THE ROLE OF WNT8 IN POSTERIOR MESODERM FORMATION

A Thesis

by

CATHRYN RENEE KELTON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Biology

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Approved by:

Chair of Committee,	Arne C. Lekven
Committee Members,	Ginger Carney
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ABSTRACT

The Role of Wnt8 in Posterior Mesoderm Formation.

(December 2008)

Cathryn Renee Kelton, B.S., Emory and Henry College

Chair of Advisory Committee: Dr. Arne Lekven

The formation of vertebrate mesoderm relies on the integration of positional information provided by several intercellular signaling pathways, including the Wnt and Bone Morphogenic Protein (Bmp) pathways. Zygotic Wnt signaling has been shown in multiple vertebrate systems to perform two functions: to restrict the size of the dorsal mesoderm structure known as the organizer, and to promote the development of posterior mesoderm that populates the trunk and tail. Importantly, the organizer is a source of secreted Bmp antagonists that regulate Bmp-dependent ventral and posterior mesoderm patterning. Because the organizer impacts Bmp signaling activity, it is not clear whether functions attributed to zygotic Wnt signaling are in fact indirectly due to reduced Bmp activity.

The objective of this thesis is to test the hypothesis that zygotic Wnt signaling plays two critical functions: to restrict the size of the organizer and to promote posterior mesoderm development in a Bmp-independent manner. To test this hypothesis, we characterized in depth the phenotypic defects of zebrafish embryos lacking Wnt8, the central ligand involved in zygotic Wnt-dependent mesoderm patterning. To identify Bmp-independent functions of Wnt8 signaling, we used double loss-of-function

conditions to elevate Bmp signaling in embryos lacking Wnt8 function. Embryos were analyzed for the expression of a comprehensive set of mesoderm markers indicative of cell fates found in all spatial positions of the embryo.

Our results show that, in addition to posterior mesoderm precursors being drastically reduced in Wnt8 morphants, anterior fates are disrupted as well. We found that increasing Bmp signaling largely has no effect on the Wnt8 morphant phenotype. However, slight rescue was observed in pronephric, heart tube, and vasculature precursors. We believe these results support the hypothesis that Wnt signaling maintains mesoderm progenitor cell populations, while Bmp signaling patterns mesoderm cell fates. Accordingly, Wnt8 signaling will appear to be epistatic to Bmp signaling during vertebrate axis patterning.

DEDICATION

To Mike for his love, Paw for his wisdom, Granny for her cards, Mom for her support, Dad for fixing things, and Christy for being gangsta

ACKNOWLEDGEMENTS

They say it takes a village to raise a child, if that is the case; it takes a city to raise a graduate student. There are many people who helped me along the way, and for that I am very grateful. First, I would like to thank my family for their support and love through this process. Without them, I would not be where I am today. To Lacy, Krithika, Silvana, Amy, Leah, Kari, Sarah, and Anand, my life is richer with you as friends, you each have a special place in my heart. Thank you for your support and the many good times we shared together. The bond of true friendship never breaks over long distances, to Susan, Coris, Kathi, Abby, and Alan, thank you for your continuing friendship over the years and miles, I miss you.

I would like to thank Dr. Kay Goldman for all of her hard work, help, and candy throughout my time in graduate school. A special thanks should be extended to Rhonda for maintaining the fish rooms, and to members of the Riley and Perkins labs for sharing reagents, equipment, and conversation. For wonderful advice and guidance, I would like to acknowledge Dr. Rita Moyes. Thank you to the members of the Lekven lab for helping me grow as a scientist. Lastly, to Mike, I love you more than Lilly loves bacon.

NOMENCLATURE

APC	Adenomatous Polyposis Coli
Bmp	Bone Morphogenic Protein
Cmlc2	Cardio Myosin Light Chain 2
Chd	Chordin
Dvl	Dishevelled
Eve1	Even-Skipped 1
Fsta	Follistatin a
GSK3β	Glycogen Synthase Kinase 3β
MHB	Midbrain Hindbrain Boundary
MO	Morpholino
MO4	Morpholino for Wnt8
Morphant	Embryo that has been injected with morpholino(s)

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CHAPTER I

INTRODUCTION

Wnt signaling

The Wnt/Wingless/Int family of proteins is a large family of secreted proteins that control embryonic patterning and cell-fate decisions in development (Eastman et al., 1999). Wnt signaling can stimulate two downstream pathways, the canonical and the non-canonical pathways. The focus of this study is the canonical Wnt/β-catenin pathway, which is named for the downstream effector β-catenin. In the absence of Wnt signaling, β-catenin is sequestered from entering the nucleus and activating TCF/LEF transcription factors by a complex made up of Axin, glycogen synthase kinase 3β (GSK3β), CK1, and adenomatous polyposis coli (APC). This complex causes β-catenin to become phosphorylated, ubiquitinated, and destroyed (Clevers, 2006).

In the presence of Wnt signaling, Wnts bind to their receptor Frizzled (Fz), a seven-pass transmembrane protein (Bhanot et al., 1996). After a Wnt protein binds to Fz, the receptor then interacts with the coreceptor LRP5/6 in vertebrates or Arrow in *Drosophila*, and activates the signaling cascade. Fz causes Dishevelled (Dvl) to become activated, and Axin is recruited to the LRP5/6 receptor thus breaking apart the β-catenin destruction complex. Once the complex is destroyed; β-catenin is free to enter the

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This thesis follows the style of Developmental Biology.

nucleus and activate TCF/LEF transcription factors (Clevers, 2006) by displacing Groucho, a repressor of TCF/LEF proteins, and interacting with the amino terminus of TCF/LEF transcription factors (Eastman et al., 1999), which turn on downstream genes important for cell proliferation and mesoderm patterning (Figure 1). Embryos lacking Wnt8 signaling exhibit a disruption in ventrolateral and posterior mesoderm formation (Lekven et al., 2001).

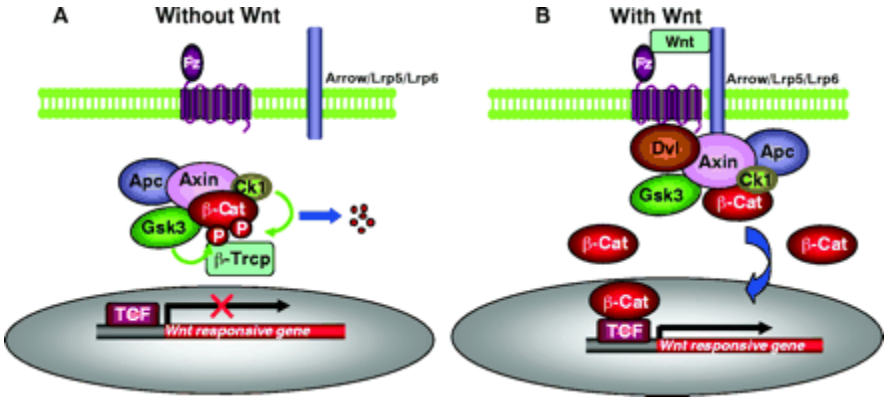

Figure 1: Canonical Wnt pathway. Binding of a Wnt ligand to its receptor Frizzled leads to the recruitment of Axin to the LRP co-receptor, breaking apart the β-catenin destruction complex. β-catenin is then free to enter the nucleus and activate TCF/LEF transcription factors. (Taken from He, 2004).

Bmp signaling

Along with Wnt8 signaling, Bmp (Bone Morphogenic Protein) signaling is also important for mesodermal patterning. Bmps are a part of the TGF-β family of cytokines that regulate cellular processes that include cell proliferation, cell differentiation, cell fate, and migration (Ross and Hill, 2008). Bmp ligands bind to the extracellular domains of type I and type II Bmp receptors, which are transmembrane proteins with intracellular

serine/theronine kinase domains (Schier and Talbot, 2005). In zebrafish, the ligand bound receptors phosphorylate three Smad transcription factors; Smad 1, 5, and 8 (Ross and Hill, 2008). Once phosphorylated, these transcription factors form a complex with Smad 4 and are able to translocate to the nucleus and regulate gene expression (Kimelman, 2006) (Figure 2).

It has been shown that Bmp signaling is required for global dorsoventral patterning during early gastrulation in zebrafish, and regulates tail development from mid-gastrulation to early somitogenesis (Stickney, 2007). Embryos lacking Bmp2b and Bmp7 have expanded trunk somitic fates, loss of tail, reduced vasculature, blood, and pronephros (Dick et al., 2000; Imai et al., 2001; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000; Shimizu et al., 2002; Stickney et al., 2007).

Mesoderm formation results in two domains with progenitors that differentiate in spatially restricted ways

Immediately after fertilization, the zebrafish embryo undergoes a series of synchronous cell divisions which produces a ball of uncommitted cells, called blastomeres, which sit atop the yolk (Kimmel, 1995). At approximately six hours after fertilization, the embryo undergoes gastrulation, in which the uncommitted blastomeres start to differentiate and segregate into three germ layers; the endoderm, mesoderm, and ectoderm. The endoderm forms the gut lining and associated organs such as the liver and pancreas; mesoderm forms organs including blood, body muscles, kidneys, heart, and vasculature; the ectoderm will ultimately give rise to the central nervous system and epidermis.

In the zebrafish, the precursors for mesoderm and endoderm comprise a mixed population of cells at the embryonic margin at the onset of gastrulation. Because of this cellular arrangement, it is often referred to as "mesendoderm" prior to segregation of the germ layers (Dougan, 2003).

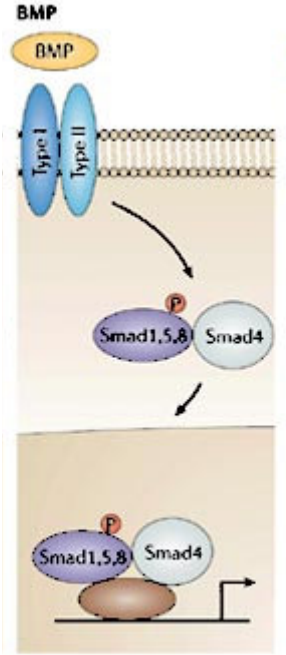

Figure 2: Bmp signaling. A Bmp ligand binds to Type I and Type II Bmp receptors which phosphorylate Smads 1, 5, and 8. The phosphorylated Smad proteins form a complex with Smad4, enter the nucleus and regulate target genes (adapted from Kimelman, 2006©).

