2003

Regulation of Zebrafish Hindbrain Development by Fibroblast Growth Factor and Retinoic Acid: A Dissertation

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REGULATION OF ZEBRAFISH HINDBRAIN DEVELOPMENT BY FIBROBLAST GROWTH FACTOR AND RETINOIC ACID

A Dissertation Presented

By

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A thesis submitted to the Department of Biochemistry and Molecular Pharmacology
and the Graduate School of Biomedical Sciences of the
University of Massachusetts, Worcester
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in
BIOCHEMISTRY AND MOLECULAR PHARMACOLOGY

Worcester, Massachusetts

2003

Part of this dissertation has appeared in the publication:

Lane, M.E., Runko, A.P, Roy, N.M and Sagerstrom, C.G. (2002). Dynamic expression and regulation by Fgf8 and Pou2 of the zebrafish LIM-only gene, *lmo4*. *Gene Expression Patterns* 2, 207-211.

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October 2003

ACKNOWLEDGEMENT PAGE

I would like to thank my advisor Dr. Charles G. Sagerstrom for his constant support throughout my years at UMASS. His constant guidance and encouragement was critical for the success of this project.

I would also like to thank the members of my thesis advisory committee: Dr. Leslie Berg, Dr. Don Chen, Dr. Kai Lin and Dr. Alonzo Ross for their helpful comments throughout the years.

I would also like to thank Dr. Mary Munson and Dr. Pamela Yelick for joining my thesis committee in short notice.

A special thanks goes out to the members of the Sagerstrom Lab, Alex Runko, Seong-Kyu Choe, Sigalit Zchut, Anusha Jayaraman and Letitiah Etheridge for their helpful discussions.

Lastly, I would like to thank my parents for their constant support through my years in college and graduate school and to Jeffrey whose support was critical in the writing of this thesis.

For M, D, J and P

ABSTRACT

Fibroblast growth factor (Fgf) and Retinoic acid (RA) are known to be involved in patterning the posterior embryo. Work has shown that Fgf can convert anterior tissue into posterior fates and that embryos deficient in Fgf signaling lack posterior trunk and tail structures. Likewise, studies performed on RA have shown that overexpression of RA posteriorizes anterior tissue, while disrupting RA signaling yields a loss of posterior fates. While it appears these signals are necessary for posterior development, the role Fgf and RA play in development of the hindbrain is still enigmatic. A detailed study of the requirements for Fgf and RA in the early vertebrate hindbrain are lacking, namely due to a deficiency in gene markers for the presumptive hindbrain at early developmental stages. In this study, we make use of recently isolated genes, which are expressed in the presumptive hindbrain region at early developmental stages, to explore Fgf and RA regulation of the early vertebrate hindbrain.

We employed both overexpression and loss of function approaches to explore the role of Fgf in early vertebrate development with an emphasis on the presumptive hindbrain region in zebrafish embryos. By loss of function analysis, we show that Fgf regulates genes expressed exclusively in the hindbrain region (*meis3* and *hoxb1a*) as well as genes whose expression domains encompass both the hindbrain and more caudal regions (*nlz* and *hoxb1b*), thus demonstrating a requirement for Fgf signaling throughout the anteroposterior axis of the hindbrain (rostral to caudal hindbrain) by mid-gastrula

stages. To further characterize early gene regulation by Fgf, we utilized an in vitro system and found that Fgf is sufficient to induce *nlz* directly and *hoxb1b* indirectly, while it does not induce *meis3* or *hoxb1a*. Furthermore, in vivo work demonstrates that Fgf soaked beads can induce *nlz* and *hoxb1b* adjacent to the bead and *meis3* at a distance. Given the regulation of these genes in vitro and in vivo by Fgf and their position along the rostrocaudal axis of the embryo, our results suggest an early acting Fgf resides in the caudal end of the embryo and signals at a distance to the hindbrain. We detect a similar regulation of hindbrain genes by RA at gastrula stages as well, suggesting that both factors are essential for early hindbrain development.

Interestingly however, we find that the relationship between Fgf and RA is dynamic throughout development. Both signals are required at gastrula stages as disruption of either pathway alone disrupts hindbrain gene expression, but a simultaneous disruption of both pathways at later stages is required to disrupt the hindbrain. We suggest that Fgf and RA are present in limiting concentrations at gastrula stages, such that both factors are required for gene expression or that one factor is necessary for activation of the other. Our results also reveal a changing and dynamic relationship between Fgf and RA in the regulation of the zebrafish hindbrain, suggesting that at segmentation stages, Fgf and RA may no longer be limiting or that they are no longer interdependent.

As we have demonstrated that an early Fgf signal is required for gastrula stage hindbrain development, we next questioned which Fgf performed this function. We have

demonstrated that the early Fgf signal required for hindbrain development is not Fgf3 or Fgf8, two Fgfs known to be involved in signaling centers at the mid-hindbrain boundary (MHB) and rhombomere (r) 4. We further show that two recently identified Fgfs, Fgf4 and Fgf24 are also insufficient alone or in combination with other known Fgfs to regulate hindbrain gene expression. However, as Fgfs may act combinatorially, we do not rule out the possibility of their involvement in early hindbrain gene regulation. However, as time passes and additional Fgfs are isolated and cloned, the elusive Fgf signal required for early hindbrain development will likely be identified.

Taken together, we propose that an early acting Fgf residing in the caudal end of the embryo regulates hindbrain genes together with RA at gastrula stages. We suggest that both Fgf and RA are required for gene expression at gastrula stages, but this requirements changes over time as Fgf and RA become redundant. We also demonstrate that the Fgf required for gastrula stage hindbrain development has yet to be identified.

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INTRODUCTION

The development of the vertebrate body axis is a complex multi-step process involving the coordinated actions of many genes and signaling molecules. Current research has elucidated many of the factors and processes involved in dorsoventral axis formation, but much remains elusive in regards to anterior posterior axis formation. Although posteriorizing signals have been studied, limited research has been performed to elucidate how these factors regulate early hindbrain development. In the following pages I will outline, using the zebrafish vertebrate model system, how the vertebrate hindbrain is regulated by posteriorizing signals during early developmental stages.

The Zebrafish System

The zebrafish, a small silvery fish with horizontal blue stripes, has come a long way from its native populace in the Ganges River in India, to common inhabitants in fish bowls and home aquariums across the US, to a rise in prominence as a mainstay in developmental biology laboratories across the world. The zebrafish, a tropical fish has catapulted itself to the forefront of developmental biology research. In fact, late in 1996, an entire issue of the journal *Development* was devoted to studies performed in zebrafish (Development 123, 1996), making researchers take note that zebrafish were on the rise. As many organisms have been studied in an attempt to unravel the mysteries of development, why then has the zebrafish risen in prominance?

The zebrafish offers several advantages as a developmental model. Unlike the fruitfly *(Drosophila melanogaster),* zebrafish are easy to keep up, inexpensive to maintain, have a short generation time and individual females produce large quantities of progeny. Unlike the mouse, zebrafish eggs develop *ex utero* and are transparent, meaning every stage of development is readily visible under the simple dissection microscope allowing the researcher easy access to all developmental stages. More importantly however the zebrafish is a vertebrate, like humans, and follows the vertebrate path of embryonic development and thus research can be applied to humans. Zebrafish researchers have amassed a collection of mutations in two large scale mutagenesis screens (one in Tubingen, Germany (Haffter et al., 1996; Hammerschmidt et al., 1996a; Kane et al., 1996; Mullins et al., 1996) and one at Massachusetts General Hospital, Boston (Solnica-Krezel et al., 1996) (Schier et al., 1996) just to cite a few of the many

references), as well as smaller screens (Artinger et al., 1999; Rubinstein et al., 2000) (Garrity et al., 2002; Golling et al., 2002; Kudoh et al., 2001) isolating genes that affect processes from early axis formation to vascular architecture to neural circuitry and have provided models of human diseases. For example, the zebrafish *sau* mutant (sauternes) has delayed erythroid maturation and abnormal globin, which phenocopies the human sideroblastic anemia (Brownlie et al., 1998). As zebrafish mutations faithfully phenocopy many human disorders, each mutation can provide candidate genes and pathways for evaluation in human disease. As a number of the zebrafish mutants are solid models of human disease, many pharmaceutical companies are utilizing zebrafish in pharmacogical trials to test candidate drugs. Currently there are models of Alzheimer disease, congenital heart disease, polycyctis kidney disease and cancer (Ward and Lieschke, 2002).

Although it is easy to illustrate the benefits of zebrafish, they, as all model systems, are not perfect. Currently, the technology to knock-out specific genes is not at hand, as we do not yet have the techniques for homologous recombination in fish. However, with recent advances in morpholino oligonucleotides, which are targeted to the 5'UTR and ATG start site of a specific gene and inhibit translation of a target mRNA, it has become possible to knock-down specific proteins of choice. Secondly, the zebrafish is genetically redundant in its genome. There are at least two copies of some paralogous chromosome segments in zebrafish, which generally correspond to orthologous chromosomes segments in mammals. This suggests one large scale gene duplication

event occurred after the divergence of fish and mammals. The redundancy of the genome may complicate the comparison of homologous developmental pathways.

Overall the zebrafish system has its advantages and disadvantages, as does any model system. However the positives far outweigh the negatives and they provide the best vertebrate model system with a powerful combination of ease, speed, cost, number, developmental access and transparency. Thus, the zebrafish has become a top choice for a developmental model system and has proven itself here to stay.

The Developing Zebrafish Embryo

From Egg to Embyro-A gross overview of zebrafish development

In order to facilitate the reading of this thesis, zebrafish development is described in general below with a particular emphasis on early developmental processes relevant to this work followed by a general overview of later stages not as pertinent to this thesis.

The Early Stages: Zygote to Late Gastrulation

The newly fertilized egg, or zygote, is alive with a great deal of activity. Cytoplasm, of the non-yolky variety, is forced to stream anterior towards the animal pole by waves of contractile forces, creating the blastodisc, the first single cell, segregating itself from the underlying yolky vegetal cytoplasm on which it sits. After the first cleavage the blastomeres, or cells, begin to divide in a meroblastic fashion at 15 minute intervals to eventually yield the embryo proper.

The blastula is the stage in which the dividing cells resemble a ball, or blastodisc resting atop the yolk cell, roughly around the 128 cell stage, after the 8th cell cycle. Two populations of cells are evident, the external layer of cells, termed the EVL or enveloping layer, and the deep cells of the blastoderm surrounded by the EVL (Figure 1B). During these early blastula stages, the marginal blastomeres which lie against the yolk cell collapse and ajoin the underlying yolk cell creating the yolk syncytial layer or YSL (Figure 1B). Thus, at this time the developing embryo consists of the blastoderm harboring roughly 1000 cells covered by the EVL and resting atop the YSL. At this stage the embryo now enters the MBT or Mid-Blastula Transition where the embryo undergoes a series of cellular changes where cell cycle time increases, zygotic transcription occurs and the beginning of cell motility or epiboly commences.

Epiboly is the process by which the cells of the blastodisc and YSL begin to thin and spread out over the yolk cell (Figure 1A). As epiboly continues, around 5 hours, the three germ layers (ectoderm, endoderm and mesoderm) of the embryo become established in a process called gastrulation. During gastrulation, which occurs concurrent with epiboly, a complex series of events needs to be coordinated, leading to the correct spatial positioning of the three germ layers along the body axis. This process begins at about 50% epiboly at the onset of involution on the future dorsal side of the embryo, a process whereby cells of the blastoderm migrate vegetally and involute upon themselves near the margin to create a sublayer of cells that migrates back anteriorly (Figure 1C,D). The new involuted layer of cells, called the hypoblast will give rise to the endoderm (future gut and associated organs) and mesoderm (future muscle and internal

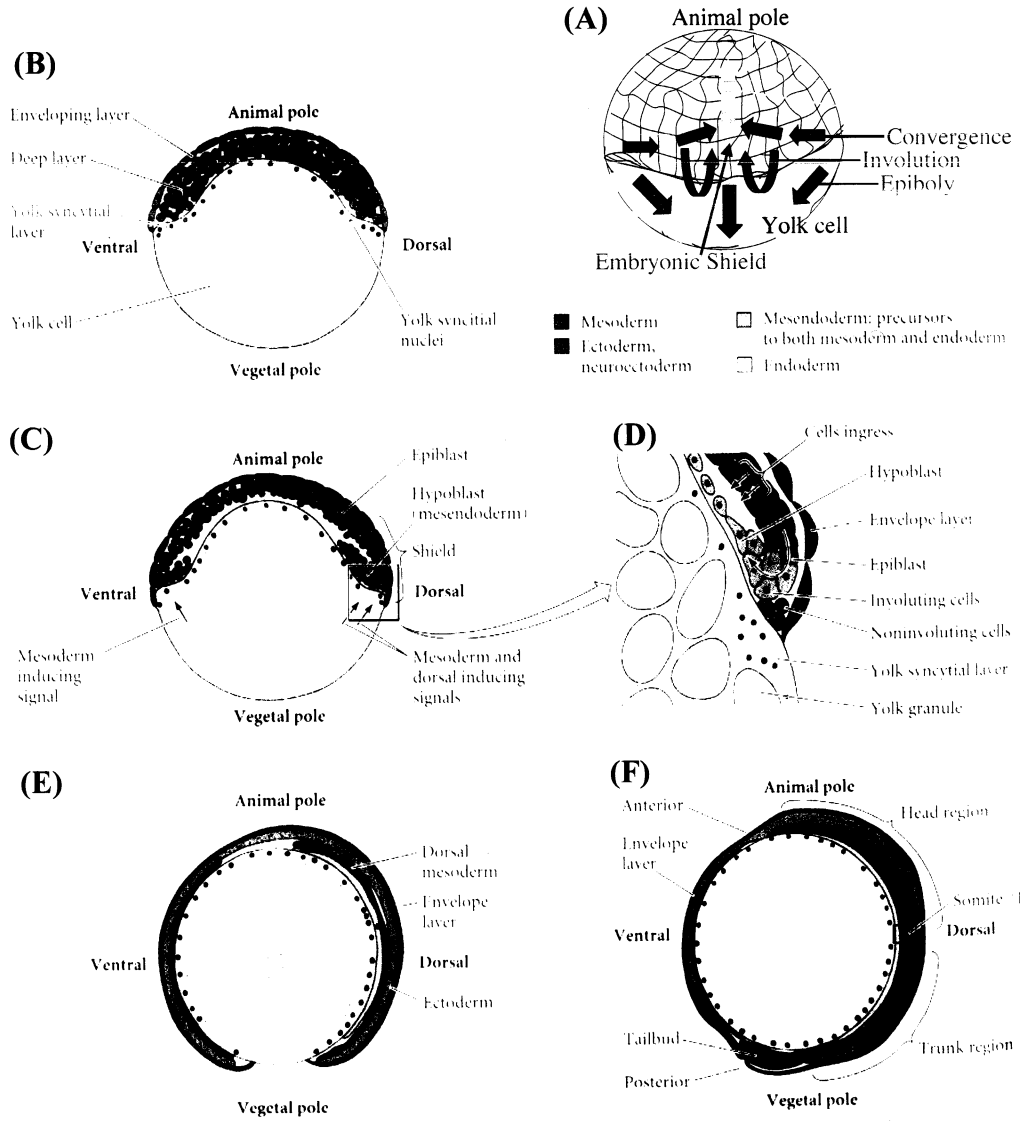

Figure 1. (A-F). Cell movements during gastrulation of the zebrafish. **A:** Movements during zebrafish gastrulation: Epiboly spreads the blastodermal cells to envelope the yolk; during involution cells of the blastoderm migrate vegetally and involute upon themselves to generate the hypoblast; during convergence and [illegible] the hypoblast and epiblast cells migrate to the dorsal side of the embryo forming the embryonic shield or "organizer." **B:** Embryo at 4 hpf illustrating the three main cell layers. **C:** Embryo at 6 hpf illustrating involution and creation of the hypoblast on the dorsal side. **D:** Close-up at the marginal region. **E:** Embryo at 9 hpf illustrating mesoderm expansion around the embryo. **F:** Embryos at 10 hpf illustrating completion of gastrualtion. Adapted from "Developmental Biology" Scott F. Gilbert, Sixth Edition.

organs). The outer layer of cells, called the epiblast will give rise to the ectoderm (future skin and nervous system). Following involution, cells of the both the hypoblast and epiblast migrate to the dorsal side of the embryo, in a process called convergence (Figure 1A). This results in the formation of a thickened region on the dorsal side of the embryos termed the embryonic shield or organizer. As more and more cells converge on the embryonic shield, the shield narrows and elongates towards the animal pole in a process called extension (Figure 1A). Thus, this is an active time in the embryo as cell movements of epiboly, gastrulation, convergence and extension all occur concurrent with one another. As time continues, the embryonic shield becomes less distinct as cells continue epiboly (Figure 1E), which comes to an end when the blastoderm completely covers the yolk by 10 hpf (Figure 1F).

Elaborating the Body Plan- Segmentation, Somitogenesis, Neurulation and Morphogenesis

By the end of epiboly when the blastoderm completely covers the yolk cell, the epiblast which is now exclusively ectodermal, begins to undergo extensive changes of segmentation and neurulation, processes which overlap quite extensively. The embryo enters the segmentation stage so called because the body plan becomes arranged in developing segments and the embryo elongates quite significantly. During the segmentation period, brain and spinal cord differentiate in a process called neurulation and bilateral pairs of somites, which flank the spinal cord, arise sequentially in the paraxial mesoderm of the trunk and tail in an anterior to posterior fashion with a

characteristic chevron shape pattern. Embryos become staged by the number of somites. There are three derivatives of the somites, the myotome, which gives rise to the body muscle segments and the sclerotome, which gives rise to the vertebral cartilage and the dermatome, which generates the connective tissue of the skin.

At the end of gastrulation, along the dorsal side of the embryo, the primordium of the central nervous system, the neural plate, becomes well delineated by prominent thickness. Neurulation refers to the process by which the brain and spinal cord are formed (Figure 2). During the very early stages of neurulation, the presumptive neuroectoderm of the gastrula epiblast converges to form the neural plate (Figure 2*a*). During the early segmentation stage, the epithelial cells of the neural plate begins to condense (Figure 2*b*) and infold at the midline to form the neural keel (Figure 2*c*), which rounds up into a solid cylindrical neural rod. Cells along the lateral sides of the neural plate move inward towards the midline to occupy a dorsal position in the neural rod, while cells of the center of the neural plate are enveloped and become the ventral neural rod. The neural tube is later formed by cavitation (Figure 2*d*). Even before cavitation occurs however, the anterior portion of the neural keel undergoes morphogenesis and eventually will become the brain. At the commencement of the segmenation period, the neural plate appears uniform in its consistency along the A-P axis (Figure 3*a*). Then, during segmentation, about 10 swellings or bulges, termed neuromeres become distinct (Figure 3*b*). The first three bulges are quite large and distinctive and will form the forebrain structures of the telencephalon, diencephalon and the mesencephalon or

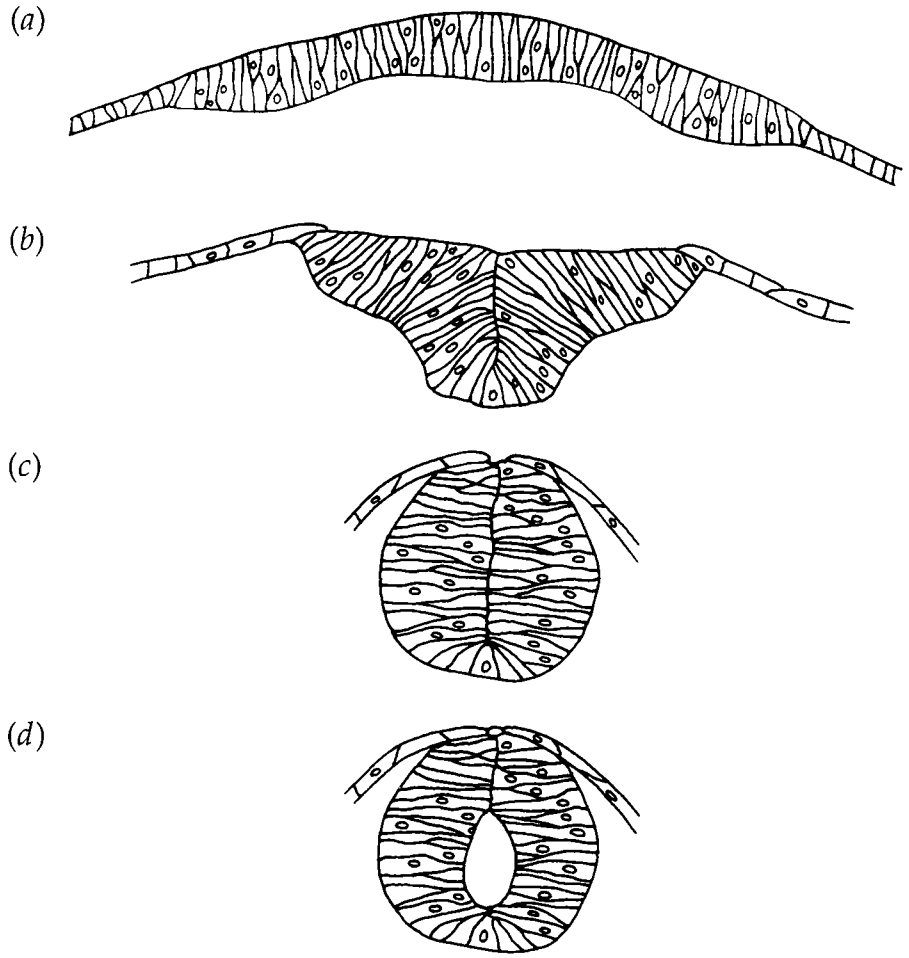

Figure 2. (*a-d*). Development of the Neural Tube. *a:* The neural plate. *b*: Infolding of the neural plate at the midline. Cells around the lateral side of the neural plate migrate inwards and become dorsally positioned. Cells at the center of the neural plate are enveloped. *c*: Continuation of infolding creates the neural keel. *d*: Cavitation forms the neural tube. Scanned from Papan and Campos-Ortega (1994).

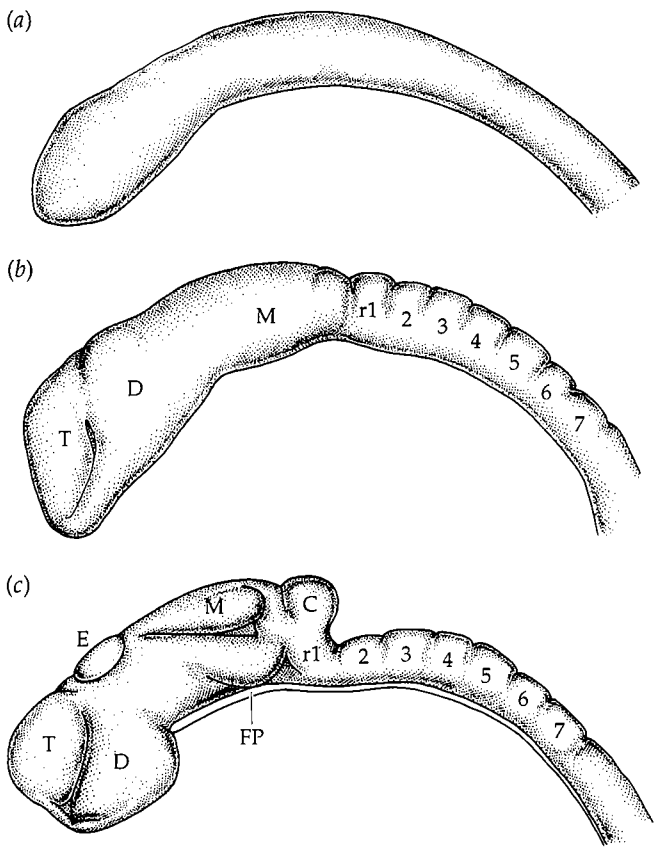

Figure 3. (*a-c*). Formation of the brain. *a*: Schematic of the zebrafish brain at 12 hpf. Note the lack of morphological subdivisions. *b*: Schematic of the brain at 18 hpf. Here the brain becomes subdivided into 10 segments or neuromeres representing the telencephalon (T), diencephalon (D) and midbrain (M) followed by seven hindbrain segments terned rhombomeres (r1-r7). *c*: Advanced brain structures are evident by 24 hpf. E: epiphasis, C: cerebellum, FP: floor plate. Adapted from "Embryology" Scott F. Gilbert.

midbrain. The beginnings of the eyes or optic primordium are also visible at this time. The remaining seven neuromeres are termed rhombomeres (r1-7) and further subdivide the hindbrain region along the AP axis with r1 being most anterior or rostral and r7 being most posterior or caudal. The first neurons begin to differentiate concurrent with early morphogenesis of the neural tube. These primary neurons develop large cell bodies and extend long axons to the hindbrain and spinal cord. Sensory neurons, which mediate tactile senses, shoot their axons between the neural tube and the periphery, and the motor neurons have axons, which grow out to meet muscle cells to elicit the muscle contractions.

