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Lmx1b is essential for survival of periocular mesenchymal cells and influences Fgf-mediated retinal patterning in zebrafish

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ABSTRACT

To gain insight into the mechanisms of Lmx1b function during ocular morphogenesis, we have studied the roles of *lmx1b.1* and *lmx1b.2* during zebrafish eye development. In situ hybridization and characterization of transgenic lines in which GFP is expressed under *lmx1b.1* regulatory sequence show that these genes are expressed in periocular tissues and in a pattern conserved with other vertebrates. Anti-sense morpholinos against *lmx1b.1* and *lmx1b.2* result in defective migration of periocular mesenchymal cells around the eye and lead to apoptosis of these cells. These defects in the periocular mesenchyme are correlated with a failure in fusion of the choroid fissure or in some instances, more severe ventral optic cup morphogenesis phenotypes. Indeed, by blocking the death of the periocular mesenchyme in Lmx1b morphants, optic vesicle morphogenesis is largely restored. Within the retina of *lmx1b* morphants, Fgf activity is transiently up-regulated and these morphants show defective naso-temporal patterning. Epistasis experiments indicate that the increase in Fgf activity is partially responsible for the ocular anomalies caused by loss of Lmx1b function. Overall, we propose zebrafish *lmx1b.1* and *lmx1b.2* promote the survival of periocular mesenchymal cells that influence multiple signaling events required for proper ocular development.

Introduction

During eye development, the optic vesicles evaginate from the forebrain neuroepithelium and come into contact with several other tissues that are required for correct ocular morphogenesis, patterning and differentiation. For instance, contact with the prospective lens epithelium is correlated with invagination of the optic vesicle to form the two-layered optic cup consisting of prospective neural retina and pigment epithelium. Several recent studies have suggested that periocular mesenchyme derived from head mesoderm and neural crest cells also plays roles in the early morphogenesis of the optic cup and differentiation of the retina (Fuhrmann et al., 2000; Matt et al., 2005; Lupo et al., 2005; Molotkov et al., 2006; Matt et al. 2008).

Periocular mesenchyme eventually gives rise to specialized structures of the anterior segment of the eye that are responsible for aqueous humor dynamics (Johnston et al., 1979; Trainor & Tam, 1995; Cvekl and Tamm, 2004; Gage et al., 2005). Consequently, in humans disruption to factors important for the survival, migration and/or differentiation of periocular mesenchymal cells can lead to dysgenesis of the ocular anterior segment, elevated intraocular pressure and increased risk of glaucoma (Gould et al., 2004).

The glaucomas are a group of complex diseases characterized by vision loss due to damage of the optic nerve. Progress has been made towards identification of a subset of the critical risk genes for glaucoma. Several of these are also essential for ocular anterior segment development. Examples include genes encoding the transcription factors *PITX2*, *FOXC1*, and *LMX1B*. Mutations in either *PITX2* or *FOXC1* result in Axenfield–Reiger syndrome, in which a subset of affected individuals develop glaucoma (Walter, 2003). The roles of *PITX2* and *FOXC1* in eye development have been explored in several species (Nishimura et al., 1998; Mears et al., 1998; Kidson et al., 1999; Smith et al. 2000; Semina et al., 1996; Gage et al., 1999; Tamimi et al., 2006). However, the role of *LMX1B* during ocular morphogenesis is less well understood.

LMX1B encodes a LIM-homeodomain transcription factor that when mutated in humans causes Nail-Patella Syndrome (NPS), a pleiotropic condition where approximately 50% of patients develop elevated IOP and glaucoma (Dreyer et al., 1998; Vollrath et al., 1998). In addition to the eye, NPS affects joint and limb development, and often disrupts function of the renal and central nervous systems (CNS). Genetic and biochemical analyses have shown that NPS is due to *LMX1B* haploinsufficiency (Lichter et al., 1997; Vollrath et al., 1998). Unlike NPS patients that are heterozygous for *LMX1B* mutations, mice heterozygous for null mutations appear normal. However, homozygous mutant mice display several abnormalities observed in NPS patients including abnormal development of renal structures, CNS patterning defects and dysgenesis of anterior ocular tissues (Chen et al., 1998; Kania et al., 2000; Guo et al., 2007;

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Pressman et al., 2000). However, it remains unknown how LMX1B regulates ocular development.

To better understand the mechanisms by which Lmx1b defects cause ocular pathology, we examined the expression and loss-of-function phenotypes of *lmx1b.1* and *lmx1b.2* in zebrafish. Analysis revealed that both genes have expression patterns conserved with other vertebrates, including within the periocular mesenchyme. Loss-of-function analyses showed Lmx1b activity is required for periocular mesenchymal cell survival, optic cup morphogenesis and choroid fissure closure. In addition, knock-down of Lmx1b in transgenic lines that express GFP under *lmx1b.1* regulatory sequence revealed migration defects in periocular mesenchyme. We also found that *lmx1b* morphants showed increased ocular FGF activity, and consistent with this, abnormal naso-temporal patterning of the retina (Picker and Brand, 2005). Preventing apoptosis of periocular mesenchyme in *lmx1b* morphants alleviated the morphogenesis defects. Furthermore, reducing Fgf activity partially restored retinal patterning. Together, these results suggest that altered Fgf signaling due to periocular mesenchymal cell death is a contributing factor to the ocular pathogeneses associated with loss of Lmx1b activity.

Materials and methods

Animals

Zebrafish embryos were raised at 28.5 °C and staged according to Kimmel et al. (1995). Phenylthiourea (PTU) was applied to embryos to prevent melanization when necessary.

