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Zebrafish Lmx1b.1 and Lmx1b.2 are Required for Maintenance of the Isthmic Organizer

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SUMMARY

The mesencephalic and metencephalic region (MMR) of the vertebrate central nervous system develops in response to signals produced by the isthmic organizer (IsO). We previously reported that the LIM homeobox transcription factor *Lmx1b* is expressed within the chick IsO where it is sufficient to maintain expression of the secreted factor *wnt1* In this paper, we show that zebrafish express two *Lmx1b* orthologs, *lmx1b.1* and *lmx1b.2* in the rostral IsO, and demonstrate that these genes are necessary for key aspects of MMR development. Simultaneous knockdown of Lmx1b.1 and Lmx1b. 2 using morpholino antisense oligos results in a loss of *wnt1*, *wnt3a*, *wnt10b*, *pax8*, and *fgf8* expression at the IsO, leading ultimately to programmed cell death and the loss of the isthmic constriction and cerebellum. Single morpholino knockdown of either Lmx1b.1 or Lmx1b.2 has no discernible effect on MMR development. Maintenance of *lmx1b.1* and *lmx1b.2* expression at the isthmus requires the function of *no isthmus/pax2.1*, as well as Fgf signaling. Transient misexpression of Lmx1b.1 or Lmx1b.2 during early MMR development induces ectopic *wnt1* and *fgf8* expression in the MMR, as well as throughout much of the embryo. We propose that Lmx1b.1 and Lmx1b.2 regulation of *wnt1*, *wnt3a*, *wnt10b*, *pax8*, and *fgf8* maintains cell survival in the isthmocerebellar region.

Keywords

Isthmus; Lmx1b; mesencephalon; metencephalon; Pax; Wnt; zebrafish

INTRODUCTION

The cerebellum and tectum develop in response to signals emanating from an organizer located at the isthmus, a constriction of the neural tube that forms between the mesencephalic and metencephalic vesicles (Joyner, 1996; Puelles et al., 1996; Wassef and Joyner 1997; Liu and Joyner, 2001). The organizing activity of the isthmus has been demonstrated by numerous classical embryonic manipulations (Nakamura et al., 1986; Alvarado-Mallart et al., 1990; Gardner and Barald, 1991; Itasaki et al., 1991; Martinez et al., 1991; Bally-Cuif et al., 1992; Marin and Puelles, 1994; Martinez et al., 1995). Several important signaling molecules and transcription factors have been shown to mediate isthmic organizer (IsO) function. These molecules coordinate the development of cells within the mes-metencephalic region (MMR), as well as provide necessary autocrine functions to maintain the IsO. Defining the signaling pathways that direct MMR cell fates and determining the mechanisms by which these pathways are regulated remain long-term goals for understanding IsO development.

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The IsO is positioned at the juxtaposition of the *otx2* and *gbx2* expression domains (Simeone et al., 1992; Millet et al., 1996; Wassarman et al., 1997; Niss and Leutz, 1998; Shamim and Mason, 1998; Li and Joyner, 2001). Coincident with this border is the expression boundary of two major signaling molecules, *wnt1* and *fgf8*. During IsO maintenance, *wnt1* is expressed at the caudal edge of the mesencephalic vesicle (Wilkinson, et al., 1987; Bally-Cuif et al., 1992; Kelly and Moon, 1995; Hidalgo-Sanchez et al., 1999), and *wnt1* mutant mice fail to maintain a number of mesencephalic and metencephalic structures (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). While Wnt1 signaling is necessary for MMR development, ectopic Wnt1 does not appear to be sufficient to globally change the fate of MMR cells (Adams et al., 2000). In contrast, isthmic *fgf8* expression is refined to the rostral edge of the metencephalic vesicle (Heikinheimo et al., 1994; Crossley and Martin, 1995; Reifers et al., 1998) and is necessary and sufficient to mediate IsO function (Brand et al., 1996; Meyers et al., 1998; Reifers et al., 1998). In fact, ectopic Fgf8 induces changes in gene expression and morphology strikingly similar to transplantation of isthmic tissue (Crossley et al., 1996; Funahashi et al., 1999; Martinez et al., 1999; Shamim et al., 1999). IsO regulation also requires a number of transcription factors that work in a coordinated fashion, including members of the Pax family (Brand et al., 1996; Favor et al., 1996; Torres et al., 1996; Lun and Brand, 1998; Pfeffer et al., 1998), the Engrailed family (Millen et al., 1994; Wurst et al., 1994), and Lmx1b.

Lmx1b is a LIM-homeodomain protein whose role in IsO patterning has only been addressed in gain-of-function studies. Originally identified as a regulator in dorsoventral limb patterning (Riddle et al., 1995; Vogel et al., 1995; Chen et al., 1998), Lmx1b has recently been shown to be required for dopaminergic and serotonergic neuron development in vertebrates (Smidt et al., 2000; Cheng et al., 2003). We originally reported that Lmx1b was expressed in the chick MMR and, using a retroviral approach, demonstrated that it was sufficient to maintain the expression of Wnt1 in the mesencephalon (Adams et al., 2000). More recently, Matsunaga et al. (2002) used an electroporation approach in the chick to demonstrate that Lmx1b induced *wnt1* cell-autonomously and *fgf8* non-cell-autonomously. However, direct evidence of a requirement for Lmx1b in IsO function has been lacking.

To further elucidate transcriptional regulation of the IsO, we have extended these studies to the zebrafish. The zebrafish provides a powerful means of studying the genetic basis of IsO formation and function. IsO regulation appears largely conserved among vertebrates, and the relative ease of gain- and loss-of-function experiments in zebrafish allows for a number of developmental studies not possible in the chick. Mutants of several major IsO genes are available in zebrafish, including *pax2.1* (*no isthmus*) (Lun and Brand, 1998) and *fgf8* (*acerebellar*) (Reifers et al., 1998). Study of these mutants and other developmental studies in fish have contributed to our understanding of a regulatory feedback loop that maintains IsO patterning function.

Here we report the isolation and functional analysis of *lmx1b.1* and *lmx1b.2*, two zebrafish orthologs of *lmx1b*. Loss- and gain-of-function studies indicate that these two closely related transcription factors have redundant functions in maintaining gene expression and cell fate at the IsO, and that these genes are necessary and sufficient for maintenance of *wnt1* and *fgf8* expression. Pax2.1 is required for maintenance of *lmx1b.1* and *lmx1b.2* at the IsO, and Lmx1b. 1 and Lmx1b.2 are required for *pax8* maintenance. We propose a model in which Lmx1b.1 and Lmx1b.2 cooperate with Pax, Wnt, and Fgf genes to maintain the IsO.

MATERIALS AND METHODS

Zebrafish methods

Zebrafish were raised under standard lab conditions as described before (Mullins et al., 1994). All injections were performed with wild-type TL embryos. Developmental stage was

determined according to Kimmel et al. (1995). *No isthmus* (*noi*) heterozygous mutants were verified by random intercrosses and homozygous mutant embryos were produced by crossing heterozygous parents.

Isolation of *lmx1b.1* and *lmx1b.2*

Based on the published sequence of hamster (German et al., 1992) and human (German et al., 1994) Lmx1a, and human (Iannotti et al., 1997), mouse (Chen et al., 1998), and chick (Tsuchida et al., 1994) Lmx1b, a single pair of degenerate PCR primers was designed using the amino acid sequences indicated in Fig.1A, that was predicted to amplify both *lmx1a* and *lmx1b* orthologs. Fragments resulting from PCR amplification of 24 hpf zebrafish cDNA were subcloned and two distinct species were isolated multiple times. Each insert was used to screen a 22-26 hpf zebrafish lambda zap cDNA library and a total of 38 purified positives was identified following screening of 1 million plaques. Multiple isolates of two distinct cDNAs were obtained, each approximately 2 kb in length and containing 300-500 bp of untranslated sequence (data not shown). Although this degenerate PCR strategy was predicted to identify zebrafish orthologs of *lmx1a* and *lmx1b*, both cDNAs isolated were closely related to *lmx1b* (Fig. 1B,C). The sequences for Lmx1b.1 (AY894989) and Lmx1b.2 (AY894990) have been submitted to GenBank. We have subsequently identified a putative *lmx1a* ortholog in the zebrafish genome database, so the failure to identify this sequence in our screen suggests that expression levels at 22-26 hpf were too low. ClustalW alignments and phylogenetic analyses were performed using MacVector (Accelrys, Inc.), and the phylogenetic tree was produced using the Neighbor Joining Method with bootstrapping (1000 replicates).