As development continues, the major vertebrate embryonic tissues are formed. By the end of segmentation, at about one day post-fertilization, the embryos contains a differentiated notochord, advanced brain morphogenesis (Figure 3*c*), segmented hindbrain, a full complement of somites, neurons and reflexive contractile responses. At this time the embryo is considered to be in pharyngeal stages where a number of body structures are further elaborated. Fins are formed, pigmentation is apparent, the heart is developed and jaws and gills become functional, yielding a free swimming, feeding, breathing fish.

As this section was just a brief overview of zebrafish development to facilitate the understanding of embryological stages used in this thesis, a more detailed description can be found at the web site: http://zfish.uoregon.edu/zf_info/zfbook/stages/stages.html.

Embryonic Axis Formation

During gastrulation, the vertebrate body plan is laid down as patterns are established along the main embryonic axes. Discussed below are the formation of the dorsoventral axis and the anteroposterior axis.

Forming the Dorsal Ventral Axis

The earliest source of patterning signals essential for induction of dorsal fate has been suggested to be derived from the yolk, the extraembryonic structure. Studies have shown that when the vegetal most portion of the yolk is removed, the embryos do not develop dorsal fates like neural tissue and in stead become ventralized, consisting mainly of epidermis (Mizuno et al., 1999; Ober and Schulte-Merker, 1999; Schier, 2001). Although this vegetally located dorsal determinant in not currently known, it is thought that the determinant/s are translocated to the future dorsal side of the embryo during the first cellular division. This translocation is thought to be mediated by the microtubule network, as studies have shown that embryos treated with microtublule destabilizing pharmacological agents lack dorsal axis specification. (Jesuthasan and Stahle, 1997). Although the determinant is not known, it brings about the stabilization and nuclear translocation of ß-catenin, a transcription factor, in the dorsal YSL and the dorsal marginal blastomeres. (Schneider et al., 1996). The Wnt siganlling pathway has been shown in both zebrafish and Xenopus to stablize and localize the ß-catenin (De Robertis et al., 2000; Moon and Kimelman, 1998). However it is still not clear what factor/s triggers the activation of the Wnt pathway on the dorsal side. Once nuclear, ß-catenin

transcriptionally activates a number of genes on the dorsal side of the embryo like *bozozok, chordin, dickkopf1* and *squint.* By this time, the "organizer" or embryonic shield (where these genes are activated on the future dorsal side of the embryo) is easily detected morphologically. The embryonic shield is a critical infrastructure for the development of the dorsal ventral axis. The shield has been shown to contain "organizing activity," that is it has the ability to induce axis formation. Studies have shown that transplanting the shield from the dorsal side of a donor embryo into the ventral side of a recipient embryo generates a complete ectopic dorsal secondary axis on the ventral side (Koshida et al., 1998; Saude et al., 2000; Shih and Fraser, 1996).

As the organizer proper is sufficient for axis formation, what exactly is its role in dorsoventral axis formation? Dorsoventral polarity of the embryo is established via interactions between the organizer-borne dorsal factors and the ventrally derived bone morphogenenic proteins or BMP's (Thomsen, 1997) (Figure 4A). BMP's are known to ventralize embryos by activating transforming growth factor ß (TGFß) family receptors and signaling via downstream SMAD proteins on the ventral side of the embryo (Itoh et al., 2000).

The organizer borne *bozozok* acts as a transcriptional repressor and appears to target ventral inducers like *BMP2b* and the *vox/vent* gene on the ventral side of the embryo (Solnica-Krezel and Driever, 2001). Mutations in *bozozok* lack dorsal neural structures. The other ß-catenin targeted gene, *chordin*, encodes extracellular antagonists to the ventral BMP's. By binding to BMPs, chordin inhibits BMP from binding to its receptor and transducing a signal. Mutations in *chordin*, the *dino* mutants, show total

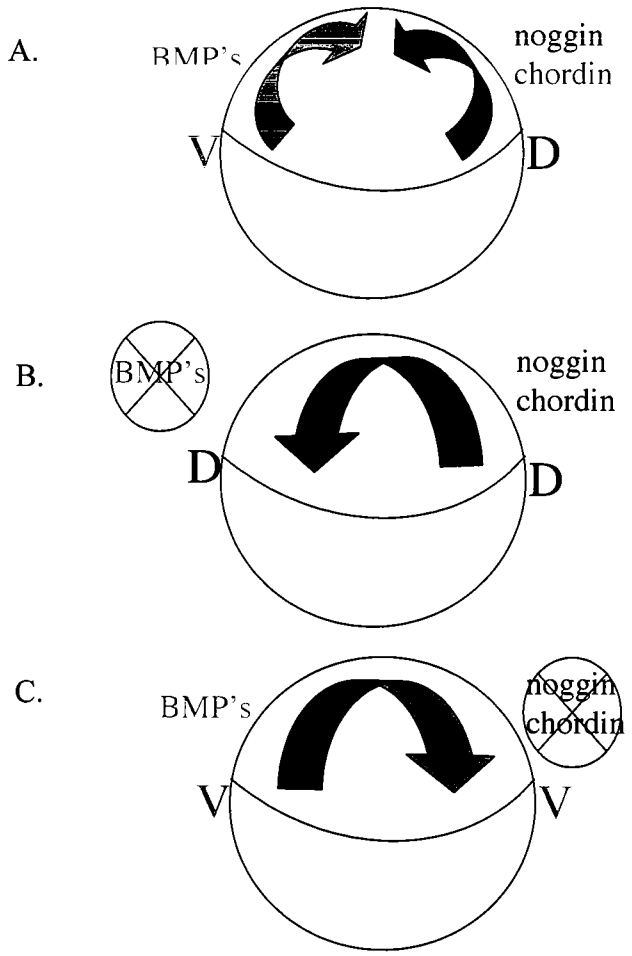

Figure 4. (A-C). DorsalVentral Axis Formation. A: Schematic of wild type embryo during early gastrula stages. BMP's on the ventral side of the embryo (V) interact with organizer borne factors of the dorsal side (D). B: Schematic of BMP mutant embryos illustrating a loss of ventral fates and an expansion of dorsal fates. C: Schematic of noggin or chordin mutant embryos illustrating a loss of dorsal fates and an expansion of ventral fates.

ventralization phenotypes, consisting of only ventral fates like blood and epidermis and a total loss or significant reduction of dorsal fates like neuroectoderm (Hammerschmidt et al., 1996b; Schulte-Merker et al., 1997) (Figure 4C). *noggin* and *follistatin*, other dorsally localized polypeptides secreted from the organizer have also been shown to antagonize BMP activity (Bauer et al., 1998; Furthauer et al., 1999). Furthermore, mutations in *bmp7 (snailhouse)* (Dick et al., 2000; Schmid et al., 2000), *bmp2 (swirl)* (Kishimoto et al., 1997) and *smad5 (somitabun)* (Hild et al., 1999; Kramer et al., 2002) result in a strong dorsalization of the embryo as a result of the loss of the ventralizing BMPs or SMAD signals (Figure 4B). Thus, the dorsoventral axis is established by the battle between ventrally derived BMP proteins with dorsally derived transcriptional repressors or secreted polypeptides.

Forming the Anteroposterior axis

Although dorsoventral patterning in the embryo has been extensively studied, less is known about how the anterior posterior axis is formed. It is thought that neuroectoderm becomes subdivided along the AP axis into forebrain, midbrain, hindbrain and spinal cord. This process occurs when signals or activators convert ectoderm to an anterior-type neural tissue subsequently followed by posteriorizing signals that modify the already neuralized ectoderm to more posterior fates. The anteroposterior (rostrocaudal) axis is established by actions of secreted factors such as cereberus and dickkopf anteriorly and retinoic Acid (RA), fibroblast growth factor (Fgf) and wnt proteins posteriorly.

*dickkopf (dkk1)*is an organizer borne gene that has been implicated in anterior specification. Overexpression of *dkk1* enhances anterior neuroectodermal development at the expense of the midbrain and enlarges eye and forebrain regions (Hashimoto et al., 2000; Shinya et al., 2000). The gene *cerberus* encodes a secreted protein that can induce ectopic heads in the absence of tail structures (Piccolo et al., 1999).

Posterior development is thought to have three main players; fibroblast growth factors (FGFs), retinoic acid (RA) and wnt proteins. Gain of function studies and dominant negative mutants in *Xenopus* embryos has suggested that Wnt, FGF and retinoids are candidates for posteriorizing transforming signals (Doniach, 1995; Kolm and Sive, 1997; McGrew 1997). In zebrafish, Wnt8 loss of function mutants have enlarged forebrains concurrent with reduced or absent caudal neural tissue (Erter et al., 2001; Lekven et al., 2001) (Figure 5C). However, ectopic activation of Wnt signaling blocks head formation (Kim et al., 2000) (Figure 5B). These data suggest Wnt signaling influences AP patterning.

The requisite roles FGF and RA play in formation of the posterior body plan were initially inferred from loss of function and gain of function studies(Blumberg et al., 1997; Cox and Hemmati-Brivanlou, 1995; Durston et al., 1989; Kengaku and Okamoto, 1993; Kengaku and Okamoto, 1995; Koshida et al., 1998; Lamb and Harland, 1995; Sive et al., 1990). For instance, over-expression of eFGF causes an anterior shift of caudal gene expression into the hindbrain in *Xenopus* (Pownall et al., 1998; Pownall et al., 1996) and misexpression of eFGF leads to development of trunk and tail derivatives at the expense of anterior structures in zebrafish (Griffin et al., 1995) (Figure 5B). Loss of function

studies generated the opposite phenotype. For instance, expression of a dominant negative FGF receptor (dnFGFR) in *Xenopus* leads to loss of *hoxB9* in the spinal cord, but does not affect *hoxb1* in rhombomere (r) r4 or *otx2* in the forebrain (Pownall et al., 1998; Pownall et al., 1996). Loss of FGF signaling in zebrafish leads to a complete loss of posterior structures from somite1 to the caudal end of the embryo, but the head and otic vesicle are unaffected (Griffin et al., 1995) (Figure 5C). Taken together, these data suggest that there is a FGF-dependent domain caudal to the hindbrain from somite1 to the caudal end of the embryo. While this thesis research was being performed, studies showed a role for FGFs at post-gastrula segmentation stages within the hindbrain. For example, Maves et al. (Maves et al., 2002) and Walshe et al. (Walshe et al., 2002) have recently defined r4 as a signaling center necessary to promote r5 and r6 development in the zebrafish hindbrain. They also demonstrated that FGF3 and FGF8 are expressed in r4 and are the essential signaling molecules in r4 mediating this effect, but how this r4 signaling center arises is still unclear. FGFs also have other roles in the hindbrain. For example, FGF8 is expressed at the mid-hindbrain boundary (MHB) where it is required for development of the rostral hindbrain (e.g. cerebellum) (Brand et al., 1996; Irving and Mason, 2000; Reifers et al., 1998) and FGFs reportedly regulate *krox20* and *kreisler* expression at late (post neurula) stages in chick (Marin and Charnay, 2000).

Retinoic acid is also necessary for posterior development, especially that of the caudal hindbrain (Blumberg et al., 1997; Conlon, 1995; Durston et al., 1989; Sive et al., 1990). Application of exogenous RA in *Xenopus* and zebrafish leads to a loss of the anterior hindbrain (r1-3), but not of the posterior hindbrain (r4-7/8) (Figure 5B).

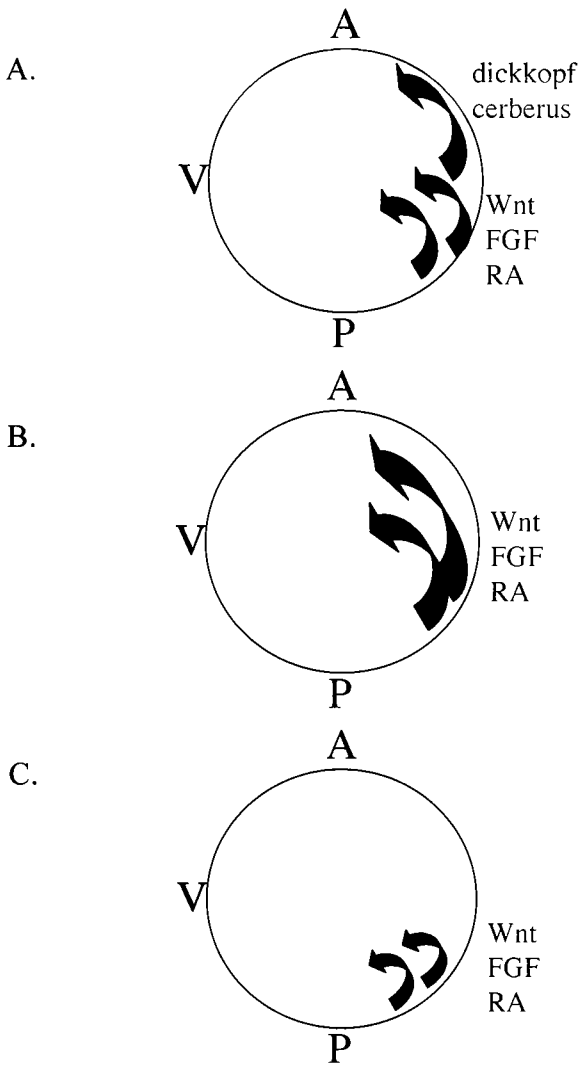

Figure 5. (A-C). Anteroposterior Formation. A: Schematic of wild type A-P patterning. B: Overexpression of posterior factors results in anterior shift in posterior gene expression. C: Loss of posterior signals results in loss of posterior structures.

Specifically, at the gene expression level, loss of *krox20* expression in r3 of the rostral hindbrain, but not in r5 was seen in *Xenopus* and zebrafish embryos treated with RA (Godsave et al., 1998; Hill et al., 1995). Other genes, e.g *Xhox3,* are also lost rostrally in response to RA (Ruiz i Altaba and Jessell, 1991). Concurrent with the loss of rostral gene expression, caudal gene expression expands. For instance, treatment with exogenous RA leads to ectopic expression of *hoxb1* in r2 in the mouse (Marshall et al., 1992). Changes in gene expression also have functional consequences. For example, Mauthner neurons normally located in r4 were ectopically detected in r2 in zebrafish treated with exogenous RA (Hill et al., 1995). These studies suggest a loss of rostral hindbrain fates, particularly in r1-r3, and an expansion of caudal hindbrain fates in response to RA treatment. RA loss of function studies using dominant negative Retinoic Acid Receptors (dnRAR) generated the opposite phenotype (Figure 5C). In *Xenopus* expressing the dnRAR, wild type *krox20* expression was detected in r3, but expression in r5 was lost. (Blumberg et al., 1997; Kolm and Sive, 1997; van der Wees et al., 1998). In addition, loss of *hoxb3* in r5/r6 was detected (van der Wees et al., 1998) as was loss of *Hoxd1* in the caudal hindbrain (Kolm and Sive, 1997). In the presence of the dnRAR, ectopic *krox20* staining was also seen in r6 and r7 (van der Wees et al., 1998) suggesting an anteriorization of the posterior hindbrain whereby r6 and r7 attained more of an r5 characteristic. Ectopic Mauthner neurons were also detected in r5 and r6 of these embryos consistent with a change in identity of r5 and r6 towards r4 (van der Wees et al., 1998). Furthermore, retinoid elimination from the diet of pregnant quail mothers lead to the loss of caudal hindbrain structures (Maden et al., 1996). Taken together, these loss of function and gain of

function studies suggest an RA dependent domain from r4 to r7 in the caudal hindbrain. This RA effect is likely mediated by Hox genes. For instance, ectopic expression of *hoxa1/hoxb1b* in zebrafish phenocopies the RA overexpression phenotype (Alexandre et al., 1996) and RA induces changes in the hindbrain Hox code resulting in a homeotic transformation of r2/3 into r4/5 (Marshall et al., 1992), suggesting that RA affects hindbrain development via its effect on Hox genes.

Goal of the Project

Although it seems likely that all three factors (Fgfs, RA and Wnts) play important roles in posteriorization, the precise roles of each factor temporally and spatially have not been well characterized. Here in this body of work, I wished to study the role of FGF and RA in early hindbrain development.

CHAPTER 1

AN EARLY FGF-SIGNAL IS REQUIRED FOR GENE EXPRESSION IN THE ZEBRAFISH HINDBRAIN PRIMORDIUM

ABSTRACT

We have explored the role of Fibroblast growth factor (Fgf)-signaling in regulating gene expression in the early zebrafish hindbrain primordium. We find that Fgf signaling is required for gene expression along the entire rostrocaudal axis of the hindbrain primordium by mid-gastrula stages. We demonstrate that this early Fgf signal is not mediated by Fgf3 or Fgf8 – two Fgf-family members known to be secreted from signaling centers at the midbrain-hindbrain boundary (MHB) and in rhombomere 4 (r4), suggesting that an as yet uncharacterized Fgf family member is acting at gastrula stages. We find that *fgf8* expression is lost upon disrupting gastrula-stage Fgf-signaling, indicating that this early Fgf-signal may in fact be required to establish the signaling

centers in r4 and at the MHB. We also find that blocking either retinoic acid (RA) or Fgf signaling disrupts hindbrain gene expression at gastrula stages, suggesting that both pathways are essential at this stage. However, both pathways must be blocked simultaneously to disrupt hindbrain gene expression at segmentation stages, indicating that these signaling pathways become redundant at later stages. Furthermore, exogenous application of RA or Fgf alone is sufficient to induce hindbrain genes in gastrula stage tissues, suggesting that the two-signal requirement can be overcome under some conditions. Our results demonstrate an early role for Fgf-signaling and reveal a dynamic relationship between the RA and Fgf signaling pathways during hindbrain development.

INTRODUCTION

The vertebrate body plan is laid down during gastrulation, as patterns are established along the main embryonic axes. The dorsoventral axis is established by the competing actions of ventrolaterally expressed bone morphogenic proteins (BMPs) and BMP-antagonists (e.g. noggin and chordin) secreted from the dorsal organizer (Lumsden and Krumlauf, 1996; Piccolo et al., 1996; Wilson and Hemmati-Brivanlou, 1997). The anteroposterior (rostrocaudal) axis is established by the actions of secreted factors such as retinoic acid (RA), fibroblast growth factor (Fgf) and wnt proteins posteriorly (Doniach, 1995; Lumsden and Krumlauf, 1996; Yamaguchi, 2001), as well as by cerberus (Bouwmeester et al., 1996; Piccolo et al., 1999) and dickkopf (Niehrs et al., 2001) anteriorly.

In terms of the anteroposterior axis, early experiments demonstrated that soluble Fgf can convert anterior neural tissue to posterior neural fates in vitro (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995) while ectopic expression of *fgf* in vivo causes anterior shifts of posterior gene expression in both *Xenopus* and zebrafish (Koshida et al., 2002; Kudoh et al., 2002; Pownall et al., 1998; Pownall et al., 1996), suggesting a role for Fgfs in patterning posterior embryonic structures. Subsequently, a dominant negative form of the Fgf receptor (dnFGFR) was used to test whether endogenous Fgf signaling is essential for posterior development. Such experiments demonstrated a requirement for Fgf signaling in development of mesodermal structures in the trunk and tail (e.g. (Griffin et al., 1995; Griffin et al., 1998; Pownall et al., 1998)). It also appears that early activation of gene expression in the spinal cord is Fgf-dependent (Pownall et al., 1998; Pownall et al., 1996), but this expression becomes Fgf-independent at later stages (Kroll and Amaya, 1996; Pownall et al., 1998). In contrast, the role of Fgf signaling in hindbrain development is less clear-cut. Some investigators report that blocking Fgf signaling disrupts expression of hindbrain genes (Holowacz and Sokol, 1999; Kolm and Sive, 1995; Koshida et al., 1998; Kudoh et al., 2002), while others find no effect (Pownall et al., 1996), and yet others find that hindbrain gene expression is lost only when Fgf signaling is disrupted simultaneously with activation of the BMP signaling pathway (Koshida et al., 2002). Recent experiments have demonstrated that both *fgf3* and *fgf8* are expressed at the blastoderm margin as well as within the hindbrain of the gastrula stage zebrafish embryo (Furthauer et al., 2001; Phillips et al., 2001; Reifers et al., 1998), consistent with regulation of caudal development by Fgfs. Indeed, simultaneous

disruption of Fgf3 and Fgf8 function leads to loss of gene expression in rhombomere 5 (r5) and r6 of the hindbrain (Maves et al., 2002; Walshe et al., 2002), but it is not clear if these Fgfs are required for hindbrain gene expression outside r5/r6. Thus, while Fgf signaling is broadly required for posterior development its detailed role in hindbrain development is not understood, and it is not clear if the activity of Fgf3 and Fgf8 is sufficient to account for all roles of Fgfs in posterior development.

Retinoic acid (RA) is also necessary for hindbrain development (Blumberg et al., 1997; Conlon, 1995; Durston et al., 1989; Sive et al., 1990). Application of exogenous RA to *Xenopus* and zebrafish embryos leads to loss of rostral hindbrain (r1-r3) fates (Godsave et al., 1998; Hill et al., 1995; Isaacs et al., 1998) and a rostralward expansion of caudal (r4-r7/8) hindbrain fates (Hill et al., 1995; Marshall et al., 1992). In contrast, disrupting endogenous RA signaling by using dominant negative Retinoic Acid Receptors (dnRAR) or by restricting dietary retinoids, leads to loss of caudal hindbrain fates (Blumberg et al., 1997; Kolm et al., 1997; Maden et al., 1996; van der Wees et al., 1998) and, occasionally, to a caudal expansion of rostral fates (van der Wees et al., 1998). RA likely mediates these effects by regulating the expression of *hox* genes. For instance, ectopic expression of *hoxb1b* in zebrafish mimics the RA overexpression phenotype (Alexandre et al., 1996) and RA induces changes in the hindbrain Hox code resulting in a homeotic transformation of r2/3 into r4/5 (Marshall et al., 1992). Taken together, these studies suggest an RA dependent domain from r4 to r7 in the caudal hindbrain, but it is not clear if RA acts only via *hox* genes in this domain, nor have the relative roles of RA and Fgf signaling been defined during hindbrain development.

Here we demonstrate that disruption of Fgf signaling blocks gene expression throughout the hindbrain primordium and that this Fgf signal is required already by mid-gastrula stages, prior to the onset of *fgf3* and *fgf8* expression. Indeed, removing Fgf3 and Fgf8 function does not affect gene expression in the hindbrain primordium at gastrula stages, but blocks expression at later segmentation stages, suggesting that an early-acting Fgf distinct from Fgf3 or Fgf8 is required for gastrula stage gene expression. Surprisingly, embryos with disrupted Fgf-signaling show only mild defects in hindbrain neuronal differentiation and we find that gene expression recovers in the hindbrain primordium of segmentation stage embryos in spite of Fgf-signaling remaining blocked. We demonstrate that blocking Fgf signaling simultaneously with blockade of RA signaling - which by itself has only a mild effect on gene expression at segmentation stages - abolishes this recovery. Thus, Fgf and RA are both required for gene expression in the hindbrain primordium at gastrula stages, but this requirement is alleviated by segmentation stages when the two pathways appear to become redundant. Lastly, while blocking either Fgf or RA signaling disrupts gastrula-stage hindbrain gene expression, exogenous application of either factor alone is sufficient to induce expression of hindbrain genes, further demonstrating that the RA and Fgf signaling pathways are dynamically related during hindbrain development.

RESULTS

Fgf signaling is required for gene expression in the entire hindbrain primordium.

