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Zebrafish *msxB*, *msxC* and *msxE* function together to refine the neural–nonneural border and regulate cranial placodes and neural crest development

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Abstract

The zebrafish *muscle segment homeobox* genes *msxB*, *msxC* and *msxE* are expressed in partially overlapping domains in the neural crest and preplacodal ectoderm. We examined the roles of these *msx* genes in early development. Disrupting individual *msx* genes causes modest variable defects, whereas disrupting all three produces a reproducible severe phenotype, suggesting functional redundancy. Neural crest differentiation is blocked at an early stage. Preplacodal development begins normally, but placodes arising from the *msx* expression domain later show elevated apoptosis and are reduced in size. Cell proliferation is normal in these tissues. Unexpectedly, Msx-deficient embryos become ventralized by late gastrulation whereas misexpression of *msxB* dorsalizes the embryo. These effects appear to involve Distal-less (Dlx) protein activity, as loss of *dlx3b* and *dlx4b* suppresses ventralization in Msx-depleted embryos. At the same time, Msx-depletion restores normal preplacodal gene expression to *dlx3b-dlx4b* mutants. These data suggest that mutual antagonism between Msx and Dlx proteins achieves a balance of function required for normal preplacodal differentiation and placement of the neural–nonneural border.

Keywords: *msx*; *dlx*; *eya*; *six*; *foxd3*; *snail2*; *sox10*; Placode; Neural crest

Introduction

The vertebrate nervous system arises from progressive refinement of the ectoderm during gastrulation. Dorsoventral signals initially split the ectoderm into two broad domains, prospective epidermis ventrally and neural ectoderm dorsally. Near the end of gastrulation, signaling interactions at the neural–nonneural border generate two additional domains, preplacodal ectoderm and neural crest (Reviewed by Baker and Bronner-Fraser, 2001; Aybar and Mayor, 2002; Riley and Phillips, 2003; Streit, 2004). Neural crest cells originate at the lateral edges of the neural plate and the dorsal neural tube and subsequently migrate to diverse locations throughout the body to generate a wide array of neural and nonneural cell types. The preplacodal ectoderm forms just beyond the edges of the neural plate in the head. Initially, a contiguous stripe of expression of *dlx* (and other) genes defines the preplacodal ectoderm, which is later subdivided into discrete patches of expression corresponding to morphologically visible placodes. Placodes then cooperate with neural crest cells to produce the paired sensory organs and cranial nerves of the head.

The mechanisms governing specification of neural crest and preplacodal ectoderm are being rapidly elucidated. Neural crest is specified along the edges of the prospective midbrain, hindbrain and spinal cord by intermediate levels of Bmp from ventral ectoderm, plus Fgf, Wnt and RA from subjacent mesoderm and posterior tissue (Reviewed by Aybar and Mayor, 2002; Huang and Saint-Jeannet, 2004). In zebrafish and *Xenopus*, Delta-Notch signaling also acts within the dorsolateral neurectoderm to specify neural crest (Cornell and Eisen, 2000; Glavic et al., 2003). A combination of transcription factors, including Snail, Slug, and Foxd3, are induced in response to the above signals and are required for the first stages of neural crest

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differentiation. Less is known about specification of the preplacodal domain but much can be inferred from regulation and function of *dlx* genes. In frog, chick and zebrafish, expression of *dlx* genes in the preplacodal domain requires Bmp, although Bmp is not sufficient (Nguyen et al., 1998; Feledy et al., 1999; Pera et al., 1999). Additional unknown signals from dorsal tissue, possibly including the neural crest domain, are also required (Artinger et al., 1999; Pera et al., 1999). Deletion or knockdown of zebrafish *dlx3b* and *dlx4b* ablates nasal, trigeminal, and otic placodes (Solomon and Fritz, 2002; Kaji and Artinger, 2004), and severely reduces lateral line and epibranchial placodes (our unpublished observations). Likewise, misexpressing a dominant-negative form of Dlx3 in *Xenopus* inhibits preplacodal gene expression (Woda et al., 2003). In contrast, misexpression of Dlx5 in chick inhibits neural crest and expands expression of a subset of preplacodal markers (McLarren et al., 2003). Other preplacodal markers, such as *six* and *eya* genes, are also important regulators of preplacodal development, but the sequence of action and epistatic relationships between these markers remains to be elucidated (Reviewed by Baker and Bronner-Fraser, 2001; Streit, 2004). In summary, while neural crest and preplacodal cells share some early features of development, such as reliance on Bmp signaling, they subsequently diverge due to activation of distinct sets of transcription factor genes.

