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WNT8 and BMP2B co-regulate non-axial mesoderm patterning during zebrafish gastrulation

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Abstract

During vertebrate mesoderm formation, fates are established according to position in the dorsoventral (D/V) axis of the embryo. Initially, maternal signaling divides nascent mesoderm into axial (dorsal) and non-axial (ventral) domains. Although the subsequent subdivision of non-axial mesoderm into multiple D/V fate domains is known to involve zygotic Wnt8 and BMP signaling as well as the Vent/Vox/Ved family of transcriptional repressors, how levels of signaling activity are translated into differential regulation of fates is not well understood. To address this question, we have analyzed zebrafish embryos lacking Wnt8 and BMP2b. Zebrafish *wnt8; swr* (*bmp2b*) double mutants display a progressive loss of non-axial mesoderm and a concomitant expansion of axial mesoderm during gastrulation. Mesoderm induction and specification of the axial domain occur normally in *wnt8; swr* mutants, but dorsal mesoderm genes eventually come to be expressed throughout the mesoderm, suggesting that the establishment of non-axial mesoderm identity requires continual repression of dorsal mesoderm factors, including repressors of ventral genes. Loss-of-function for Vent, Vox, and Ved phenocopies the *wnt8; swr* mutant phenotype, consistent with Wnt8 and BMP2b maintaining non-axial mesoderm identity during gastrulation through the regulation of these three transcriptional repressors. We postulate that timely differentiation of the mesoderm requires the maintenance of non-axial mesoderm identity by Wnt8 and BMP2b at the onset of gastrulation followed by subdivision of the non-axial mesoderm into different functional domains during gastrulation.

Keywords: Wnt8; BMP; Mesoderm; Zebrafish; *vent*; *vox*; *ved*

Introduction

During embryogenesis, a complex series of regulated events leads to the formation of the three germ layers: ectoderm, mesoderm, and endoderm. The mesoderm gives rise to structures such as notochord, muscles, kidneys, and blood in a distinguishable pattern along the dorsoventral (D/V) axis (reviewed in Schier, 2001; Kimelman and Griffin, 2000; Weng and Stemple, 2003). While many aspects of mesoderm induction and development are well understood, less is known about the molecular events leading to the subdivision of the mesoderm into D/V domains.

The overlapping activities of the Nodal and maternal β-catenin pathways induce two initial mesodermal domains: axial (or dorsal mesoderm), which constitutes the dorsal organizer and gives rise to the prechordal plate anteriorly and notochord posteriorly, and non-axial (or ventrolateral mesoderm), which is the remainder (reviewed in Kimelman and Schier, 2002; Hibi et al., 2002). Once established by Nodal and β-catenin activity, the axial and non-axial mesoderm domains are further delineated and maintained through mutual repression systems. For instance, the dorsally expressed protein Bozozok (Boz) prevents the transcription of the ventrally expressed genes *bmp2b* and *vox* (Kawahara et al., 2000b; Leung et al., 2003; Melby et al., 2000; Shimizu et al., 2002). Ventrally, the Vent, Vox, and Ved homeodomain transcriptional regulators prevent the transcription of *boz* and other dorsal genes such as *goosecoid* (*gsc*) and *chordin* (*chd*) (Imai et al., 2001; Kawahara et al., 2000a,b; Melby et al., 2000; Shimizu et al., 2002). Although the mechanisms that define axial mesoderm identity are becoming well defined, it is less clear how the non-axial mesoderm becomes progressively subdivided into para-axial, intermediate, and lateral plate domains.

In *Xenopus*, it has been postulated that graded BMP signaling activity establishes nested domains of *Xvent-1* and

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Xvent-2 expression which establish zones within non-axial mesoderm corresponding to paraxial and lateral plate regions (Dosch et al., 1997; Onichtchouk et al., 1998). In support of a role for Vent proteins in regulating the subdivision of non-axial mesoderm, Xvent-1 was shown to directly repress *Xmyf-5* in the ventral non-axial mesoderm (Polli and Amaya, 2002). Thus, Xvent-1 and Xvent-2 may establish two gross domains within non-axial mesoderm (referred to as dorsolateral and lateroventral in Dosch et al., 1997) in response to graded levels of BMP activity. However, though attractively simple, this model neglects the input of signaling by Wnt8, another known input into the mesoderm patterning process and a known regulator of *vent/vox/ved* genes (Friedle and Knochel, 2002; Ramel and Lekven, 2004). Studies in *Xenopus* have suggested that Xwnt-8 and BMP4 cooperatively pattern the mesoderm and zygotic Wnt/β-catenin signaling can directly regulate *Xvent-1b* (Friedle and Knochel, 2002; Hoppler and Moon, 1998; Marom et al., 1999). However, the relative roles of Wnt8 and BMP in this process, as well as their epistatic relationship, remain unclear. Furthermore, it is unclear if the association between *vent/vox/ved* expression and non-axial mesoderm subdivisions is generally true for vertebrates since an association between zebrafish *vent* (most similar to *Xvent-1*) and *vox* (most similar to *Xvent-2*) and specific non-axial mesoderm domains has not been established.