We set out to examine how Fgf signaling regulates gene expression in the hindbrain primordium. Since several Fgf family members are expressed in the developing zebrafish embryo (e.g. *fgf3*, *fgf8* and *fgf17*; (Phillips et al., 2001; Reifers et al., 2000; Reifers et al., 1998; Walshe et al., 2002)), and other Fgfs may also be expressed during early embryogenesis (e.g. *fgf4* (Draper, 1999; Grandel et al., 2000)) it is possible that Fgfs may act combinatorially or redundantly to regulate gene expression in the hindbrain primordium. We therefore employed a dominant negative FGF receptor (dnFGFR) construct (Amaya et al., 1991; Amaya et al., 1993) that blocks signaling by most Fgf family members (Ueno et al., 1992). mRNA encoding the dnFGFR was injected at the 1-4 cell stage and the injected embryos raised to late gastrula stage (9-10 hpf) when they were assayed for changes in gene expression. We find a reduction in *caudal* expression in dnFGFR expressing embryos (Fig. 1A. b; 94% of embryos affected; Table 1), consistent with Fgf signaling regulating development of the zebrafish trunk and tail as previously demonstrated (Griffin et al., 1995; Griffin et al., 1998). The effect varies from unilateral reduction to complete bilateral loss of gene expression, likely dependent on the distribution of the injected dnFGFR mRNA. We also observe a loss or reduction of *hoxb1b* expression (normally found in the caudal embryo and the caudal hindbrain up to the r3/r4 boundary (Alexandre et al., 1996; Sagerstrom et al., 2001)) in dnFGFR-injected embryos (Fig. 1A. d; 95% of embryos affected), in accordance with a recent report (Kudoh et al., 2002). To explore this effect further, we examined *nlz*, which is expressed in a similar pattern to *hoxb1b* (Sagerstrom et al., 2001), and find that *nlz* expression is also repressed by the dnFGFR construct (Fig. 1A. f; 93% of embryos affected). We next

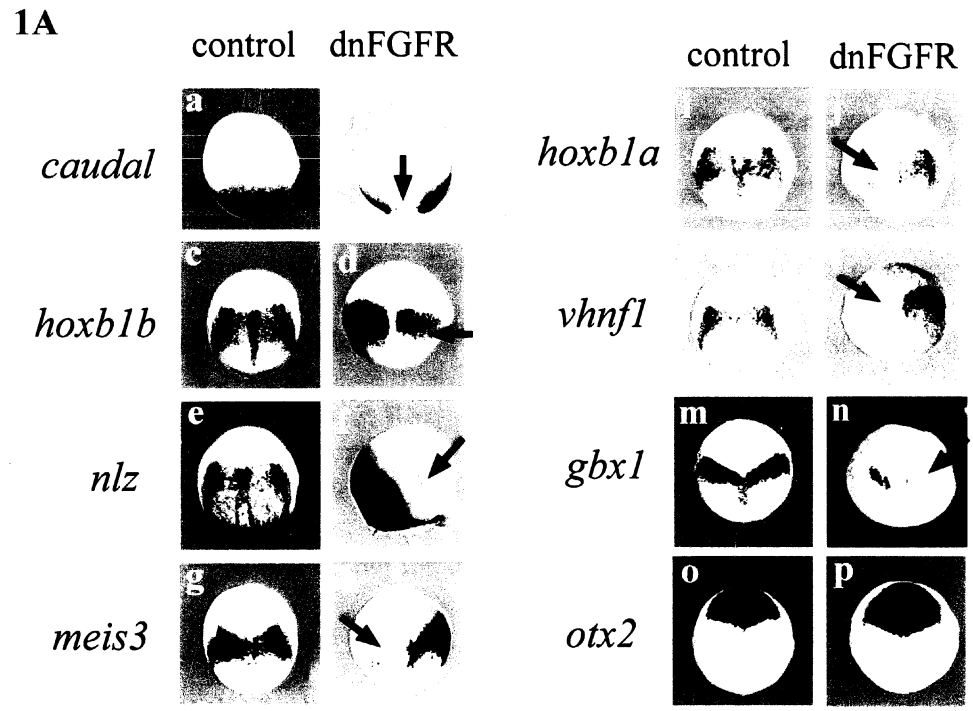

Figure 1A. Early hindbrain genes are FGF responsive. Embryos were injected at the one to two cell stage with 500 pg LacZ control mRNA (a, c, e, g, i, k, m, o) or 500 pg dnFGFR mRNA (b, d, f, h, j, l, n, p), fixed at 10 hpf and analyzed by wholemount *in situ* hybridization for expression of *caudal* (a, b), *hoxb1b* (c, d), *nlz* (e, f), *meis3* (g, h), *hoxb1a* (i, j), *vhnf1* (k, l), *gbx1* (m, n) and *otx2* (o, p). All panels are dorsal views with anterior to the top.

examined *meis3* and *hoxb1a*, which are expressed between the r3/r4 boundary and the caudal end of the hindbrain (Prince et al., 1998b; Sagerstrom et al., 2001), but not further caudally. We find that both *meis3* (Fig. 1 A. h; 88% affected) and *hoxb1a* (Fig. 1A. j; 86% affected) expression is lost in dnFGFR expressing embryos, as is *vhnf1* expression (Fig 1A. l; 87% affected), which is normally found in r5/r6 (Sun and Hopkins, 2001). These results demonstrate that Fgf signaling is required for gene expression in the caudal hindbrain primordium, at least up through presumptive r4.

We next addressed whether Fgf signaling is required also for gene expression in the rostral hindbrain. Specifically, we examined if expression of *gbx1*, which is normally found in r1-r3 (Itoh et al., 2002; Rhinn and Brand, 2001), or *otx2*, which is expressed rostral to r1, is affected by the dnFGFR construct. We find that *gbx1* expression is lost in tailbud stage embryos expressing the dnFGFR construct (Fig. 1A. n; 73% affected), but *otx2* expression is unaffected (Fig. 1A. p), indicating that Fgf signaling is required for gene expression throughout the hindbrain, but not rostral to the midbrain-hindbrain boundary, at this stage of development. Thus, our experiments confirm that *hoxb1b* expression in the hindbrain is dependent on Fgf signaling, as reported previously (Koshida et al., 1998; Kudoh et al., 2002), but extend this data by demonstrating that four other genes expressed in the caudal hindbrain primordium, as well as a gene expressed in the rostral hindbrain, are also Fgf-dependent.

To confirm that the observed effects are due to disruption of Fgf signaling, we also interfered with the function of Ras, which acts at a receptor proximal step in the Fgf signaling cascade (Widmann et al., 1999). To this end we employed a dominant negative

form of Ras (dnRas) (Feig and Cooper, 1988) and we find that dnRas disrupts expression of *caudal*, *hoxb1b*, *nlz*, *meis3* and *hoxb1a* in a manner similar to dnFGFR (not shown), further confirming that Fgf signaling is required for early gene expression in the hindbrain.

Fgf receptors have also been shown to interact with other receptor tyrosine kinases, namely the PDGF (platelet derived growth factor) (Russo et al., 2002). To ensure that the observed effect we see is not due to any cross interactions between FGF and other receptors, we utilized an inhibitor specific to Fgf receptors, SU5402 (Mohammadi et al., 1997). SU5402 is a member of a new class of protein tyrosine kinase inhibitors, which has been shown to specifically inhibit the tyrosine kinase activity of FGFRs by inducing a conformational change in the nucleotide-binding loop (Mohammadi et al., 1997). Embryos were soaked at 4 hpf in a 0.2 mg/ml solution of SU5402 in 1X MBS until 10 hpf when they were fixed and processed for in situ analysis. Upon treatment with SU5402, we were able to phenocopy the dnFGFR injected results perfectly for all genes tested, *caudal* (Fig. 1B. a,b), *hoxb1b* (Fig. 1B. c-e), *nlz* (Fig. 1B. f-h), *meis3* (Fig. 1B. i-k), *hoxb1a* (Fig. 1B. l-n) and *otx2* (Fig. 1B. o,p). 100% of embryos were affected very strongly ranging from a total loss of gene expression to very strong equivalent reduction or unilateral reduction. It was interesting that the drug could phenocopy both unilateral (Fig 1B. d,g,j,m) and bilateral reduction (Fig. 1B. e,h,k,n) in gene expression, as we expected a more uniform distribution of drug compared to the injected dnFGR mRNA. However, this could be due to uneven diffusion of the drug or perhaps may reveal a more complex underlying left-right asymmetry mechanism. Thus,

1B

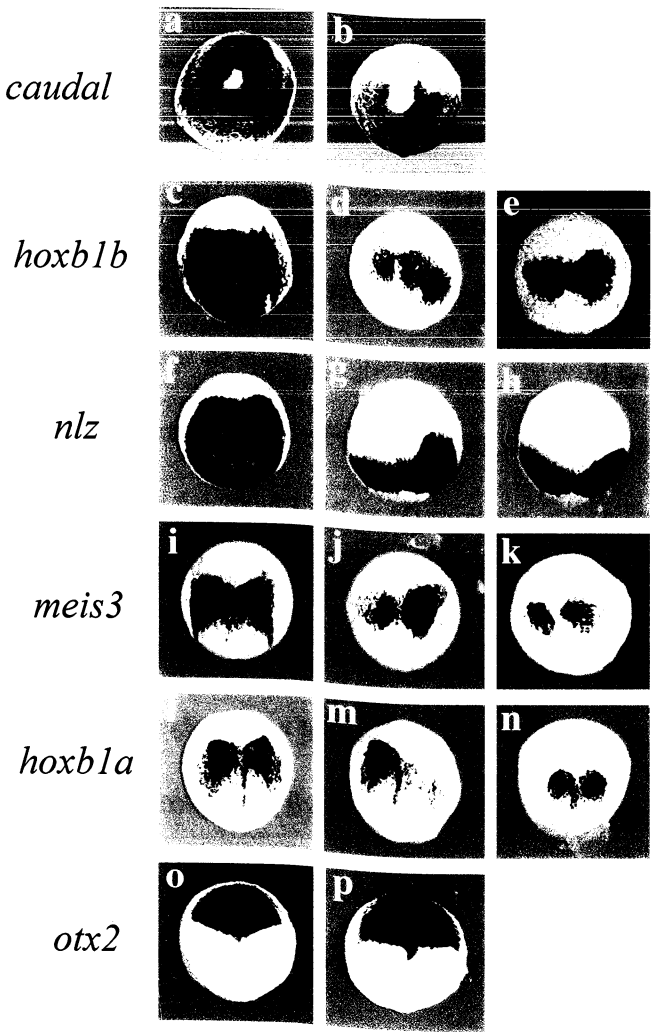

Figure 1B. Early hindbrain genes are FGF responsive. Embryos were manually dechorionated and soaked in 1X MBS until 4 hpf when they were transferred to fresh 1X MBS (a, c, f, i, l, o) or 0.2 mg/ml SU5402 in 1X MBS (b, d, e, g, h, j, k, m, n, p) until 10 hpf when they were fixed and processed for *in situ* hybridization for expression of *caudal* (a, b), *hoxb1b* (c-e), *nlz* (f-h), *meis3* (i-k), *hoxb1a* (l-n) or *otx2* (o, p). All panels are dorsal views with anterior to the top except panels a and b which are vegetal views.

we conclude that the effect on hindbrain genes we detect in dnFGFR expressing embryos is not due to cross interactions of the dnFGFR with PDGF or EGF.

We were also able to test the time window of Fgf requirement for hindbrain gene regulation with SU5402. Embryos were soaked in SU5402 starting at 4 hpf until 10 hpf, from 6 hpf to 10 hpf and from 8 hpf to 10 hpf when they were fixed and processed for in situ analysis. We find that the 4-10 hpf treatments phenocopied the dnFGFR results (Fig. 1B) and treatments at 6-10 hpf or 8-10 hpf yielded mostly wild type embryos (not shown). To more closely determine the FGF-required time window, we then soaked embryos in SU5402 at 4 hpf for 15, 30, 45 or 60 minutes, extensively washed the embryos and allowed them to develop in buffer until 10 hpf when they were fixed and processed for in situ hybridization for *nlz* expression. We find that after 15 minute treatments, 54% of embryos showed a loss of *nlz* gene expression. The percentage of embryos that were affected rose steadily from 54% after 15 minutes, to 67% after 30 minutes, to 79% after 45 minutes to 92% by the 60 minute time interval. Thus, we conclude that the early Fgf signaling required for hindbrain development occurs in a time window between 4-5 hpf.

Fgf signaling is required prior to establishment of the signaling centers in r4 and the MHB.

Two Fgf-producing organizing centers have been described in the hindbrain; one at the midbrain-hindbrain boundary (MHB) and one in r4. The MHB expresses *fgf8* and *fgf17* and regulates development of the caudal midbrain and the rostral hindbrain (reviewed in

(Rhinn and Brand, 2001)) while r4 expresses *fgf3* and *fgf8* and regulates development of r5/r6 (Maves et al., 2002; Walshe et al., 2002). Thus, our observation that Fgf-signaling is required for gene expression throughout the hindbrain primordium might be explained by the actions of Fgf8/Fgf17 secreted from the MHB and/or Fgf3/Fgf8 secreted from r4.

fgf17 is not expressed at the zebrafish MHB until the 8 somite stage (Reifers et al., 2000), but the dnFGFR construct disrupts hindbrain gene expression already at tailbud stage (Fig. 1), demonstrating that Fgf17 cannot regulate early gene expression in the hindbrain primordium. In contrast, *fgf8* and *fgf3* are expressed in the zebrafish hindbrain primordium during gastrula stages. *fgf8* is expressed in the anterior half of the hindbrain primordium already at 70% epiboly and resolves into separate expression domains at the MHB and in r4 by tailbud stage (Maves et al., 2002; Reifers et al., 1998), while *fgf3* becomes expressed in r4 at 90% epiboly (Maves et al., 2002; Walshe et al., 2002). However, we find that the dnFGFR construct represses *gbx1* expression already at 70% epiboly (67% affected; not shown), suggesting that Fgf8 and Fgf3 secreted from the MHB and/or r4 may not be required for early gene expression in the hindbrain primordium.

To further explore the role of *fgf3* and *fgf8* in regulating hindbrain gene expression, we disrupted Fgf3 and Fgf8 function using antisense morpholino oligos (MOs), as previously reported (Maves et al., 2002; Walshe et al., 2002). Since Fgf3 and Fgf8 may act redundantly, their function was disrupted simultaneously by co-injecting anti-Fgf3 and anti-Fgf8 MOs. We do not observe any changes in *hoxb1b*, *nlz*, *meis3*, *hoxb1a*, or *gbx1* expression in the hindbrain primordium of late gastrula stage embryos

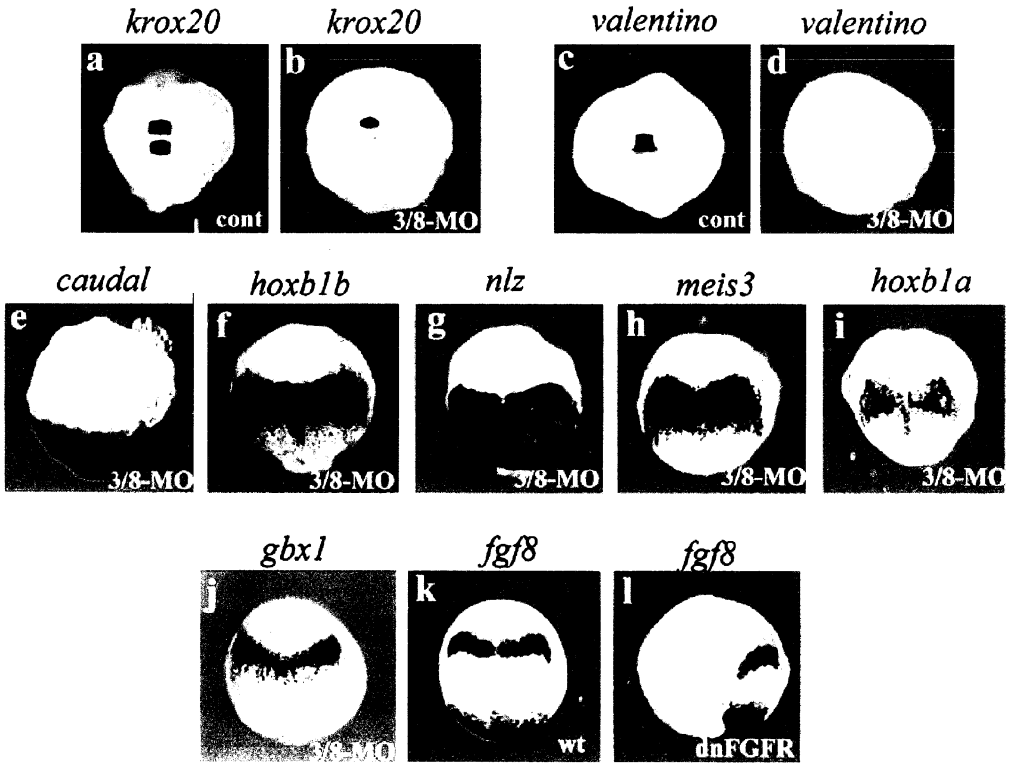

Figure 2 (a-l). Fgf3 and Fgf8 are not responsible for induction of early hindbrain gene expression. Embryos were injected at the one to two cell stage with control MOs (a, c), anti-Fgf3/antiFgf8 (b, d, e-j) morpholino antisense oligonucleotides or dnFGFR (l) and raised to 10 hpf (e-l) or 16 hpf (a-d). Embryos were analyzed by wholemount *in situ* hybridization for expression of *krox20* (a, b), *valentino* (c, d), *caudal* (e), *hoxb1b* (f), *nlz* (g), *meis3* (h) *hoxb1a* (i), *gbx1*(j) and *fgf8* (k, l). All panels are dorsal view with anterior to the top.

co-injected with anti-Fgf3 and anti-Fgf8 MOs (Fig. 2f-j), suggesting that Fgf3 and Fgf8 are not responsible for the induction of early gene expression in the hindbrain primordium. Two previous reports have demonstrated that co-injecting anti-Fgf3 and anti-Fgf8 MOs blocks gene expression in r5 and r6 during segmentation stages (Maves et al., 2002)(Walshe et al., 2002). Consistent with these reports, we find that embryos co-injected with anti-Fgf8 and anti-Fgf3 MOs display loss of *krox20* expression in r5 and *valentino* expression in r5/r6 (Fig. 2a-d; 91% and 86% affected respectively) during segmentation stages. However, expression of *vnhf1*, which is thought to regulate *krox20* and *valentino* expression in r5/r6 (Sun and Hopkins, 2001), is not repressed by Fgf3/Fgf8 MOs at late gastrula stages (not shown). These findings suggest that an early Fgf signal, acting prior to the establishment of the MHB and r4 signaling centers, is required for hindbrain development. In fact, we find that *fgf8* expression is lost in the rostral hindbrain of embryos expressing the dnFGFR (Fig. 2l; 81% affected), indicating that this early Fgf signal may be required to establish the r4 and/or MHB signaling centers. The differing effects of the dnFGFR construct and the MOs are not due to quantitative differences in their efficacy, since both approaches block Fgf signaling in the rostral hindbrain primordium at tailbud stages (Fig. 2m-o; Fgf signaling detected by staining for di-phosphorylated ERK (dp-ERK), an antibody which reacts specifically to the di-phosphorylated form of MAP kinase (ERK-1 and ERK-2). The antibody does not recognize the non-phosphorylated or the mono-phosphorylated forms of the MAP kinase molecule or the di-phosphorylated forms of JNK and p38 MAP kinase (Umbhauer et al., 1995)). Also, anti-*fgf3* and anti-*fgf8* MOs do not affect trunk or tail development (e.g.

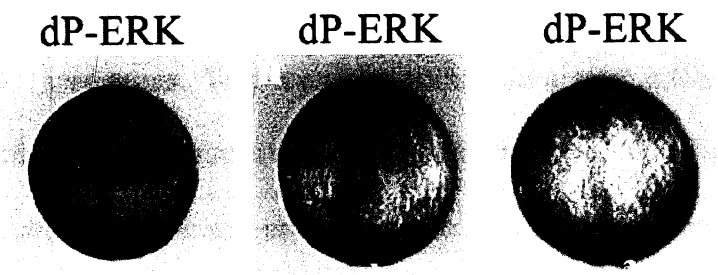

Figure 2. (m-o). Fgf3 and Fgf8 are not responsible for induction of early hindbrain gene expression. Embryos were injected at the one to two cell stage with control morpholino (m), dnFGFR mRNA (n) or antiFgf3/antiFgf8 morpholino antisense oligonucleotides (o) and raised to 10 hpf. ERK activation was detected by anti-dPERK antibody staining (m-o). All panels are dorsal views with anterior to the top.

caudal expression, Fig. 2e; (Maves et al., 2002; Walshe et al., 2002), in contrast to the dnFGFR, further suggesting the involvement of an early-acting Fgf signal. Taken together, our results demonstrate that Fgf signaling is required in the hindbrain primordium at an earlier stage and in a broader domain than previously reported.

Fgf signaling is sufficient to induce expression of some caudal genes in vitro

Our experiments demonstrate that Fgf signaling is required for gene expression in the hindbrain primordium. We next tested whether Fgf signaling is sufficient to induce expression of these genes. To this end we utilized an in vitro differentiation assay (cartoon in Fig 3A; (Sagerstrom et al., 1996)) that also enabled us to test if Fgf acts directly or indirectly to induce gene expression. In particular, a small group of cells was explanted from the animal pole of several embryos at 5 hpf and aggregated together in vitro in the presence of Fgf. We utilized bFgf, which is functionally interchangeable with other Fgfs, e.g. eFgf/Fgf4 (Kimelman et al., 1988; Lamb and Harland, 1995; Slack, 1989) to induce Fgf signaling. After 4 hours in culture, the explants were subjected to RT-PCR analysis to detect gene expression. We find that Fgf signaling significantly enhances the expression of *hoxb1b*, *caudal* and *nlz*, while expression of *hoxb1a* and *meis3* is unaffected (Fig. 3B; lane 2). We next treated explants with Fgf in the presence of cycloheximide (which inhibits protein synthesis in the explants by 80%; not shown) to determine if Fgf induces gene expression directly or indirectly. We find that induction of *nlz* expression is unaffected by cycloheximide (Fig. 3B, compare lanes 2 and 3), suggesting that Fgf signaling regulates *nlz* expression directly. In contrast,

3A

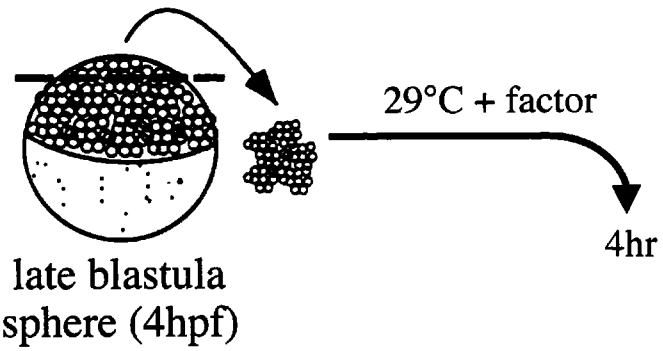

Figure 3A. Schematic representation of animal cap dissection in culture. Animal caps were taken from sphere stage embryos, aggregated into groups of 30, and cultured *in vitro* for 4 hours in control buffer (1X MBS), 1 μg/ml FGF or 1 μg/ml FGF + 5 μg/μl cycloheximide in 1X MBS.

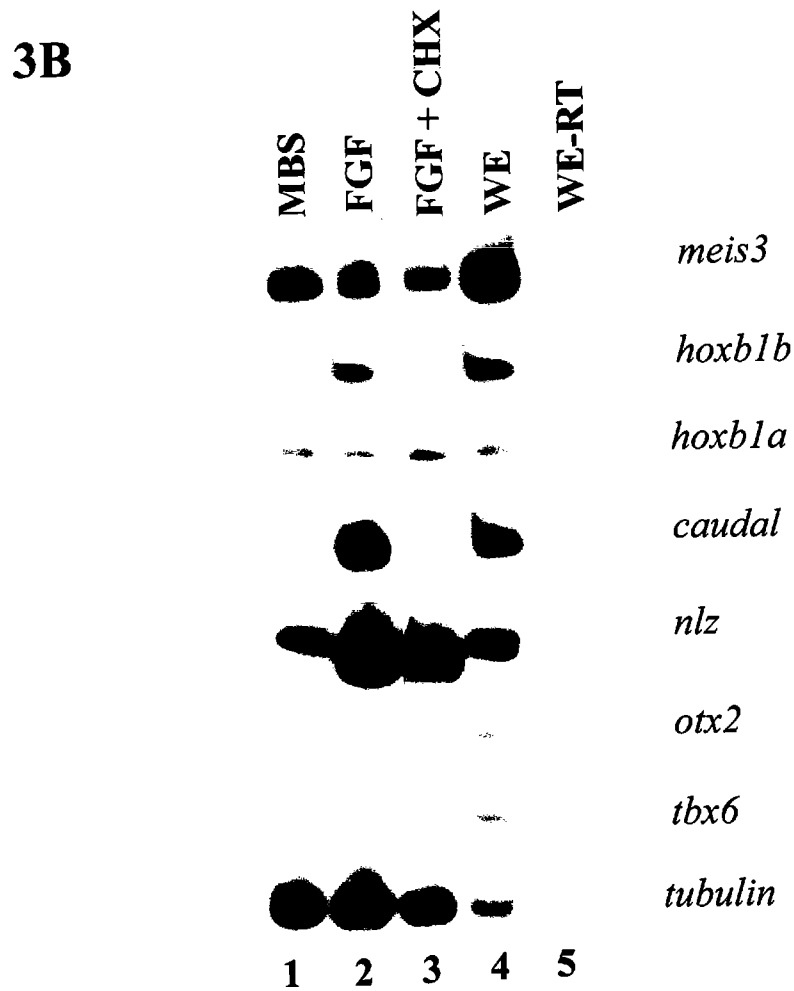

Figure 3B. RT-PCR analysis of gene expression in explanted animal caps and whole embryos. Explants (lanes 1-3) were harvested after 4 hour treatments in 1X MBS (lane 1), 1 μg/ml FGF (lane 2), or 1 mg/μl FGF + 5 μg/μl cycloheximide (lane 3) and analyzed for expression of hindbrain markers *meis3*, *hoxb1b*, *hoxb1a*, *caudal* and *nlz*. *otx2* was used as an anterior marker, *tbx6* as a mesodermal marker and *tubulin* as a loading control. RT-PCR was performed as described in Materials and Methods. Whole embryos (lanes 4 and 5) were harvested at 10 hpf and analyzed for the same genes. RT-PCR reactions for whole embryos were also performed without Reverse Transcriptase (RT) (lane 5) as a control.

cycloheximide blocks Fgf-mediated induction of *hoxb1b* and *caudal* expression (Fig. 3B, compare lanes 2 and 3), suggesting that Fgf induces expression of these genes indirectly.

nlz and _hoxb1b_ expression is induced near the Fgf source, while _meis3_ is induced at a distance.