Transgenic and mutant lines

Tg(*h2afx*:H2A-mCherry)^{mw3} (this study)
Tg(−5 kb*lmx1b.1*:GFP)^{mw10,11,12,13} (this study)
Tg(*foxd3*:GFP)^{zf15} (Gilmour et al. 2002)
Tg(−7.2*sox10*:EGFP)^{zf77} (Hoffman et al., 2007)
Tg(fli1a:EGFP)^{y5} (Lawson and Weinstein, 2002)
Tg(*dusp6*:EGFP)^{pt6} (Molina et al., 2007)
Tg(*Bactin*:HRAS-EGFP)^{vu119} (Cooper et al., 2005)
fgf8a^{ti282} (*acerebellar*) (Reifers et al., 1998).

Isolation of full-length lmx1b.1 and lmx1b.2 cDNA

Total RNA was extracted from adult zebrafish eyes using RNAeasy Mini Kit (Qiagen). RT-PCR combined with 5′ and 3′ RACE was performed to identify full-length *lmx1b.1*. To isolate *lmx1b.1* full-length sequence, the following primers were used:

*lmx1b.1*flF 5′ATGTTGGACGGTATAAAAATCGAA
*lmx1b.1*flR 5′TCATGAGGCGAAATAGGAGCTC.

Full-length *lmx1b.2* sequence was isolated by RT-PCR using the following primers:

*lmx1b.2*flF 5′AGTAGACATGCTGGACGGAAT
*lmx1b.2*flR 5′GAGCTCCTACTTCACCTCCTGA.

Accession tags

Zebrafish *lmxb.1* (AY551077) and *lmx1b.2* (AY551078) sequences have been deposited into GenBank.

Histology

Embryos were dechorionated and fixed overnight at 4 °C in 2.5% gluteraldehyde/1% paraformaldehyde in phosphate-buffered sucrose, pH7.4. The next morning embryos were dehydrated and infused with Epon. Transverse sections were 1 μm thick, heat-mounted on gelatin coated glass slides, and stained with 1% toluidine blue.

In situ hybridization

Whole mount in situ hybridization was performed as previously described (Thisse and Thisse, 2004) with one modification. Following LiCl precipitation, anti-sense RNA probe was further purified using a ProbeQuant G-50 micro spin column (GE Healthcare).

Morpholino oligonucleotides

Morpholino oligonucleotides (GeneTools, Inc.) were targeted to splice site junctions between exon 1 and intron 1 (splice-MOs) and the translation start site (ATG-MOs) for each *lmx1b.1* or *lmx1b.2*. The following morpholino sequences were used:

lmx1b.1—ATG, 5′CTTCGATTTTTATACCGTCCAACAT (O'Hara et al., 2005)
lmx1b.2—ATG, 5′GATTCCGTCCAGCATGTCTACTTGA
lmx1b.1—splice, 5′TTGAAGGACTTACCGAGCATAACTC
lmx1b.2—splice 5′GTGTGTGTGAAACTCACCCAGCATC.

Mismatch controls had sequences matching those of the splice-inhibiting morpholinos with the exception of 5 bases denoted in lowercase letters:

5 mismatch *lmx1b.1*—splice 5′TTcAAcGACTTAgCGAGgATAAgTC
5 mismatch *lmx1b.2*—splice 5′GTcTGTcTGAAAgTCACCgAGgATC.

For each *lmx1b* gene, translation-inhibiting and pre-mRNA splice-inhibiting morpholinos were injected at the one-cell stage with 1% phenol red (Nasevicius and Ekker, 2000). For *lmx1b.1* and *lmx1b.2*, both translation- and splice-inhibiting morpholinos produced similar phenotypes. As controls, morpholinos with 5 base-pairs mismatched relative to the splice-disrupting oligos did not produce phenotypes when injected at similar concentrations to those used for the experimental morpholinos. Each splice-disrupting morpholino inhibited normal transcript processing while mismatch control morpholinos did not. Quantitative real-time RT-PCR determined that the *lmx1b* splice-inhibiting morpholinos functioned as predicted. For *lmx1b* morphant experiments described in the Results section, splice-inhibiting morpholinos were used and for most analyses, embryos showing moderate phenotypes were analyzed (see Fig. 2 and Table S1 for details).

Additional morpholinos were used as previously published:

fgf8a MO 5′TGAGTCTCATGTTTATAGCCTCAGT3′ (Albertson and Yelick, 2005)
fgf3 MO2 5′GGTCCCATCAAAGAAGTATCATTTG3′ (Maves et al., 2002)
fgf3 MO3 5′TCTCGCTGGAATAGAAAGAGCTGGC3′ (Maves et al., 2002)
p53 MO 5′GCGCCATTGCTTTGCAAGAATTG (Robu et al., 2007).

Lmx1b.1 promoter and cDNA expression constructs

RT-PCR was used to isolate −5 kb of the *lmx1b.1* promoter using the following primers designed from HTG sequence clone CH211-81G2:

Lmx1b.1–5 kb F 5′GGGGACAACTTTGTATAGAAAAGTTGA-TCACGTGCTTTTGGGTTTC and Lmx1b.1–5 kb R 5′GGGGACTGCCTT-TTTTGTACAAACTTGGCGGATGATCTTCGATTTT. Gateway technology (Invitrogen) using Tol2-kit reagents (Kwan et al. 2007) was employed to generate tol2:−5*lmx1b.1*:lmx1b:ires:GFP and tol2:−5*lmx1b.1*:GFP. Expression of the tol2 constructs was achieved by co-injection of transposase mRNA as previously described (Kawakami 2005).