Whole mount in situ hybridization and histology

Digoxigenin- or fluorescein-labelled probes were generated from linearized templates using an RNA labeling and detection kit (Roche). Hybridization and detection with alkaline phosphatase-conjugated antibodies is described in Odenthal and Nüsslein-Vollhard (1998). Stained embryos were cleared in benzyl alcohol:benzyl benzoate (2:1), mounted in Canada balsam, and photographed using a Magnafire SP digital camera (Optronics). Alternatively, embryos were photographed in PBS using a Micropublisher digital camera (Qimaging). To obtain histological sections, embryos were embedded in JB-4 plastic resin (Polysciences, Inc.). 5-micron sections were prepared with glass knives using a Leica RM2155 microtome. Sections were affixed to glass slides and stained with Methylene Blue/Azure II.

Morpholinos and microinjections

Antisense morpholino oligonucleotides were obtained from Gene Tools, LLC (Corvallis, OR). Morpholino sequences were CTTCGATTTTTATACCGTCCAACAT for *lmx1b.1*-MO (B1-MO) and CCTCAATTTTGATTCCGTCCAGCAT for *lmx1b.2*-MO (B2-MO). Mismatch oligonucleotides were designed for use as negative controls: CGTCTATTTTCATGCCGTCCATCAT for *lmx1bN*-MO (BN-MO), and CATCCATTTTAATCCCGTCCACCAT for *lmx1bX*-MO (BX-MO). An unrelated control oligonucleotide (CON-MO) was provided by the manufacturer. Morpholino microinjections were performed on 1-4 cell embryos. Morpholino solutions (4mg/mL in Danieu buffer) were back loaded into glass needles before injection, and 1.5-2.5 nL of morpholino solution were delivered to the yolk of each embryo. To verify morpholino specificity, in vitro translation analysis was performed using the TNT Coupled Transcription/Translation system (Ambion, Inc.) with 0.5 μg of plasmid DNA and 0.5 μg of morpholino oligonucleotide. ^{35}S-methionine was incorporated and labeled proteins were resolved by 10% SDS-PAGE and visualized by autoradiography.

Immunohistochemistry and TUNEL staining

For immunohistochemistry, a mixture of two mouse monoclonal anti-GFP antibodies was used (Roche) and detected with biotinylated anti-mouse secondary antibody. Phosphohistone H3 protein was detected with a rabbit polyclonal antibody (Upstate) and biotinylated anti-rabbit antibody. Color reaction was carried out using Vectastain ABC kit (Vector) and FAST DAB (Sigma). For TUNEL staining, the ApopTag Peroxidase Detection kit (Chemicon) was used, but anti-digoxigenin-AP was substituted for anti-digoxigenin-HRP. Color reaction was carried out using NBT/BCIP.

Visualization of cranial motor neurons

A previously described transgenic line allows for the visualization of cranial motor neurons that express GFP under control of the Islet1 promotor (Higashijima et al.,2000). Transgenic embryos were injected with both morpholino oligos, and expression of GFP was observed in live embryos at 12-hour intervals beginning at 24 hpf. For images shown in Fig. 7, embryos were fixed at 48 hpf and processed for immunohistochemical visualization of GFP.

Pharmacological inhibition of FGF signaling

To block FGF signaling, tailbud stage embryos were incubated in an 8 μM solution of SU5402 (Calbiochem) in E3/DMSO for four hours. Control embryos were incubated in E3/DMSO alone.

Hsp 70-Driven Misexpression of Lmx1b.1 and Lmx1b.2

To misexpress Lmx1b.1 and Lmx1b.2, cDNAs containing the complete coding regions were cloned into pBluescript SK-plasmids downstream of the *hsp70* promoter. Injection solutions were prepared at a concentration of 10 ng/μL in 0.2M KCl and phenol red, and 1-3 nL of solution was injected directly into the cytoplasm of one-cell stage embryos. Embryos were then reared until early somitogenesis (1-5 somites). At that time, embryos were heat shocked at 37 degC for 45 minutes. After heat shock, embryos were allowed to further develop for 2-29 hr before fixation.

RESULTS

Zebrafish has two Lmx1b orthologs, Lmx1b.1 and Lmx1b.2

Degenerate PCR primers were designed based on Lmx1a and Lmx1b sequence information from a number of species (see methods), and two distinct zebrafish orthologs of Lmx1b were isolated (Fig. 1A). Comparison of the putative amino acid sequences of these cDNAs, designated *lmx1b.1* and *lmx1b.2*, with other Lmx proteins in the published databases (Fig. 1A,B) shows that both zebrafish cDNAs encode proteins that are most similar to chicken Lmx1b (Lmx1b.1 88% identity, and Lmx1b.2 80% identity). These sequences also exhibit a high degree of identity with mouse and human Lmx1b proteins. Comparison of the two zebrafish Lmx1b genes reveal a relatively high degree of divergence (Fig. 1B). While mammalian Lmx1b orthologs are very highly conserved (97-99% identity), zebrafish Lmx1b.1 and Lmx1b.2 share only 77% identity. Somewhat lower identity is observed when comparing Lmx1a and Lmx1b sequences (64-68% identity), whether within a species or between distantly related species.

While our cloning strategy did not yield a Lmx1a homolog, we have identified a putative zebrafish Lmx1a by performing a BLAST search of available zebrafish genomic sequences. Phylogenetic analysis supports the grouping of this sequence with other known Lmx1a proteins, while both zebrafish sequences obtained in our screen cluster with Lmx1b proteins (Fig. 1C).

Expression of Lmx1b.1 and Lmx1b.2 in the zebrafish brain

We next determined when and where each gene was expressed. While the focus of these experiments is on the developing CNS, both genes are also expressed elsewhere, including the developing limb field (data not shown). *Lmx1b.2* expression initiates first at the margin of 90% epiboly embryos (Fig. 2A). Soon thereafter, *lmx1b.1* and *lmx1b.2* are both expressed at the tailbud stage in chevron-shaped domains corresponding to the presumptive MMR (Fig. 2B,C). Both genes are also expressed in distinct, overlapping domains at the midline, but only *lmx1b.1* is expressed in the presumptive otic anlage. Expression of *lmx1b.1* and *lmx1b.2* in the MMR becomes refined to a narrow ring by the 5-somite stage (Fig. 2D,E). By the 13-somite stage, both *lmx1b.1* and *lmx1b.2* are strongly expressed in the MMR and in the ventral diencephalon (Fig. 2F,G). Additionally, *lmx1b.1* is expressed in the developing otic placodes and the dorsal diencephalon, while *lmx1b.2* is expressed in the dorsal midline of the hindbrain. The expression patterns of *lmx1b.1* and *lmx1b.2* are thus dynamic and different in some aspects, but both are strongly expressed in the developing MMR.

Early expression of *lmx1b.1* and *lmx1b.2* overlaps extensively with *pax2.1*, a marker and regulator of the IsO. By the tailbud stage, the expression of *lmx1b.1* at the presumptive MMR is contained entirely within the expression domain of *Pax2.1* (Fig. 3A). *Lmx1b.2* has an identical expression domain at the MMR (data not shown). By the 13-somite stage, *pax2.1* expression at the IsO becomes refined to a ring that appears to encompass the expression domains of *lmx1b.1* and *lmx1b.2* (Fig. 3B,C), and extend further caudally.

At 24 hpf, *lmx1b.1* and *lmx1b.2* are expressed at the caudal limit of the mesencephalon abutting the isthmic constriction in a pattern similar to the *wnt1* expression domain (Fig. 3D-F). All three genes are also expressed in the dorsal midline of the mesencephalon and metencephalon. During IsO patterning, the MMR expression of *lmx1b.1* and *wnt1* is contained within the anterior domain of *pax2.1* expression (Fig. 3G,H), as is *lmx1b.2* (data not shown).

Lmx1b.1 and Lmx1b.2 are required for maintenance of MMR structure and gene expression

To determine what developmental events require Lmx1b.1 and Lmx1b.2, we performed a series of morpholino knockdown experiments. Antisense morpholino oligos were designed to specifically block translation of Lmx1b.1 (B1-MO) or Lmx1b.2 (B2-MO). An in vitro translation assay to asses oligo efficacy and specificity demonstrated that B1-MO effectively blocked only Lmx1b.1 translation, and B2-MO blocked only Lmx1b.2 translation. Mismatch control oligos (see methods) did not block translation of either (Fig. 4A). Injection of embryos with single morpholinos directed against either Lmx1b.1 or Lmx1b.2 does not produce a discernible morphological phenotype (data not shown), nor does injection of any of the three control morpholinos, although one control, BN-MO, causes a small degree of nonspecific toxicity. Embryos injected with morpholinos targeting both Lmx1b.1 and Lmx1b.2 (B1B2-MO) fail to maintain the isthmic constriction or cerebellum (Fig. 4). At 24 hpf, experimental and control embryos are morphologically similar, but the loss of the isthmic constriction and cerebellum in B1B2-MO-injected embryos is easily observed by 30 hpf (92%, N=60). The affected embryos subsequently develop circulatory problems and severe cardiac edema by 48 hpf and die soon thereafter.