To further explore the role of Fgf signaling in hindbrain gene expression, we next implanted Fgf-soaked agarose beads (same bFGF solution utilized for in vitro differentiation assay) at the animal pole of 5 hpf zebrafish embryos. Bead-implanted embryos were raised to tailbud stage when they were fixed and analyzed by in situ hybridization. We find that *caudal*, *nlz* and *hoxb1b* expression is ectopically induced over the implanted Fgf bead (white arrows in Fig. 4A panel b, 83%; f and g, 89%; and d, 89%). In contrast, Fgf-soaked beads induce *meis3* expression not over the bead, but rather in a ring-like domain at a distance (Fig. 4A, panel j; 37%). Double in situ hybridizations revealed that *caudal* expression (red stain) is found immediately surrounding the Fgf bead up to the *meis3* expression border (blue stain; Fig. 4B, panel b). As the red staining is diffuse in nature, we cannot determine to what extent *caudal* expression overlaps with *meis3* expression, but the domains appear to be in close proximity. We also note that when Fgf-soaked beads are found within the endogenous *meis3* expression domain, *meis3* expression immediately adjacent to the bead is lost and replaced with *caudal* (Fig 4B, panel c) or *nlz* (Fig. 4B, panel e) expression. Lastly, cross sections through ectopic expression domains induced by Fgf-soaked beads show expression of *caudal, nlz* and *hoxb1b* immediately adjacent to the transplanted bead (Fig

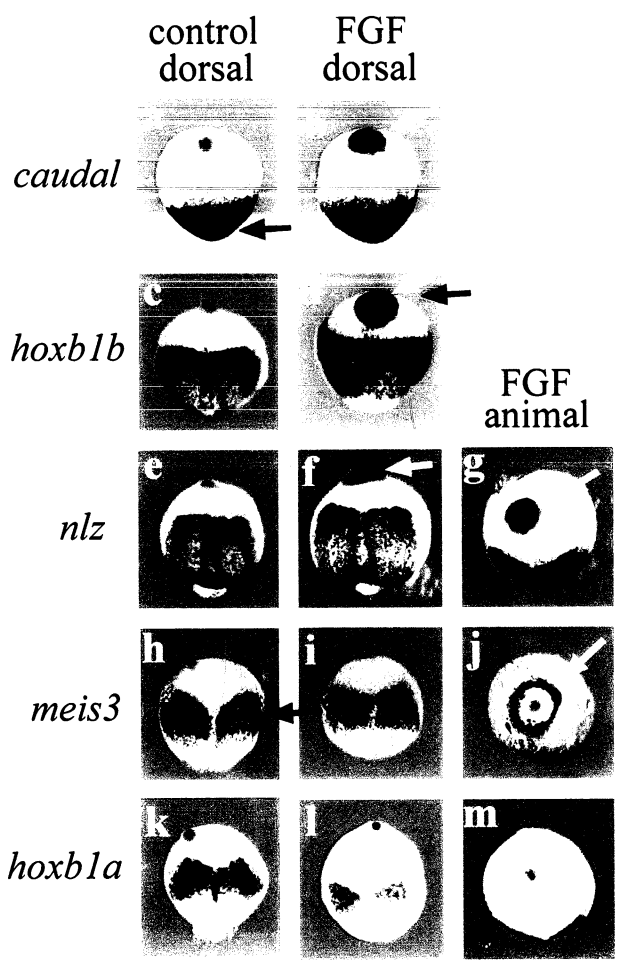

Figure 4A. Early hindbrain genes are FGF responsive. Beads soaked in 1X MBS (a, c, e, h, k) or 0.5 mg/ml bFGF (b, d, f, g, i, j, l, m) were implanted into the animal pole of 5 hpf embryos and analyzed by wholemount *in situ* hybridization for expression of *caudal* (a, b), *hoxb1b* (c, d), *nlz* (e-g), *meis3* (h-j) and *hoxb1a* (k-m). All panels are dorsal views with anterior to the top, except panels g, j and m which are animal pole views. Black arrow in panels a, d and h represent location of sections in Figure 4C (black arrow in panel a shows location of section caudal to the hindbrain for *caudal*, black arrow in panel h shows location of sections through the hindbrain primordium for *nlz*, *meis3* and *hoxb1b* and black arrow in panel d shows location of animal pole section for all genes). White arrow represents ectopic gene expression in response to implanted bead.

4B

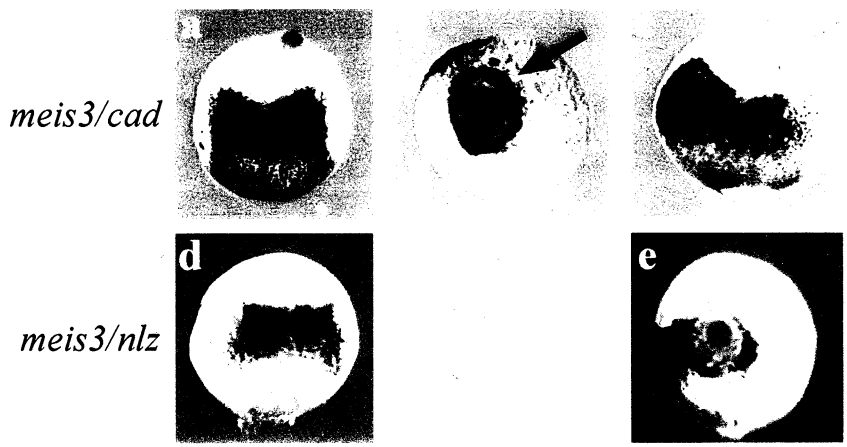

Figure 4B. Early hindbrain genes are FGF responsive. Double in situ hybridizations. Bead implants were performed as described for Figure 4A, except double *in situ* hybridizations were performed with *meis3* detected in blue (a-e), *caudal* in red (a-c) and *nlz* in red (d, e). Panels a, c and d are dorsal views with anterior to the top. Panel b is an animal pole view and panel e is a lateral view with dorsal to the left. Arrow points to ectopic meis3 expression in b.

4C

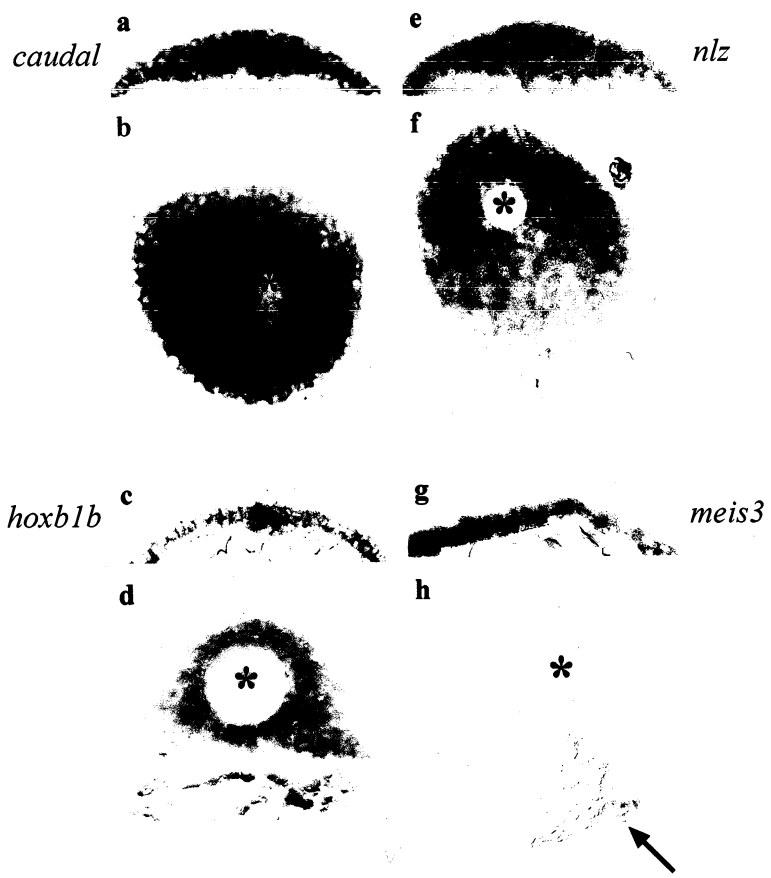

Figure 4C. Early hindbrain genes are FGF responsive. Sections of FGF bead implanted embryos. Embryos implanted with FGF soaked beads (as described in Figure 4A.) were cross sectioned to determine germ layer distribution of *caudal* (a, b), *hoxb1b* (c, d), *nlz* (e, f) and *meis3* (g, h). Panels c, e and g represent sections through the hindbrain primordium (Fig 4A. panel h, black arrow) and panel a represents a section through the caudal domain of the embryo (Fig 4A. panel a, black arrow). Dorsal to the top. Panels b, d, f and h represent sections through the anterior pole of the embryo where the FGF bead was implanted showing ectopic gene expression (Fig 4A. panel d, black arrow). Arrow in h points to ectopic *meis3* expression. Asterisks show location of implanted bead.

4C, panels b, d, f) while *meis3* expression is found at a distance (Fig. 4C, arrow in panel h; the *meis3* expression ring was often incomplete, making it difficult to obtain sections of more than a partial *meis3* ring in a given plane). We conclude that *hoxb1b, caudal* and *nlz* expression is induced adjacent to the Fgf source, but that *meis3* expression is induced at a distance and that regions close to the Fgf source are incompatible with *meis3* expression.

Fgf signaling is required for branchiomotor neuron migration in the hindbrain.

To further explore the role of Fgf signaling in hindbrain development we next examined neuronal differentiation in dnFGFR-expressing embryos. In particular, we monitored branchiomotor (BM) neuron differentiation using a transgenic line expressing Green Fluorescent Protein (GFP) under control of the motorneuron specific promoter/enhancer from the *islet-1* (*isl1*) gene (Higashijima et al., 2000). BM neurons of the cranial nerves differentiate in rhombomere-specific patterns such that the cells of the facial (VIIth) nerve differentiate in r4 and r5 at 16 hpf, projecting their axons out through r4 and innervating the second pharyngeal arch. By 19 hpf, these cells undergo a posterior migration from r4/r5 to r6/r7 and by 36 hpf this migration is complete. In contrast, BM neurons of the trigeminal (Vth) nerve do not migrate, but differentiate as two groups, a major anterior group in r2 (Va) and a smaller posterior group in r3 (Vp). Both Va and Vp project axons out r2 and innervate the first pharyngeal arch (Chandrasekhar et al., 1997; Higashijima et al., 2000; McClintock et al., 2002). Lastly, BM neurons of the vagal

(Xth) nerve differentiate in the caudalmost hindbrain (caudal to r7) and innervate the gill arches.

We observe large numbers of GFP positive cells in r5, and sometimes r4 (Fig. 5b, c) of dnFGFR-injected embryos at 24 hpf. This is in contrast to control embryos that lack GFP positive cells in r4 and r5 as a result of nVII neurons migrating to r6/r7. This suggests that nVII neurons do not migrate properly in dnFGFR expressing embryos, but instead 'stall' in r4 or r5. Overall, 57% of dnFGFR-expressing embryos display nVII neuron migration defects at 24 hpf. By defining the 'stalling position' as the rostral-most rhombomere showing clumping of cells, we find that 44% of embryos show stalling in r4, 7% in r5 and 6% in r6. To confirm that these defects are not the consequence of dnFGFR expressing embryos developing more slowly than control embryos, we examined dnFGFR-expressing embryos at 36 hpf. At 36 hpf, migration of nVII neurons has not improved as 76% of embryos display defects (Fig. 5e, f) with 44% stalling in r4 (an extreme case is shown in Fig 5, panel g), 26% in r5 and 6% in r6. As this defect was seen at both time points, we conclude that it is not the result of delayed development but is a consequence of disrupting Fgf signaling. We also note that nVa neurons in r2 differentiate normally in dnFGFR expressing embryos (Fig. 5b, c, e, f). Differentiation of nVp neurons in r3 was more variable (partially lost in 33% of embryos; Fig. 5b, c, e, f), suggesting a minor effect on r3. We also find that nX BM neurons are lacking in dnFGFR-expressing embryos (shown in Fig. 5f; 59%), consistent with Fgf signaling being required for trunk and tail development caudal to the hindbrain. In contrast, the

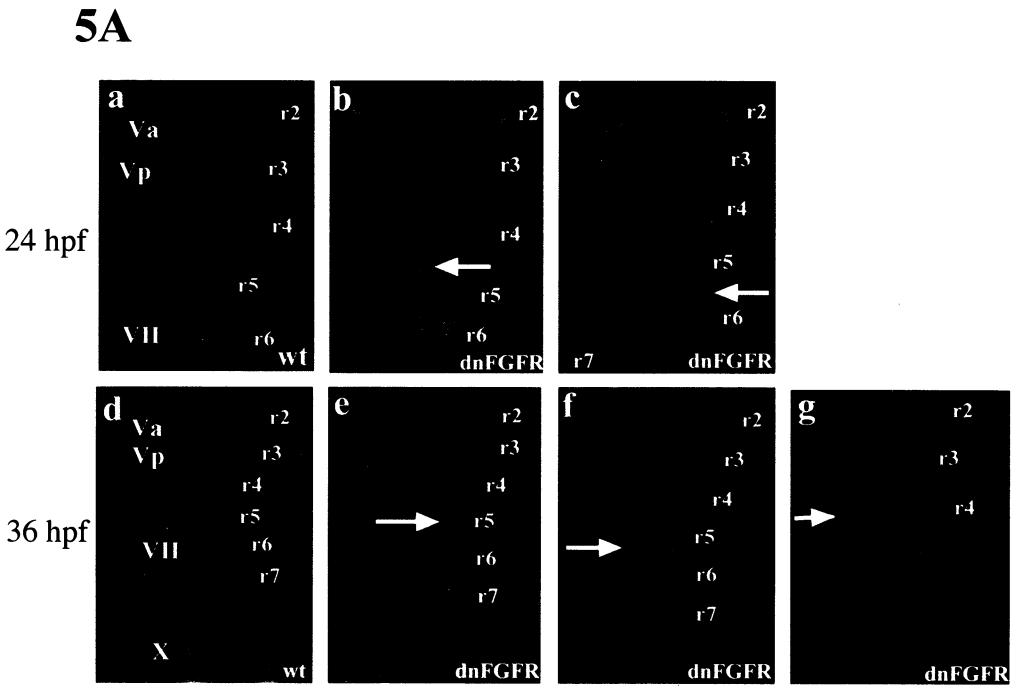

Figure 5A. FGF is required for branchiomotor neuron migration in the hindbrain. a-g: control (a, d) or dnFGFR (b, c, e, f, g) mRNA was injected into one to two cells of Isl1-GFP transgenic fish embryos and fixed at 24 hpf (a-c) or 36 hpf (d-g), deyolked and flatmounted. Arrows point to groups of BM neurons that do not migrate, but are stalled at rostral positions. Panel g shows total migration failure and clumping in r4.

5B

Figure 5B. FGF is not required for primary reticulospinal neuron development. h and i: Control (h) or dnFGFR (i) mRNA was injected into one to two cells of wild type embryos and fixed at 28 hpf. Mauthner neurons were visualized via immunohistochemistry with the 3A10 antibody (see Materials and Methods).

dnFGFR has no effect on Mauthner neuron differentiation in r4, as 95% of injected embryos show wild type arrangement of Mauthner neurons (Fig 5, panels h and i).

We wished to verify that the migration defects we detected at 36 hpf were in fact a consequence of blocking FGF signaling at early gastrula stages and not due to the persisting dnFGFR blocking other Fgf's required later in hindbrain development. Thus, we soaked embryos in SU5402, an Fgf inhibitor (Sawada et al., 2001; Shinya et al., 2001) at 4 hpf for one hour, extensively washed the embryos and examined BM neuron migration at 36 hpf. Indeed we detect the same migration failure and stalling of cells within the hindbrain (not shown) confirming that early gastrula stage Fgf signaling is required for BM neuron migration.

Hindbrain gene expression recovers in later stage dnFGFR expressing embryos

The effect of the dnFGFR construct on differentiation of BM and reticulospinal neurons was surprisingly mild given the effect on gene expression in the hindbrain primordium at 10 hpf. We therefore examined hindbrain gene expression in dnFGFR embryos at a later stage (14 hpf) and find that they are significantly less affected at 14 hpf than at 10 hpf (Table 1). In particular, at 10 hpf *nlz* expression is affected in 93% of embryos, whereas at 14 hpf, only 9% show a decrease in *nlz* expression and *hoxb1a* is affected in 86% of embryos at 10 hpf, but only in 24% at 14 hpf and the phenotype is less severe at 14 hpf. Similarly, *meis3* is affected in 88% of embryos at 10 hpf and in 44% at 14 hpf, but the phenotype is milder at 14 hpf.

Table 1. Co-operative actions of Fgf and RA in regulating hindbrain gene expression[a]

	dnFGFR		dnRAR		dnFGFR/dnRAR
	10 hpf	14 hpf[b]	10 hpf	14 hpf[b]	14 hpf[b]
nlz	93%(78/84)	9% (5/56)	58% (43/74)	18% (6/33)	70% (37/53)
meis3	88%(91/103)	44%(28/64)	69% (44/64)	9% (5/54)	94% (51/54)
hoxb1a	86% (61/71)	24% (18/74)	72% (56/77)	20% (7/35)	61% (28/46)
hoxb3	nd	18%(19/105)	nd	23% (10/43)	77% (41/63)
valentino	nd	16% (23/142)	nd	23% (22/93)	47% (22/47)
krox20	nd	23% (30/133)	nd	35%(30/85)	59% (27/46)
hoxd4	nd	90% (48/53)	nd	10% (4/41)	nd
caudal	94% (62/66)	93% (25/27)	0% (0/61)	0% (0/28)	nd
ntl	82% (18/22)	65% (32/49)	nd	nd	nd
hoxb1b	95% (81/85)	nd	77%(41/53)	nd	nd
vhnf1	87%(90/104)	nd	nd	nd	nd
gbx1	73%(27/37)	nd	nd	nd	nd
fgf8	81%(90/111)	nd	nd	nd	nd

a). mRNA encoding dnFGFR or dnRAR were injected into embryos as outlined in Materials and Methods. Embryos were harvested at 10 hpf or 14 hpf and analyzed by in situ hybridization for expression of the genes indicated at left. Embryos showing reduction or loss of gene expression were scored as affected and are indicated as a percent of total embryos. Absolute numbers of embryos are given in parentheses for each experimental condition. Embryos injected with an equivalent amount of ß-gal mRNA were >95% normal. nd = not done

Other hindbrain markers tested at 14 hpf could not be compared to 10 hpf as they are not expressed that early, but these genes also show only mild defects in dnFGFR-expressing embryos. Specifically, *hoxb3* and *valentino* show moderate reductions in r5 and r6 in 16-18% of dnFGFR-expressing embryos and *krox20* expression in r3 and r5 is affected in only 23% of dnFGFR-expressing embryos. The only exception is *hoxd4,* normally expressed caudal to the r6/r7 border, that is affected in 90% of dnFGFR-expressing embryos at 14 hpf. The *hoxd4* expression domain appears shorter along the anteroposterior axis, but double in situ hybridizations reveal that the gap between the *hoxd4* expression domain and *krox-20* expression in r5, or *hoxb1a* expression in r4, remains normal (not shown). We interpret this to mean that *hoxd4* expression is not affected in r7, but is lost caudal to the hindbrain. In agreement with this, we find that *caudal* and *ntl* remain affected caudal to the hindbrain at 14 hpf (93% at 14 hpf versus 94% at 10 hpf for *caudal* and 65% at 14 hpf versus 82% at 10 hpf for *ntl*). Thus, gene expression in the hindbrain and in more caudal domains is affected in dnFGFR expressing embryos at 10 hpf. This effect is largely reverted in the hindbrain at 14 hpf, but persists caudal to the hindbrain. This effect is not due to reduced efficacy of the dnFGFR at these stages since Fgf signaling remains blocked (Fig. 7A panels a, b).

Retinoic Acid regulates early hindbrain gene expression similar to Fgf.

We reasoned that the restoration of gene expression at 14 hpf may be mediated by the actions of other signaling molecules. A likely candidate for this role is RA, which

regulates expression of hindbrain genes (particularly *hox* genes (Gavalas, 2002; Gavalas and Krumlauf, 2000; Marshall et al., 1996)). To test if RA regulates expression of the Fgf-dependent genes defined in this study, we utilized a dominant negative form of retinoic acid receptor α (dnRARα), which carries a C- terminal truncation blocking its ability to dissociate from corepressors (Chen and Evans, 1995; Damm et al., 1993; Kolm et al., 1997)). dnRARα mRNA was microinjected at the 1-2 cell stage and changes in gene expression scored by in situ hybridization at tailbud stage. As expected, *hoxb1b* and *hoxb1a* expression was lost (Fig. 6A, panels j and o; 77% and 72% respectively) in dnRARα-injected embryos. *meis3* expression was also lost (Fig. 6A, panel e; 69%) demonstrating that *meis3* is regulated by RA in the caudal hindbrain. In contrast, while *nlz* expression was also lost, this effect appeared restricted to the caudal hindbrain (approximately r4-r7; asterisk in panel y; 58%) and *nlz* expression was not affected caudal to the hindbrain (Fig 6A, panel y). *caudal* gene expression was unaffected (Fig. 6A, panel t).