Mesendoderm progenitors form above the yolk syncytial layer (YSL), an extraembryonic tissue located between the developing embryo and the yolk that plays important roles in mesoderm induction and in driving the morphogenetic movements of

epiboly during gastrulation (Figure 3) (Kimmel and Law, 1985; Solnica-Krezel and Driever 1994; Kimelman et al., 2000; Kimelman and Griffin, 2000 Kimmel; Chen et al., 2006).

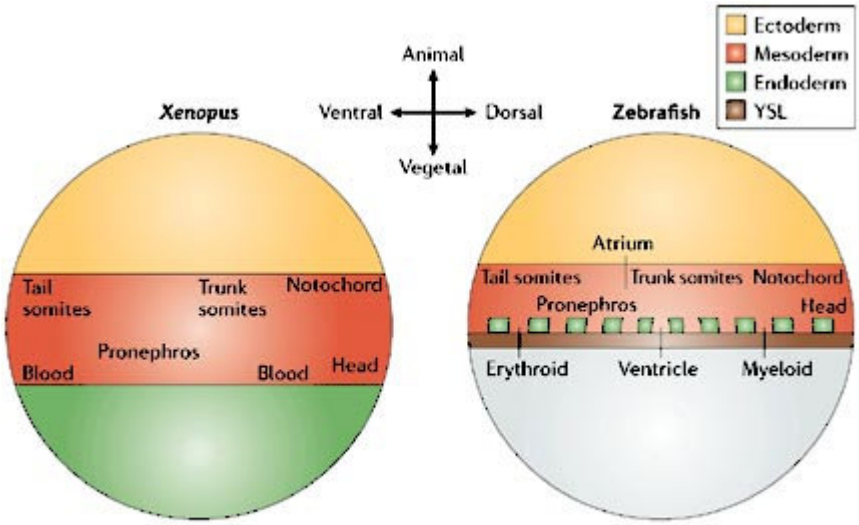

Figure 3: Fate map of mesoderm and endoderm precursors. Mesoderm precursors (red) are distinct from endoderm precursors (green) in *Xenopus* embryos, while in zebrafish both groups of precursors are mixed along the margin. (Adapted from Kimelman, 2006©).

Mesoderm induction in zebrafish occurs in response to signals secreted by the YSL (Kimelman, 2006). Upon induction, the mesoderm is divided into two gross domains referred to as dorsal and ventrolateral domains (Figure 4A). The dorsal domain will differentiate into axial structures such as the notochord. The ventrolateral domain comprises progenitors for mesoderm structures that form away from the dorsal axis, such as blood, kidney and body muscles (Kimmel et al., 1990). The dorsal mesoderm domain contributes to a visible thickening of the zebrafish embryonic margin, called the embryonic shield, at the onset of gastrulation. The shield corresponds to the zebrafish

equivalent of the amphibian dorsal blastopore lip, also known as "Spemann's Organizer". Previous embryological experiments discovered that the organizer regulates embryonic axis patterning because it secretes antagonists to Wnt and Bmp ligands (Ramel et al., 2004; Sokol et al., 1999; Xanthos et al., 2002). Interactions of antagonists secreted by the organizer and Wnt and Bmp ligands expressed on the opposite side of the embryo establish positional information that is responsible for guiding the differentiation of mesoderm progenitors into position-specific cell fates (Kimelman, 2006) (Figure 4B).

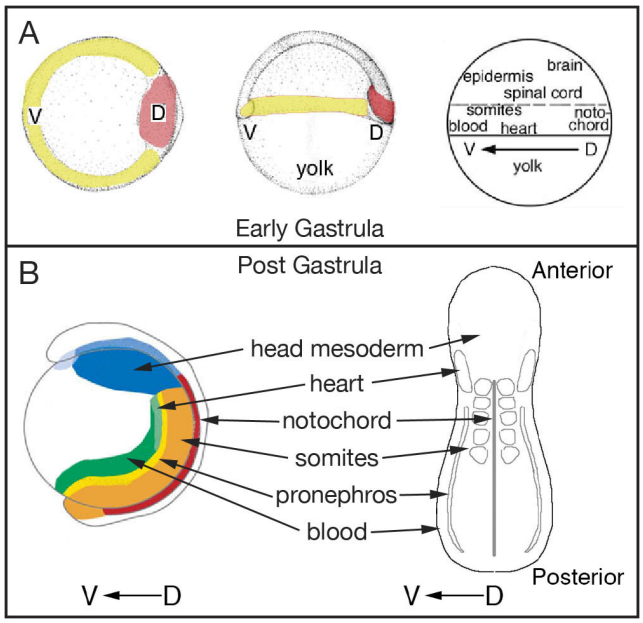

Figure 4: Fate map of ventral lateral domains. Ventrolateral mesoderm, also called the margin, yellow, gives rise to more posterior fates. Dorsal mesoderm, red, gives rise to more anterior fates (A). A fate map of an embryo after gastrulation. Mesoderm is organized into D/V domains and A/P domains. Anterior domains are in blue (B). (Adapted from Schier and Talbot, 2005©).

Embryonic axis patterning is regulated by zygotic Wnt and Bmp signaling

Early studies on the organizer showed that cell fate specification in the anteroposterior axis is intertwined with patterning of the dorsoventral axis. For example, in zebrafish, the absence of Wnt8 signaling results in an expanded organizer (dorsoventral patterning defect) and a shortened body axis (anteroposterior patterning defect) (Hoppler et al., 1996; Lekven et al, 2001). Likewise, zebrafish embryos lacking Bmp2b and Bmp7 have expanded trunk somites, reduced vasculature, blood, and pronephros (dorsoventral patterning defects) and loss of tail (anteroposterior patterning defect) (Dick et al., 2000; Imai et al., 2001; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000; Shimizu et al., 2002; Stickney et al., 2007). Thus, Wnt and Bmp signaling are essential to both dorsoventral and anteroposterior axis patterning.

Wnt signaling regulates dorsoventral patterning through the organizer

The organizer secretes both Wnt and Bmp antagonists. These antagonists create a gradient of Wnt and Bmp signaling that is higher in the ventral domain of the embryo and lower in dorsal domain. Wnt antagonists secreted by the organizer can be classified into two families based on how they perform. The first group consists of the secreted Frizzled-related proteins (sFRPs) which include *frzb-1*, *sFRP-2* and *crescent*. These proteins inhibit Wnt signaling by binding to the Wnt ligands and preventing ligand/receptor interactions. The second group includes the Dkks, which interact with the LRP5/6 coreceptor to prevent Wnt binding and activation (reviewed in De Robertis et al., 2000). Overexpression of Wnt antagonists produces embryos that have a dorsalized phenotype such as enlarged eyes, enlarged head, expansion of the organizer,

and a shortened tail (Glinka et al., 1998; Hoppler et al., 1996; Leyns et al., 1997). Chordin, Noggin, and Follistatin are all Bmp antagonists which, like the Wnt antagonists, produce a dorsalized phenotype when overexpressed. Thus, Wnt signaling restricts organizer size and thereby limits the expression of Wnt and Bmp antagonists from the organizer. As a consequence, this function may impact both dorsoventral and anteroposterior embryo axis patterning. While these observations show that anteroposterior and dorsoventral patterning are coordinately regulated, whether anteroposterior and dorsoventral fate specification are an output of separable molecular mechanisms has not been determined.

Wnt8 is expressed in ventrolateral mesoderm

Wnt8 is expressed in the ventrolateral embryonic margin of zebrafish (Kelly et al., 1995). Wnt8 signaling is required to maintain high levels of *vent*, *vox* and *ved* expression in the ventrolateral margin during gastrulation (Ramel and Lekven, 2004). Vent, Vox, and Ved act as repressors to prevent the expression of dorsal genes in the ventral region of the embryo (Melby et al., 2000; Imai et al., 2001; Shimizu et al., 2002; Ramel et al., 2004; and Ramel et al., 2005). Thus, Wnt8 regulates dorsoventral patterning through Vent, Vox and Ved-dependent organizer regulation. This leaves open the question of whether Wnt8 signaling has a direct role in specifying anteroposterior mesoderm fates.

There is evidence that in addition to regulating dorsoventral fates, Wnt8 plays a direct role in patterning anteroposterior fates. It has been found that *sp5l*, a gene that functions in tail development, is downstream of *wnt8* (Thorpe et al., 2005).

Interestingly, expression of the posterior genes *cdxla* and *cdx4* was reduced in *wnt8* mutants, but not Bmp mutants (Shimizu et al., 2005). This evidence suggests that Wnt8 has a separate anterior-posterior patterning function that is independent of Bmp-dependent dorsoventral patterning.

Summary and focus of research

How dorsoventral and anteroposterior patterning is controlled by Wnt8 signaling in the zebrafish embryos is poorly understood. It is known that Wnt8 and Bmp play a role in patterning dorsoventral mesoderm. Mutants deficient in *wnt8* and *bmp* lack various structures that arise from dorsoventral mesoderm. Interestingly, there is evidence that these genes have different functions when it comes to anteroposterior patterning. Zebrafish embryos that lack *wnt8* signaling show a dramatic loss of posterior mesodermal structures. To gain a better understanding of Wnt8 function, various mesodermal markers were analyzed by *in situ* hybridization in wild type and Wnt8 loss-of-function embryos produced by morpholino antisense oligonucleotide (MO) gene knockdown.

