Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest

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Summary
The neural crest is a multipotent, migratory cell population that contributes to a variety of tissues and organs during vertebrate embryogenesis. Here, we focus on the function of Msx1 and Msx2, homeobox genes implicated in several disorders affecting craniofacial development in humans. We show that Msx1/2 mutants exhibit profound deficiencies in the development of structures derived from the cranial and cardiac neural crest. These include hypoplastic and mispatterned cranial ganglia, dysmorphogenesis of pharyngeal arch derivatives and abnormal organization of conotruncal structures in the developing heart. The expression of the neural crest markers Ap-2α, Sox10 and cadherin 6 (cdh6) in Msx1/2 mutants revealed an apparent retardation in the migration of subpopulations of preotic and postotic neural crest cells, and a disorganization of neural crest cells paralleling patterning defects in cranial nerves. In addition, normally distinct subpopulations of migrating crest underwent mixing. The expression of the hindbrain markers Krox20 and EphA4 was altered in Msx1/2 mutants, suggesting that defects in neural crest populations may result, in part, from defects in rhombomere identity. Msx1/2 mutants also exhibited increased Bmp4 expression in migratory cranial neural crest and pharyngeal arches. Finally, proliferation of neural crest-derived mesenchyme was unchanged, but the number of apoptotic cells was increased substantially in neural crest-derived cells that contribute to the cranial ganglia and the first pharyngeal arch. This increase in apoptosis may contribute to the mispatterning of the cranial ganglia and the hypoplasia of the first arch.

Key words: Neural Crest, Calvaria, Craniofacial, Cranial Ganglia, Cardiac outflow tract, Msx1, Msx2, Mouse embryo

Introduction
The neural crest, a population of multipotent, migratory cells, plays a variety of crucial roles in vertebrate organogenesis (Gammill and Bronner-Fraser, 2003; Santagati and Rijli, 2003). Neural crest cells are first specified through an interaction between the neural plate and the adjacent non-neural ectoderm (Knecht and Bronner-Fraser, 2002; Meulemans and Bronner-Fraser, 2004). They later delaminate from the neural folds and migrate through the embryo laterally and ventrally, contributing to elements of the craniofacial apparatus, the peripheral nervous system, the cardiac outflow septum, and the endocrine glands. They are capable of differentiating into many tissue and cell types, including smooth muscle, bone, cartilage, tendon and melanocytes (Noden, 1983; Tan, 1986; Miyagawa-Tomita et al., 1991; Bronner-Fraser, 1993; Le Douarin and Kalcheim, 1999).

Combined human-genetic and molecular approaches have uncovered several genes required for neural crest development in humans and mice. These include transcription factors, cell adhesion molecules and molecules involved in cell-cell signaling (Wilkie and Morriss-Kay, 2001; Gammill and Bronner-Fraser, 2003; Halloran and Berndt, 2003; Santagati and Rijli, 2003). Most of the major signaling pathways, including Bmp, Wnt, Fgf, Shh, RA (retinoic acid) and ET (endothelin), have roles in neural crest development. Although these roles are increasingly well understood (Knecht and Bronner-Fraser, 2002; Gammill and Bronner-Fraser, 2003), it remains unclear how neural crest cells respond to and integrate signals from these pathways. One approach to this problem is to investigate the functions and interactions of the transcription factors that mediate the signals from these various pathways. Here, we focus on the Msx genes – effectors of Bmp, Wnt and Fgf signaling – and their roles in the development of subpopulations of neural crest that contribute to the craniofacial apparatus, cranial nerves, and cardiac outflow septum.