To achieve ectopic RA signaling, we next treated embryos with RA between sphere and tailbud stage and scored changes in gene expression by in situ analysis at tailbud stage. As expected, *hoxb1b* and *hoxb1a* expression expands anteriorly in response to RA treatment (Fig. 6A, panels h, i, m and n; 97% and 90% respectively). *meis3* and *nlz* expression is also expanded anteriorly in the presence of exogenous RA (Fig. 6A, panels c and d; 96% and panels w and x; 100%), while *caudal* expression is unaffected (Fig 6A, panels r and s). In all affected cases, the expansion of gene expression extended from the caudal border of the endogenous expression domain to the

6A

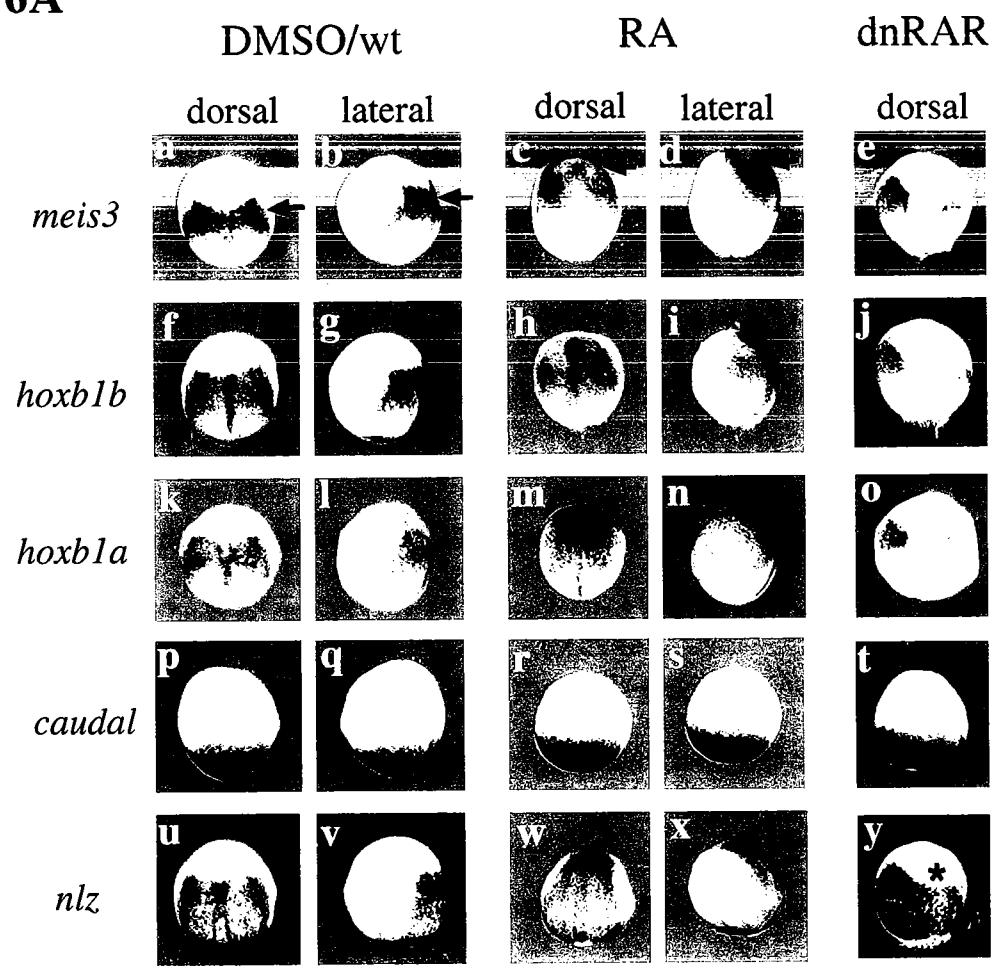

Figure 6A. Early hindbrain genes are RA responsive. Embryos were cultured in 1X MBS + 0.001% DMSO (a, b, f, g, k, l, p, q, u, v) or 1X MBS+ 1μM RA and 0.001% DMSO (c, d, h, i, m, n, r, s, w, x) from sphere (4 hpf) to tailbud stage (10 hpf), or injected with dnRAR mRNA at the one to two cell stage and raised to 10 hpf (e, j, o, t, y). Embryos were analyzed by wholemount *in situ* hybridization for expression of *meis3* (a-e), *hoxb1b* (f-j), *hoxb1a* (k-o), *caudal* (p-t) and *nlz* (u-y). Panels are dorsal views with anterior to the top (a, c, e, f, h, j, k, m, o, p, r, t, u, w, y) or lateral views with dorsal to the right and anterior to the top (b, d, g, i, l, n, q, s, v, x).

anterior pole of the embryo. No ectopic or expanded gene expression was detected ventrally, laterally or posteriorly.

Our results correlate well with RA treatments of *Xenopus* and zebrafish embryos (Holder and Hill, 1991; Papalopulu et al., 1991) which showed a dose dependent truncation of the anterior axis of the embryo at later stages. To examine if such RA mediated truncations correlate with our observed changes in gene expression, we examined later stage RA treated embryos (14 and 24 hpf) and found that at 14 hpf *hoxb1b, hoxb1a* and *meis3* gene expression was still expanded rostrally (Fig 6B, panels a-f). This effect persisted at 24 hours, as expression of *hoxb1a* remained strongly upregulated in the head region (Fig 6B, panels i and j) and coincided with a loss of *otx2* forebrain gene expression (Fig 6B, arrows in panels g and h). Thus, application of exogenous RA expands endogenous gene expression from r4-r7 to the anterior pole of the embryo and causes anterior truncations.

We analyzed embryo sections to determine which germ layers express endogenous *hoxb1b, hoxb1a, meis3* and *nlz* expression, and whether exogenous RA induced ectopic gene expression in other germ layers. Cross sections through the hindbrain primordium show *hoxb1b* and *nlz* gene expression in the outer (likely corresponding to ectoderm) and inner (likely corresponding to mesendoderm) layers for both RA and control treated embryos (Fig. 6C, panels e,g,m and o). *hoxb1a* expression was weak at this stage in untreated embryos, but appears primarily in the inner layer both in treated and untreated embryos (Fig 6C, panels i and k). *meis3* gene expression in control embryos was found in both inner and outer layers. However, RA treated embryos

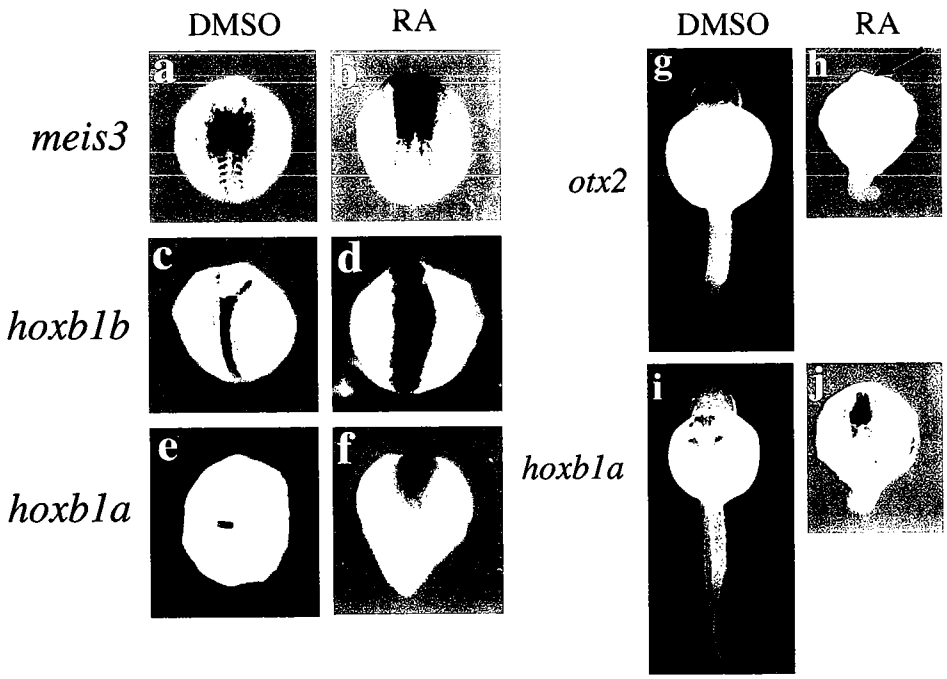

Figure 6.B. RA mediated anterior truncations correlate with changes in gene expression. a-j. Embryos were treated with1X MBS + 0.001% DMSO (a, c, e, g, i) or 1X MBS + 1μM RA and 0.001% DMSO (b, d, f, h, j) from sphere to tailbud stage, washed and allowed to develop until 14 hpf (a-f) or 24 hpf (g-j) and analyzed by wholemount in situ hybridization for expression of *meis3* (a, b), *hoxb1b* (e, d), *hoxb1a* (e, f, i, j) or *otx2* (g, h). All panels are dorsal views with anterior to the top. Black arrows in g and h represent loss of *otx2* forebrain expression.

showed *meis3* gene expression most strongly primarily in the inner layer (Fig 6C, panels a and c). In anterior sections, representing expanded gene expression in response to RA treatment, *hoxb1b* and *nlz* expression was detected again in both layers (Fig 6C, panels h and p) and *hoxb1a* was again detected only in the inner layer (Fig. 6C, panel l). In the expanded *meis3* domain, inner layer staining was again detected (Fig 6C, panel d). Thus, the presence of exogenous RA does not alter the germ layer distribution of *hoxb1b, hoxb1a* or *nlz* expression, but *meis3* germ layer gene expression seems to be altered by RA treatment.

Using our in vitro differentiation assay, we find that regulation of *nlz* and *hoxb1a* is protein synthesis independent (Fig 6D, lanes 2, 3, 7, 8). In contrast, induction of *meis3* and *hoxb1b* expression is protein synthesis dependent (Fig 6D, lanes 2, 3, 7, 8), although *hoxb1b* may be less dependent on protein synthesis shortly after RA addition (compare ratio between lanes 2 and 3 to ratio between lanes 7 and 8).

Lastly, we find that dnRARα expressing embryos show a "stalling" phenotype similar to that observed for dnFGFR expressing embryos (not shown). Specifically, 28% of embryos showed a wild type migration pattern of nVII BM neurons, while 52% showed stalling defects in r4 and 20% in r5. No stalling defects were observed further caudally and nX neurons were not affected, consistent with RA signaling not being required for trunk and tail development. Differentiation of nVp neurons in r3 was affected in 20% of embryos. This data suggest that RA, like Fgf, is required for branchiomotor neuron migration within the hindbrain.

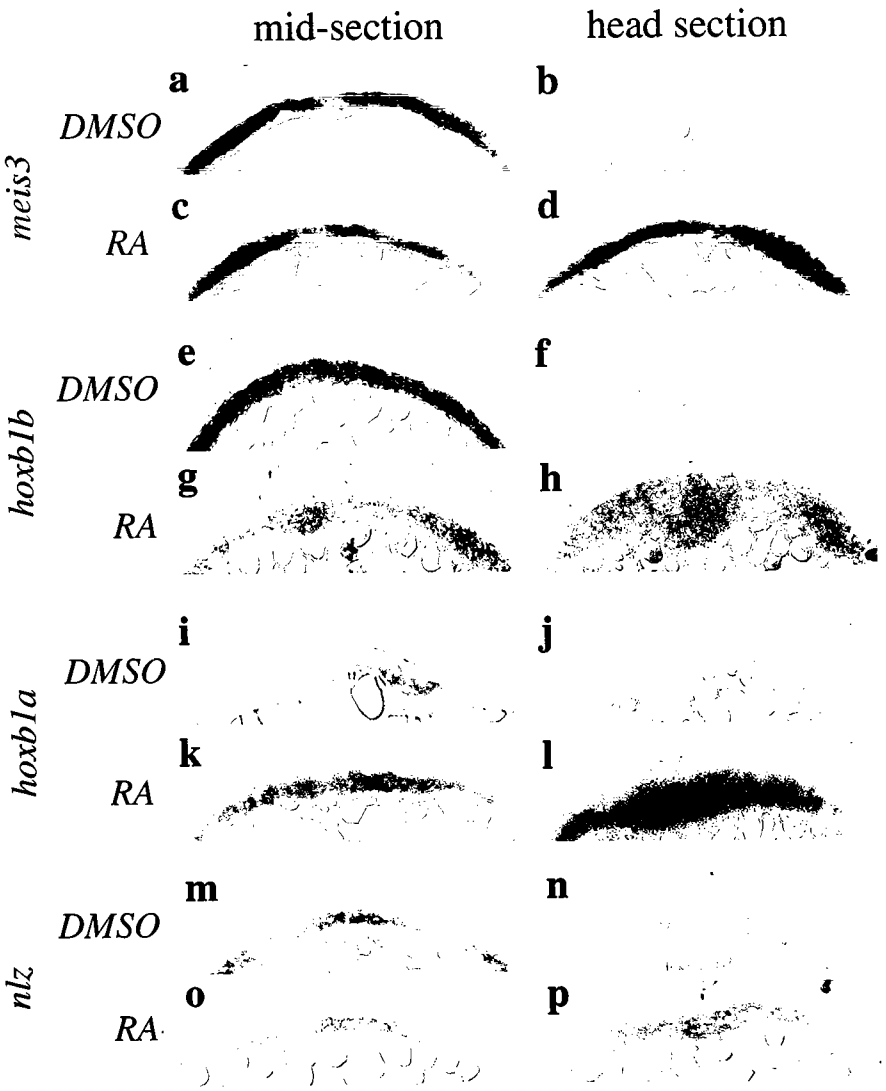

Figure 6.C. Sections of RA treated embryos. Embryos were treated with 1X MBS + 0.001% DMSO (a, b, e, f, i, j, m, n) or 1X MBS + 1μM RA and 0.001% DMSO (c, d, g, h, k, l, o, p) as in Figure 6.A. and cross sectioned to analyze germ layer expression of *meis3* (a-d), *hoxb1b* (e-h), *hoxb1a* (i-l) and *nlz* (m-p). Panels a, c, e, g, i, k, m, o represent cross sections through the hindbrain primordium, panels b, d, f, h, j, l, n, p are cross sections through a more anterior region displaying ectopic gene expression in response to RA treatment. Dorsal to the top.

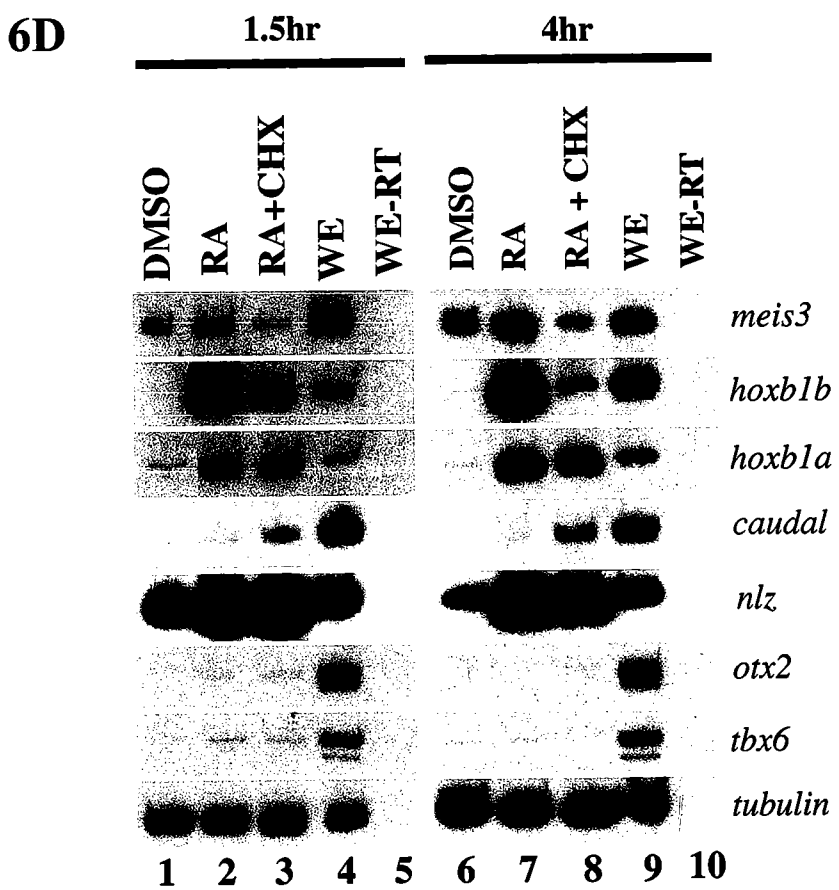

Figure 6D. Early hindbrain genes are RA responsive. RT-PCR analysis of gene expression in explanted animal caps and whole embryos. Animal caps were taken from sphere stage embryos (as shown in cartoon in Fig. 3A), aggregated into groups of 30 and cultured *in vitro* for 1.5 or 4 hours in 1X MBS with 0.001% DMSO, 1μM RA in 0.001% DMSO or 1μM RA + 5μg/μl cycloheximide in 0.001% DMSO. Explants (lanes 1-3, 6-8) were harvested after 1.5 hrs (lanes 1-3) or 4 hrs (lanes 6-8) treatments in DMSO (lanes 1, 6), 1μM RA (lanes 2, 7) or 1μM RA + 5μg/μl cycloheximide (lanes 3, 8) and analyzed for expression of hindbrain markers *meis3*, *hoxb1b*, *hoxb1a*, *caudal* and *nlz*. *otx2* was used as an anterior marker, *tbx6* as a mesodermal marker and *tubulin* as a loading control. RT-PCR was performed as described in Materials and Methods. Whole embryos (lanes 4, 5, 9, 10) were harvested at 10 hpf and analyzed for the same genes. RT-PCR reactions for whole embryos were also performed -RT (lanes 5 and 10) as a control.

RA and Fgf cooperate to regulate hindbrain gene expression at segmentation stages. We hypothesized that RA signaling might compensate for the loss of Fgf signaling during segmentation stages. We first examined hindbrain gene expression at 14 hpf in dnRARα expressing embryos and detect only a mild effect, suggesting that gene expression recovers also in dnRARα expressing embryos (Table 1). In particular, at 10 hpf, *nlz* expression is affected in 58% of embryos, whereas at 14 hpf, only 18% show a slight decrease in *nlz* expression. Similarly, *hoxb1a* is affected in 72% of embryos at 10 hpf, but only 20% show a slight decrease in *hoxb1a* stain at 14 hpf and *meis3* is affected in 69% of embryos at 10 hpf, while only 9% show disruption at 14 hpf. *caudal* was never effected at 10 hpf, and shows no effect at 14 hpf. Later stage hindbrain markers were also tested in dnRAR-expressing embryos. Specifically, *hoxb3* and *valentino* showed very mild decrease in expression in r5 and r6 (23% each) while *krox20* expression in r3 and r5 was affected in 35% of dnRAR-expressing embryos. *hoxd4* expression was mildly effected in 10% of dnRAR-expressing embryos at the r6/r7 border, but not affected in its expression domain further caudally.

We next co-injected dnFGFR and dnRAR at the one cell stage and analyzed hindbrain gene expression at 14 hpf. We find that gene expression remains affected in dnFGFR/dnRAR co-expressing embryos at 14 hpf. In particular, while *nlz* expression is mildly reduced in 9% of dnFGFR-expressing embryos and 18% of dnRARα-expressing embryos at 14 hpf, 70% of embryos are affected in dnFGFR/dnRAR co-expressing embryos (Fig. 7B, panels a, b; Table 1). A similar increase in affected embryos (ranging from 47-94%; Table 1) in the presence of both dominant negatives was detected for

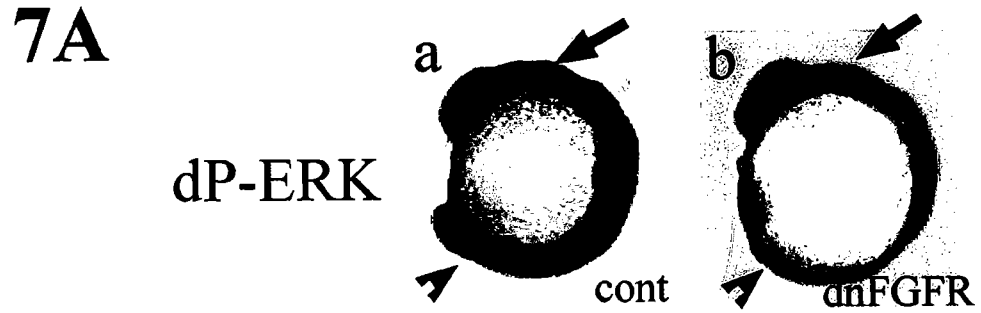

Figure 7A. dnFGFR still functions at 14hpf. Embryos were injected with 500 pg LacZ control (a) or dnFGFR (b), fixed at 14 hpf and processed for immunohistochemistry with anti-dP-ERK antibody. Arrows represent loss of dP-ERK staining at the MHB and arrowheads show loss of tail structures in dnFGFR expressing embryos. Views are lateral with anterior to the top.

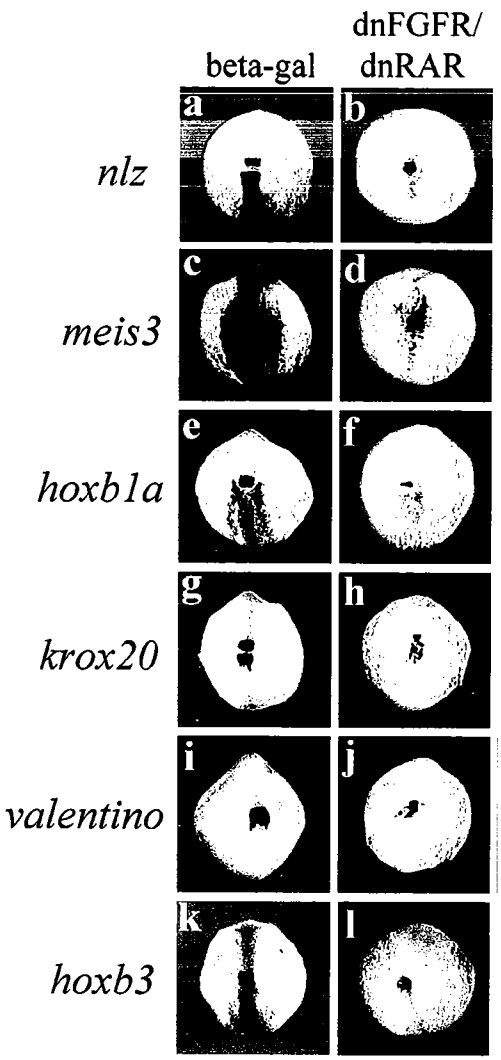

Figure 7B. Hindbrain gene expression in dnFGFR/dnRAR co-expressing embryos is affected at 14 hpf. Embryos were injected at the one to two cell stage with 500 pg LacZ control (a, c, e, g, i, k) or 500 pg dnFGFR/500 pg dnRAR mRNA (b, d, f, h, j, l), fixed at 14 hpf and analyzed by wholemount *in situ* hybridization for *nlz* (a, b), *meis3* (c, d), *hoxb1a* (e, f), *krox20* (g, h), *valentino* (i, j) and *hoxb3* (k, l) expression. All views are dorsal with anterior to the top.

hoxb1a, meis3, hoxb3, valentino and *krox20* (Fig. 7B, panel c-l). This data suggest that Fgf and RA alone are sufficient to induce hindbrain gene expression at 14 hpf and only when both signals are blocked is hindbrain gene expression affected. Thus, Fgf and RA cooperate to regulate hindbrain gene expression at segmentation stages.

DISCUSSION

Previous work has demonstrated that Fgf signaling is required for gene expression in the caudal embryo, including the caudal hindbrain (Holowacz and Sokol, 1999; Kolm and Sive, 1995; Koshida et al., 1998; Kudoh et al., 2002), an effect potentially attributable to *fgf3* and *fgf8* (Maves et al., 2002; Walshe et al., 2002). We have further explored the role for Fgf signaling and find that it is required for gene expression throughout the hindbrain primordium already by mid-gastrula stages. We demonstrate that this early Fgf signal is not provided by Fgf3 or Fgf8, suggesting that an as yet uncharacterized Fgf family member is acting at gastrula stages. As expected, Fgf3 and Fgf8 regulate gene expression in the caudal hindbrain during segmentation stages (as part of a signaling center in r4 (Maves et al., 2002; Walshe et al., 2002)), but we find that *fgf8* expression is lost upon disrupting gastrula stage Fgf-signaling, indicating that early Fgf-signaling may be required to establish the signaling center in r4. We also demonstrate that although both Fgf and RA signaling is required for gene expression in the gastrula stage hindbrain primordium, application of either exogenous RA or Fgf is sufficient to induce hindbrain gene expression in gastrula stage tissues. Furthermore, while signaling by both factors is required for gastrula stage gene expression, this requirement is alleviated by

segmentation stages when the two pathways appear to become redundant. Together, these results reveal a dynamic relationship between RA and Fgf signaling during hindbrain development.

Which Fgf family member regulates early hindbrain development?

Our results reveal an early role for Fgf signaling in regulating hindbrain gene expression at gastrula stages, but it is unclear which Fgf family member is at work. While *fgf3* and *fgf8* are expressed in the germring by the 50% epiboly stage and in presumptive r4 by 80%-90% epiboly (Phillips et al., 2001; Reifers et al., 1998; Walshe et al., 2002), potentially consistent with their regulating early gene expression in the hindbrain primordium, we note marked differences between disrupting Fgf3 and Fgf8 function on the one hand and disrupting all Fgf signaling on the other hand. Specifically, using antisense morpholino oligonucleotides to disrupt Fgf3 and Fgf8 function does not affect gastrula stage gene expression, while the dnFGFR construct blocks *nlz*, *meis3*, *hoxb1b*, *hoxb1a*, *caudal* and *gbx1* expression throughout the hindbrain primordium. This is particularly apparent in r4, which is largely unaffected by disrupting Fgf3 and Fgf8 function, but at least four genes expressed in r4 are lost upon blocking Fgf signaling by the dnFGFR construct. Thus, it is unlikely that *fgf3* and/or *fgf8* regulate early gene expression in the hindbrain primordium. *fgf17* has recently been isolated in zebrafish, but unlike *fgf3* or *fgf8*, it is not expressed during gastrulation (Reifers et al., 2000) and therefore is not a candidate for an early role. Similarly, a zebrafish *fgf4* gene was reported recently (Grandel et al., 2000), but we find that *fgf4* is not expressed until 11 hpf

(not shown). Since the Fgf-family contains at least 23 members (Ford-Perriss et al., 2001) and the expression pattern of these genes has not been exhaustively analyzed during embryogenesis, it appears possible that at least one family member will be expressed at gastrula stages. Lastly, we cannot exclude the possibility that the early Fgf-signal is mediated by a maternally deposited Fgf.