The research presented here was designed to test the hypothesis that Wnt8 signaling regulates Bmp-dependent and Bmp-independent patterning. Embryos lacking Wnt8 have expanded organizers that secrete elevated levels of Bmp antagonists that reduce Bmp signaling. Thus, *wnt8* mutants have reduced Wnt8 signaling as well as reduced Bmp signaling. As a consequence, to reveal Wnt8-specific fate specification, it is necessary to restore Bmp signaling activity within *wnt8* loss-of-function embryos.

Mesoderm fates that fail to be specified under these conditions must require Wnt8 signaling but not Bmp.

We have taken a double loss-of-function approach to restore Bmp signaling activity in embryos lacking Wnt8. The Bmp antagonist Chordin (*chd*) is produced from the organizer. We used morpholino antisense oligonucleotides to simultaneously reduce both Wnt8 and Chordin expression, and then we analyzed the effect on multiple mesoderm markers. Our results show that, in addition to posterior mesoderm precursors being drastically reduced in Wnt8 morphants, anterior fates are disrupted as well. We found that increasing Bmp signaling largely has no effect on the Wnt8 morphant phenotype. However, slight rescue was observed in pronephric, heart tube, and vasculature precursors. These results support the hypothesis that Wnt signaling maintains mesoderm progenitor cell populations, while Bmp signaling patterns mesoderm cell fates. Accordingly, Wnt8 signaling will appear to be epistatic to Bmp signaling during vertebrate axis patterning.

CHAPTER II

MATERIALS AND METHODS

Fish maintenance and strains

Fish were maintained as described in (Westerfield, 2000). Fish used in this study were AB x TL. To generate AB x TL fish, AB wild-type fish were crossed with TL wild-type fish. Progeny were raised and crossed to produce embryos used in this study.

Injections and morpholinos

A combination of four morpholinos (MOs; Genetools, LLC) was used to block splicing of *wnt8* pre-mRNA. The sequence of each morpholino has been previously described (Ramel et al., 2005). The *chordin* (*chd*) MO has been previously described (Nasevicius and Ekker, 2000). MOs were diluted in 1X Danieau's buffer and injected into one to four cell stage wild-type embryos. To generate $wnt8^{MO}$;chd^{MO} embryos, each MO was individually injected into the same wild-type embryo. In all injections, the volume of MO injected per embryo was approximately 3 nL.

In situ hybridizations and probes

In situ hybridizations were essentially preformed as described in (Jowett, 2001). The probes used were: *cardiac myosin light chain-2* (*cmlc2*; Huang et al., 2003), *even-skipped-1* (*eve1*; Joly et al., 1993), *fli1* (Brown et al., 2000), *follistatin a* (Bauer et al., 1998), *gata1* (Dietrich et al., 1995), *hgg1* (Vogel and Gerster, 1997), *myf5* (Rescan, 2001), *myoD* (Weinberg et al., 1996), *neurogenin-1* (Blader et al., 1997), *pax2.1* (Abdelilah et al., 1996), and *T-box24* (*tbx24*; Nikaido et al., 2002).

CHAPTER III

CLASSIFYING THE WNT8 PHENOTYPE

Wnt8 is required to promote the development of posterior mesoderm fates

To better understand the role Wnt8 plays in patterning, wild-type embryos were injected with *wnt8* morpholinos, fixed at the 5 to 9 somite stage (approximately 12 to 13 hours after fertilization) and analyzed by in situ hybridization to detect several mesodermal markers. The morpholinos used are a cocktail of four morpholinos designed to block splicing of *wnt8* pre-mRNAs (Ramel et al., 2005; embryos injected with this morpholino cocktail are referred to as *wnt8*^{MO4} embryos). Because the *wnt8* phenotype is characterized by a severe lack of posterior mesoderm, we began by examining the expression of four paraxial and presomitic mesoderm markers: *myf5, myoD, mesogenin,* and *tbx24*. In all cases, the expression domains of these markers were decreased in length and width in the majority of the morphants (Figure 5 A-H). This result suggests that Wnt8 is required for the maintenance or specification of presomitic mesoderm progenitors.

The reduction in somitic progenitors observed would be predicted to result in smaller somites. At the 5 to 6 somite stage, *follistatin a* (*fsta*) expression marks anterior somites. Consistent with the above results, the expression domain of *fsta* was reduced in 50% of *wnt8*^{MO4} embryos, (Figure 6 A-D, n=24), and was barely visible or undetectable in 41% of the embryos. The results of these in situs indicate that Wnt8 is required for normal presomitic and somitic mesoderm development.

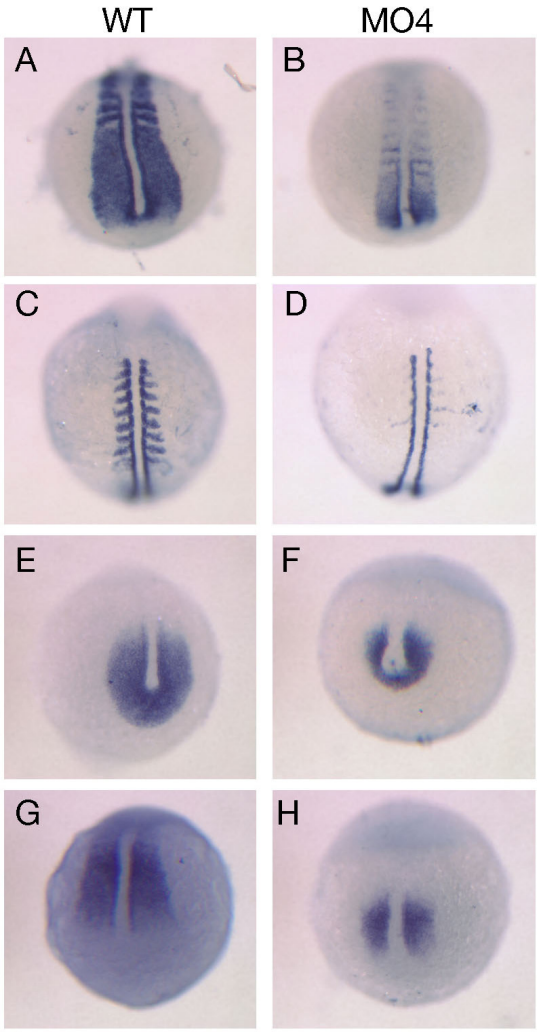

Figure 5: Absence of *wnt8* causes a reduction in paraxial and presomitic mesoderm. All views are posterior views, all embryos were fixed at the 5-9somite stage. Wild type expression of *myf5* (A). Expression of *myf5* in *wnt8* morphant is reduced (B). Wild type expression of *myoD* (C). Reduction of expression of *myoD* in *wnt8 morphant* (D). Wild type expression of *mesogenin* (E). *mesogenin* expression is reduce in the absence of *wnt8* (F). Wild type expression of *tbx24* (G). Reduction of *tbx24* in *wnt8* morphant (H).

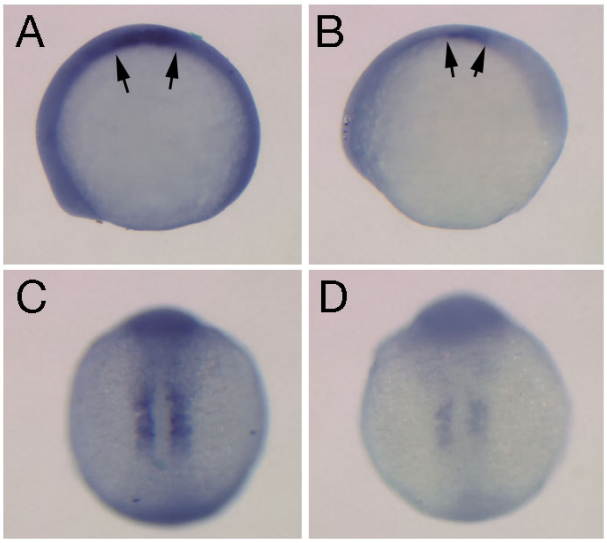

Figure 6: Expression of *follistatin a* at 5-6 somites. Expression of Follistatin is reduced in *wnt8* morphants at the 5-6 somite stage. Anterior/Posterior view (A,B). Dorsal view (C,D). Wild-type embryos (A,C). Wnt8 morphants (B,D).

Posterior mesoderm comprises several tissues in addition to somites, including intermediate and lateral plate mesoderm. In zebrafish, intermediate mesoderm encompasses hematopoietic and pronephric progenitors (Rohde et al., 2007). To determine whether Wnt8 signaling regulates all posterior mesoderm fates equivalently, we examined the expression of intermediate mesoderm markers in *wnt8*^{MO4} embryos.

To examine the fate of red blood cell progenitors, we assayed the expression of *gata1*, the first red blood cell specific marker to be expressed (Lyons et al., 2002, Rohde et al., 2004). A reduction in *gata1* expression was observed in 62% of *wnt8*^{MO4} embryos, and *gata1* expression was totally absent in 32% of the injected embryos (Figure 7 A-D, n=32).