We have recently shown that zebrafish Wnt8 prevents the expansion of the organizer though the direct transcriptional regulation of *vent* and *vox* (Ramel and Lekven, 2004). Our analysis further showed that BMP2b has a supporting role in co-regulating *vent* and *vox* at the onset of gastrulation. In other words, *wnt8; swr* double mutants fail to repress the anterior axial mesoderm marker *gsc* in the nascent ventrolateral mesoderm. This finding raises the possibility that Wnt8 and BMP2b might both sit at the top of a genetic hierarchy required for establishing non-axial mesoderm fates. To test this possibility, we have characterized the *wnt8; swr* double mutant phenotype. Our results show that, downstream of mesoderm induction, Wnt8 and BMP2b function in parallel throughout gastrulation to establish the non-axial mesoderm and its early subdivisions. Wnt8 and BMP2b perform this function through the combined regulation of the transcriptional repressors Vent, Vox, and Ved. We postulate that Vent, Vox, and Ved may be further involved in subdividing the non-axial mesoderm into D/V domains during mid- to late gastrulation.

Materials and methods

Fish maintenance and strains

Animals were maintained as described previously (Westerfield, 2000). Wild-type fish were AB. The strains used were: *Df(LG14)wnt8*^{w8}/+ (also called *Df*^{w8}/+ or *wnt8*−/+ in this study; Lekven et al., 2001), *swr*^{TC300}/+ (*swr*/+; Mullins et al., 1996), *Df*^{ST7}/+ (*vent*−/+; *vox*−/+ in this study; Imai et al., 2001). All mutants are considered to be null or strong loss-of-function (Lekven et al., 2001; Nguyen et al., 1998; Imai et al., 2001). To generate *wnt8*−/+; *swr*/+ animals, a *wnt8*− heterozygote was crossed to a *swr* heterozygote. Progeny were individually screened for *wnt8*−/+ and *swr*/+. Double mutant embryos were confirmed by PCR genotyping as previously described (Ramel and Lekven, 2004).

In situ hybridization and probes

In situ hybridizations were performed essentially as described (Jowett, 2001). The probes used were: *even-skipped-1* (*eve1*; Joly et al., 1993), *cdx4* (previously *cad1*; Joly et al., 1992), *tbx6* (Hug et al., 1997), *T-box24* (*tbx24*; Nikaido et al., 2002), *ved* (Shimizu et al., 2002), *vox* (Melby et al., 2000), *chd* (Miller-Bertoglio et al., 1997), *floating head* (*flh*; Talbot et al., 1995), *boz* (also called *dharma*, Yamanaka et al., 1998), *gsc* (Stachel et al., 1993), *myoD* (Weinberg et al., 1996), and *no tail* (*ntl*; Schulte-Merker et al., 1992).

Injection and morpholinos

Because previously reported translation blocking morpholinos (MOs) targeted against *wnt8* produce *wnt8*− phenotypes of variable penetrance and expressivity (Lekven et al., 2001), MOs designed to block the splicing of *wnt8* pre-mRNAs were utilized. Sequences are as follows (5′ to 3′):

orf1 E1i1 MO: AATATGACTGTACCATGCTGTTGAC
orf1 exon3 MO: ATATTTAACTTACCACTCCGCAGGC
orf2 E4i4 MO: AACTGTTCTTACCAAGTCTGCCGTT
orf2 exon3 MO: CTTATGAATATCTTACCACTTCTCA.

Simultaneous injection of the four splice blocking MOs (2.5 ng/nL each) gave results comparable to the translation blocking MOs but with higher penetrance and expressivity as well as lower lethality. Furthermore, the phenotypic effects of the splice blocking MOs were rescued by RNA injection (see Supplemental figure). The *ved* MO has previously been described (Shimizu et al., 2002). MOs were diluted as described in Danieau's buffer (Genetools, LLC) and injected into one- to four-cell stage embryos. To replicate the *wnt8*−*; swr* phenotype, the progeny from a cross between *swr* heterozygotes were injected with *wnt8* MOs to obtain *wnt8* MO; +/+, *wnt8* MO*; swr*/+, and *wnt8* MO*; swr*/*swr* embryos. To achieve a *vent*/*vox*/*ved* knockdown, embryos obtained from a cross between *Df*^{ST7} heterozygotes were injected with *ved* MO (10 ng/nL). For the rescue of *tbx24* expression in *wnt8; swr* double mutants, the progeny from 3 to 4 *wnt8*/+*; swr*/+ intercrosses were injected with *vent* RNA (7 ng/μL; higher concentrations can be toxic to embryos and produce gastrulation defects, our unpublished observations). After in situ hybridization, the embryos were individually photographed and genotyped by PCR to identify the double mutants. In all injections, a volume of approximately 3 nL was injected per embryo.

Results

Wnt8 and BMP2b act in parallel to control non-axial mesoderm identity

Previous studies in *Xenopus* and zebrafish have suggested that Wnt8 and BMP signaling interact during D/V patterning and that they share common transcriptional targets (Ramel and Lekven, 2004; Agathon et al., 2003; Hoppler and Moon, 1998; Marom et al., 1999; Szeto and Kimelman, 2004). However, there is also evidence that Wnt8 and BMP regulate independent transcriptional targets. For instance, *myf5* expression in *Xenopus* is dependent on Xwnt8 but not on BMP (Marom et al., 1999). If both pathways are required for unique as well as combined patterning functions, then one would expect to detect this when comparing single and double mutant phenotypes. To this aim, we analyzed the phenotype of zebrafish embryos that lack functional Wnt8 and BMP2b.

We first compared the bud stage morphology of embryos resulting from *wnt8* MO injection in the progeny of *swr* heterozygotes (Fig. 1). *swr* mutant embryos are distinguished