Several genes have been identified that are expressed at the MMR and are necessary for MMR development. To determine if Lmx1b.1 and Lmx1b.2 loss-of-function affects gene expression in the MMR, we injected B1B2-MO and then determined the effects on the temporal and spatial expression of MMR genes. Based on the previously reported relation between chick Lmx1B and Wnt1, (Adams et al., 2000; Matsunaga et al., 2002) we first examined *wnt1* regulation. Initiation and early expression of *wnt1* is unaffected by knockdown of Lmx1b.1 and Lmx1b.2 (N=18, Fig. 5A,B). However, *wnt1* expression at the MMR is lost in B1B2-MO-injected

embryos at the 15- to 18-somite stage (100%, N=37). In contrast, *wnt1* expression persists in the midline and forebrain (Fig. 5C,D). Embryos injected with single morpholinos against Lmx1b.1 or Lmx1b.2 exhibit normal *wnt1* expression as late as 24 hpf (data not shown). Knockdown of Lmx1b.1/2 also affects *wnt3a* and *wnt10b* expression (data not shown). At the 18-somite stage, *wnt3a* is normally expressed in the dorsal midline of the midbrain, with ventral extensions into the MMR. In embryos injected with B1B2-MO, the ventral extensions of *wnt3a* expression at the MMR are missing at the 18-somite stage (100%, N=12). *Wnt3a* expression is normal in embryos injected with BX-MO (100%, N=13). *Wnt10b* is expressed at the MMR and the dorsal midline of the mesencephalon, hindbrain, and spinal cord at the 18-somite stage, and its expression strongly resembles that of *wnt1*. Injection with B1B2-MO results in loss of *wnt10b* expression at the MMR at the 18-somite stage (100%, N=9), and injection of BX-MO does not affect expression of *wnt10b* (N=8).

The effect of Lmx1b.1 and Lmx1b.2 knockdown on *pax* gene expression at the MMR was also examined. At the 15-to 18-somite stage, knockdown of Lmx1b.1 and Lmx1b.2 results in complete loss of *pax8* expression (100%, N=10, Fig. 5E,F). Expression of *pax2.1*,*pax2.2*, and *pax5* is unaffected until 24 hpf. *Fgf8* expression was unaffected by morpholino injection at the 18-somite stage. However, knockdown of both Lmx1b.1 and Lmx1b.2 causes *fgf8* expression at the MMR to fade by the 19- to 22-somite stage (93%, N=14, Fig. 5G,H). The loss of *fgf8* expression in the MMR proceeds from ventral to dorsal. At the 19-to 22-somite stage in knockdown embryos, *fgf8* is lost ventrally and consistently retained dorsally, but by 24 hpf, this dorsal expression is almost completely absent (data not shown).

The MMR deletion caused by knockdown of Lmx1b.1/2 affects cells at the isthmus and cerebellum, but not the tectum or ventral rhombomere 1 (R1). At 24 hpf, *pax5* expression marks the caudal limit of the mesencephalon, the isthmic constriction, and the cerebellar anlage (Pfeffer et al., 1998). Knockdown of Lmx1b.1/2 causes a strong reduction of *pax5* expression at the MMR (100%, N=10), while injection of BX-MO has no effect (Fig. 6A,B). *Pax2.1* (100%, N=36),*pax2.2*(100%, N=12), and *pax8* (100%, N=10), markers of the isthmus but not the cerebellum, are strongly reduced by 24 hpf at the MMR following knockdown of Lmx1b.1/2 (data not shown). *Otx2* marks the mesencephalon, and its expression is neither deleted nor expanded in Lmx1b.1/2 knockdown embryos at 24 hpf (N=28) (Fig. 6C,D). Nor is an affect on *otx2* expression observed after 36 hpf (N=18, data not shown). *Mab21l2* is an additional marker of the mesencephalon that is unaffected by knockdown of Lmx1b.1/2 (N=20) (Fig. 6E,F). *EphA4a* expression marks several regions of the developing CNS, including the portion of R1 caudal and ventral to the cerebellum. *EphA4a* expression is unaffected in Lmx1b.1/2 knockdown embryos at 24 hpf (N=20) (Fig. 6G,H). *En3* expression marks both the tectum and the isthmocerebellar region. Knockdown of Lmx1b.1/2 results in a partial deletion of *en3* expression limited to the ventral isthmocerebellar region (50%, N=18) (Fig. 6I,J), while expression in the tectum and the dorsal isthmocerebellar region is unaffected. Therefore, Lmx1b.1/2 knockdown results in a loss of gene expression at the isthmocerebellar region, but not in the adjacent mecencephalon or rhombomeres. This suggests that anteroposterior patterning and specification is not altered in the absence of Lmx1b.1/2, but that cells fated to form the IsO and cerebellum are not maintained.

Lmx1b.1 and Lmx1b.2 loss-of-function results in cell death and loss of neuronal subtypes

To determine the consequences of altered IsO gene expression that cause the deletion of recognizable isthmic and cerebellar structures, we examined cell death and cell proliferation. We performed TUNEL staining to visualize apoptotic cells. At 24 hpf, uninjected embryos contain a low level of apoptotic cells distributed uniformly throughout the CNS (100 %, N=11) (Fig. 7A). Injection of the BX-MO control results in a low and variable increase in cell death throughout the embryo (100%, N=19) (Fig. 7B). In contrast, injection of B1B2-MO results in

a dramatic increase in the number of apoptotic cells in the MMR (83%, N=24) (Fig. 7C). Interestingly, the region of increased apoptosis is centered caudal to the *Lmx1b.1/2* expression domain. Conversely, in *noi* mutant embryos, cell death is visible at the same stages, but is centered more rostral to the isthmus (20%, N=20) (Fig. 7D). At 18 hpf (18 somites), embryos injected with B1B2-MO do not exhibit increased apoptosis around the isthmus, and therefore, the onset of increased cell death occurs between 18 and 24 hpf. In addition to examining cell death, we used antibodies against phosphohistone H3 (pH3) to determine if knockdown of Lmx1b.1/2 affected mitosis at the MMR. When embryos injected with B1B2-MO (N=35) were compared to embryos injected with BX-MO (N=20) at 24 hpf, no significant difference in pH3-positive cells at the MMR was observed (data not shown). Therefore, loss of isthmic and cerebellar identity in Lmx1b.1/2 knockdown embryos is associated with increased levels of apoptosis, but not altered cell proliferation.

We next examined the consequences of Lmx1b.1 and Lmx1b.2 loss-of-function on neuronal differentiation. Cranial motor neurons (CMNs) III and IV flank the isthmus, and thus serve as markers of neuronal differentiation at the MMR. A transgenic islet1-GFP line allows for the visualization of Islet1-expressing CMNs in the developing zebrafish brain (Higashijima et al., 2000). GFP expression in the cell bodies of these neurons was observed as early as 36 hpf. Live B1B2-MO-injected embryos were examined through 54 hpf, and CMNIII and CMNIV were never observed. CMNs were also visualized in fixed embryos using an antibody against GFP, which yielded better images of CMNs, and these data are presented (Fig. 7E,F). Islet1-GFP embryos were injected with BX-MO or B1B2-MO and fixed at 36, 48, and 72 hpf. Antibody staining for GFP revealed that while CMNs developed normally in BX-MO-injected embryos, CMNIII and CMNIV were not detected in B1B2-MO-injected embryos at any stage examined.

MMR expression of *lmx1b.1* and *lmx1b.2* requires Pax2.1 and FGF signals

To test the requirement for Pax2.1 in *lmx1b.1* and *lmx1b.2* expression, we performed whole mount in situ hybridization on *noi* mutant embryos (Table 1). At the one-somite stage, all embryos expressed *lmx1b.1* (N=17) and *lmx1b.2* (N=17) normally. However, at subsequent developmental stages (5, 7, 12, and 18 somites) *lmx1b.1* and *lmx1b.2* expression at the MMR was absent in approximately 25% of embryos (59/234 and 64/214, respectively). This corresponds to the predicted one quarter of embryos homozygous for the *pax2.1* mutation. Significantly, this loss of *lmx1b.1* and *lmx1b.2* expression occurs earlier than the loss of *wnt1* (first affected at 6 somites) and *fgf8* (first affected at 9 somites) in *noi* mutant embryos (Lun and Brand, 1998). Therefore, Pax2.1 is not required for the initiation of *lmx1b.1* or *lmx1b.2* expression, but soon thereafter is required for their maintenance at the IsO in a manner independent of Wnt1 and Fgf8.