What is the source of Fgf at gastrula stages?

Although the source of the early-acting Fgf is not clear, clues might be derived from variations in Fgf-responsiveness among the genes analyzed. In particular, although *meis3*, *hoxb1a*, *nlz* and *hoxb1b* all require Fgf signaling in vivo, only *hoxb1b* and *nlz* are induced by Fgf in vitro. Furthermore, *hoxb1b* and *nlz* are induced adjacent to implanted Fgf beads while *meis3* is induced at a distance and *hoxb1a* not at all. These observations suggest that *nlz* and *hoxb1b* may be expressed near the Fgf source in vivo, while *meis3* and *hoxb1a* expression might be found at a distance. This prediction correlates with the expression of these genes along the rostrocaudal axis in vivo - *nlz* and *hoxb1b* expression extends to the caudal end of the embryo while *meis3* and *hoxb1a* expression is found further rostrally – suggesting that the caudal end of the embryo may be the source of Fgf. As discussed, both *fgf3* and *fgf8* are expressed caudally at gastrula stages, but are unlikely to represent the essential Fgf signal. It is instead possible that the source of Fgf lies outside the embryo proper, perhaps within the yolk, as it has been demonstrated that the yolk can induce posterior gene expression in gastrula stage zebrafish embryos (Koshida et al., 1998).

As noted, induction of *meis3* expression appears to depend on the distance from the Fgf-bead. This could simply be due to *meis3* expression requiring a particular concentration of Fgf, perhaps corresponding to the concentration of Fgf found in the caudal hindbrain in vivo. Furthermore, since endogenous *meis3* is repressed when a Fgf-bead is located within the hindbrain primordium (Fig 4B, panel c, e), it is possible that a factor regulated by Fgf represses *meis3* expression adjacent to the Fgf source (see model described in Co-operative actions of Fgf and RA).

Co-operative actions of Fgf and RA

We find that disruption of either RA or Fgf signaling blocks hindbrain gene expression at gastrula stages, suggesting that both factors are necessary at this stage. However, RA or Fgf alone is sufficient to induce expression of at least some hindbrain genes when applied exogenously to gastrula stage ectodermal explants or intact embryos. How do we explain this discrepancy?

Our data support a model in which Fgf and RA each induce some factor x, which is present at a limiting concentration at gastrula stages. Hindbrain genes can only be induced when both Fgf and RA signaling produce a combined threshold level of factor x. Factor x will, in turn, induce hindbrain genes (Figure 8A, a). Perhaps *meis3* expression is only turned on at a distance because some repressor of *meis3* is expressed directly around the Fgf source. Upon injection of either dominant negative, the required threshold concentration of factor x is not achieved and a loss of hindbrain gene expression detected (Figure 8A, b and c). Exogenous application of either factor alone in the animal pole

(beads or caps) may induce hindbrain gene expression because the high concentration of exogenously applied Fgf or RA can induce significantly higher levels of factor x. (Figure 8B, b and c).

Our results also indicate that the relationship between RA and Fgf signaling changes by segmentation stages. In particular, blocking RA or Fgf signaling alone has only a minor effect on hindbrain gene expression at segmentation stages, while simultaneous disruption of RA and Fgf signaling severely blocks gene expression (Table 1). Based on our model proposed above, the endogenous levels of RA and Fgf may no longer be limiting and thus, factor x can be produced by either factor alone at the appropriate threshold concentration (Figure 8C, a-c). In support of this segmentation stage model, it has been demonstrated in *Xenopus* that Fgf signaling increases as the embryos develops (as detected by increase in MAPK activity) (LaBonne and Whitman, 1997) and that RA metabolism increases directly after gastrula stages in zebrafish and chick (Costaridis et al., 1996; Maden et al., 1998).

8A

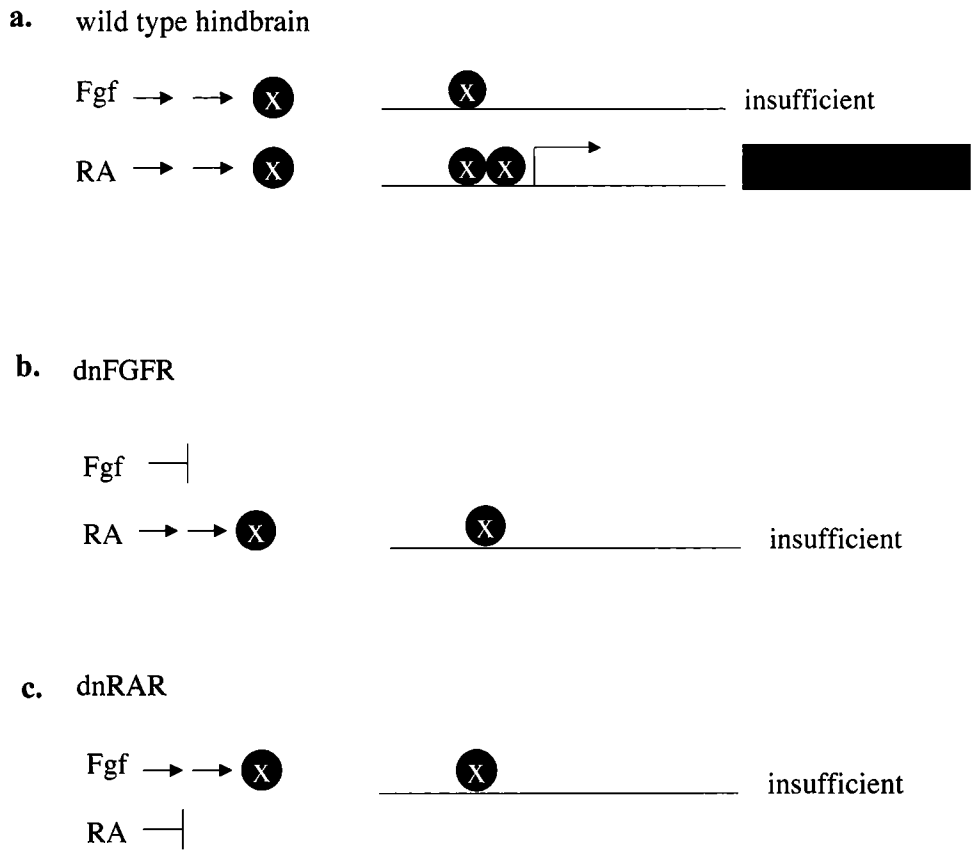

Figure 8A. Endogenous hindbrain domain. a. Both Fgf and RA induce expression of factor x at some basal level within the hindbrain. Only when a threshold concentration of factor x is reached will hindbrain gene expression be induced. **b.** In the presence of the dnFGFR, only RA can induce expression of factor x, which is insufficient to induce hindbrain genes. **c.** In the presence of the dnRAR, only Fgf can induce expression of factor x, which is insufficient to induce hindbrain genes.

8B

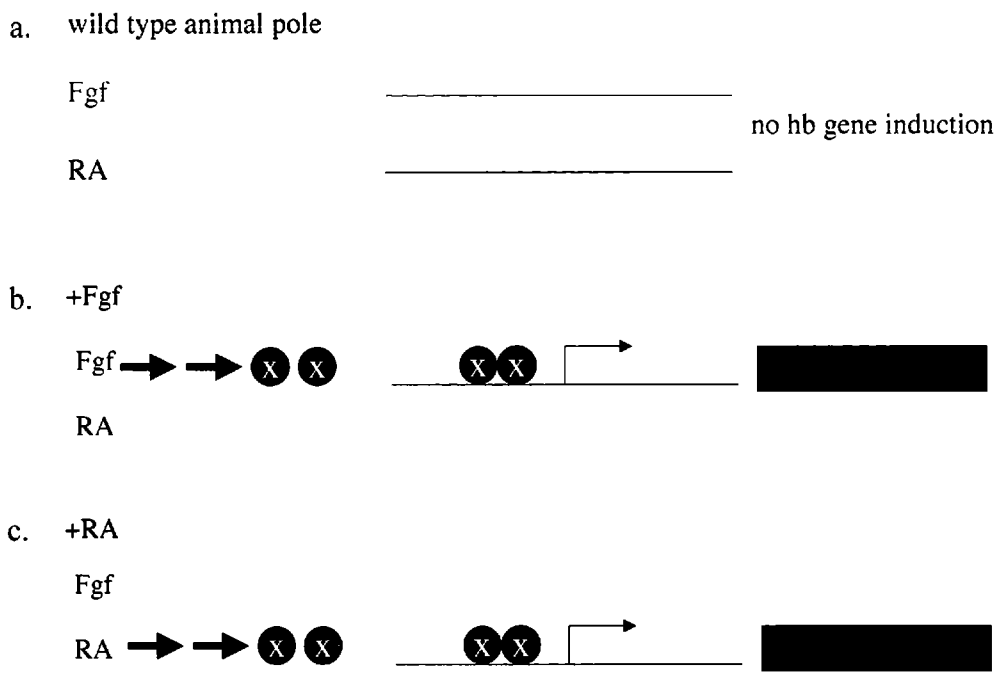

Figure 8B. The animal pole a. There is no Fgf or RA signaling in the animal pole of zebrafish embryos and thus no production of factor x. **b.** In the presence of high exogenous levels of Fgf, factor x is produced in abundance and even without the levels of factor x produced by RA, Fgf alone can induce hindbrain genes. **c.** In the presence of high exogenous levels of RA, factor x is produced in abundance and even without levels of factor x produced by Fgf, RA alone can induce hindbrain genes.

8C.

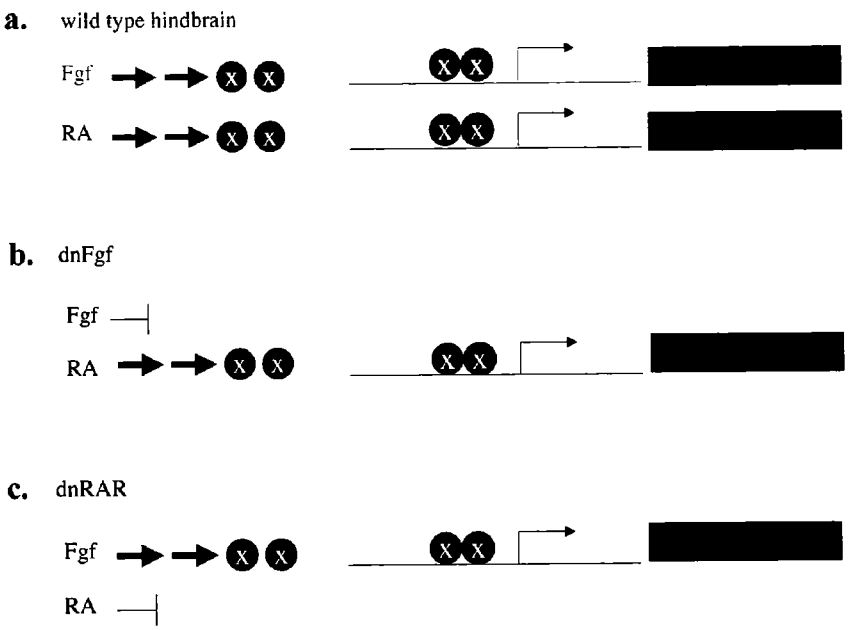

Figure 8C. Segmentation stage hindbrain. a. By 14hpf enogenous levels of Fgf and RA signaling have increased in the endogenous hindbrain region. Now Fgf signaling alone or RA signaling alone can induce factor x at sufficinet levels to induce hindbrain genes. **b.** In the presence of the dnFGFR, hindbrain gene expression is still induced as RA signaling alone is sufficient to induce hindbrain genes. **c.** In the presence of the dnRAR, hindbrain gene expression is still induced as Fgf signaling alone is sufficient to induce hindbrain genes.

MATERIALS AND METHODS

Fibroblast growth factor and retinoic acid treatments

For bead implants, embryos were manually dechorionated, allowed to develop to sphere stage (4 hpf) in 1X MBS + G (Modified Barth's Saline + gentamicin 50μg/μl) (Sagerstrom et al., 1996) and transferred to 3% methyl cellulose (Sigma) in 1X MBS. BioRad Affi-Gel Blue Beads (100-200mesh) were washed several times in water, transferred to a 0.5 mg/ml bFGF solution (Invitrogen) or 1X MBS control solution and incubated for 2 hours. Beads were then implanted into the animal pole of embryos and allowed to recover in the methyl cellulose for 1 hour after which they were transferred to 1X MBS until 10 hpf. Embryos were then fixed and processed for in situ hybridization studies. Retinoic Acid (Sigma) was dissolved in DMSO and diluted in 1X MBS. Embryos were transferred into a solution of 1 uM RA or an equivalent amount of DMSO (0.001%) in 1X MBS at 4 hpf and left until 10 hpf when they were washed several times in 1X MBS and fixed in 4% paraformaldehyde. Staging was performed according to (Kimmel et al., 1995).

SU5402 Drug Soaks

SU5402 (Calbiochem) treatments were performed on manually dechorionated fish embryos at 4 hpf for various time periods as described in Results Section. SU5402 stock solution was prepared by dissolving SU5402 in DMSO and diluted before use to 0.2 mg/ml in 1X MBS. After appropriate soaking period, embryos were washed extensively

in 1X MBS and allowed to develop to 10 hpf for in situ analysis or 36 hpf to monitor BM neuron migration.

Microinjections

Templates were generated by digesting pXFD/XSS (dnFGF) (Amaya et al., 1991) with EcoRI and pSP6nucβ-gal with XhoI and pSP6-RARαΔ403 (dnRAR) (Chen and Evans, 1995; Damm et al., 1993; Kolm et al., 1997) with EcoRI. mRNA was generated with the mMessage Machine Kit (Ambion). 1-4 cell stage embryos were microinjected into one blastomere with 500 pg of either mRNA or 500 pg of dnFGFR plus 500 pg dnRAR. Control embryos received and equivalent amount of ß-gal mRNA. Embryos were allowed to develop to 10 hpf or 14 hpf, fixed in 4% paraformaldehyde overnight and then stored in MeOH prior to in situ hybridization or processed for immunohistochemistry with anti-dP-ERK antibody as previously described (Shinya et al., 2001).

Morpholino injections

Antisense morpholino oligonucleotides (MOs) *fgf8*, 4-MIS *fgf8*, *fgf3*B, *fgf3*C, 5-MIS*fgf3*B and 5-MIS*fgf3*C were ordered from GeneTools based on published reports (Maves et al., 2002; Walshe et al., 2002). 1-2 cell stage embryos were injected with a cocktail of approximately 8.5 ng *fgf8*, 4.0 ng *fgf3B* and 4.0 ng *fgf3C* MOs or an equivalent amount of 4-MIS *fgf8*, 5-MIS*fgf3*B and 5-MIS*fgf3*C control MOs. Embryos were allowed to develop until 10-16 hpf, fixed in 4% paraformaldehyde overnight and

then stored in MeOH prior to in situ hybridization or processed for immunohistochemistry as described above.

Explants and RT-PCR

Animal caps were dissected as previously described (Sagerstrom et al., 1996). Aggregates were cultured in 1X MBS, 1X MBS with 1 μg/ml bFgf, 1X MBS with 1 μg/ml bFgf plus 5 μg/μl cycloheximide, 1X MBS with 0.001% DMSO, 1X MBS with 1 μM RA or 1X MBS with 1 μM RA plus 5 μg/μl cycloheximide. RNA was isolated using the ToTally RNA Isolation Kit (Ambion). cDNA synthesis, PCR reaction and sample purification was performed as previously described (Sagerstrom et al., 1996). Samples were run on a 5% acrylamide gel and analyzed autoradiographically. Primers for *otx2* were previously described (Sagerström et al., 1996). Other primers used were: *meis3*: 5'-GCGCCGCTAATGCTGGATAC-3' and 5' TTTGGTCCCGGTGGTGTTTC-3'. *hoxb1a*: 5'-AGAAGAAGCGAGAGAAGGA-3' and 5'-TTCACATTTTTCGCCTGT –3'. *hoxb1b*: 5'-TACTTGCCAAGTGCTTGTGCAAGT-3' and 5'-TATGATTGATAGTGGCTTGCAGA-3'. *tbx6*: 5'-GGCCCGGTTAGAAGAGGTGT-3' and 5'-GGTATTTTTGCGGTTGAGTTGC-3'. *tubulin*: 5'-CTGTTGACTACGGAAAGAAGT-3' and 5'-TATGTGGACGCTCTATGTCTA. Inhibition of protein synthesis by cycloheximide was determined through ^{35}S incorporation. An 80% reduction of ^{35}S incorporation was detected in explants treated with cycloheximide.

Whole mount in situ hybridizations

Single and double in situ hybridizations were performed as previously described (Sagerstrom et al., 1996). In situ probes for the following genes were used: *meis3* and *hoxb1b* (Vlachakis et al., 2000), *hoxb1a* and *valentino* (Prince et al., 1998b), *ntl* (Schulte-Merker et al., 1994), *krox20* (Oxtoby and Jowett, 1993), *hoxd4* (Prince et al., 1998a), *hoxb3* (Isaacs et al., 1998), *nlz* (Sagerstrom et al., 2001) and *cad* (Joly et al., 1992). To prepare sections, whole in situ stained embryos were embedded in JB-4 media following protocol in PolySciences JB-4 Mini Kit. 8 micron sections were taken using a microtome with a dry glass knife and mounted in crystal mount (Biomeda MO3).

Branchiomotor and Primary Reticulospinal Neuron visualization

dnFGF mRNA was injected into embryos derived from natural crosses between female and male GFP+*Islet1* transgenic fish (Higashijima et al., 2000) at the one to two cell stage. Injected embryos were kept in the dark to prevent photobleaching. At 18 hpf embryos were transferred to 1X phenylthiourea (PTU) media to prevent melanocyte formation and then fixed in 4% paraformaldehyde at 24 or 36 hpf. Embryos were dechorionated and washed in 0.1 M phosphate buffer ($Na_2HPO_4 \cdot {}_7H20$ and $NaH_2PO_4 \cdot H_20$) and transferred to 90% glycerol overnight. Embryos were deyolked and flatmounted. Visualization of GFP positive cells was performed with a Nikon Eclipse E600 microscope with a mercury/xenon lamp with filters set for FITC. Reticulospinal neurons were detected using the same protocol as (Hatta, 1992). Staining for HRP was followed

according to instruction in Vector Laboratories DAB Substrate Kit for Peroxidase (SK-4100).

ACKNOWLEDGEMENTS

We wish to thank M. Whitman for the dnRas construct, E. Amaya and H. Sive for the dnFGFR construct, V. Prince for the *hoxd4* probe, R. Karlstrom and H. Okamato for the Isl1-GFP transgenic fish and members of the Sagerstrom lab for helpful discussions. This work was supported by grant NS38183 to CGS.

CHAPTER II

FGF4 AND FGF24 IN ZEBRAFISH HINDBRAIN DEVELOPMENT

ABSTRACT

As illustrated in Chapter 1, an unidentified Fgf, acting early in development, regulates gastrula stage hindbrain development. We could infer from the data that there was an early acting Fgf signal and speculate as to its location, but were unable to determine which specific Fgf or Fgfs performed this function. Currently 24 members of the Fgf superfamily are known (Ford-Perriss et al., 2001; Ornitz and Itoh, 2001), but many have not been extensively characterized. Thus far only Fgf8, Fgf3, Fgf4, and Fgf17/18 have been characterized in terms of expression pattern and function in the zebrafish. It has already been determined that neither Fgf3,8 or 17/18 could regulate our early hindbrain genes of interest (Chapter 1). Here, we demonstrate that disruption of Fgf4 alone or in combination with Fgf8 and Fgf3 is not sufficient to phenocopy the loss of hindbrain gene expression we detect in dnFGFR expressing embryos. Recently *fgf24*

was cloned and shown to be expressed during early gastrulation, but we demonstrate the disruption of Fgf24 alone or in combination with Fgf8 is unable to phenocopy the disrupted hindbrain gene expression phenotype seen in dnFGFR expressing gastrula stage embryos. Thus, we conclude that Fgf4 and Fgf24 alone or in combination with other identified Fgfs are insufficient to be the early Fgf-signal required for gene expression in the zebrafish hindbrain primordium.

INTRODUCTION

Currently 24 Fgf family members of the teleost *fgf* gene family have been isolated (Ford-Perriss et al., 2001). In zebrafish only a few of these genes, *fgf3* (Maves et al., 2002; Phillips et al., 2001; Walshe et al., 2002), *fgf8* (Draper et al., 2003; Irving and Mason, 2000; Maves et al., 2002; Walshe et al., 2002), *fgf17/18* (Phillips et al., 2001; Reifers et al., 2000) and *fgf4* (Grandel et al., 2000) have been characterized. Fgf4 in zebrafish was recently cloned (Grandel et al., 2000) and its expression pattern reported in fin bud development post 35 hours, but early gastrula stage expression of Fgf4 was not described. However, an abstract (Draper, 1999) had suggested that Fgf4 was expressed during gastrulation in zebrafish. This is in agreement with Fgf4 work that was performed in chick. Shamim et al. reported that *fgf4* expression in chick was detected in the primitive streak at the onset of gastrulation and was later found in the presumptive posterior hindbrain. At later stages *fgf4* was also detected in the mesoderm underlying the hindbrain, in the somites, branchial arches, limb bud and the tail bud (Shamim and Mason, 1999).

Fgf24 was recently cloned and characterized and shown to have a role in promoting posterior mesodermal development together with Fgf8 (Draper et al., 2003). *fgf24* transcripts were detected as early as 4 hpf in the dorsalmost cells of the blastula margin and by 6 hpf extends completely around the margin. By the end of gastrulation, *fgf24* was localized to the tail bud. Morpholino-based gene knockdown of Fgf24 alone had very minor effects on posterior mesodermal development, but when Fgf24 morpholinos were injected into *acerebellar* fish (mutated *fgf8* (Reifers et al., 1998)) generating a knockdown of Fgf24 in an *fgf8* mutant background, they demonstrated a loss of most posterior mesodermal structures. However, expression of early hindbrain genes was not analyzed in this study.

The temporal expression patterns of these newly isolated zebrafish Fgf's suggest that they could play a role in early hindbrain development and thus, we sought to test the involvement of Fgf4 and Fgf24 in early hindbrain development. Here we demonstrate that Fgf4 knockdown experiments show no effect on hindbrain development, alone or in combination with other known Fgf's. Accordingly, we show that loss of Fgf24 alone or in combination with Fgf8 is unable to phenocopy the dnFGFR effect demonstrated in Chapter 1. Thus, we conclude that Fgf4 and Fgf24 alone or in combination with other known Fgfs is insufficient to be the early acting Fgf signal required for hindbrain development.

RESULTS

Zebrafish Fgf4 most closely resembles chick and mouse Fgf4

The zebrafish Fgf4 was previously cloned (Grandel et al., 2000)(AF283555) and we designed primers to the published sequence to clone Fgf4 as well (see Materials and Methods). Conceptual translation of the open reading frame yielded a 553 amino acid polypeptide. We tested the homology of zebrafish Fgf4 to Fgf4 in other species and determined that zebrafish Fgf4 is most closely related to Fgf4 in chick and mouse (Fig. 1A,B). Our phylogenetic tree also illustrates that zebrafish Fgf8, Fgf17, Fgf18 and Fgf24 are closely related in agreement with reports that suggest Fgf24 is a member of the Fgf8/Fgf17/Fgf18 subclass of Fgfs (Fig. 1B) (Draper et al., 2003; Reifers et al., 2000).

In situ analysis of FGF4 expression

Expression of *fgf4* is first detected at 11 hpf on the dorsal side of the embryo overlapping with the developing tail bud (Fig. 2a). By 13 hpf very weak expression of *fgf4* can be found at the MHB (arrow in Fig. 2b), as shown by double *in situ* analysis with the MHB marker *pax2* (Fig. 2c). Expression at this time also becomes very robust in the tail tip (Fig. 2b). This strong expression in the tail tip will persist until post 48hrs. The expression at the MHB may initially be weak (Fig2 b-e), but becomes quite strong by 18 hpf (Fig. 2f) and maintains its intensity until post 48hours. By 30 hpf *fgf4* is detected in the branchial and pharyngeal arches (Fig. 2g) and by 36 hpf is weakly detected in the forming fin bud (Fig. 2h). This expression pattern is in agreement with *fgf4* studies in chick.