To examine hematopoietic precursors, we assayed *scl*, which is expressed earlier than *gata1* in bilateral stripes that represent both blood and endothelial precursors (Rohde et al., 2004). In addition to the posterior expression domains, *scl* is expressed

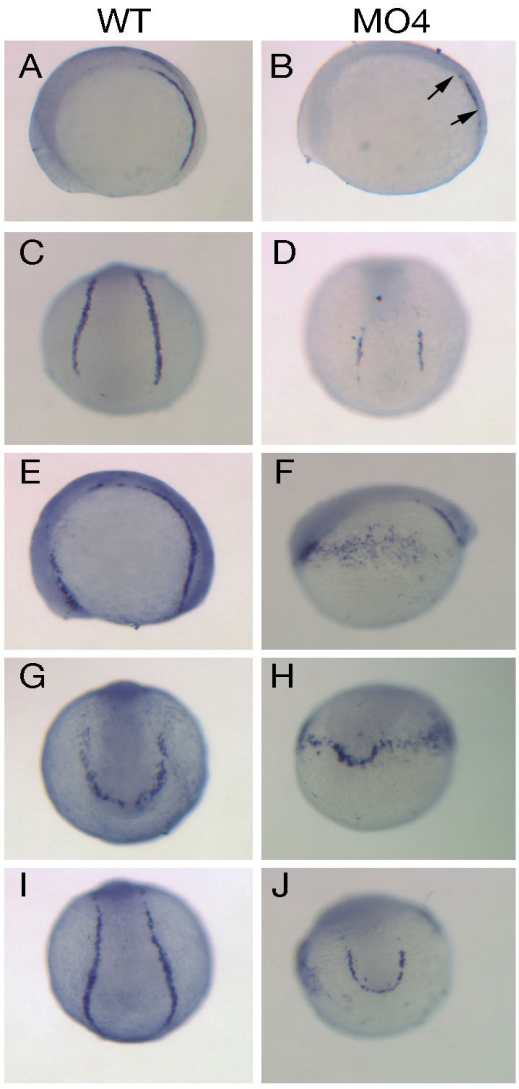

Figure 7: *Wnt8* morphants show a disruption in blood precursors. Wild type posterior view of *gata1* expression at 5-6 somites (A). Lateral view, anterior to left, of reduced *gata1* expression, between arrows, in *wnt8* morphants (B). Wild type lateral view of *gata1* expression, anterior to left (C). Posterior view of reduced *gata1* expression in *wnt8* morphant (D). Wild type *scl* expression at 5-6 somites (E,G,I). Lateral view, anterior to left, (E) Anterior view, (G) Posterior view (I). Disrupted *scl* expression in *wnt8* morphants (F,H,J). Lateral view, anterior to left. Notice reduction of posterior domain and expansion of rostral blood islands around the middle of the embryo (F). Anterior view of rostral blood islands (H). Posterior view (J).

in bilateral stripes in anterior lateral plate mesoderm that represent the rostral blood islands, which comprise myeloid precursors (Hogan et al., 2006), . The anterior and

posterior expression domains of *scl* are separated by a gap and do not touch. Consistent with the reduction of *gata1* expression, a reduction in the posterior expression domain of *scl* was observed in *wnt8*^{MO4} embryos. In contrast, the expression marking the rostral blood islands, normally restricted to the anterior end of the embryo, expanded almost to the posterior pole of *wnt8*^{MO4} embryos. This phenotype was observed in 67% of the morphants (Figure 7 E-J, n=28). Thus, *wnt8*^{MO4} embryos display reduced paraxial and intermediate mesoderm progenitors, and this is accompanied by a significant expansion of anterior lateral plate mesoderm progenitors.

We next examined expression of *pax2.1* at the 5 to 6 somite stage. At this stage, *pax2.1* is expressed in pronephric progenitors in the intermediate mesoderm, the presumptive eye field, the midbrain-hindbrain boundary and the otic placode (Abdelilah et al., 1996). *wnt8*^{MO4} embryos fell into three phenotypic classes. The strongest class (33%, n=13) showed a loss of pronephric and otic placode *pax2.1* expression. The midbrain-hindbrain boundary had formed a ring around the posterior end of the embryo, and the presumptive eye field was significantly expanded and not separated into two discrete domains (Fig. 8D,H). The second phenotypic class was slightly less severe than the first, with some pronephric *pax2.1* expression present and a narrower neural plate than in the strongest class (Fig. 8C,G, n=16). The third phenotypic class (23% of total) was the weakest, and was close to the wild-type phenotype (Fig. 8B,F, n=9).

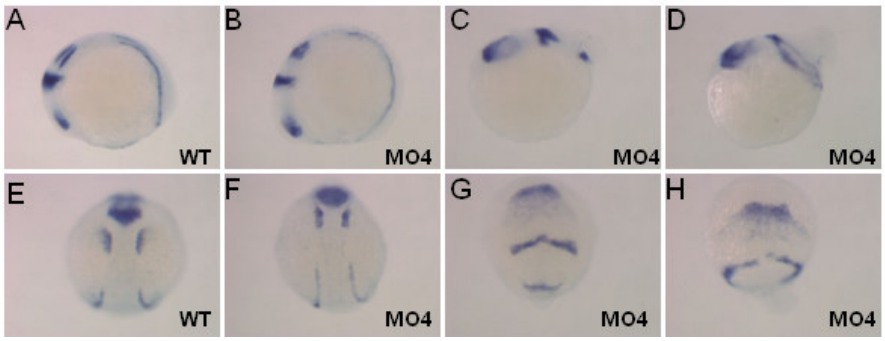

Figure 8: Pax2.1 expression in Wnt8 morphants. Lateral view of wild type embryo (A). Lateral view of Wnt8 morphants (B,C,D). Dorsal view of wild type embryo (E). Dorsal view of Wnt8 morphants (F,G,H).

Wnt8 is required for cardiac progenitor specification

The previous results showed that Wnt8 promotes posterior mesoderm development and antagonizes anterior lateral plate mesoderm and neurectoderm specification. The cardiogenic mesoderm is situated between anterior and posterior mesoderm. Recent studies have shown that myocardial specification in zebrafish is under both positive and negative regulation by Wnt signaling (Ueno et al., 2007): Wnt signaling prior to gastrulation promotes cardiogenic mesoderm specification while Wnt signaling during and after gastrulation antagonizes cardiogenic mesoderm. We predicted that Wnt8 may be responsible for the early heart-promoting activity.

To test this, we examined the myocardial markers *nkx2.5* and *cmlc2* at the 21 somite stage (~19 hours post fertilization). Myocardial progenitors comprise bilateral fields of cells that migrate to the dorsal midline and fuse to form the heart tube. *cmlc2* is expressed throughout the heart tube in zebrafish (Yelon et al., 1999). In wild type embryos proper tube patterning is represented by a circle of *cmlc2* expression just

ventral to the developing hindbrain. This pattern of expression was significantly disrupted in *wnt8*^{MO4} embryos (Fig. 9). 42% of *wnt8*^{MO4} embryos displayed bilateral *cmlc2*, which indicates the failure of heart progenitors to merge and form the heart tube (Figure 9 A, B, n=40). In 53% of morphants, *cmlc2* was also expressed in bilateral domains but the expression level was at most barely detectable. Similar results were obtained when we examined *nkx2.5* (Keegan et al., 2005) expression: 64% of the morphants exhibited unfused bilateral myocardial domains (Figure 9 C, D, n=35).

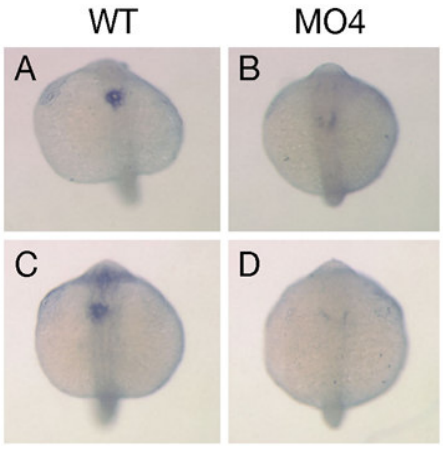

Figure 9: Loss of Wnt8 causes reduction in heart and vasculature precursors. Cmlc2 expression (A,B). Nkx2.5 expression (C,D).

Wnt8 promotes anterior paraxial mesoderm specification

The results presented above, with previously published results, suggest Wnt8 signaling is essential for establishing a balance within the mesoderm between anterior and posterior progenitor fates: Wnt8 promotes posterior fate specification and antagonizes anterior mesoderm specification. To extend this analysis, we examined

hgg1, a marker for the anterior prechordal plate, and *follistatin a*, a marker for cephalic paraxial mesoderm during gastrulation.

At bud stage (~10 hours post fertilization), the anterior prechordal plate is an oval shaped group of cells at the anterior pole of the embryo (Figure 10 A). *wnt8*^{MO4} embryos fell into two phenotypic classes. In the more severe class, *hgg1* expression expanded to encircle the embryo (54%, n=18; Fig. 10D). In the less severe class, the *hgg1* expression domain was slightly widened (33%, n=11; Fig. 10B,C). This result is consistent with previous observations of *gsc* expression, which marks an overlapping population of axial mesoderm cells (Ramel and Lekven, 2004; Ramel et al., 2005).

Considering the expanded domains of anterior prechordal plate (*hgg1*) and anterior lateral plate mesoderm (*scl*) in *wnt8*^{MO4} embryos, we predicted that cephalic paraxial mesoderm would show a similar response to Wnt regulation. To test this, *wnt8*^{MO4} embryos were fixed at 90% epiboly (~9 hours post fertilization), and *in situ* hybridizations were preformed to detect *follistatin a (fsta)*. In contrast to our expectations, we found that there was an overall decrease in the intensity of *fsta* expression, and the area encompassing *fsta* expressing cells shifted from its normal location in the anterior half of the embryo to a ring around the middle of the embryo (Figure 10 E-H). Thus, paraxial mesoderm appears to require Wnt8 signaling throughout the anteroposterior axis.

Summary and conclusions

To better understand the role Wnt8 plays in mesoderm patterning, we characterized the *wnt8*^{MO4} phenotype with a panel of cell fate markers. The data from

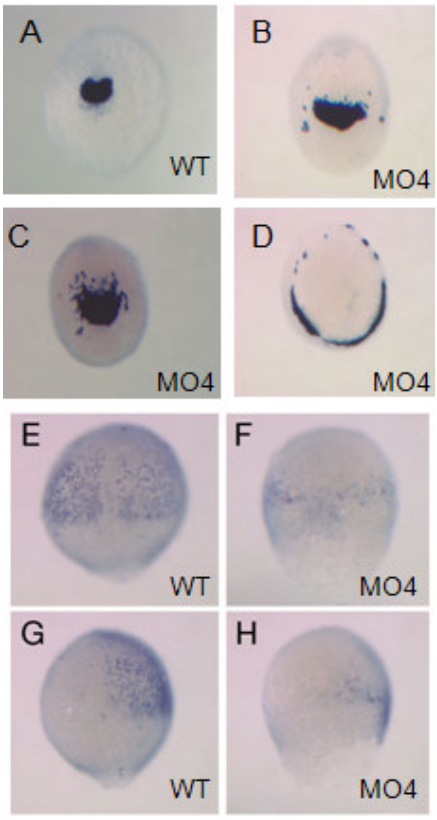

Figure 10: Hgg1 and follistatin expression in Wnt8 morphants. Hgg1 (A,B,C,D), Follistatin (E,F), lateral view (G,H).