To test the dependence of *lmx1b.1* and *lmx1b.2* expression on FGF signaling, we treated embryos with SU5402, a drug that inhibits the function of the FGFR1-4 receptors, and effectively eliminates the signaling of FGF8 and other FGFs (Mohammadi et al., 1997). Exposure of embryos to SU5402 in a DMSO solution at tailbud stage resulted in a loss of *lmx1b.1* and *lmx1b.2* expression at the MMR within 4 hours, while expression at the midline was retained (N=15) (Fig. 8A,B). Expression of *lmx1b.1* and *lmx1b.2* was not affected in embryos treated with DMSO alone (N=15) (Fig. 8C,D). Therefore, maintenance of *lmx1b.1* and *lmx1b.2* expression at the MMR requires FGF signaling.

Lmx1b.1 and Lmx1b.2 induce expression of *wnt1* and *fgf8*

To determine which IsO genes are regulated by Lmx1b.1 and Lmx1b.2, we induced ectopic expression using constructs containing the *lmx1b.1* or *lmx1b.2* coding sequence under the control of the *hsp70* promoter. Embryos were injected with plasmid at the one-cell stage and

were heat-shocked during early somitogenesis to induce expression of *lmx1b.1* (Fig. 9A,B) or *lmx1b.2* (data not shown). Ectopic expression was highly variable, and the number of expressing clones varied from one to hundreds. The *lmx*-positive clones were distributed randomly throughout most tissues of heat-shocked embryos, but the cells surrounding the yolk seemed most sensitive to the treatment. Wild type embryos were heat-shocked, resulting in no ectopic induction of *lmx1b.1* (N=26) or *lmx1b.2* (N=20). Control embryos injected with *hsp70-lmx1b.1*, but not heat shocked, did not express ectopic *lmx1b.1* (N=13) or *wnt1* (N=12). Embryos injected with *lmx1b.2-hsp70* and not heat-shocked did not express ectopic *lmx1b.2* (N=10) or *wnt1* (N=19).

Both Lmx1b.1 and Lmx1b.2 induced ectopic expression of *wnt1* (Fig. 9C,D). Hsp70-Lmx1b.1 induced mosaic ectopic expression of *wnt1* in 36% of embryos (N=141), and Hsp70-Lmx1b.2 induced *wnt1* in 71% of embryos (N=17). *Wnt1* expression was induced in all regions of the embryo. Both Lmx1b.1 and Lmx1b.2 also induced *fgf8* (Fig. 9E,F). Hsp70-Lmx1b.1 induced *fgf8* in 81% of embryos (N=69) and Hsp70-Lmx1b.2 induced *fgf8* in 96% (N=52).

Since the Hsp70-Lmx1b.2 construct was more effective, we used it to test Lmx1b regulation of other IsO genes. We injected one-cell stage embryos with *hsp70-lmx1b.2*, heat shocked during early somitogenesis, and fixed embryos 2, 13, or 29 hours after treatment. Embryos were then examined for *wnt1*,*fgf8*,*pax2.1*, and *pax8* expression. At each time point, ectopic expression of *wnt1* (9/19, 4/14, 13/33) and *fgf8* (8/20, 8/21, 2/29) was observed. Although strong endogenous expression was detected, no ectopic expression of *pax2.1* (0/21, 0/20, 0/20) or *pax8* (0/20, 0/18, 0/26) was detected. Therefore, while Lmx1b.1 and Lmx1b.2 are sufficient to induce *wnt1* and *fgf8*, Lmx1b.2 is not sufficient to induce *pax2.1* or *pax8*.

DISCUSSION

In this study we identify two zebrafish orthologs of *lmx1b*,*lmx1b.1* and *lmx1b.2*, and show that both genes are expressed at the isthmus and are necessary for maintenance of the MMR. The functions of Lmx1b.1 and Lmx1b.2 at the isthmus are redundant; both proteins must be knocked down to disrupt MMR development, and either gene is sufficient to induce *wnt1* and *fgf8*. The requirement for Lmx1b.1 and Lmx1b.2 at the isthmus is likely a result of their roles in maintaining expression of *wnt1*, *wnt3a*, *wnt10b*, *pax8*, and *fgf8*. Here we discuss the interaction of Lmx1b.1 and Lmx1b.2 with other transcription factors and secreted factors to maintain the function of the IsO (Fig. 10).

Lmx1b.1 and Lmx1b.2 are required to maintain the IsO

Loss of Lmx1b.1 and Lmx1b.2 function causes the degeneration of the isthmus and cerebellum by 30 hpf. This morphological effect is preceded by a substantial increase in cell death at the MMR between 18 and 24 hpf. It is possible that the primary role of Lmx1b.1 and Lmx1b.2 at the isthmus is to maintain cell survival in the isthmocerebellar region. Alternatively, Lmx1b.1 and Lmx1b.2 may play an indirect role in the cell death; loss of Lmx1b.1 and Lmx1b.2 may result in a failure of IsO autoregulation, which leads to loss of maintenance of downstream trophic factors necessary for cell survival.

The requirement of Lmx1b.1 and Lmx1b.2 for maintenance of cell survival is likely tied to their regulation of Wnt1 and Fgf8. Wnt1 has been shown to have a proliferative role in the CNS (Dickinson et al., 1994; Matsunaga et al., 2002; Megason and McMahon, 2002; Panhuysen et al., 2004), and *Wnt1*$^{-/-}$ mice have ectopic cell death at the MMR (Serbedzija et al., 1996; Chi et al., 2003). Simultaneous loss of signaling from Wnt1, Wnt10b, and Wnt3a results in increased apoptosis at the MMR (Buckles et al., 2004). Similarly, knockout of MMR *fgf8* results in ectopic apoptosis in mice (Chi et al., 2003). In zebrafish, *ace* mutants lacking functional Fgf8 exhibit increased cell death concentrated above the rostral metencephalon from

midsomitogenesis on, and a marked decrease in mitosis at the MMR at 57 hpf (Jászai et al., 2003). Cell death observed in the MMR of B1B2-MO injected embryos could thus be attributed to the loss of *wnt1/3a/10b* and *fgf8*, and cell death may be initiated when cells fail to receive these essential proliferative signals.

A close regulatory relationship between Lmx proteins and Wnt1 at the IsO is conserved among vertebrates. Lmx1b can induce expression of *wnt1* in the chick (Adams et al., 2000; Matsunaga et al., 2002). The gain-of-function experiments in the present study demonstrate that this regulatory relation is conserved in zebrafish, with *wnt1* being regulated by both Lmx1b.1 and Lmx1b.2. Morpholino knockdown experiments demonstrate that Lmx1b.1 and Lmx1b.2 are required for maintenance of *wnt1*, but only at the IsO and only after the 18-somite stage. This function is preserved in the mouse, as *Lmx1b* null mice fail to maintain *wnt1* expression at the IsO beyond E9.5, and display a subsequent failure of MMR development (R. Johnson, personal communication).

As in the chick, the expression of *lmx1b.1* and *lmx1b.2* at the isthmus most closely resembles the expression of *wnt1/10b* during IsO development. Combined with our loss- and gain-of-function results, we conclude that *wnt* expession at the IsO is fundamentally linked to Lmx1b. 1 and Lmx1b.2 activity during this developmental period. Whether this transcriptional control is direct requires further study. Interestingly, knockdown of Wnt1 results in only a slight reduction of gene expression at the isthmus in zebrafish (Lekven et al., 2001), while simultaneous loss of Wnt1, Wnt10b, and Wnt3a results in a complete loss of the isthmic constriction (Buckles et al., 2004). Consistent with these observations, Lmx1b.1 and Lmx1b. 2 are required to maintain *wnt1*,*wnt3a* and *wnt10b* in the MMR and may regulate other *wnt* genes.

Regulatory interactions between Pax and Lmx genes at the IsO

A clear positive feedback relation exists between Lmx and Pax genes at the IsO, but we are only beginning to understand the details of these interactions. *Pax2.1*,*pax2.2*,*pax5*, and *pax8* are not maintained at the isthmus in the absence of functional Lmx1b.1/2 activity, and early maintenance of *lmx1b.1* and *lmx1b.2* requires Pax2.1. The failure of *pax2.1*,*pax2.2*, and *pax5* maintenance past 24 hpf in B1B2-MO-injected embryos is consistent with the hypothesis that loss of Lmx1b.1/ 2 function results in a breakdown of IsO signaling, and precipitates programmed cell death at the isthmus. In contrast, the loss of *pax8* expression by the 15-somite stage may reveal a more direct interaction between Pax8 and Lmx1b.1/2.