Msx genes form a subfamily within the Nk-like homeobox gene family (Gauchat et al., 2000; Pollard and Holland, 2000). Mammals possess three Msx genes, Msx1, Msx2 and Msx3 (Davidson, 1995; Shimeld et al., 1996; Wang et al., 1996). In vertebrates, Msx1 and Msx2 are known to act in a variety of cell types to control cell proliferation, differentiation (Woloshin et al., 1995; Liu et al., 1999;
Odelberg et al., 2000; Hu et al., 2001; Han et al., 2003; Ishii et al., 2003) and survival (Marazzi et al., 1997). From work in several vertebrate embryos and various organ systems, it has been shown that Msx genes function as downstream effectors of the Bmp pathway (Vainio et al., 1993; Marazzi et al., 1997; Bei and Maas, 1998; Hollnagel et al., 1999; Sirard et al., 2000; Daluiski et al., 2001; Brugger et al., 2004). In addition, in some tissues, they serve as effectors of the Wnt and Fgf pathways (Chen et al., 1996; Montero et al., 2001; Willert et al., 2002; Hussein et al., 2003). Msx1 and Msx2 are expressed in premyelatory and migratory neural crest, as well as in the neural crest-derived mesenchyme of the pharyngeal arches and median nasal process (Davidson, 1995; Bolland and Abate-Shen, 2000; Maxson et al., 2003). Recent studies in Xenopus and chicken showed that the forced expression of Msx1 can induce neural crest marker expression in the dorsal aspect of embryos (Tribulo et al., 2003; Liu et al., 2004).

Mice homozygous for a targeted mutation in Msx1 exhibit agenesis of the teeth, a cleft palate, and abnormalities of the cranial skeleton (Satokata and Maas, 1994). Tissue recombination experiments have shown that Msx1 plays an essential role in epithelial-mesenchymal interactions during the tooth development (Chen et al., 1996; Bei and Maas, 1998). Han et al. provided evidence that Msx1 also controls cell proliferation in the dental mesenchyme (Han et al., 2003). A defect in the development of the frontal bone is evident in mice homozygous for a targeted mutation in Msx2, mimicking key features of Familial Parietal Foramina (Satokata et al., 2000). We showed recently that the cause of this frontal bone defect includes deficiencies in the differentiation and proliferation of neural crest-derived calvarial osteogenic cells (Ishii et al., 2003). Mice with homozygous mutations in both Msx1 and Msx2 die in late gestation with severe craniofacial malformations, including encephalycephaly, cleft palate, agenesis of teeth, and unossified calvarial bones (Bei and Maas, 1998; Satokata et al., 2000). Although this spectrum of anomalies in Msx1/2 mutants suggests a deficiency in the cranial neural crest, an analysis of neural crest development in such embryos has been lacking. Here, we report that Msx1–/–; Msx2–/– embryos have defects not previously described in derivatives of the neural crest and cranial neural crest. These include hypoplasia and mis-patterning of the cranial ganglia, and craniofacial and cardiac neural crest. These include defects not previously described in derivatives of the neural crest development, and in different neural crest subpopulations, to control the patterning of the craniofacial apparatus and cardiac outflow tract.

**Materials and methods**

**Genotyping of Msx1+/–; Msx2+/- mutant mice**

Msx1 and Msx2 mutant mice have been described (Satokata and Maas, 1994; Satokata et al., 2000). Compound Msx1, Msx2 heterozygotes (mixed genetic background of BALB/c and CD-1) were crossed to produce Msx1+/–; Msx2+/- embryos. The noon copulation plug was counted as embryonic day 0.5. DNA was prepared either from yolk sac (embryos) or from tails (postnatal mice). Msx1 and Msx2 knockout alleles were identified by PCR. Primers and PCR conditions were as described (Satokata and Maas, 1994; Satokata et al., 2000).

**Immunostaining, analysis of skeletal morphology and detection of apoptotic cells**

Whole-mount immunohistochemistry was carried out according to Mark et al. (Mark et al., 1993), using the 2H3 anti-neurofilament monoclonal antibody (1:500, Developmental Studies Hybridoma Bank). Primary antibody was followed by HRP-conjugated goat anti-mouse IgG antibody (1:100, Calbiochem). 4-chloro-1-naphthyl chromogenic substrate (Sigma) was used for signal detection. Whole heads of E15.5 embryos were stained in PBS containing 1 μg/ml DAPI for 30 minutes and photographed under UV light. Detection of alkaline phosphatase and counterstaining with Nuclear Fast Red were as described (Liu et al., 1999). Whole-mount analysis of skeletal morphology was performed as described by McLeod (McLeod, 1980) and Hogan et al. (Hogan et al., 1994). For TUNEL identification of apoptotic cells, embryos were fixed in 4% paraformaldehyde and cryosectioned (10 μm). The In Situ Cell Death Detection Kit (Roche) was used according to the manufacturer’s instructions. Sections were then treated with anti-phosphorylated Histone H3 polyclonal antibody (1/100, Upstate), then incubated with rhodamine-conjugated anti-rabbit IgG (1/100, Molecular Probes). Nuclei were counter-stained with DAPI. After Prolong (Molecular Probes) mounting, signals were photographed under fluorescence. Cell death was detected in whole embryos by Nile blue sulfate (Sigma), as described (Trumpp et al., 1999).