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these in situ hybridization experiments suggest that Wnt8 signaling is required for specification of paraxial mesoderm progenitors throughout the anteroposterior axis. Further, intermediate mesoderm fates in the posterior embryo are positively regulated by Wnt8 signaling. Of anterior mesoderm fates, axial mesoderm and lateral plate mesoderm progenitors are antagonized by Wnt8 signaling, thus these populations expand in the absence of Wnt8 signaling.

These results lead to several unanswered questions. What is happening to the mesoderm progenitors that would normally be fated to contribute to the posterior embryo? There is clearly a reduction, but is it because fewer posterior mesoderm progenitors are specified, because the cells die early, or because they undergo fewer rounds of cell division? Because the dorsoventral axis is established before the anteroposterior axis, are the patterning defects we see in *wnt8*^{MO4} embryos due to an earlier defect in dorsoventral patterning? Clearly any of these possibilities would result in fewer cells contributing to the posterior mesoderm. In the next chapter, I describe experiments that test whether reduced Bmp signaling lies behind the reduction in posterior mesoderm specification in *wnt8*^{MO4} embryos.

CHAPTER IV

IDENTIFYING BMP-INDEPENDENT FUNCTIONS OF WNT8

Introduction

It can be suggested that the reason there is an anterior/posterior patterning defect in Wnt8 morphants is because there is an early dorsoventral patterning defect. That is, embryos lacking Wnt8 have expanded organizers that secrete elevated levels of Bmp antagonists, such as Chordin, that reduce Bmp signaling levels. Thus, the full extent of the effects of *wnt8* loss of function may be masked by Bmp-dependent effects. To ascertain if reduced Bmp signaling contributes to the *wnt8* loss-of-function phenotype, we simultaneously knocked down Wnt8 and the Bmp antagonist Chordin with morpholino antisense oligos. Reducing Chordin protein levels should promote elevated Bmp activity in the context of Wnt8 loss of function. If the phenotype of the double morphants looks like a wild-type embryo, then it would suggest that increased Bmp signaling can compensate for the loss of Wnt8, and a defect in early Bmp signaling might explain at least part of the Wnt8 loss-of-function phenotype.

Chd knockdown partially suppresses the wnt^{MO4} phenotype

To begin, we injected wild type embryos with morpholinos targeting *wnt8* and *chordin*, and then examined morphological phenotypes at 24 hours post fertilization. *wnt8*^{MO4} embryos fall into phenotypic classes that can be categorized according to a classification scheme devised for Bmp mutants (Kishimoto et al., 1997). According to this scheme, C4 and C5 represent the most severe phenotypes and are characterized by

an absent yolk extension, severe axis truncation and severe head disorganization. C3 embryos also fail to make a yolk extension but show only a mildly disorganized head and only moderate axis truncation. C2 embryos form a partial yolk extension, head development is relatively normal, and the tail is only slightly shortened but lacks the ventral tail fin. C1 embryos lack the ventral tail fin but are otherwise wild-type. *wnt8*^{MO4} embryos typically show a range of phenotypes (Table 1) including C4/C5 (strongly dorsalized) to C3/C2 (moderately/weakly dorsalized). Knockdown of Chordin consistently produces embryos that phenocopy *chordin* genetic mutants (97% of embryos). Simultaneous knockdown of Wnt8 and Chordin resulted in no significant

Table 1: Phenotypes of single *Wnt8* MO and *Chd* MO and double *Wnt8* + *Chd* injected embryos.

	phenotypes							
morpholino injected	wild type	strongly dorsalized	moderate/weakly dorsalized	*chordin* phenotype	curled-up	dead	unclassified	n
wnt8 MO	0	**30%**	48%	0	**0**	13%	9%	69
chordin MO	0	**0**	0	97%	**0**	3%	0	69
wnt8+chordin MO	0	**27%**	0	22%	**30%**	7%	14%	41

change in the percent of strongly dorsalized embryos from Wnt8 knockdown alone, but the class of moderately to weakly dorsalized embryos observed upon Wnt8 knockdown was not observed in the double knockdown group. Instead, a new phenotypic class emerged characterized by a curled-up tail, absent yolk extension and ventral tail fin and a conspicuous cell mass on the side of the yolk opposite the embryo (“yolk cell mass”)

(Figure 11 A-D). This result suggests the strongest *wnt8*^{MO4} phenotype cannot be altered by elevated Bmp signaling, but embryos with low levels of Wnt8 signaling are responsive to elevated levels of Bmp.

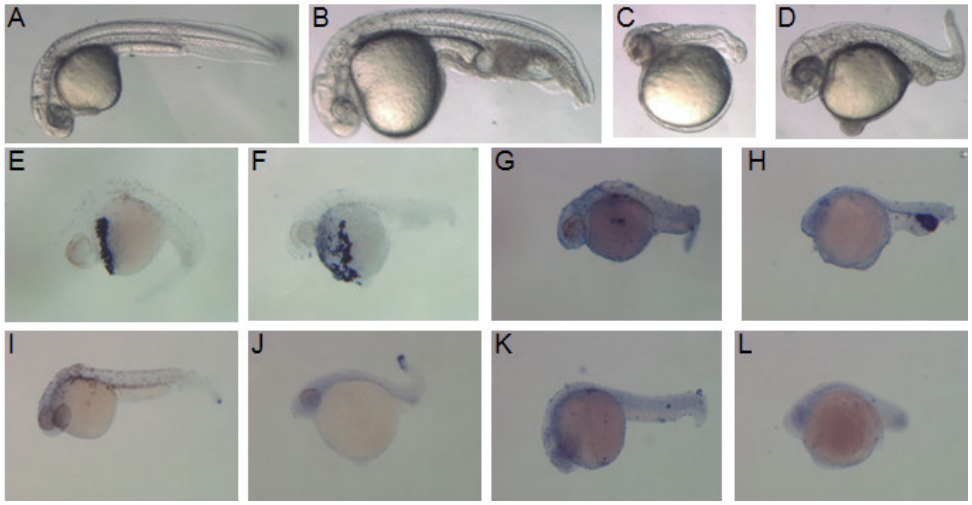

Figure 11: 24 hour phenotype of Din/MO4 morphants. WT 24 hour phenotype (A). Chd 24 hour phenotype (B). MO4 24 hour phenotype (C). Double morphant 24 hour phenotype exhibiting yolk cell mass (D). Hgg1 at 24 hours (E,F). Gata1 at 24 hours (G,H). Eve1 at 24 hours (I,J). Neurogenin1 at 24 hours (K,L).

To determine the identity of cells contributing to the yolk cell mass, we performed a series of *in situ* hybridizations on this new class of embryos. The first gene we examined was *hgg1*, a marker of the hatching gland, a structure found on the anterior end of the embryo that secretes enzymes that help dissolve the corion. While a few cells expressed *hgg1*, the majority of the cell mass did not (Figure 11 E, F). We then examined *gata1*, a blood cell marker, *eve1*, a tail bud progenitor marker, and *neurogenin*, a neural marker. None of these genes were expressed in the yolk cell mass

(Figure 11 G-L). While the identity of these cells remains undetermined, they may be similar to a smaller population of apoptotic cells found ventral to the yolk extension of *chordin* mutant embryos (Hammerschmidt et al., 1996).

Paraxial mesoderm is compromised in wnt8^{MO4};*chd*^{MO} *embryos*

We fixed *wnt8*^{MO4};*chd*^{MO} embryos between the 5 and 9 somites stages and performed *in situ* hybridizations using the posterior paraxial mesoderm markers, *myf5*, *myoD*, and *tbx24* to ascertain the effects of increased Bmp in a *wnt8* loss of function background. All three genes are markers of presomitic mesoderm but are not expressed in tailbud progenitors. Thus, their expression at the 5-6 somite stage marks paraxial mesoderm that will contribute to the trunk somites (Holley and Takeda, 2002). All three genes show reduced expression in *wnt8*^{MO4} and *chd*^{MO} embryos at the 5-6 somite stage. In *wnt8*^{MO4} embryos, this may be explained by a role for Wnt8 in promoting paraxial mesoderm formation in general (Yamaguchi, 2001). In *chd*^{MO} embryos, reduced expression of presomitic mesoderm markers may reflect the diversion of trunk paraxial mesoderm progenitors toward a tail fate (Szeto and Kimelman, 2006), since cells that express *myf5*, *myoD* and *tbx24* at the 5-6 somite stage will contribute to trunk somites, not tail somites.

myf5 expression was reduced in the wnt8 morphants, but still showed some somite expression. It was also reduced in the chd morphants; however somite expression had been lost. *tbx24* expression was similarly reduced in *wnt8*^{MO4}, *chd*^{MO} and *wnt8*^{MO4};*chd*^{MO} embryos (Figure 12). *myoD* is expressed in somites and adaxial cells. *wnt8*^{MO4} embryos showed decreased *myoD* expression, but still had somite expression,

while the *chd*^{MO} embryos showed no somite expression and light expression in the adaxial stripes (Figure 12). The double morphants showed an additive phenotype: no somite expression as in *chd*^{MO} embryos but short adaxial stripes as in *wnt8*^{MO4} (Figure 12E-H).