During IsO initiation,*pax*,*fgf*, and *lmx* gene expression is not yet interdependent, but by the mid- to late-somitogenesis stages, the IsO is regulated by a positive feedback loop in which these genes are mutually dependent for maintenance of expression. Regulation of *pax8* by Lmx1b.1 and Lmx1b.2 may represent an intermediate stage in IsO development, between initiation and maintenance. Since *fgf8* is still expressed at the 19- to 22-somite stage in B1B2-MO-injected embryos, regulation of *pax8* by Lmx1b.1 and Lmx1b.2 must occur via an Fgf8-indepentent mechanism that is yet to be defined. The early requirement for Pax2.1 in *lmx1b. 1/2* expression is further evidence of a Fgf8-independent regulatory pathway required for IsO development. Further supporting this idea, in *noi* mutant embryos *lmx1b.1* and *lmx1b.2* become dependent on Pax2.1 by the 5-somite stage, while *fgf8* is maintained until the 9-somite stage (Lun and Brand, 1998).

While Lmx1b.1 and Lmx1b.2 are necessary for expression of multiple *pax* genes, they do not appear to be sufficient. Ectopic Lmx1b.2 induced *wnt1* and *fgf8*, but not *pax2.1* or *pax8*. This result is surprising given that *fgf8* is induced in this assay. Since Fgf8 gain-of-function was sufficient to induce *pax2.1* and other IsO genes in chick (Crossley et al., 1996), we expected that misexpression of Lmx1b.2 would result in induction of *pax2.1*, either directly or through

Fgf8 induction. Failure to detect ectopic *pax2.1* suggests that either the level of Fgf8 induced in this experiment was insufficient to subsequently induce *pax2.1*, or that, unlike in the chick, Fgf8 is not sufficient to induce *pax2.1* in the zebrafish.

Anteroposterior and dorsoventral patterning of the MMR

The results presented are consistent with a hypothesis that ascribes a polarizing activity to the IsO (Lee et al., 1997; Reifers et al., 1998; Picker et al., 1999). We propose that Lmx1b.1 and Lmx1b.2 participate by maintaining cell survival in the region just posterior to the *Lmx1b.1/2* expression zone. During the maintenance phase of the IsO, MMR gene expression is codependent. Loss-of-function for a number of individual transcription factors and secreted factors results in loss of expression of the other regulators, increased cell death, and the degeneration of the MMR. However, loss-of-function for individual IsO regulators has differing consequences for anteroposterior patterning of the MMR. While *lmx1b.1* and *lmx1b.2* are expressed more rostral to the isthmic constriction, knockdown of Lmx1b.1 and Lmx1b.2 results in increased apoptosis caudal to the constriction. On the other hand, *pax2.1* is expressed in a domain that flanks the isthmus, but apoptosis in the *noi* homozygous mutants is concentrated rostrally and extends well into the midbrain. This indicates that both Lmx1b.1/2 and Pax2.1 have asymmetric, non-cell-autonomous effects on cell survival. This is likely due to their regulation of trophic factors. While a number of factors may be involved, we hypothesize that maintenance of the MMR depends on asymmetric responses to signaling from the expression boundary of Fgf8 and Wnt1/3a/10b, and that Lmx1b.1/2 mediate this regulation by maintaining Wnt1. Electroporation studies in chick suggest that Lmx1b.1/2 may have an additional role. Matsunaga et al. (2002) proposed that *fgf8* was cell-autonomously repressed by Lmx1b, but induced non-cell-autonomously by Lmx1b through Wnt1/3a/10b. However, we have not observed a repressive effect of Lmx1b.1 or Lmx1b.2 on *fgf8* in zebrafish.

Our results also indicate a fine degree of specificity in dorsoventral patterning at the IsO. Morpholino knockdown of Lmx1b.1 and Lmx1b.2 affects ventral expression of IsO genes more than dorsal expression. *Pax2.1*,*pax2.2*, and *fgf8* expression was often lost throughout the MMR, but in some cases, a variable amount of dorsal expression was retained. The observed pattern of dorsal retention of IsO gene expression is also seen in the *fgf8* mutant, *acerebellar* (Pfeffer et al., 1998), while ventral retention is seen in the *pax2.1* mutant, *noi* (Lun and Brand, 1998). These results indicate that maintenance of the IsO is controlled differently in dorsal and ventral domains, though there is as yet no known mechanism that regulates dorsoventral patterning of the MMR. Further studies of Lmx1b.1 and Lmx1b.2 function at the IsO may help answer this and other questions concerning patterning and cell fate decisions in the MMR.

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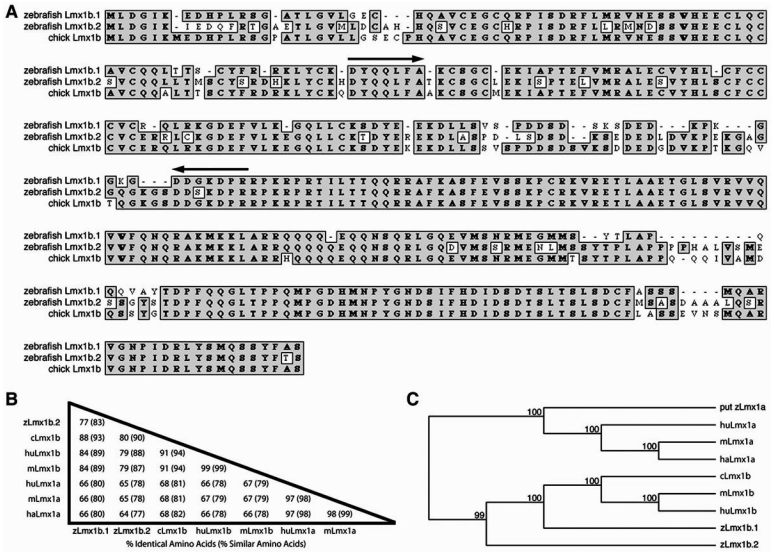

Fig. 1.

(A) ClustalW alignment of the predicted amino acid sequences of zebrafish Lmx1b.1, Lmx1b.2, and chick Lmx1b. Identical residues are shaded, and dashes indicate predicted gaps. Arrows indicate the positions of the residues against which degenerate primers were designed. (B) Matrix of amino acid sequence comparisons among Lmx sequences from zebrafish (z), chick (c), human (hu), mouse (m), and hamster (ha). Percent identities are given, followed by percentage of similar residues. (C) Phylogenetic relationships among several Lmx protein sequences. Both zebrafish lmx genes obtained in our screen cluster with other Lmx1b orthologs. A putative zebrafish Lmx1a ortholog (put zLmx1a) found in the Ensembl Zebrafish Genome Server (ENSDARP00000006532) clusters with Lmx1a orthologs. Numbers represent bootstrap values for each branchpoint. Accession numbers: zLmx1b.1, AY894989; zLmx1b.2, AY894990; cLmx1b, P53413; huLmx1b, CAI40918; mLmx1b, NP034855; huLmx1a, CAH73258; mLmx1a, Q9JKU8; haLmx1a, X81406.

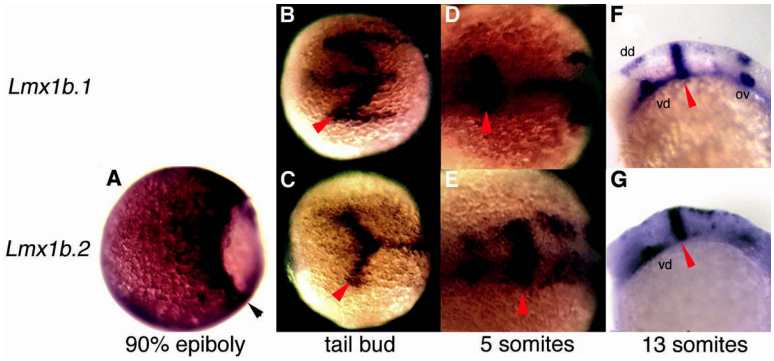

Fig. 2.

