp2b expression, we knocked down a Nodal co-receptor gene oep, or overexpressed lef1 in wild-type embryos (Gritsman et al., 1999). We then performed in situ hybridization to examine the effects of these manipulations on bmp2b transcripts at the shield stage. Knockdown of oep led to an increase in bmp2b expression not only in the organizer but also in the ventral domain (Fig. 3B). Approximately 65% of embryos in which left1 was overexpressed showed a similarly increased pattern of bmp2b expression. The gfp expression in the organizer of Tg(-2.272bmp2b:gfp) transgenic embryos was also enhanced by oep knockdown or left1 overexpression (Fig. 3C), consistent with the results from wild-type embryos and the cell reporter assays. These data indicate that organizer-specific bmp2b is inhibited by Nodal signal and activated by canonical Wnt signal. This suggests that the 0.273-kb minimal promoter contains responsive elements for Nodal and Wnt signals.

Transcription of organizer-specific bmp2b is directly down-regulated by Nodal and up-regulated by Wnt signal

We next asked whether Nodal and Wnt signaling regulate organizer-specific bmp2b transcription directly. To address this question, we searched for Nodal/Smad2/3-responsive and Wnt/β-catenin-responsive elements in the 0.273-kb minimal promoter. The 0.273-kb proximal promoter region harbors one putative consensus Smad-binding element (SBE) (CAGAC, -36–32 bp) (Feng and Derynck 2005) and one putative Lef/Tcf-binding element (LBE) (AGATAA, -263–258 bp) (Blaufkamp et al., 2008). When the SBE or LBE in the 0.273-kb proximal promoter region
was mutated, luciferase reporter expression in embryos failed to respond to overexpression of constitutively active smad2 (casmad2) (Liu et al., 2013) or to lef1 overexpression (Fig. 4A). Thus, the SBE and LBE are required for the response of the bmp2b promoter to Nodal repression and Wnt activation, respectively.

Finally, we used chromatin-immunoprecipitation (ChIP) to investigate whether endogenous Smad2 or β-catenin could bind the SBE and LBE in the 0.273-kb proximal promoter region in zebrafish embryos. ChIP assays revealed that DNA immunoprecipitated using an anti-Smad2 antibody or an anti-β-catenin antibody could be amplified using specific primers spanning those elements (Fig. 4B). This indicates that in embryos, the SBE and the LBE regions are bound by Smad2 and β-catenin, respectively.

Taken together, our data indicate that expression of bmp2b in the organizer is regulated negatively by Nodal/Smad2/3 signaling and positively by Wnt/β-catenin signaling in zebrafish embryos.

Discussion

In this study, we identified a 2.272-kb bmp2b promoter that drives stable and specific GFP expression in the dorsal organizer region in transgenic gastrulas. In addition, we used molecular dissection to show that a 0.273-kb minimal promoter was sufficient to express bmp2b in the organizer at the shield stage. This 0.273-kb minimal promoter contained a Smad2 binding site and a Lef1 binding site and was directly repressed by Nodal signal and activated by Wnt signal (Fig. 5).

In zebrafish, bmp2b mRNA is observed in the organizer region beginning at around 5.7 hpf, at the onset of organizer formation. bmp2b mRNA is further enriched in the organizer region and the dorsal side of the embryo throughout the gastrula stage. As mentioned earlier in the text, boz, a gene downstream of the Wnt signaling pathway, acts as the earliest repressor of bmp2b tran-
Nodal and Wnt signal oppositely regulate organizer-specific bmp2b

Fig. 5. Molecular regulatory model of organizer-specific bmp2b. Top image shows Bmp2b distribution at the shield stage. Embryos are viewed laterally with dorsal to the right. Bmp2b forms a gradient distribution along the ventral-dorsal axis, with the highest expression ventralmost, a gradual reduction in expression on the dorsal side, and specific enrichment at the dorsal organizer. Bottom image shows transcriptional regulation of organizer-specific bmp2b is mediated through activation by canonical Wnt signaling via Lef/Tcf1 binding, and repression by Nodal signaling via Smad2 binding on its promoter.

The 0.273-kb bmp2b promoter initiated gfp reporter gene expression in the dorsal side of the embryo at 30% epiboly stage (Fig. 1C). This was earlier than initiation of endogenous dorsal bmp2b expression (Xue et al., 2014), suggesting that the 0.273-kb promoter lacks the binding elements of transcriptional repressor such as Boz, which is necessary for inhibition of dorsal bmp2b transcription at the early gastrula stage through targeting its first intron (Leung et al., 2003). We also deleted a 50 bp region (-123--74 bp) of the 0.273-kb promoter to further explore its regulation. This deletion led to the permanent absence of gfp expression (data not shown), suggesting that an important enhancer is embedded within this 50 bp sequence. As Fgf signal and other factors are essential for organizer formation (Kuo et al., 2013; Maegawa et al., 2006), it is likely that other signals or transcriptional factors work together with Nodal and Wnt signals to regulate organizer-specific bmp2b transcription. This hypothesis should be investigated in future studies.

One interesting result was that mutation of SBE included in the 0.273-kb bmp2b promoter caused almost loss of responsiveness to casmind2 repression. While, this mutation also reduced the luciferase reporter expression in embryos at basal level (Fig. 4A). This phenomenon may be explained that perhaps SBE not only functions as a repressor, but also an enhancer for some other factors, that is, there may exist some co-activators essential for transcriptional activity, which can form complex with Smad2/3 to function. Mutation of SBE both suppresses the binding of the complex to SBE and down-regulates the transcriptional activity of reporter gene.