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1   M S - - - - V Q S A L L P I L V L G L M - T S S V R C A FGF4 zebrafish.
1   M L - - - - S A A A L L P A L L L G L L W P G A V R G R FGF4 Chick
1   M A K R G P T T G T L L P R V L L A L V V A L A D R G T FGF4 Mouse

70  P L P G G - H S G P V E R R W E T - L Y S R S L A R I P FGF4 zebrafish.
73  P P P G R L P P G P R Q R R W D A A L F A R S V A R L P FGF4 Chick
85  A A P N G T R H A E L G H G W D G - L V A R S L A R L P FGF4 Mouse

148 G E - - - K R D I S R D S - - D Y L T G I K R L R R L Y FGF4 zebrafish.
157 A E - - - R R D A A R D G - - D Y L L G Y K R L R R L Y FGF4 Chick
166 V A A Q P P Q A A V R S G A G D Y L L G L K R L R R L Y FGF4 Mouse

217 C N V G I G F H L Q V L P G G K I T G V H N E N R Y S L FGF4 zebrafish.
226 C N V G I G F H I Q V L P D G R I D G I H S E N R Y S L FGF4 Chick
250 C N V G I G F H L Q V L P D G R I G G V H A D T R D S L FGF4 Mouse

301 L E I S P V E R G V V T L F G V R S G L F V A M N S K G FGF4 zebrafish.
310 L E I S P V E R G V V S I F G V R S G L F V A M N S K G FGF4 Chick
334 L E L S P V Q R G V V S I F G V A S R F F V A M S S R G FGF4 Mouse

385 K L Y G S E Q F T N E C K F R E K L L A N N Y N A Y E S FGF4 zebrafish.
394 K L Y G S T H V N D E C K F K E I L L P N N Y N A Y E S FGF4 Chick
418 K L F G V P F F T D E C K F K E I L L P N N Y N A Y E A FGF4 Mouse

469 L A H P G M Y I G L S K A G K T K K G N R V S T S M T M FGF4 zebrafish.
478 R I Y P G M Y I A L S K N G R T K K G N K V S P T M T V FGF4 Chick
502 Y A Y P G M F M A L S K N G R T K K G N R V S P T M K V FGF4 Mouse

553 T H F L P R I .                                       FGF4 zebrafish.
562 T H F L P R I .                                       FGF4 Chick
586 T H F L P R L .                                       FGF4 Mouse
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Figure 1A. Alignment of zebrafish with chick and mouse Fgf4. The amino acid sequences of zebrafish Fgf4, chick Fgf4 and mouse Fgf4 were aligned with the Clustal Method in the Lasergene Package from DNAStar, using PAM 250 residue weight table. Yellow shaded areas are identical to zebrafish Fgf4. Dashes indicate spaces inserted for better alignment.

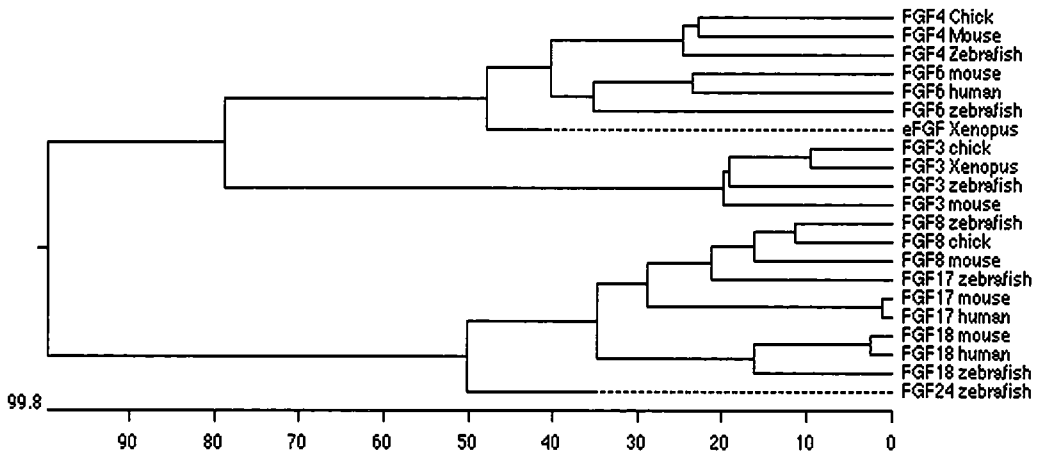

Figure 1B. Phylogenetic Tree of Fgfs. Phylogenetic analysis of various Fgfs in zebrafish, mouse, chick, *Xenopus* and human species.

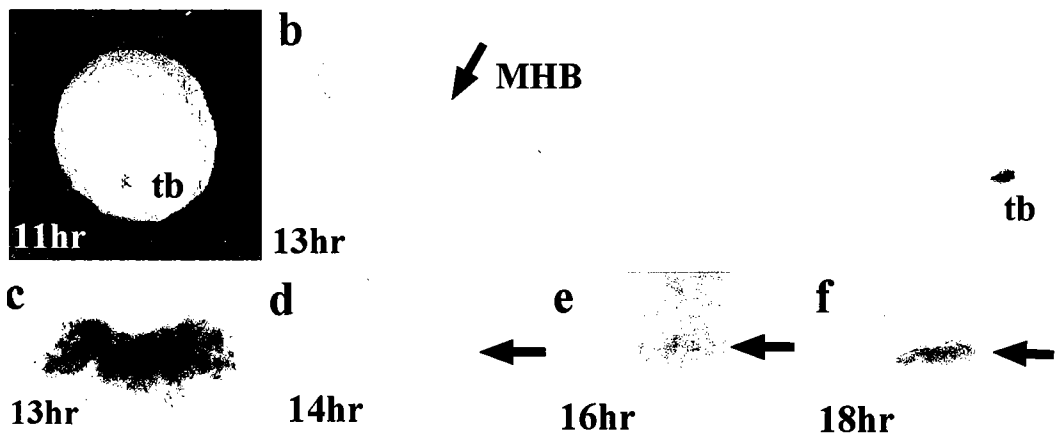

Figure 2. (a-f). Expression pattern of *fgf4* during zebrafish development. An *fgf4* probe was hybridized to embryos at 11 hpf (a), 13 hpf (b,c), 14 hpf (d), 16 hpf (e) and 18 hpf (f). Panel a is a dorsal view of a wholemount *in situ* hybridization. Panels b-f are flatmounts. Panel b is a flatmount with anterior to the left. Arrow shows weak exression of *fgf4* in the MHB. Panels c-f are close-up views of the MHB of flatmounted embryos. Arrow show increase in *fgf4* expression over time. Anterior is to the top. Panel c is a flatmount of a double *in situ* hybridization with *fgf4* in blue and *pax2* in red marking the MHB. MHB:Mid-hindbrain boundary, tb: tail bud.

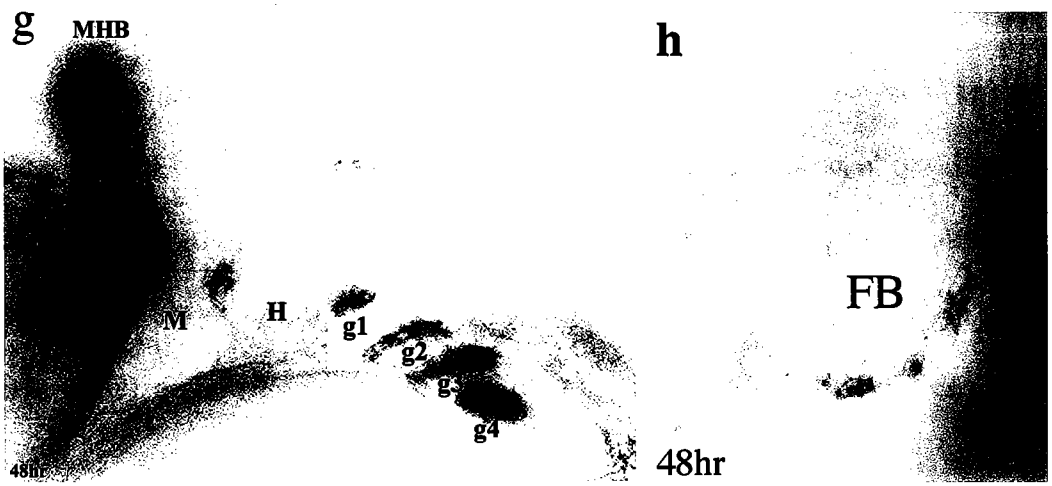

Figure 2. g-h. Expression pattern of *fgf4* during zebrafish development. An *fgf4* probe was hybridized to embryos at 48 hpf. Panel g is a close-up of a lateral view of the head. Panel h is a close-up of the fin bud. Anterior to the top. MHB: Mid-hindbrain boundary, M: mandibular arch, H: hyoid arch, g: gill arch, FB: fin bud.

Fgf4 overexpression studies

As demonstrated above, we do not detect expression of *fgf4* in zebrafish embryos until 11 hpf. However, others report *fgf4* expression during gastrula stages and suggest a role for Fgf4 with Fgf8 in mesodermal patterning at gastrula stages (Draper, 1999). This early expression discrepancy may simply be a result of a less sensitive in situ protocol utilized by this lab. Even though we do not detect *fgf4* expression during gastrula stages, we still wished to determine if Fgf4 was involved in hindbrain development. Thus, Fgf4 was overexpressed and gastrula stage hindbrain gene expression analyzed. In particular, 25 pg *fgf4* mRNA was injected in the one to two cell stage embryo, allowed to develop in 1X MBS until 10 hpf when they were fixed and processed for in situ analysis. Upon overexpression, we detect a rostralward expansion of hindbrain genes *nlz* (Fig. 3 a,b; 90%, n=69), *meis3* (Fig. 3 d,e; 89%, n=56), *hoxb1b* (Fig. 3 g,h; 100%; n=66), *hoxb1a* (Fig. 3 j,k; 97%; n=59) and of the caudal gene marker *caudal* (Fig. 3 m,n; 98%; n=61) over control levels. This is consistent with other Fgf overexpression studies described in the introduction of this thesis in that there is a rostralward expansion of posterior fates upon overexpression of Fgfs (see Introduction section: "Forming the Anteroposterior Axis").

Injection of anti-Fgf4 morpholino oligonucleotides can not phenocopy the dnFGFR results.

Our overexpression studies suggest that Fgf4 acts as a typical Fgf in promoting posterior fates in the early gastrula stage embryo, but our in situ analysis suggests that

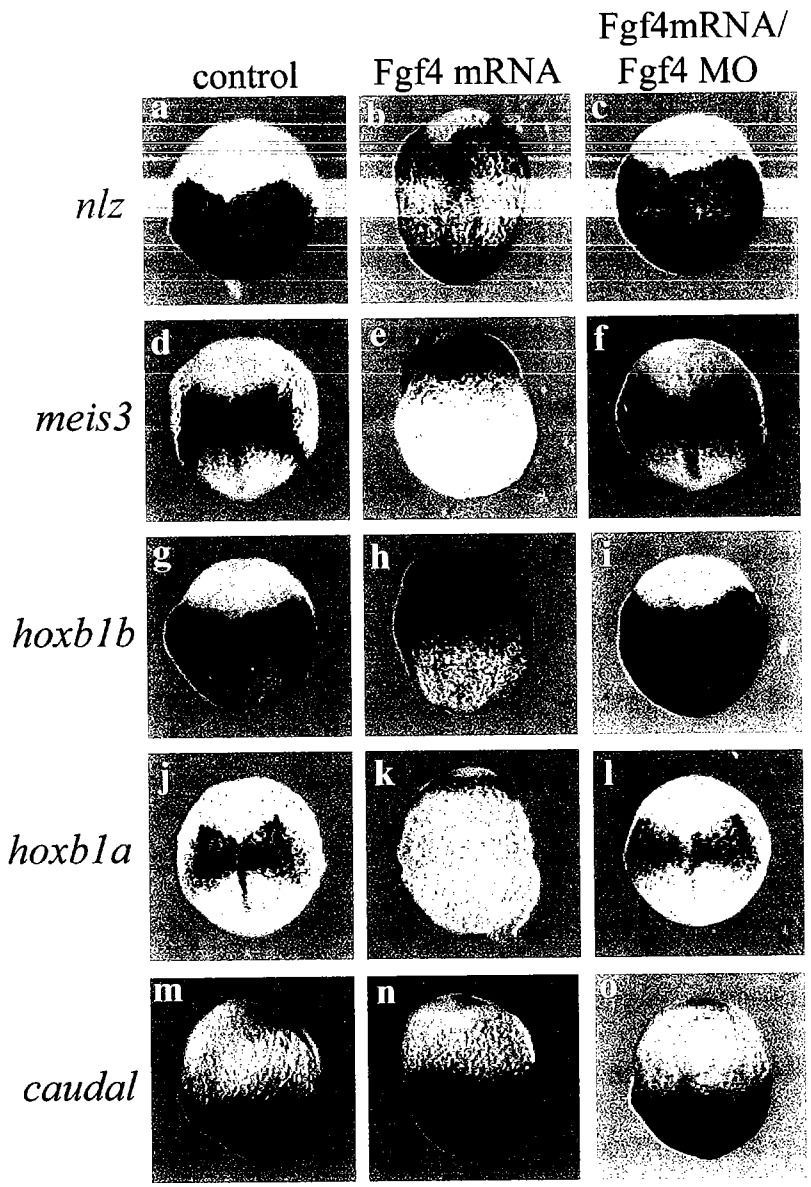

Figure 3. Fgf4-MO is functional in vivo. Embryos were injected at the one to two cell stage with control MO (a, d, g, j, m) or 25 pg *fgf4* mRNA (b, e, h, k, n) or 25 pg *fgf4* mRNA + 15 ng *fgf4*-MO, fixed at 10 hpf and analyzed by wholemount *in situ* hybridization for expression of *nlz* (a-c), *meis3* (d-f), *hoxb1b* (g-i), *hoxb1a* (j-i) and *caudal* (m-o). All panels are dorsal views with anterior to the top.

fgf4 is not expressed until 11 hpf. However, given the reported earlier expression of Fgf4 by others (Draper, 1999) and our demonstrated overexpression phenotype, we thus set out to examine if knocking down Fgf4 protein via morpholino injection was sufficient to phenocopy the dnFGFR effect seen in Chapter 1.

To first determine if the Fgf4 morpholino was functional and could specifically block translation of *fgf4,* we performed an in vitro transcription/translation assay. Namely, in vitro translated *fgf4* mRNA was incubated with lysate mix (see materials and methods) and incubated in the presence or absence of 40 μM Fgf4 or control morpholinos. We detect that in the presence of *fgf4* RNA with no added morpholino that a 21 kDA band corresponding to Fgf4 was translated (Fig 4, lane2). However, in the presence of *fgf4* RNA and 40 μM anti-Fgf4 MO, we detect a total loss of *fgf4* translation (Fig 4, lane 3). The ability to block *fgf4* translation was specific to the anti-Fgf4 MO as a block in translation is not detected with the Fgf4 control morpholino, Fgf4 inverse MO, or with two other random morpholinos (Fig 4. lanes 4-6). These results demonstrate the specificity of the anti-Fgf4 morpholino and show that in an in vitro assay, Fgf4 translation can effectively be inhibited upon addition of anti-Fgf4 MO.

Once the morpholino was determined to be functional and specific in vitro, we next determined if knockdown of Fgf4 could phenocopy the dnFGFR effect in vivo. Thus anti-Fgf4 morpholinos were injected into the one cell stage and changes of gene expression determined by in situ analysis. No change in early hindbrain gene expression was noted even at extremely high concentrations (34 ng) of Fgf4 morpholino (not shown). No change in MHB marker genes was noted as well, but this could clearly be a

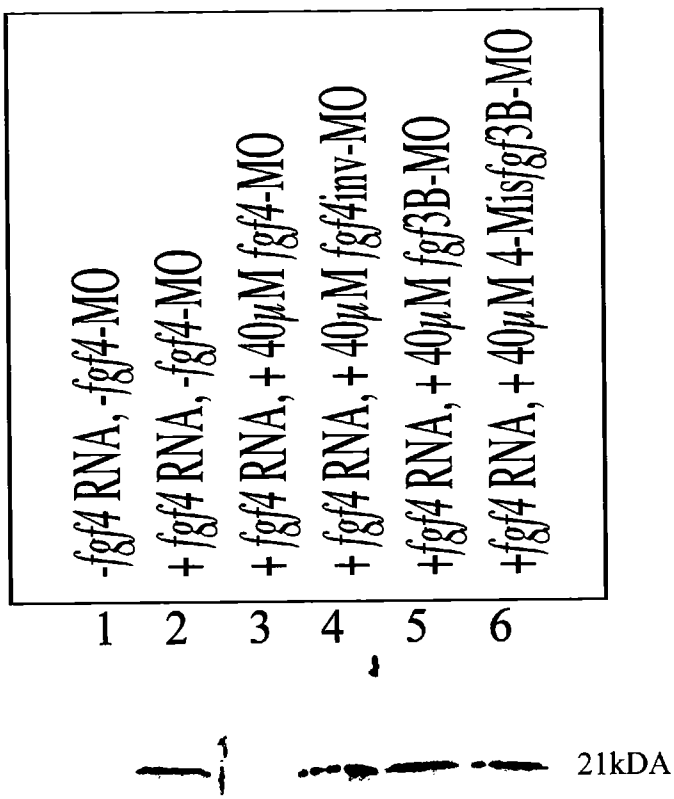

Figure 4. Fgf4-MO is functional in vitro. In vitro TNT's of: Lane 1: Control lane with no RNA or morpholino. Lane 2: 100 ng *fgf4* RNA with no morpholino. Lane 3: 100 ng *fgf4* RNA incubated with 40 μM fgf4-MO. Lane 4: 100 ng *fgf4* RNA incubated with 40 μM fgf4inv-MO, the inverse sequence to *fgf4*, as a control. Lane 5: 100 ng *fgf4* RNA incubated with 40 μM fgf3B-MO, a control. Lane 6: *fgf4* RNA incubated with 40 μM 4-MISfgf3B-MO, another control. The Fgf4 translation product is 21 kDA.

result of compensation by other Fgf's expressed there, namely Fgf8. As we did not see a visible phenotype upon injection of Fgf4-MO, we wished to confirm that the morpholino was in fact functional in vivo. To test this we overexpressed *fgf4* mRNA and attempted to rescue the overexpression phenotype (as seen in Fig. 3 b,e,h,k,n) by coinjection with anti-Fgf4 MO. As described earlier, upon overexpression of *fgf4* mRNA, we detect a rostral-ward expansion of hindbrain genes *nlz, meis, hoxb1b, hoxb1a* and of the caudal gene marker *caudal* (Fig. 3, panels b,e,h,k,n). However, upon co-injection of 25 pg *fgf4* mRNA and 15 ng of anti-Fgf4 MO, this effect is largely reverted back to a wild type phenotype for *nlz* (Fig. 3 panel c, 93% wild type, n=55), *meis3* (Fig. 3 panel f, 87% wild type, n=54), *hoxb1b* (Fig. 3 panel i, 92% wild type, n=61), *hoxb1a* (Fig. 3 panel l, 86% wild type, n=69) and *caudal* (Fig. 3 panel o, 100% wild type, n=53). This confirms that *in vivo* the anti-Fgf4 MO is functional.

Although no effect on hindbrain genes could be detected upon injection of anti-Fgf4 MO alone, it does not rule out the possibility that Fgf4 is involved in early hindbrain gene regulation. It is possible that Fgf4 function is required along with other Fgf's, and these other Fgf's may compensate for a loss of Fgf4 in hindbrain development in the presence of the anti-Fgf4 MO. In order to test this, we co-injected the anti-Fgf4 MO with morpholinos targeted to the other known Fgf's, namely Fgf3 and Fgf8. Upon co-injection of a cocktail of all four morpholinos (fgf3B, fgf3C, fgf4, and fgf8), we detected a loss of *krox20* expression in r5 (97% affected, n=27) and *valentino* expression in r5/r6 (100% affected, n=8) at somite stages. These results suggest that the anti-*fgf8* and anti-*fgf3* morpholinos were functional in agreement with previously published work (Maves et

al., 2002; Walshe et al., 2002). Again however, no change in early hindbrain genes was detected (not shown). Thus, the combined knockdown of Fgf4, Fgf3 and Fgf8 has no effect on early hindbrain gene development. Thus, we can conclude that Fgf4 alone or Fgf4 in combination with Fgf3 and Fgf8 are insufficient to phenocopy our dnFGFR results.

Injections of anti-Fgf24 morpholino oligonucleotides can not phenocopy the dnFGFR result.

Recently zebrafish Fgf24 was cloned and its role in posterior mesodermal development characterized (Draper et al., 2003). This study reports that *fgf24* is co-expressed with *fgf8* in mesodermal precursors during gastrulation. Inhibition of Fgf24 alone has very minor effects on mesodermal development, but co-injection of both anti-Fgf8 and anti-Fgf24 morpholinos blocks the formation of posterior mesodermal structures. Thus, both Fgf8 and Fgf24 are required to promote posterior mesodermal development. More importantly, Draper et al. (Draper et al., 2003) show *fgf24* is expressed as early as 4 hpf in the dorsalmost cells of the blastula margin. By 6 hpf, *fgf24* was detected completely around the margin and continued to be expressed in the marginal cells throughout gastrulation. By 80% epiboly, *fgf24* was detected in the developing neuroectoderm and by the end of gastrulation *fgf24* is localized to the tail bud. This early temporal and spatial expression pattern of *fgf24* suggests that Fgf24 is a prime candidate to regulate early hindbrain gene expression in the hindbrain primordium.

In order to determine if Fgf24 regulates early hindbrain genes, anti-Fgf24 morpholinos were injected at the one to two cell stage and changes in hindbrain gene expression were detected by in situ analysis at 10 hpf. We find no effect on any of our early hindbrain genes, *caudal* (Fig. 5A. panel b), *hoxb1b* (Fig. 5A. panel e), *nlz* (Fig. 5A. panel h), *meis3* (Fig. 5A. panel k), *hoxb1a* (Fig. 5A. panel n), the forebrain marker *otx2* (Fig. 5A. panel q) or a mesodermal marker *ntl* (Fig. 5A. panel t). These results suggest that Fgf24 alone does not regulate early hindbrain genes. We next co-injected the Fgf24 morpholino along with an anti-Fgf8 morpholino. Knockdown of both Fgf's has been reported to block posterior mesodermal development (Draper et al., 2003). However, in the presence of both anti-Fgf24 and anti-Fgf8 morpholinos, we again detected no change in early expression of the hindbrain genes *caudal* (Fig. 5A. panel c), *hoxb1b* (Fig. 5A. panel f), *nlz* (Fig. 5A. panel i), *meis3* (Fig. 5A. panel l), *hoxb1a* (Fig. 5A. panel o) or the forebrain marker *otx2* (Fig. 5A. panel r). *ntl*, a mesodermal marker, was used as a control and was downregulated (Fig. 5A. panel u) in the presence of both morpholinos in agreement with previously published work (Draper et al., 2003). This illustrates the morpholinos were functional. After morpholino injection, sibling embryos were incubated to 14 hpf or 24 hpf and were also examined. At 14 hpf, *ntl* is extremely downregulated (75% lost, 24% extremely reduced) in the mesodermal precursors of the tailbud in Fgf24/Fgf8 knockdown embryos (Fig. 5B. panel c) compared to control (Fig. 5B. panel a) or the single knockdown of Fgf24 (Fig. 5B. panel b). By 24 hpf, Fgf24/Fgf8 morpholino injected embryos show little paraxial mesoderm development and significantly shorter tails (97% affected)(Fig. 5C. panel c and d) than control-MO

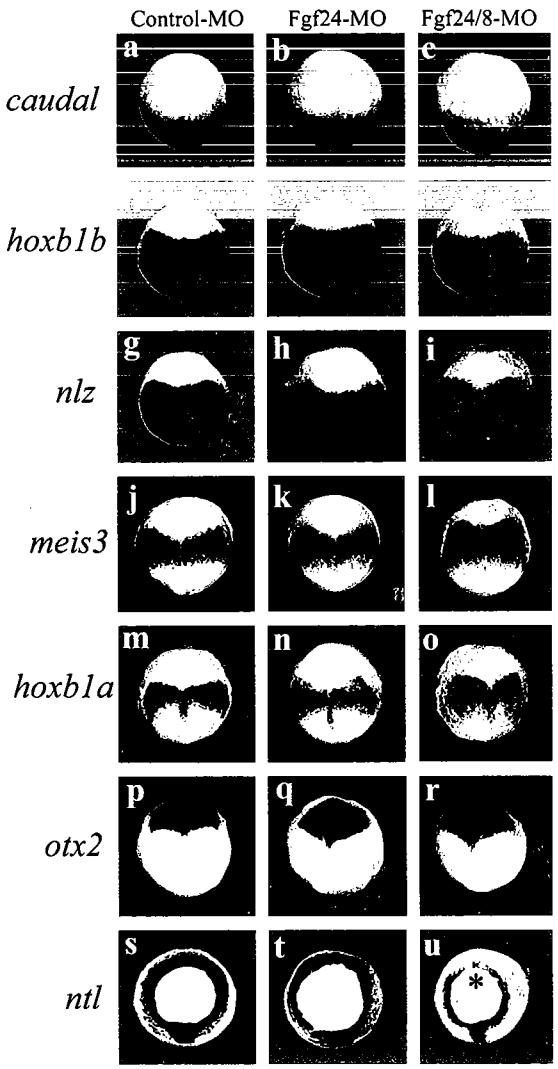

Figure 5.A. Fgf 24 and Fgf8 do not regulate gastrula stage hindbrain genes expression. Embryos were injected at the one to two cell stage with control MOs (a, d, g, j, m, p, s), anti-Fgf24 MOs (b, e, h, k, n, q, t) or anti-Fgf24/anti-Fgf8 MOs (c, f, i, l, o, r, u) raised to 10 hpf and analyzed by wholemount in situ hybridization for expression of *caudal* (a-c), *hoxb1b* (d-f), *nlz* (g-i), *meis3* (j-l), *hoxb1a* (m-o), *otx2* (p-r) and *ntl* (s-u). All panels are dorsal views with anterior to the top except for panels s-u which are vegetal pole views. Asterisks indicates loss of mesodermal precursors compared to control.