Expression of *lmx1b.1* and *lmx1b.2* in wild type embryos. (A) Lateral view of a gastrula at 90% epiboly, animal pole left. *lmx1b.2* is expressed in the blastoderm margin. Dorsal views with anterior to the left are shown (B-E). (B, C) At the tailbud stage, *lmx1b.1* and *lmx1b.2* are both expressed in chevron-shaped domains at the presumptive MMR. Both are also expressed in the midline, with *lmx1b.1* extending more rostrally, and *lmx1b.2* extending more caudally. (D, E) By the 5-somite stage expression of *lmx1b.1* and *lmx1b.2* are refined to rings at the MMR. *Lmx1b.1* is now expressed at the developing otic placode, while *lmx1b.2* is expressed in the diencephalic region and in cells of the hindbrain that are converging on the dorsal midline. (F, G) Lateral views, rostral left. By the 13-somite stage, *lmx1b.1* and *lmx1b.2* are both stably expressed at the MMR, the ventral diencephalon, and the dorsal midline of the caudal CNS. Lmx1b.1 is also stably expressed in the otic placodes. Black arrowhead indicates *lmx1b.2* expression at the blastoderm margin and red arrowheads indicate the MMR. ov, otic vesicle; dd, dorsal diencephalon; vd, ventral diencephalon.

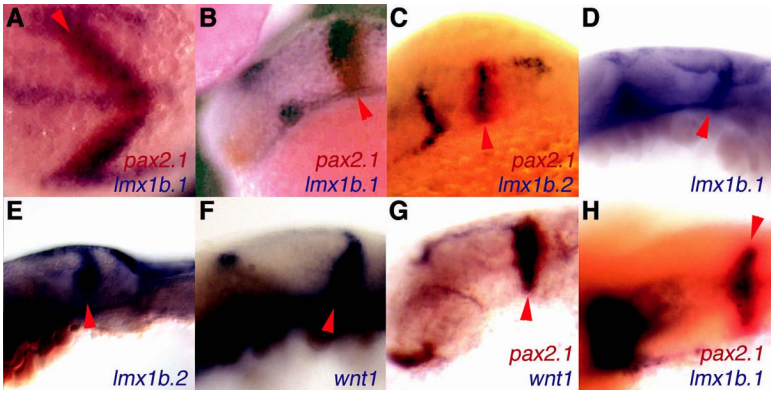

Fig. 3.

Lmx1b.1, *lmx1b.2*,*wnt1*, and *pax2.1* are expressed in overlapping domains at the isthmus. Lateral views, with dorsal up and anterior left are shown, except where noted.(A) Dorsal view, with anterior left. At the 1-somite stage, *lmx1b.1* is expressed in the midline and in the precursors of the MMR and the otic placodes. At the presumptive IsO, *pax 2.1* expression completely overlaps with *lmx1b.1* expression and the *pax2.1* domain is slightly broader. At the 13-somite stage, *pax2.1* expression at the IsO overlaps the expression of *lmx1b.1* (B) and *lmx1b.2* (C), and extends posterior. At 24 hpf, the expression domains of *lmx1b.1* (D), *lmx1b.2* (E), and *wnt1* (F) are found in the posterior mesencephalic vesicle, abutting the metencephalic vesicle. At 24 hpf, *pax2.1* expression overlaps both *wnt1* (G) and *lmx1b.1* (H, dorsolateral view), and extends more posterior. Red arrowheads indicate the MMR.

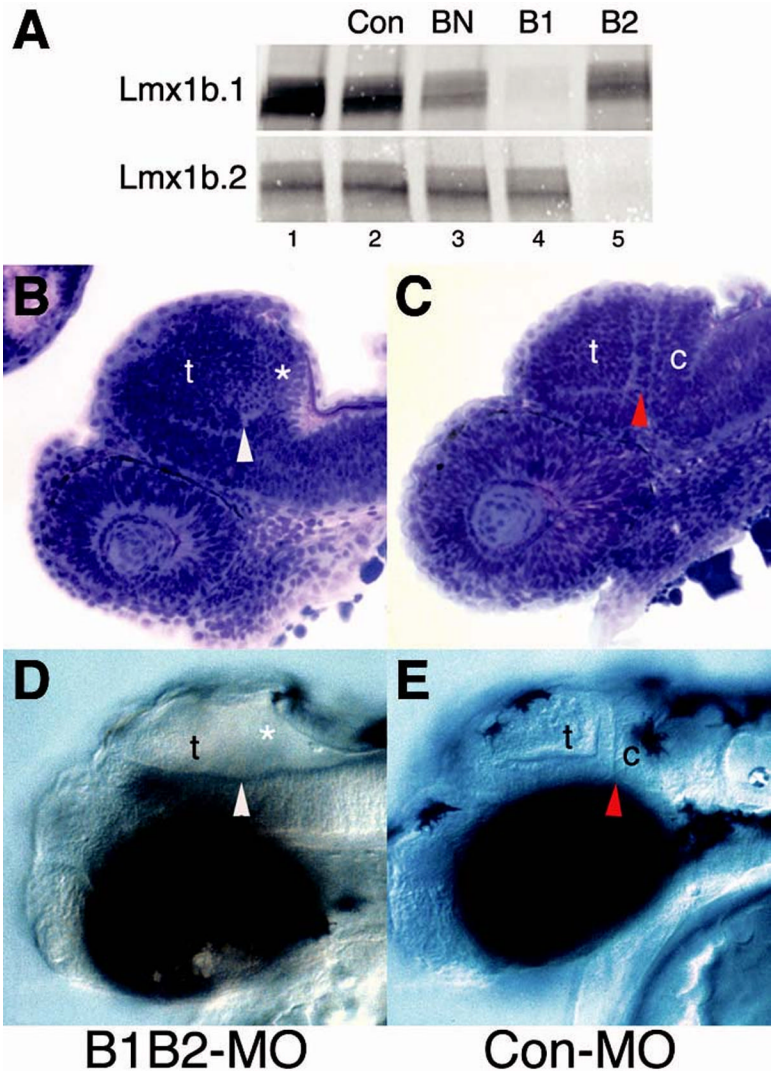

Fig. 4.

Simultaneous knockdown of Lmx1b.1 and Lmx1b.2 results in a loss of isthmic and cerebellar structures. (A) Specific inhibition of Lmx1b.1 and Lmx1b.2 translation by morpholino oligonucloetides. In vitro translation of Lmx1b.1 (top panel) and Lmx1b.2 (bottom panel) in the absence of oligonuclotide (lane 1), or with addition of an unrelated oligonuclotide (CON-MO, lane 2), a mismatch oligonucleotide (BN-MO lane 3), or oligonucleotides specific for Lmx1b.1 (B1-MO, lane 4) or Lmx1b.2 (B2-MO, lane 5). Lateral views of anterior CNS, with dorsal up and anterior left. Microtome sections at 30 hpf (B,C) and whole mounts at 36 hpf (D,E). Red arrowheads indicate the position of the isthmic constriction and white arrowheads indicate the missing isthmic constriction. t, tectum; c, cerebellum; *indicates missing structures.

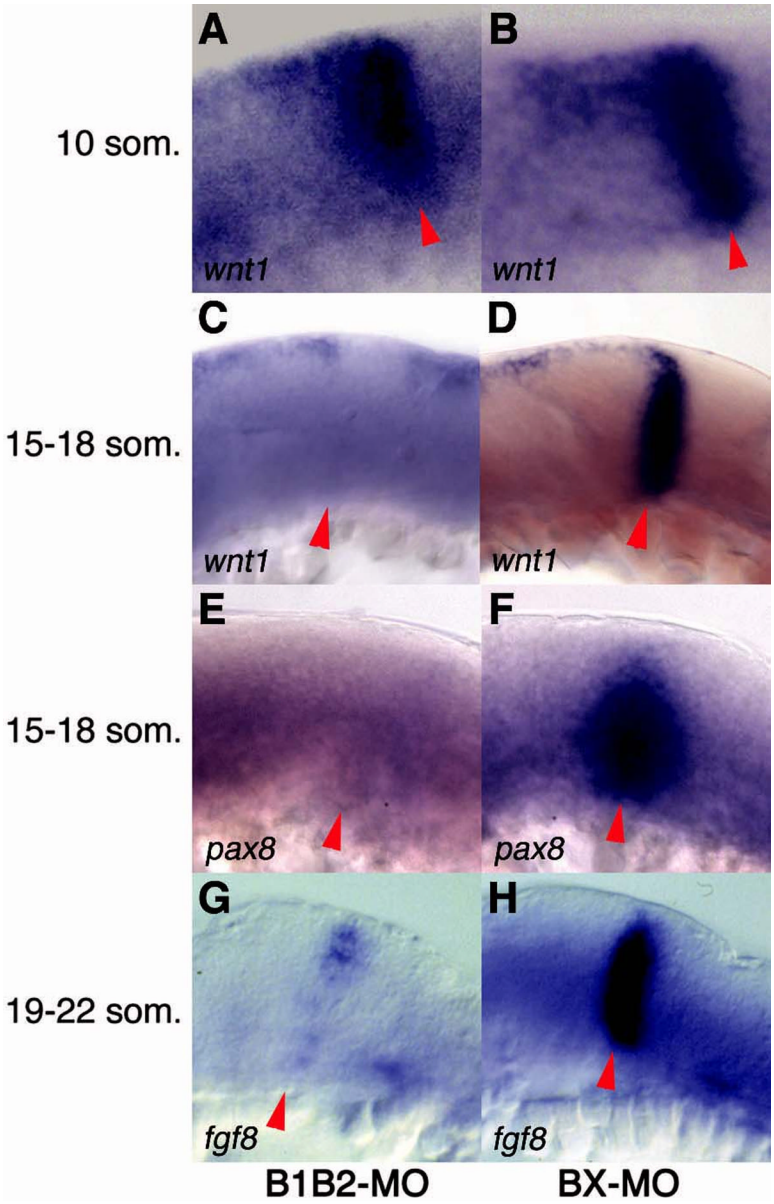

Fig. 5.