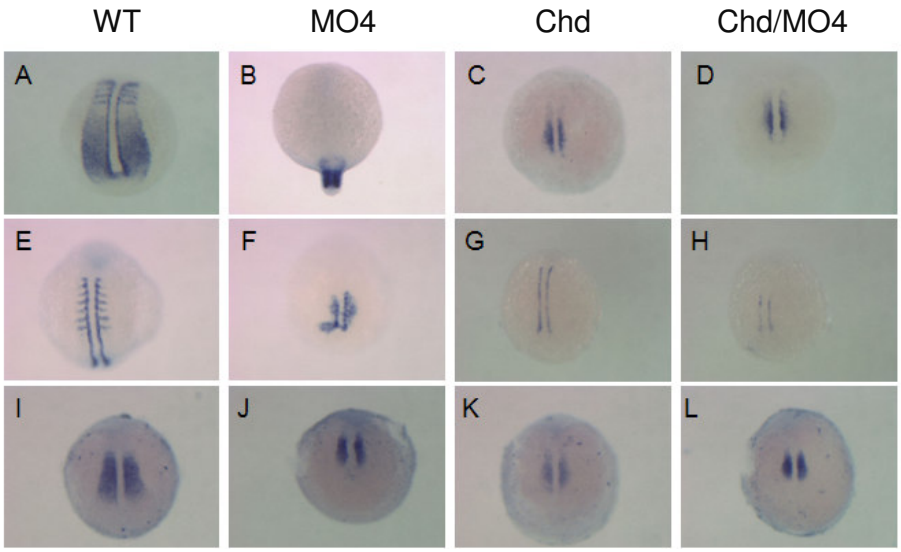

Figure 12: Posterior markers in Chd/MO4 morphants. All views are posterior. Myf5 expression (A,B,C,D). MyoD expression (E,F,G,H). Tbx24 expression (I,J,K,L).

Intermediate mesoderm patterning is rescued in wnt8^{MO4};*chd*^{MO} *embryos*

We next examined whether Chordin knockdown could rescue intermediate mesoderm fate specification in *wnt8* morphants. As shown in Chapter III, *pax2.1*-expressing intermediate mesoderm is either absent or almost absent in *wnt8*^{MO4} embryos (Figure 8 , Figure 13). Because *pax2.1* is also expressed in the neural plate and otic vesicles at the 5-6 somite stage, we could also use its expression to evaluate the extent of neural induction in the knockdown conditions.

Embryos deficient in Wnt8 showed a loss of pronephric expression and otic placode, the midbrain hindbrain boundary (MHB) thinned and expanded into a ring encircling the embryo (Figure 13), and the forebrain expression was disorganized (Figure 13 A,B,F,G). In *chd*^{MO} embryos, *pax2.1* expression domains are present but are shifted in the anterior embryo toward the dorsal midline. This shift reflects suppressed neural induction by elevated Bmp (in the neural plate) and the consequence of reduced trunk paraxial mesoderm that separates intermediate mesoderm from the dorsal midline. In contrast, the pronephric region of the embryos had widened around the posterior end (Figure 13 C,H), reflecting increased numbers of tail mesoderm progenitors.

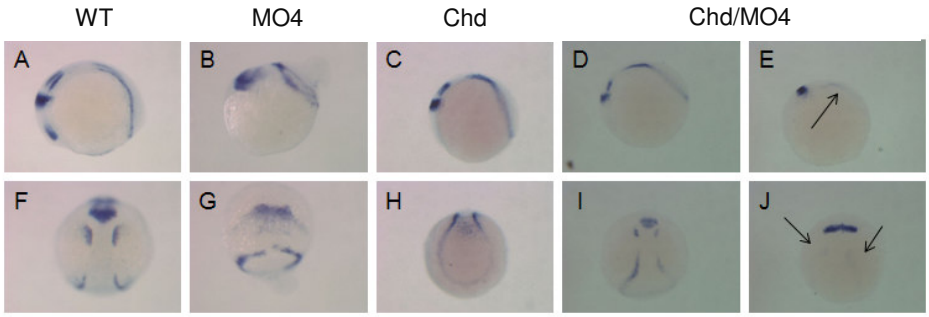

Figure 13: *pax2.1* expression in Chd/MO4 morphants. Lateral view (A,B,C,D,E). Dorsal/Posterior view (F,G,H,I,J). Wild type (A,F). *wnt8*^{MO4} (B,G). *chd*^{MO} (C,H). Double morphants (D,E,I,J). Arrows show very slight return of pronephric expression.

wnt8^{MO4};*chd*^{MO} embryos could be classified into two phenotypic groups. The weakly affected group resembled very closely the *chd*^{MO} phenotype: a relatively normal anterior end, slightly reduced otic placode expression, a slightly narrower midbrain-hindbrain boundary (MHB), and diminished expression in the pronephric region.

Interestingly, *wnt8*^{MO4};*chd*^{MO} embryos display a MHB width midway between wild-type and the *wnt8*^{MO4} phenotype, but the distribution of *pax2.1*$^{+}$ pronephric progenitors resembled that of *chd*^{MO} embryos, although expression was significantly reduced (n=27). Thus, Chordin knockdown only slightly suppressed the expanded neural induction of Wnt8 knockdown, but rescued the patterning of intermediate mesoderm progenitors (Figure 13 D,E,I,J).

To extend this result to another intermediate mesoderm marker, we examined *fli1* expression, which labels both head and body vasculature, at the 17 somite stage in the double morphants. *fli1* in Wnt8 morphants showed truncated posterior expression and the anterior expression domain formed one solid line of expression. Chordin morphants had relatively normal anterior expression, but the posterior expression domain had expanded from one line of expression down the axis of the embryo to a band of expression around the posterior end. The double morphants (n=22) exhibited anterior expression that was partially restored to normal, and the posterior expression resembled the pattern in the Chordin morphants (Figure 14 A-D).

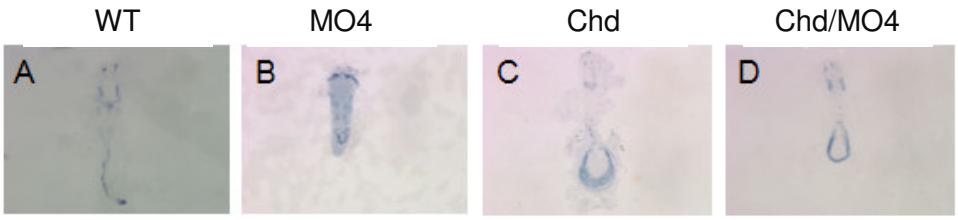

Figure 14: Fli1 expression in Chd/MO4 morphants. Wild type (A). MO4 (B). Chd (C). Chd/MO4 (D).

Cardiac mesoderm is not rescued in wnt8^{MO4};chd^{MO} embryos

In the Wnt8 deficient embryos expression of *cmlc2* was in bilateral stripes instead of the normal circular pattern, and in the most affected embryos, the expression of *cmlc2* was completely gone. Likewise, the *chd* morphants showed expression in bilateral stripes and in the most affected embryos expression of *cmlc2* was abolished altogether. The double morphants exhibited two groups of phenotypes, the first had faint bilateral stripes of expression (n=5), and the second group had scattered cells that expressed *cmlc2* (Figure 15, n=22).

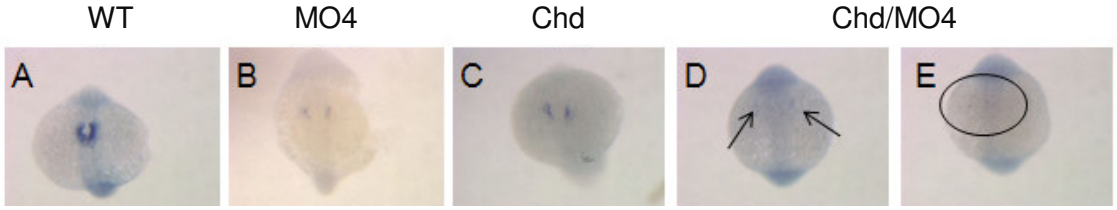

Figure 15: Cmlc2 expression in Chd/MO4 morphants. All views are dorsal. Wild type (A). MO4 (B). Chd (C). Chd/MO4 (D,E). Arrows point to slight return of Chd expression, most severely affected MO4 and Chd morphants had no expression of Cmlc2. Circle shows scattered expression.

Summary and conclusions

It was our goal to determine if increasing Bmp signaling would compensate for the loss of Wnt8 signaling. We double injected wild-type embryos with morpholinos designed against *wnt8* and *chd* and performed whole mount in situ hybridizations to determine if there were any changes in expression in the double morphants versus the single morphants. We first examined the 24 hour phenotype of the double mutants and

noticed that a peculiar cell mass developed on the bottom of the yolk. We tried to determine the nature of the cell mass by performing *in situ* hybridizations using the probes *hgg1*, *gata1*, *eve1*, and *neurogenin1*. While a few cells in the cell mass turned out to be part of the hatching gland, we did not find a marker that would label the entire or majority of the cells in the cell mass. These findings lead us to believe that the yolk cell mass probably consists of undifferentiated cells.

Next we tested the fates of anterior and posterior cell populations when we increased Bmp signaling by knocking down its antagonist chordin and knocked down Wnt8 signaling at the same time. We first examined the posterior markers *myf5*, *myoD*, and *tbx24* and saw in each case that increasing Bmp signaling did not restore the *wnt8* phenotype. The case of *myf5* and *myoD* the double morphants both have lost somite expression like that of the Din morphants, and the adaxial expression is still truncated like that of the Wnt8 morphants. We would have expected somite expression and longer adaxial expression if increasing Bmp would have restored some or all of the *wnt8* phenotype. When we examined *tbx24* expression the double morphants' expression looked very similar to both the individual morphants' expression. If Bmp signaling could have restored *tbx24* expression in a Wnt8 morphant, we would expect to see a larger area of expression in the double morphants.