As we mentioned in the introduction, maternal and zygotic Wnt signal function differently during dorsoventral patterning, previous study suggested that Wnt signal is inactive at the dorsal margin during late gastrulation (Lu et al., 2011; Ramel and Lekven 2004; Shimizu et al., 2012). One interesting issue is if Wnt signal continuously activates the expression of bmp2b at dorsal margin after shield stage? To explore this, we incubated wild-type embryos with a Wnt inhibitor, IWR-1-endo (1:5000, Selleck) (Burridge et al., 2014) from 64-cell stage. Compared with control group treated with DMSO, 100% of embryos treated with IWR showed observably reduced bmp2b expression in organizer region at shield stage (Fig. S1A), while not clearly decreased at dorsal margin at 75% epiboly stage (Fig. S1B). These results suggested that Wnt-signal is essential forbmp2b expression in organizer region but becomes weak and contributes less to bmp2b expression at dorsal margin during late gastrulation. We speculate that other genes expressing in this region compensate for the dorsal bmp2b maintenance at these stages.

In conclusion, we showed that organizer-specific bmp2b, which is essential for maintenance of the Bmp2b activity gradient, and more broadly, for dorsoventral patterning, was regulated by Nodal and Wnt signals in an opposite manner, thereby maintaining a balance of bmp2b expression in the dorsal organizer.

Materials and Methods

Zebrafish embryos and cell lines

Zebrafish embryos, the human 293 cell line, and the Hep3B cell line were used in this study. All animal studies were performed in accordance with guidelines approved by the institutional Human Ethics Review Committee and the Animal Care and Use Committee of Tsinghua University.

Molecular cloning

A sequence between base pairs -2272 to +348 of the bmp2b locus was amplified by PCR from zebrafish genomic DNA and cloned into the pGL3 basic vector (Promega) to form the construct pGL3(-2.272bmp2b: luc). This insert was then subcloned into a modified version of the pGL3 basic vector in which Luciferase was replaced by the enhanced green fluorescent protein (GFP) coding sequence to generate the construct Tgf(-2.272bmp2b: gfp) for transient expression and deletion analysis. The primer sequences used for PCR amplification of DNA were the same as those used previously (Xue et al., 2014).

To create the construct for antisense lef1 mRNA, lef1 coding sequence was amplified by PCR from cDNA, then was reversely inserted into pXT7
vector. The primer sequences were: Forward-Spel: 5'-GGACTAGTATcgc-ccagtgtcagcctga-3'; Reverse-EcoRI: 5'-CCGGAATTCTcagtgactgct-cttcatc-3'.

Zebrafish strains and transgenesis

The tuebingen zebrafish strain was used in this study. To generate the transgenic line, the bmp2b promoter was cloned into a modified Tsg vector (a map of the modified Tsg vector used in this study can be provided upon request) (Han et al., 2011). The plasmid Tsgp-2.72Tbmp2b (gfp) was then co-injected with Tol2 transposase mRNA into one-cell wild-type embryos. These founder fish were then mated to wild-type fish, and progeny that carried the transgene were identified by GFP expression. Embryos born from male founders showed specific but weak GFP expression that could be detected by in situ hybridization, while offspring of female founders displayed strong maternal GFP expression.

RNA synthesis, morpholinos, microinjection and whole-mount in situ hybridization

mRNAs were synthesized in vitro from corresponding linearized plasmids using the mMessage mMachine kit (Ambion). Digoxigenin-UTP-labeled antisense RNA probes were generated by in vitro synthesis with a linearized plasmid as a template (Roche).

Whole-mount in situ hybridization was performed as previously described (Xiong et al., 2006). Stained embryos were cleared in glycerol and photographed using a digital camera (SPOT Insight) under a Nikon SMZ 1500 microscope. The images were adjusted using Adobe Photoshop software.

The morpholinos used in this study were synthesized by Gene Tools, LLC and was as follows: oep-MO, 5'-GCCAATAAAACTCCAAAACACTCGA-3' (Nasevicus and Ekker 2000). standard-MO, 5'-AAGGAAAAACGAAATG-GAAAGGAT-3'.

Approximately 1-1.5 nl of morpholino solution or DNA was injected into the yolk or cell of each embryo at the one-cell stage using MPPI-2 quantitative injection equipment (Applied Scientific Instrument Co.).

Luciferase reporter and chromatin immunoprecipitation assay

Cell culture, transfection, and luciferase reporter assays were performed as previously described (Liu et al., 2013). Shield stage embryos were harvested for embryonic reporter assays. The chromatin-immunoprecipitation (ChIP) assay was performed as described previously (Liu et al., 2011). For this experiment, approximately 1500 embryos at shield stage were harvested. A rabbit anti-Smad2/3 antibody (Cell Signaling, 1:50) and a rabbit anti-β-catenin antibody (Abcam, 1:200) were used. The primer sequences for ChIP-PCR were: bmp2b-forward: 5'-ATACAAATGTAGATAATTTA-3'; bmp2b-reverse: 5'-TTTTTGTTGTCTGAGTGTGATGT-3'; control-forward: 5'-ACACCGTTGTTGTCAGGAC-3'; control-reverse: 5'-AGCTCTCTGATACCTTCTC-3'. The ChIP-PCR products were examined by gel electrophoresis and band intensity was quantified using ImageJ software.

Statistical analysis

Student's t-tests (two-tailed, unequal variance) were used to determine p-values of all groups compared in this study. Significance levels were donated as *p<0.05 and **p<0.01.

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Author Contributions

Conceived and designed the experiments: YX CX AM YP. Performed the experiments: YX CX CC WZ JX. Analyzed the data: YX CX AM. Contributed reagents/materials/analysis tools: YX CX AM YP. Wrote the paper: YX CX AM.

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