Following knockdown of Lmx1b.1 and Lmx1b.2, IsO genes are initiated normally, but some are not maintained. Lateral views of anterior CNS, with dorsal up and anterior left. *Wnt1* is initiated normally (A,B), but fades at the MMR by 15-18 somites (C,D). *Pax8* expression fades by 15-18 somites (E,F), and *fgf8* fades by 19-22 somites, with partial dorsal retention (G,H). Red arrowheads indicate the MMR.

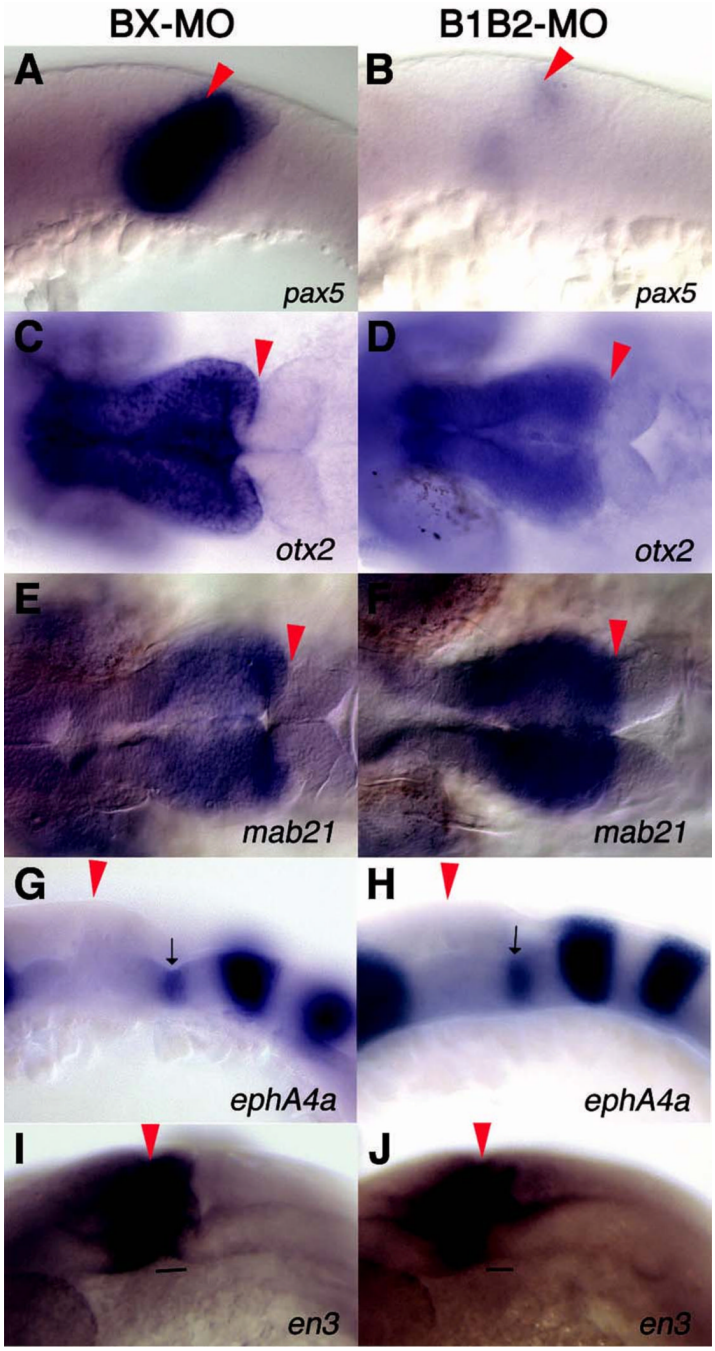

Fig. 6.

Knockdown of Lmx1b.1 and Lmx1b.2 affects cell fate in the isthmocerebellar region, but not the mesencephalon or ventral rhombomere 1 (R1). Lateral (A,B,G,H), dorsal (C-F), or dorsolateral views (I,J), with anterior left. *Pax5* expression, which marks the isthmus and cerebellum, is strongly reduced at 24 hpf in Lmx1b.1/2 knockdown embryos (A,B). Expression of *otx2* (C,D) and *mab21* (E.F), markers of the mesencephalon, is unaffected Lmx1b.1/2 knockdown embryos. EphA4a expression marks several regions of the developing brain, including the ventral region of R1, and EphA4a expression is unaffected in Lmx1b.1/2 knockdown embryos (G,H). Expression of *en3* marks both the tectum and the isthmocerebellar region. Knockdown of Lmx1b.1/2 results in a loss of *en3* expression at the ventral isthmus,

but normal expression is retained in the tectum and dorsal isthmus. Red arrowheads indicate the position of the MMR, black arrows indicate the ventral R1 staining of *epha4a*, and line segments indicate the ventral isthmic staining of *en3*.

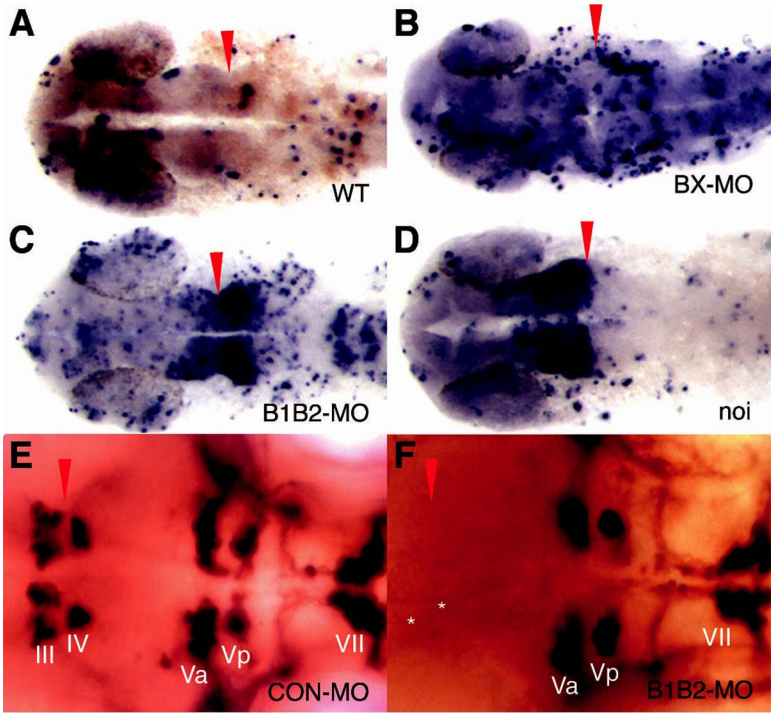

Fig. 7.

Knockdown of Lmx1b.1 and Lmx1b.2 results in increased cell death around the IsO, with a concomitant failure of neural differentiation. (A-D) A TUNEL assay stains apoptotic cells blue. Dorsal views with anterior left. (A) A low level of randomly distributed apoptosis is detected in wild type embryos. (B) Injection of BX-MO results in a nonspecific increase in apoptosis. (C) Injection of B1B2-MO sharply increases apoptosis in the IsO, especially caudally. Nonspecific apoptosis is also increased. (D) Increased apoptosis is detected around the IsO in embryos homozygous for the *pax2.1*/*no isthmus* mutation, especially rostrally. (E, F) Cranial motor neurons were visualised by expressing GFP under the control of the Islet-1 promoter and staining with an antibody against GFP. (E) Cranial motor neurons III and IV flank the IsO. (F) Neurons III and IV fail to form in B1B2-MO-injected embryos, while other neurons are unaffected. Red arrowheads indicate position of the MMR and asterisk mark normal position of missing neurons.