We then examined *pax2.1*, *cmlc2*, and *fli1*, which mark anterior cell populations, and in the case of pax2.1, posterior cells as well. Interestingly, *pax2.1* expression in the pronephros in Wnt8 morphants is completely absent. In the double morphants, we saw very light pronephric expression. The only anterior cell population that still expressed

Pax2.1 was the MHB which had thinned and lengthened horizontally. Because of the light pronephric staining we believe that increasing Bmp signaling can slightly reverse the loss of pronephric cells due to a reduction in *wnt8* signaling. We then looked at *cmlc2* and *fli1* markers for heart precursors and vasculature respectively.

In both instances, the double morphants showed signs that the *wnt8* phenotype is being reversed. In the single morphants, the heart tube is expressed as two bilateral lines instead of a nice round structure, in the most affected embryos; heart tube expression is completely absent. In the double morphants we saw two groups of embryos. The first group exhibited slight expression of *cmlc2* in bilateral stripes, indicating that the increase in Bmp signaling can reverse the phenotype due to a loss of *wnt8*. Interestingly, in the second group, *cmlc2* expression was discovered to be in scattered cells with no clear pattern. Whether this case represents embryos that did not get as much of the Din MO as the other ones, resulting in lesser amount of Bmp signaling so that a small amount of cells are being fated to become heart tube, is not clear and needs to be studied more.

The last marker we examined was *fli1*. The MO4 injected embryos showed a loss of posterior and a disruption in anterior vasculature, while the Din morphants had relatively normal anterior expression, and expanded posterior expression. The double morphants showed a partial restoration of the anterior expression, and a gain of posterior expression. This leads us to believe that increasing Bmp signaling in the absence of *wnt8* can partially restore the *wnt8* loss of function phenotype.

CHAPTER V

SUMMARY AND CONCLUSIONS

It was the goal of this research to better characterize the *wnt8* phenotype and to investigate if increasing Bmp signaling could restore the *wnt8* phenotype to wild type. To accomplish these goals we first injected wild type embryos with a morpholino designed to knock down *wnt8* signaling. We fixed embryos at various time points and examined several probes by whole mount *in situ* hybridization. Embryos deficient in *wnt8* signaling lack the posterior region, because of this we first looked at various posterior markers, *myf5, myoD, mesogenin, tbx24, follistatin,* and *gata1,* to examine if expression was reduced or totally absent. In all cases the markers were either severely reduced, or completely absent. It was apparent that *wnt8* is essential for proper expression of paraxial mesoderm, presomitic mesoderm, and blood precursors. We next examined expression of *pax2.1* which not only marks posterior pronephric mesoderm, but also structures derived from anterior mesoderm. In addition to *pax2.1*, we investigated expression of *scl*, which is a hematopoietic marker that has anterior and posterior expression.

Not surprisingly, we saw a decrease in the posterior expression of *pax2.1*, but surprisingly we saw a disruption in anterior expression as well. The otic placode was either reduced or completely missing; the forebrain expression had expanded from a tight formation to an unorganized field, and the MHB had thinned and had formed a ring that circled what was left of the posterior end. We saw similar results in the *scl in situs*

as well. The anterior portion of expression was disrupted, and the posterior expression was absent. The disruption in the anterior region of the *wnt8* morphants prompted us to examine other anterior mesoderm markers to determine if other anterior cell populations were disrupted as well. We first examined the heart and vasculature makers, *cmlc2*, *nkx2.5*, and *fli1*. We saw improper heart tube formation, and a disruption in vasculature formation, indicating that not only is *wnt8* important for posterior mesoderm, it is also important for cardiac mesoderm. The last two anterior markers we examined were *hgg1* and *follistatin*. They mark anterior prechordal plate and at an early stage presumptive head mesoderm respectively. In data that was consistent with our previous results, we saw a disruption in these anterior mesoderm precursors, leading us to conclude that not only is *wnt8* important for forming posterior mesoderm, it is important for proper pattering of anterior mesoderm as well.

It is possible that the reduction in posterior fates is due to the cells being fated properly but dying early, or the cells might be undergoing fewer rounds of cell division. To answer these questions, TUNEL and BrdU assays can be performed to detect dying and proliferating cells. If the cells were being fated to become anterior precursors, we would expect to see an increase in anterior fates, which we did not. The anterior results were interesting and need further investigation to understand what is happening to these cells. One way we propose to understand what happens to posterior cells and why there are anterior patterning problems is to perform a series of fate mapping experiments to learn what happens to posterior mesoderm cells in Wnt8 morphants. Exactly how *wnt8* controls posterior mesoderm development is still unknown. It is known that *fgf8* and

fgf24 play important roles in patterning the posterior mesoderm, embryos deficient in both genes look remarkably similar to *wnt8* mutants (Draper et al, 2003). It also should be noted that the Fgf pathway and the Wnt pathway share a downstream effector, GSK3β (Dailey et al, 2005). Finding the relationship between Wnt and Fgf signaling pathways will help in understanding how *wnt8* can pattern both dorsoventral and anteroposterior patterning.

It is known that Bmp signaling plays a role in dorsoventral patterning (Stickney, 2007), and mutants lacking in Bmp signaling show a reduction in posterior fates (Dick et al., 2000; Imai et al., 2001; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000; Shimizu et al., 2002; Stickney et al., 2007). We next asked the question can over expressing Bmp signaling compensate for the loss of Wnt signaling. To answer this question we injected wild type embryos with MOs designed against Wnt8 and Din to create single controls. We then injected both MOs into the same embryos, fixed at various time points, and performed *in situ* hybridizations using various markers to ascertain any changes in different mesodermal precursors. We first examined the double morphants at 24 hours after fertilization to examine morphology changes. We saw an array of phenotypes; however, the majority of embryos exhibited a cell mass on the bottom of the yolk. After performing *in situ* hybridizations using markers for *hgg1, eve1, gata1,* and *neurogenin1* we failed to type the cell mass. This data lead us to believe that the cell mass consists mainly of undifferentiated cells.

We next examined expression of the posterior markers: *myf5, myoD,* and *tbx24*. We did not see a rescue of the *wnt8* phenotype in any of the double morphants. We then

examined expression of *pax2.1* which has anterior and posterior expression. The posterior pronephric expression is completely lost in Wnt8 morphants, but not in Din morphants. Interestingly, we saw a partial rescue of pronephric precursors when we examined *pax2.1* expression in the double morphants. There was a slight return of pronephric expression in the double morphants. This result was exciting because it indicated that increasing Bmp signaling in the absence of Wnt8 can slightly reverse the *wnt8* phenotype.

We then examined expression of *cmlc2* and *fli1* which are markers for the heart tube and vasculature respectively. In both the individual morphants we saw a complete loss of *cmlc2* expression in the most severely affected embryos, while the normally round heart tube structure had failed to form normally, instead was in two bilateral stripes in the lesser affected embryos. The embryos injected with both MOs showed one of two phenotypes. In the first we saw light bilateral expression, indicating a slight restoration of heart tube precursors. In the second group, the *cmlc2* expressing cells were scattered around the embryo. Lastly, we examined expression of *fli1*, a marker for vasculature precursors. Again, we witnessed partial restoration of the *wnt8* phenotype in the double morphants. The anterior region which was disrupted in the Wnt8 morphants was relatively normal in the double injected embryos, and the posterior region, which had mostly disappeared in the Wnt8 morphants, had returned, but had the phenotype of a Din morphant.

Taken together we believe this data supports the theory that in general Wnts are involved in maintaining mesodermal states, and Bmps are responsible for patterning the

mesoderm (Kimelman, 2006). It has been found that Bmp functions during late blastula stage and early gastrula stage to pattern more dorsal mesoderm, and it functions during later gastrula stages to pattern more ventral mesoderm (Pyati et al, 2005; Tucker et al, 2008). Because it is thought Wnts maintain mesoderm and Bmps pattern mesoderm, this could be why we do not see a better rescue of mesodermal fates by increased Bmp signaling in Wnt8 morphants. If *wnt8* is not there to maintain the mesoderm, Bmp cannot pattern mesoderm that is not there. While our research sheds some light on how *wnt8* functions to regulate mesoderm precursors, there are still many more questions that need to be answered. Further research needs to be performed to fully understand how *wnt8* regulates posterior mesoderm development.

REFERENCES

Abdelilah, S., Mountcastle-Shah, E., Harvey, M., Solnica-Krezel, L., Schier, A.F., Stemple D.L., Malicki, J., Neuhauss, S.C., Zwartkruis, F., Stainier, D.Y., Rangini, Z., Driever, W., 1996. Mutations affecting neural survival in the zebrafish danio rerio. Development 123, 217-227.

Arce, L., Yokoyama, N.N., and Waterman, M.L., 2006. Diversity of lef/tcf action in development and disease. Oncogene 25(57), 7492-7504.

Bauer, H., Meier, A., Hild, M., Stachel, S., Economides, A., Hazelett, D., Harland, R.M., and Hammerschmidt, M., 1998. Follistatin and Noggin are exluded from the zebrafish organizer. Dev Biol 204, 488-507.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R., 1996. A new member of the frizzled family from drosophila functions as a wingless receptor. Nature 382, 225-230.

Blader, P., Fischer, N., Gradewchl, G., Guillemut, F., and Strahle, U., 1997. The activity of neurogenin1 is controlled by local cues in the zebrafish embryo. Development 124, 4557-4569.

Brown, L.A., Rodaway, A.R.F., Schilling, T.F., Jowett, T., Ingham, P.W., Patient, R.K., and Sharroks, A.D., 2000. Insights into early vasculogenesis revealed by expression of the ets domain transcription factor fli-1 in wild type and mutant zebrafish embryos. Mech Dev 90(2) 237-252.

Chen, G.D., Chou, C.M., Hwang, S.P., Wang, F.F., Chen, Y.C., Hung, C.C., Chen, J.Y., and Huang, C.J., 2006. Requirement of nuclear localization and transcriptional activity of p53 for its targeting to the yolk syncytial layer (ysl) nuclei in zebrafish embryo and its use for apoptosis assay. Biochem Biophys Res Commun 344(1), 272-282.

Clevers, H., 2006. Wnt/β-catenin signaling in development and disease. Cell 127, 469-480.