Although most neural crest and preplacodal markers do not show extensive overlap in their spatial expression, several members of the *muscle segment homeobox* (*msx*) gene family are transiently expressed in both domains as gastrulation nears completion (Ekker et al., 1997; Suzuki et al., 1997; Feledy et al., 1999; Streit, 2002; Streit and Stern, 1999; reviewed by Riley and Phillips, 2003). Within the neural crest lineage, *msx* function appears crucial. Activation of an inducible *msx1* construct at the end of gastrulation in *Xenopus* results in upregulation of neural crest markers, whereas expression of putative dominant-negative forms of Msx reduce their expression (Tribulo et al., 2003). Likewise, morpholino-mediated knockdown of *Xenopus msx1* inhibits expression of all early neural crest markers (Monsoro-Burq et al., 2005). These studies suggest that *msx* function regulates specification or early differentiation of neural crest. However, Msx proteins have also been found to regulate proliferation or cell death in various developing tissues (Hu et al., 2001; Tribulo et al., 2004; Reviewed by Bendall and Abate-Shen, 2000). It is not clear whether these processes contribute to the effects of *msx* function on early neural crest development. The potential role of *msx* genes in regulating preplacodal development has never been tested, but the essential role of *dlx* genes in preplacodal development suggests that *msx* genes might also be involved. Msx and Dlx are closely related protein families that are often coexpressed during development, but they appear to have opposing activities. All studies to date suggest that Msx proteins act as transcriptional repressors, often associated with growth, whereas Dlx proteins are transcriptional activators associated with differentiation (Bendall and Abate-Shen, 2000). Furthermore, Msx and Dlx proteins can functionally antagonize each other through heterodimer formation (Zhang et al., 1997). Hence, the overlapping expression of *msx* and *dlx* genes in the preplacodal domain could reflect the need to balance growth and differentiation within this domain.

Here, we analyze the roles of *msxB*, *msxC* and *msxE* in zebrafish (Ekker et al., 1997). All three genes are expressed in partially overlapping domains along the neural–nonneural boundary during gastrulation, and there are also unique domains for each gene. To test gene function, we injected antisense morpholinos to block splicing of *msxB*, *msxC* or *msxE* transcripts, and also examined the phenotype of a deletion mutation that removes *msxB*. Disrupting gene functions individually or in various combinations revealed both gene-specific and redundant functions. Our data confirm that *msx* genes regulate both neural crest and preplacodal development, but by different tissue-specific mechanisms. We also provide the first in vivo evidence that *msx* and *dlx* genes act in opposition to influence the position of the neural–nonneural boundary.

Materials and methods

Strains and developmental conditions

The wild-type strain was derived from the AB line (Eugene, OR). Embryos were developed in an incubator at 28.5°C in fish water containing 0.008% Instant Ocean salts. The *w8* deletion removes *dlx3b* and *dlx4b* and was induced by γ irradiation (Fritz et al., 1996). The *Df(LG1)msxB*^{x8} (or *x8*) mutation was induced by ENU (B. Riley, unpublished data). PCR and linkage analysis shows that *x8* is a deletion with one end just distal to *msxB* and the other end near *lef1*, a distance of 2–8 cM (pending resolution of the physical map).

Morpholino oligomer injections

Morpholino oligomers obtained from Gene Tools Inc. were diluted in Danieaux solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca$(NO_3)_2$, 5.0 mM *N*-[2-Hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) pH 7.6 (Nasevicius and Ekker, 2000)) to a concentration of 4–5 μg/μl. Filtered green food coloring was added to a concentration of 3% to visualize fluid during injections. Approximately 1 nl (5 ng MO) was injected into the yolk of one- to two-cell stage embryos. Embryos were injected and allowed to briefly recover in fish water. Morpholino sequences were as follows. *msxB*, 5′TATACTTACGAGGAGGAGATGTGAA3′; *msxC,* 5′ATTATTGCTGAGGTGCTTACTTGGC3′; *msxE* 5′CCGAGCATCACTGTTACCACTGGG 3′.

MsxB misexpression

msxB cDNA was cloned into pCS2+ expression vector. To misexpress msxB, 12–25 pg of plasmid DNA was injected into wild-type embryos at the 1–2 cell stage.

RT-PCR

Primers used to amplify various sequences are as follows (5′–3′):

msxB b1 GAAAAGTCTGAGTCGGACAGCG, b2 TGCTTGCGTAAGGTGCACGG, b3 GGGTTTGTGGAACTGGGTAGG;
msxC c1 CTGGACCAGAGTACAGTTCAGG, c2 TGGACTTGAATGCCTTGGCGG, c3 CATTCCGTAGGTCACTGGCC;
msxE e1 GGGAATTCATGGCCCCAGTGGTCACC, e2 TGTTTTCTCAGAGGGCACGCG, e3 CCGTCAGACTCAGTCTAGATCC;
ornithine decarboxylase (*odc*) GGATGTCCTGAAGCACCT, CCCACTGACTGCACGAT. Primers for *odc* were designed by Draper et al. (2001).