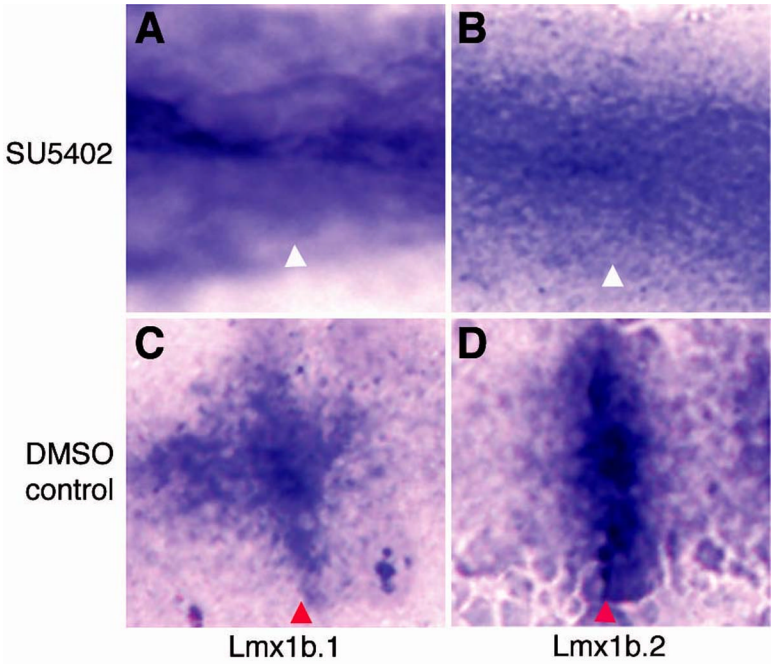

Fig. 8.

Blocking of FGF receptor activity results in loss of *lmx1b.1* and *lmx1b.2* expression at the developing IsO. Four hours of exposure to SU5402 beginning at the tailbud stage eliminates expression of both *lmx1b.1* and *lmx1b.2* at the developing IsO, while expression at the midline is unaffected (A,B). Exposure to DMSO alone does not affect expression (C,D). Red arrowheads indicate position of the MMR and white arrowheads indiacte missing MMR staining.

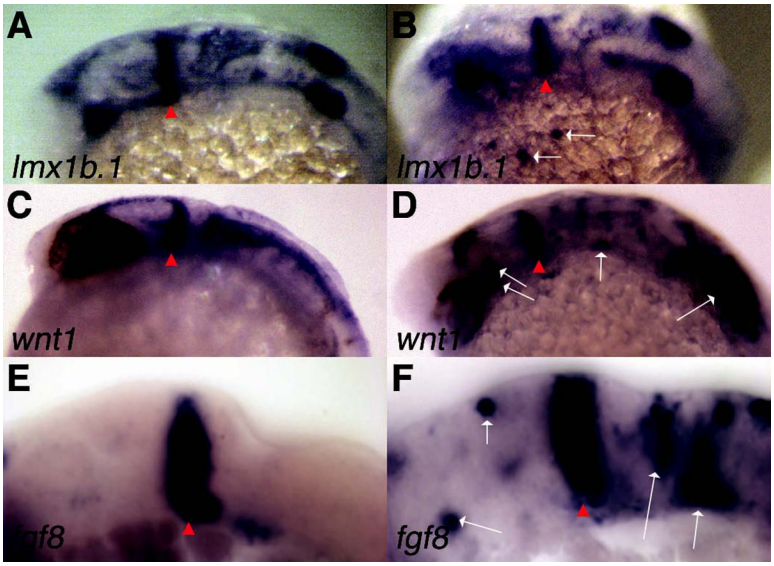

Fig. 9.

Heat shock-induced ectopic expression of *lmx1b.1*, *wnt1*, and *fgf8*. One-cell stage embryos were injected with Hsp70-Lmx1b.1 (B,D) or Hsp70-Lmx1b.2 (F), heat shocked during early somitogenesis, and fixed approximately 12 hours after heat shock. Embryos were then examined for *lmx1b.1*, *wnt1*, or *fgf8* expression by whole mount in situ hybridization. Uninjected, heat shocked embryos were used as controls (A,C,E). Lateral views with anterior left and dorsal up are shown magnified 200X (A-D) or 400X (E, F). Red arrowheads indicate position of MMR and white arrows indicate ectopic clones.

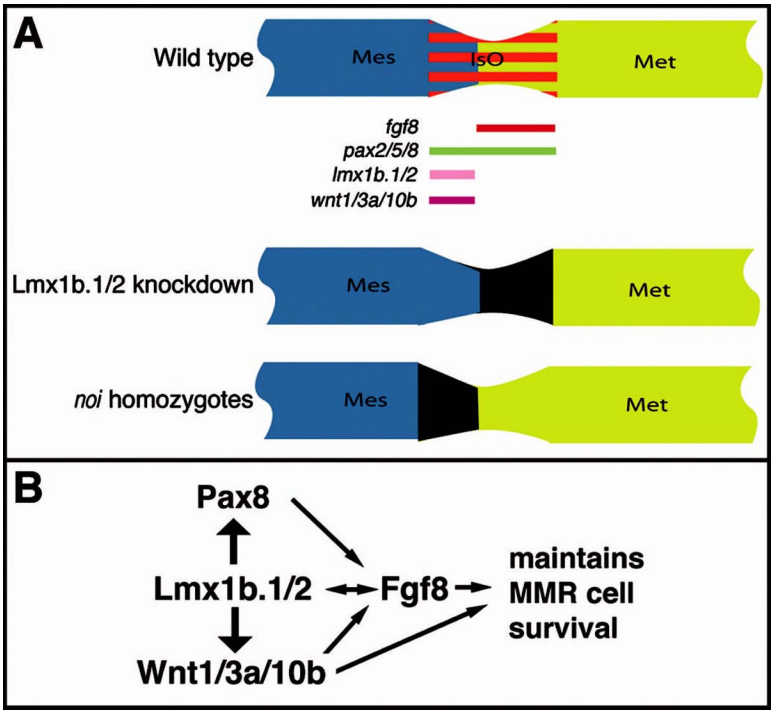

Fig. 10.

Lmx1b.1 and Lmx1b.2 cooperate with other IsO genes to maintain cell survival in the caudal MMR. (A) During IsO maintenance in wild type embryos, Lmx1b.1 and Lmx1b.2 maintain *wnt1*, *wnt3a*, and *wnt10b* expression at the caudal edge of the mesencephalic vesicle. *Fgf8* is expressed in an adjacent domain in the rostral metencephalic vesicle. *Pax2.1*,*pax5*, and *pax8* are expressed in a broader domain that encompasses the caudal mesencephalon and rostral metencephalon. When Lmx1b.1/2 activity is knocked down with morpholinos, increased apoptosis is observed, not in the *Lmx1b.1/2* expression domain, but in the adjacent portion of the MMR, including the normal *fgf8* domain. In contrast, loss of Pax2.1 function in *noi* mutant embryos results in increased apoptosis concentrated in the rostral portion of the MMR. These data indicate that the IsO produces signals that maintain cell survival in an asymmetric, non-cell-autonomous manner. (B) The data presented are consistent with a model in which Lmx1b.1 and Lmx1b.2 maintain cell survival at the MMR by regulating *fgf8*,*wnt1*, *wnt10b*, and *wnt3a*, secreted factors whose loss precipitates apoptosis in the MMR (Buckles et al., 2004; Jászai et al., 2003). Lmx1b.1/2 may maintain *fgf8* by regulating expression of *wnt1*, *wnt3a*, *wnt10b*,*pax8*, or an alternative mechanism. Fgf8, in turn, functions in a positive feedback loop to maintain *Lmx1b.1/2* expression.

Table 1:

IsO expression of *lmx1b.1* and *lmx1b.2* in offspring of *no isthmus* heterozygotes.

	Lmx1b.1			Lmx1b.2		
Stage	**Normal**	**Absent**	**%Absent**	**Normal**	**Absent**	**%Absent**
1 somite	17	0	0.0	17	0	0.0
5 somite	52	10	16.1	33	10	23.3
7 somite	30	15	33.3	33	17	34.0
12 somite	63	24	27.6	69	28	28.9
18 somite	30	10	25.0	15	9	37.5

Heterozygous parents were crossed and offspring were fixed at several different stages and examined for expression of *lmx1b.1* or *lmx1b.2*. Absence of expression in 25% of the embryos corresponds to the predicted frequency of *no isthmus* homozygous offspring.