Dailey, L., Ambrosetti, D., Mansukhani, A., Basilico, C., 2005. Mechanisms underlying differential responses to fgf signaling. Cytokine Growth Factor Rev 16, 233-247.

Dick, A., Hild, M., Bauer, H., Imai, Y., Maifeld, H., Schier, A.F., Talbot, W.S., Bouwmeester, T., and Hammerschmidt, M., 2000. Essential role of bmp7

(snailhouse) and its prodomain in dorsoventral patterning of the zebrafish embryo. Development 127, 343-354.

De Robertis, E.M., Larrain, J., Oelgeschlager, M., Wessely, O., 2000. The establishment of spemann's organizer and patterning of the vertebrate embryo. Nat Rev Genet 1 (3), 171-181.

Detrich, H.W. III, Kieran, M.W., Chan, F.Y., Barone, L.M., Yee, K. Rundstadler, J.A., Pratt, S., Ransom, D., and Zon, L.I., 1995. Intraembryonic hematopoietic cell migration during vertebrate development. Proc. Natl. Acad. Sci. U.S.A. 92, 10713-10717.

Dougan, S.T., Warga, R.M., Kane, D.A., Schier, A.F., and Talbot, W.S., 2003. The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. Development 130(9), 1837-1851.

Draper, B.W., Stock, D.W., and Kimmel, C.B., 2003. Zebrafish fgf24 functions with fgf8 to promote posterior mesodermal development. Development 130, 4639-4654.

Eastman, Q., and Grosschedl, R., 1999. Regulation of lef-1/tcf transcription factors by wnt and other signals. Curr Opin Cell Biol 11, 233-240.

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Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., Niehrs, C., 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391 (6665), 357-362.

Hammerschmidt, M., Pelegri, F., Mullins, M.C., Kane, D.A., Brand, M., van Eeden, F.J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C.P., Jiang, Y.J., Kelsh, R.N., Odenthal, J., Warga, R.M., Nusslein-Volhard, C., 1996. Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, danio rerio. Development 123, 143-151.

He, X., Semenov, M., Tamai, K., and Zeng, X., 2004. LDL receptor-related proteins 5 and 6 in wnt/b-catenin signaling: arrows point the way. Development 131, 1663-1677.

Holley, S.A., and Takeda, H., 2002. Catching a wave: the oscillator and wavefront that create the zebrafish somite. Semin Cell Dev Biol 13(6), 481-488.

Hoppler, S., Brown, J.D., and Moon, R.T., 1996. Expression of a dominant-negative wnt blocks induction of myoD in xenopus embryos. Genes Dev 10 (21), 2805-2817.

Huang, C.J., Tu, C.T., Hsiao, C.D., Hsieh, F.J., and Tsai, H.J., 2003. Germ-line transmission of a myocardium specific gfp transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. Dev Dyn 228 (1), 30-40.

Imai, Y., Gates, M.A., Melby, A.E., Kimelman, D., Schier, A.F., and Talbot, W.S., 2001. The homeobox genes vox and ved are redundant repressors of dorsal fates in zebrafish. Development 128, 2407-2420.

Joly, J.S., Joly, C., Schulte-Merker, S., Boulekbache, H., and Condamine, H., 1993. The ventral and posterior expression of the zebrafish homeobox gene eve1 is perturbed in dorsalized and mutant embryos. Development 119, 1261-1275.

Jowett, T., 2001. Double in situ hybridization techniques in zebrafish. Methods 23(4), 345-358.

Keegan, B.R., Feldman, J.L., Begemann, G., Ingham, P.W., and Yelon, D., 2005. Retinoic acid signaling restricts the cardiac progenitor pool. Science 307, 247-249.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203(3), 253-310.

Kimmel, C.B., and Law, R.D., 1985. Cell lineage of zebrafish blastomeres. II. Formation of the yolk syncytial layer. Dev Biol 108(1), 86-93.

Kimmel, C.B., Warga, R.M., and Schilling, T.F., 1990. Origin and organization of the zebrafish fate map. Development 108(4), 581-594.

Kimelman, D., 2006. Mesoderm induction: from caps to chips. Nature Rev Gen 7, 360-372.

Kimelman, D., and Griffin, K.J.P., 2000. Vertebrate mesendoderm induction and patterning. Curr Opin Gen Dev 10, 350-356.

Kishimoto, Y., Lee, K.H., Zon, L., Hammerschmidt, M., Schulte-Merker, S., 1997. The molecular nature of zebrafish swirl: bmp2 function is essential during early dorsoventral patterning. Development 124(22), 4457-4466.

Lekven, A.C., Thorpe, C.J., Waxman, J.S., and Moon, R.T., 2001. Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. Dev Cell 1(1), 103-114.

Leyns, L., Bouemeester, T., Kim, S.H., Piccolo, S., and De Robertis, E.M., 1997. Frzb-1 is a secreted antagonist of wnt signaling expressed in the spemann organizer. Cell 88(6), 747-756.

Melby, A.E., Beach, C., Mullins, M., and Kimelman, D., 2000. Patterning the early zebrafish by the opposing actions of bozozok and vox/vent. Dev Biol 224, 275-285.

Nasevicius, A., and Ekker, S.C., 2000. Effective targeted gene 'knockdown' in zebrafish. Nat Genet 26(2), 216-220.

Nguyen, V.H., Schmid, B., Trout, J., Connors, S.A., Ekker, M., and Mullins, M.C., 1998. Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. Dev Biol 199(1), 93-110.

Nikaido, M., Kawakami, A., Sawada, A., Furutani-Seiki, M., Takeda, H., and Araki, K., 2002. Tbx24, encoding a t-box protein, is mutated in the zebrafish somite-segmentation mutant fused somites. Nat Genet 31(2), 195-199.

Pyati, U.J., Webb, A.E., and Kimelman, D., 2005. Transgenic zebrafish reveal stage-specific roles for bmp signaling in ventral and posterior mesoderm development. Development 132, 2333-2343.

Ramel, M.C., and Lekven, A.C., 2004. Repression of the vertebrate organizer by wnt8 is mediated by vent and vox. Development 131, 3991-4000.

Ramel, M.C., Buckles, G.R., Baker, K.D., and Lekven, A.C., 2005. Wnt8 and bmp2b co-regulate non-axial mesoderm patterning during zebrafish gastrulation. Dev Biol 287, 237-248.

Rescan, P.Y., 2001. Regulation and functions of myogenic regulatory factors in lower vertebrates. Biochem Physiol B Biochem Mol Biol 130(1), 1-12.

Rohde, L.A., Oates, A.C., and Ho, R.K., 2004. A crucial interaction between embryonic red blood cell progenitors and paraxial mesoderm revealed in spadetail embryos. Dev Cell 7, 251-262.

Ross, S., and Hill, C.S., 2008. How the smads regulate transcription. Int J Biochem Cell Biol 40(3), 383-408.

Schier, A.F., and Talbot, W.S., 2005. Molecular genetics of axis formation in zebrafish. Annu Rev Genet 39, 561-613.

Schmid, B., Furthauer, M., Connors, S.A., Trout, J., Thisse, B., Thisse, C., and Mullins, M.C., 2000. Equivalent genetic roles for bmp7/snailhouse and bmp2b/swirl in dorsoventral pattern formation. Development 127(5), 957-967.

Shimizu, T., Bae, Y.K., Muraoka, O., and Hibi, M., 2005. Interaction of wnt and caudal-related genes in zebrafish posterior body formation. Dev Biol 279, 125-141.

Shimizu, T., Vamanaka, Y., Ncjima, H., Yabe, T., Hibi, M., and Hirano, T., 2002. A novel repressor-type homeobox gene, ved, is involved in dharma/bozozok mediated dorsal organizer formation in zebrafish. Mech Dev 118, 125-138.

Sokol, S.Y., 1999. Wnt signaling and dorso-ventral axis specification in vertebrates. Curr Opin Genet Dev 9(4), 405-410.

Solnica-Krezel, L., and Driever, W., 1994. Microtubule arrays of the zebrafish yolk cell: organization and function during epiboly. Development 120(9), 2443-2455.

Stickney, H., Imai, Y., Draper, B., Moens, C., and Talbot, W.S., 2007. Zebrafish bmp4 functions during late gastrulation to specify ventroposterior cell fates. Dev Biol 310(1), 71-84.

Szeto, D.P., and Kimelman, D., 2006. The regulation of mesodermal progenitor cell commitment to somitogenesis subdivides the zebrafish body musculature into distinct domains. Genes Dev 20(14), 1923-1932.

Thorpe, C., Weidinger, G., and Moon, R., 2005. Wnt/β-catenin regulation of the sp1-related transcription factor sp5l promotes tail development in zebrafish. Development 132, 1763-1772.

Tucker, J.A., Mintzer, K.A., and Mullins, M.C., 2008. The BMP signaling gradient patterns dorsoventral tissues in a temporally progressive manner along the anteroposterior axis. Dev Cell 14(1), 108-119.

Vogel, A.M., and Gerster, T., 1997. Expression of a zebrafish cathepsin l gene in anterior mesendoderm and hatching gland. Dev Genes Evol 206(7), 477-479.

Weinberg, E.S., Allende, M.L., Kelly,C.S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O.G., Grunwald, D.J., and Riggleman, B., 1996. Developmental regulation of zebrafish myoD in wild-type, no-tail, and spadetail embryos. Development 122, 271-280.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 4th ed. University of Oregon Press, Eugene.

Xanthos, J.B., Kofron, M., Tao, Q., Schaible, K., Wylie, C., and Heasman, J., 2002. The roles of three signaling pathways in the formation and function of the spemann organizer. Development 129(17), 4027-4043.

Yamaguchi, T.P., 2001. Heads or tails: wnts and anterior-posterior patterning. Curr Biol 11(17), 713-724.

Yelon, D., Horne, S.A., and Stainier, D.Y.R., 1999. Restricted expression of cardia myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol 214, 23-37.

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