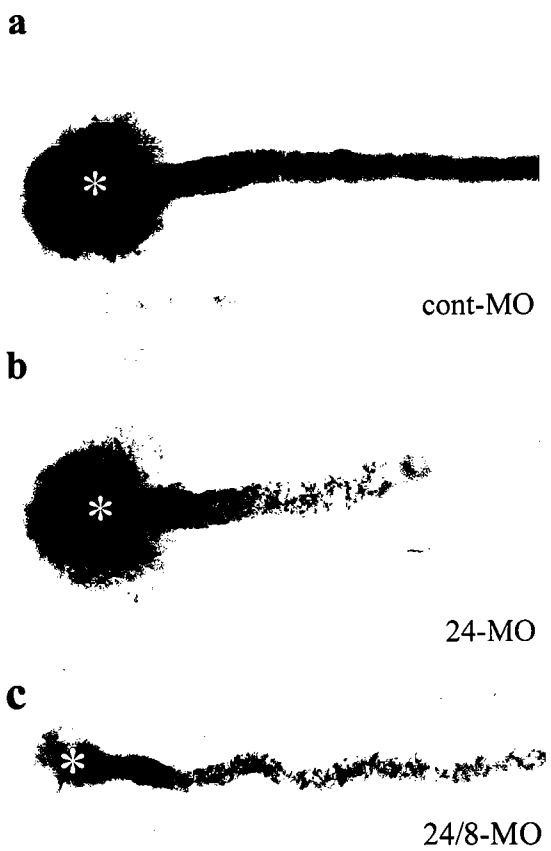

Figure 5.B. Fgf24/Fgf8-MO injected embryos do not have mesodermal precursors in the tail. Emrbyos were injected at the one to two cell stage with control morpholino (a), anti-Fgf24 morpholino (b) or anti-Fgf24 and anti-Fgf8 morpholino (c) and raised to 14 hpf when they were fixed and processed for in situ analysis for expression of *ntl*. All panels are flatmounts with tail to the left. Asterisks indicate mesodermal precursors in the tailbud which are reduced in anti-Fgf24/anti-Fgf8 MO expressing embryos.

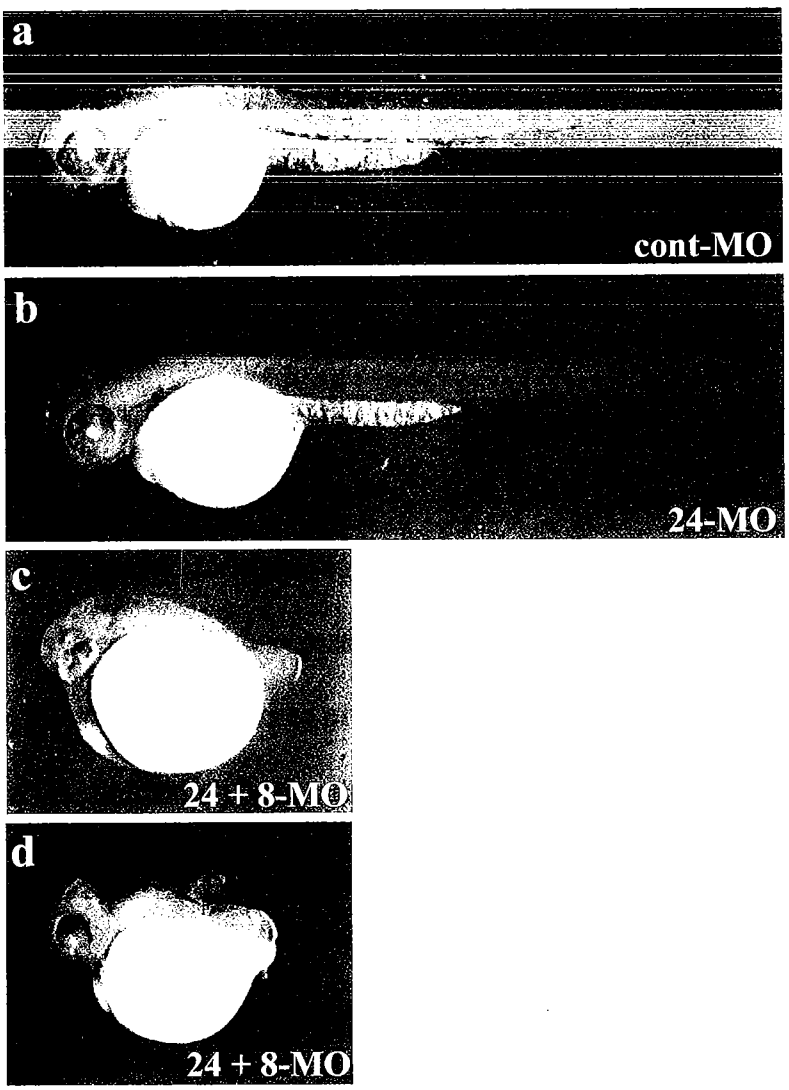

Figure 5.C. Fgf24/Fgf8 Morpholino injected embryos show lack of paraxial mesodermal structures. Embryos were injected at the one to two cell stage with control morpholino (a), anti-Fgf24 morpholino (b) or anti-Fgf24 and anti-Fgf8 morpholinos, raised to 48 hpf and fixed. All panels are lateral views with anterior to the left.

injected (Fig. 5C. panel a) or Fgf24-MO injected siblings (Fig. 5C. panel b), again demonstrating the morpholinos were functional. Thus, Fgf24 alone or in combination with Fgf8 are not responsible for early hindbrain gene regulation.

DISCUSSION

In Chapter 1 of this thesis, we clearly identify a need for early acting Fgf signaling for gene expression throughout the hindbrain primordium at early gastrula stages. We demonstrate here that this early Fgf signal is not provided by Fgf4 or Fgf24 alone or by a combination of either factor with known Fgfs.

Given the post-gastrula expression pattern detected for Fgf4 in this thesis, we were not surprised that knockdown of Fgf4 alone or in combination with Fgf8 and Fgf3 showed no effect on hindbrain gene expression. Unlike *fgf4*, *fgf24* is expressed as early as 4 hpf and therefore was a prime candidate for the Fgf which regulates the hindbrain genes studied in this thesis. Therefore, we were surprised that Fgf24 knockdowns alone or in combination with Fgf8 were insufficient to phenocopy the dnFGFR effect we defined in this study.

During the course of our Fgf knockdown experiments, we have suggested that neither Fgf4 nor Fgf24 alone or in combination with other known Fgfs tested are responsible for early hindbrain gene expression. However, we can not rule out the possibility that these Fgf's are involved with another essential as yet unidentified Fgf. We also cannot test all permutations of all known Fgf's via morpholino knockdown

technology, as injected more than four morpholinos per injection is extremely difficult, if not impossible. Thus, we can not rule out the possibity that early hindbrain genes are regulated by a combination of multiple Fgfs. We also cannot conclude that the morpholino approach knocks down all expression of the targeted protein. Although we were able to faithfully phenocopy the loss of trunk and tail mesodermal derivatives in Fgf24 knockdown experiements, we simply may not see an effect on the hindbrain genes because the hindbrain is more sensitive to residual protein than is mesoderm.

The zebrafish *fgf6* gene has been identified and catalogued in the Genbank (AF516334), but work on Fgf6 has yet to be published. Thus, the expression pattern of *fgf6* is not yet known in zebrafish. However, *fgf6* in mouse has been reported and found specifically localized to the skeletal muscle at post-gastrulation stages (deLapeyriere et al., 1993). No work on *fgf6* in *Xenopus* or chick has been published. However, given the late expression pattern in mouse and the lack of *fgf6* expression in any neural tissue, *fgf6* may not be a potential candidate for the FGF which regulates early hindbrain gene expression.

Thus, the future of this work lies first and foremost with cloning more members of the Fgf family. As Fgfs share approximately 30-60% amino acid homology (Ford-Perriss et al., 2001), it is possible to create degenerate PCR primers for the amplification of more Fgfs. In fact, this is how the known Fgfs were first cloned. For instance, *fgf3* was cloned using degenerate PCR primers corresponding to a conserved region of protein found in mouse and human homologues (Kiefer et al., 1996). Perhaps the early acting Fgf that we suggest regulates early hindbrain development could be isolated in this

manner. Secondly, the impact of the zebrafish genome sequencing cannot be overlooked. In February 2001, the Sanger Center, the Max-Planck Institute for Developmental Biology and Harvard Medical School joined forces and began sequencing the zebrafish genome by using BAC libraries as well as by a "shotgun" strategy to produce many short stretches of sequence that, in the end, would be assembled. It is hoped that the complete annotated sequence of the genome will be available to researchers at the end of 2003. As the zebrafish genome is sequenced to completion, unknown Fgfs will certainly be discovered.

MATERIALS AND METHODS

Cloning FGF4

The FGF4 sequence was obtained from GENBANK (AF283555) (Grandel et al., 2000). Gene specific primers were designed to PCR amplify the sequence from 10 somite staged total RNA (Qiagen RNeasy Kit according to manufactures protocols). 5'primer: 5'-ATGAGTGTCCAGTCGGCCCTCTT-3", 3'primer: 5'-TCAAATTCTAGGCAAGAAATGTGTCATCGTCA-3'. PCR for 30 cycles as follows: cycle 1: 94°C for 30 seconds, cycle 2: 55°C for 20 seconds, cycle 3: 72°C for 2 minutes. The resulting PCR product was subcloned via EcoR1 and Xho1 sites engineered into the primers into pCRII (NOT2.1 Invitrogen) for *in situ* probe and pCS2+ for mRNA overexpression and sequenced.

Fgf4 *in situ* hybridization

To prepare an antisense probe, the Fgf4 pCRII plasmid was digested with Not1 and transcribed with SP6 RNA polymerase. For sense probe, Fgf4 plasmid was digested with BamHI and transcribed with T7 RNA polymerase. In situ hybridization was performed as previously described (Sagerstrom et al., 1996).

Fgf4 mRNA injection

mRNA was synthesized from Not1-linearized pCS2+ plasmids using the SP6 Message Machine kit (Ambion) and purified with the RNeasy Mini Kit (Qiagen). For

injections, 25-50 pg of mRNA was injected at the 1-2 cell stage and fixed at 10 hpf for *in situ* analysis.

FGF4 *in vitro* transcription and translation

In vitro translation of synthetic mRNA was performed by using Promega's Rabbit reticulolysate system according to manufacturer's protocols. A total of 100 ng of *fgf4* mRNA was added to 5.25 μl of lysate mix with [^{35}S]Methionine, RNasin (Promega) and amino acid mix (Promega) and was incubated at 30°C for 90 minutes in the presence or absence or morpholino ranging from 20-400 μM. The translation products were electrophoresed through a 15% agarose SDS-PAGE gel, transferred to Watman paper, dried and exposed for 2 hours.

Morpholino injections

Antisense morpholino oligonucleotides (MO's) were ordered from GeneTools as follows: *fgf4-MO*: 5'-GCAAGAGGGCCGACTGGACACTCAT-3'; *fgf4inverse-MO*: 5'-TACTCACAGGTCAGCCGGGAGAACG-3'. 1-2 cell stages embryos were injected and allowed to develop until 10 hpf, fixed in 4% paraformaldehyde and stored in MeOH prior to in situ hybridization. *fgf8*, 4-MIS*fgf8*, *fgf3*B, *fgf3*C, 5-MIS*fgf3*B and 5-MIS*fgf3*C were ordered based on published reports (Maves et al., 2002; Shinya et al., 2001). Embryos were injected at the 1-2 cell stage with a cocktail of approximately 12 ng *fgf4*, 4.25 ng *fgf3*B, 4.25 ng *fgf3*C and 11 ng *fgf8* or an equivalent amount of *fgf4*inverse, 4-MIS *fgf8*, 5-MIS*fgf3*B, 5-MIS*fgf3*C and processed as described above.

For *fgf24*, morpholinos were ordered as described in (Draper et al., 2003). *fgf8*-MO#2 was utilized for this set of experiments (Maves et al., 2002).

ACKNOWLEDGMENTS

We wish to thank Dr. Phillip DiIorio for assistance in creating the PCR primers to clone Fgf4 and for performing the PCR reaction. This work was supported by grant NS38183 to CGS.

CONCLUSION

LOOKING TOWARDS THE FUTURE: OUTLOOK FOR FGFS IN NEURAL DEVELOPMENT

This Thesis has demonstrated a role for Fgfs in early hindbrain development. In addition to this work, many research articles have demonstrated roles for specific Fgfs throughout a wide range of early and late neural developmental processes. Currently in zebrafish Fgfs 3, 4, 8 and 17 have been shown to play critical roles in neural developmental (Holzschuh et al., 2003; Irving and Mason, 2000; Lam et al., 2003; Maves et al., 2002; Phillips et al., 2001; Reifers et al., 2000; Shinya et al., 2001; Walshe et al., 2002; Walshe and Mason, 2003).

In other vertebrate species, namely chick and mouse, up to 10 Fgfs have been shown to play a role in brain development from the early stages of neural induction to late stage neuronal connectivity, spanning a vast spatial and temporal period in neural development (Ford-Perriss et al., 2001). Complicating the matter further is the

expression in vertebrates of four different Fgf receptors (FGFR), three of which have multiple isoforms expressed in a highly specific but dynamically changing manner throughout development (Ford-Perriss et al., 2001). The combination of multiple Fgfs, multiple FGFR isoforms and varying temporal and spatial expression patterns of them both creates a complex web of Fgf regulation in brain development.

In mouse, Fgf-FGFR interactions have been studied primarily in cell lines. Reports in mouse cell lines have demonstrated that Fgf3 binds with high affinity to FGFR-1 IIIb and FGFR-2 IIIb isoforms (Mathieu et al., 1995) and FGFR-3 IIIc was preferentially activated by Fgf1 (Lin et al., 1997). In vivo studies are sparse, but one report in mouse has demonstrated that Fgf10 signals through the FGFR-2 IIIb isoform for formation of the otic placode (Ornitz et al., 1996; Pirvola et al., 2000) and one report in *Xenopus* has shown that Fgf8 signals through FGFR-4 in mesodermal induction (Hardcastle et al., 2000). However, much about specific ligand receptor pairs still remains elusive.

Although knowledge of Fgf ligand and receptor interactions in cell lines and in vivo is limited, our knowledge of Fgfs and their cognate receptors role in neural development in zebrafish pales in comparison. There are significant gaps in our understanding of Fgfs, FGFRs and neural development in zebrafish. Namely, isolation of Fgfs in zebrafish is still in its infancy. This lack of a full complement of Fgfs significantly reduces our understanding of neural development as illustrated by this Thesis work. Although we can conclude that Fgfs are involved in the critical processes of early hindbrain development, we cannot specifically determine which Fgf or Fgfs

perform this function. As time passes however, more Fgfs will be isolated, cloned and studied and roles for other Fgfs in neural development elucidated. Secondly the four FGFRs present in zebrafish have only been cloned within the past two years and initial expression studies have only been performed (Sleptsova-Friedrich et al., 2001; Thisse et al., 1995; Tonou-Fujimori et al., 2002). No studies have been performed to determine the specifics of Fgf-FGFR interactions in zebrafish.

Thus, the future of Fgfs in neural development lies with isolating more Fgfs, determining their expression patterns and characterizing their functions, determining more extensively the expression patterns of FGFRs as well as characterizing the unique combinations of FGFR isoforms with specific Fgfs both temporally and spatially throughout neural development. This data is essential to elucidate the processes of Fgf signaling in neural induction, neural patterning, neural proliferation and neuronal specification.

APPENDIX 1

FGF REGULATES ZEBRAFISH *lmo4* GENE EXPRESSION

The zebrafish *lmo4* (limb-only domain) is a LIM containing protein (Grutz et al., 1998) (Kenny et al., 1998) (Racevskis et al., 1999) (Tse et al., 1999) that was recently cloned and its expression pattern temporally and spatially characterized during the first 48 hours of development (Lane et al., 2002). LMO proteins serve to help form transcription regulatory complexes by binding to LIM containing proteins as well as other proteins (e.g. bHLH and GATA proteins (Rabbitts, 1998)). Zebrafish *lmo4* was found to be expressed in the somites, branchial arches, otic vesicle, pectoral fin buds, developing eye as cardiac tissue at late post-gastrula stages (Lane et al., 2002). During early gastrula stages, zygotic *lmo4* expression was detected by 6 hpf in the germ ring and by 8 hpf becomes distributed in the dorsal ectoderm. By the bud stage (10 hpf), *lmo4* expression is restricted to the rostral hindbrain. This expression profile of *lmo4* overlaps with that of

the early gastrula stage Fgf- dependent domain characterized in this thesis work. This suggests that *lmo4* is regulated by Fgf.

To test if *lmo4* is regulated by Fgf, we first injected dnFGFR mRNA (as described in Chapter 1) at the one to two cell stage and the injected embryos were raised to late gastrula stage (9-10 hpf) when they were fixed and processed for wholemount in situ hybridization analysis. We find a reduction in *lmo4* expression in dnFGFR expressing cells varying from unilateral (Fig. 1b) to complete bilateral loss (Fig. 1c) of gene expression most likely dependent on distribution of injected dnFGFR mRNA. To further explore Fgfs role in regulating *lmo4*, we implanted Fgf-soaked beads at the animal pole (as described in Chapter 1) of 4 hpf wild type embryos, raised the embryos to tailbud stage when they were fixed and processed for in situ analysis with an *lmo4* probe. We find that *lmo4* is ectopically induced quite strongly over the implanted Fgf bead (Fig. 1e). Thus, we conclude that early gastrula stage *lmo4* expression is Fgf dependent. This is in agreement with work that shows a decrease of *lmo4* expression in the MHB region (Lane et al., 2002) of *acerebellar* mutant fish, a fish mutation in the *fgf8* gene required for maintenance of the MHB (Reifers et al., 1998).

We also analyzed cross sections of 10 hpf wild type embryos to determine germ layer distribution of *lmo4*. We detected weak expression of *lmo4* in the tail in all germ layers (Fig. 1f) but very robust expression in all germ layers in the presumptive hindbrain region (Fig. 1g). No expression of *lmo4* was detected in the head region (Fig. 1h).

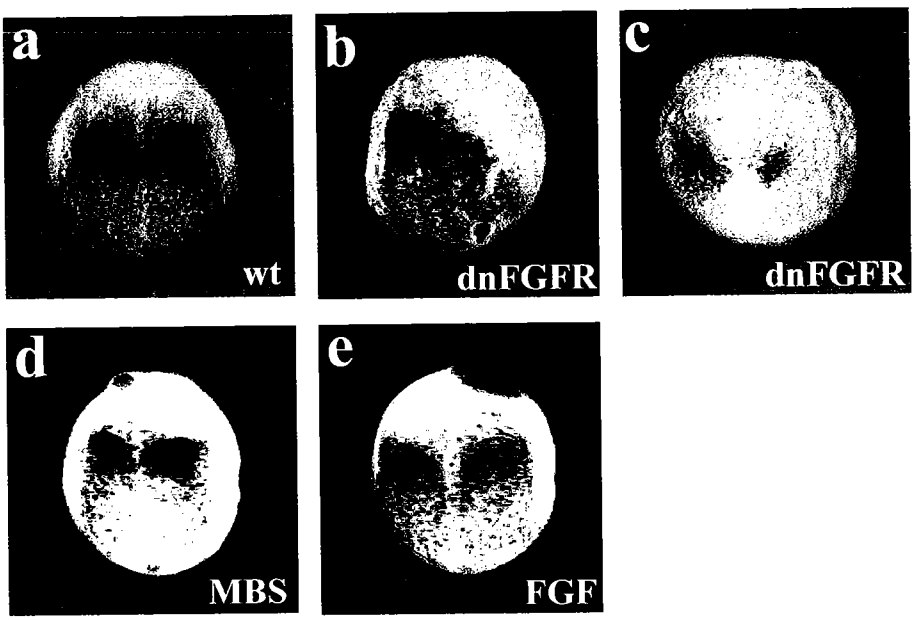

Figure 1 (a-e). Fgf regulates lmo4 gene expression. Embryos were injected at the one to two cell stage with 500 pg LacZ control (a) or 500 pg dnFGFR mRNA (b, c) or beads soaked in 1X MBS (d) or 0.5 mg/ml bFGF (e) were implanted into the animal pole of 5 hpf embryo, fixed at 10 hpf and analyzed by wholemount *in situ* hybridization for expression of *lmo4* (a-e). All panels are dorsal with anterior to the top.

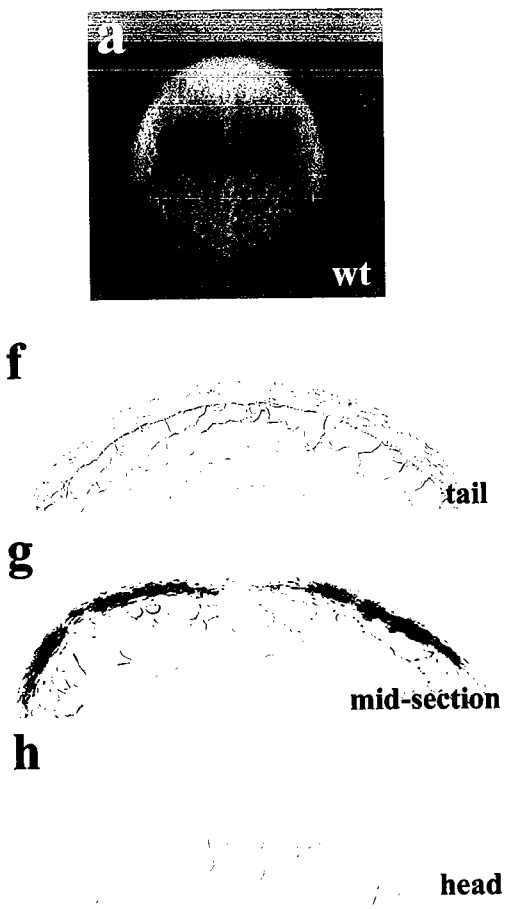

Figure 1. (a,f-g): Maximum *lmo4* expression is detected in the presumptive hindbrain. Wild type embryos were subjected to wholemount *in situ* hybridization with an *lmo4* probe at 10 hpf (a). Transverse sections of those embryos were subsequently taken (f-g). (f): Transverse section taken of caudal domain. (g): Transverse section taken through presumptive hindbrain region. (h): Transverse section taken through presumptive head region.

Parts of this work have appeared in separate publication:

Lane, M.E., Runko, A.P., Roy, N.M., and Sagerstrom, C.G. (2002). Dynamic expression and regulation by Fgf8 and Pou2 of the zebrafish LIM-only gene, *lmo4*. *Gene Expression Pattens* 2, 207-211.

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