**In situ hybridization**

Whole-mount in situ hybridization was performed according to Hogan et al. (Hogan et al., 1994). Conclusions were based on at least two independent experiments. Digoxigenin-labeled anti-sense RNA probes were visualized by BM-purple substrate (Roche). An Msx1 1.2 kb Xhol-Xbal cDNA fragment was subcloned into pSP72. An RNA probe was synthesized from the T7 promoter. Other RNA probes were generated as reported previously: Ap-2α (Mitchell et al., 1991), Bmp4 (Wu et al., 2003), ecb6 (Inoue et al., 1997), Crabp1 (Stoner and Gudas, 1989), Dlx5 (Depew et al., 1999), Fgf8 (Crossley and Martin, 1995), Eph4α (Nieto et al., 1992), Hoxb1, Hoxd4 (Jiang et al., 2002), Krox20 (Wilkinson et al., 1989), Msx2, Twist (Ishii et al., 2003), Sox10 (Kuhlbrodt et al., 1998), Tbx1 (Bollag et al., 1994) and Wnt1 (Parr et al., 1993).

**Wnt1-Cre/R26R reporter assay**

Msx1 and Msx2 mutants were crossed with Wnt1-Cre or R26R lines, producing embryos with the genotype Msx1+/–; Msx2+/-; Wnt1-Cre+/-; R26R+. β-Galactosidase analysis was carried out as described (Chai et al., 2000; Jiang et al., 2000).

**Results**

**Gross morphological defects in Msx1/2 mutant embryos**

We crossed Msx1+/–; Msx2+/- mutants and examined Msx1+/–; Msx2+/- embryos at various stages of development (Fig. 1). A neural tube closure defect was apparent by E9.5, and some E9.5 embryos exhibited a flattened median nasal prominence. At E12.5, additional defects were visible, including thoraco-
abdominoschisis (open body wall), malformed limb buds (data not shown), and a failure of the median nasal prominence to fuse with the lateral nasal and maxillary prominences. E15.5-E16.5 embryos had a hypoplastic maxilla, a cleft and shortened mandible (Fig. 1A-E), and spina bifida (with a penetrance of approximately 1 in 8; data not shown). At E15.5, embryos were pale and edematous, and had enlarged hearts. Few embryos survived to E18.5. Given that one out of 16 offspring of Msx1+/–; Msx2+/– matings should have had the genotype Msx1–/–; Msx2–/–, we expected five such double homozygous mutants in the 87 embryos examined. Instead, we found only one, suggesting that most Msx1/2 mutants die during embryogenesis (Table 1).

Fig. 1. Craniofacial dysmorphogenesis and skeletal abnormalities in Msx1/2 compound mutant mice. (A-E) Craniofacial morphology of control (A,C) and Msx1/2 double-mutant embryos (B,D,E) at E15.5. A and B show frontal views; C,D and E lateral views. Msx1–/–; Msx2–/– embryos exhibit exencephaly, a hypoplastic maxilla, cleft mandible, and defects in the fusion of the median lateral, nasal and maxillary prominences. Arrows in B, D and E indicate the whisker pad. (F–I) Alkaline phosphatase stain of coronal sections of control (F,H) and mutant (G,I) embryos. Boxed areas in F and G are enlarged in H and I. Msx1/2 mutants exhibit cleft palate (asterisk in G). Arrows in H and I indicate vibrissa follicles. Note, the mutant has divided whisker pads (brackets in I). (J–S) Skeletal analysis of Msx1/2 compound mutants. (J–Q) E18.5 Alizarin Red/Alcian Blue-stained bone and cartilage. (J,L,N,P) Normal controls; (K,M,O,Q) Msx1/2 mutants. (J,K,P,Q) Lateral views; (L,M) dorsal views; (N,O) ventral views. Note that a significant portion of the cranial skeletal components, including neural crest derivatives, is either missing or severely affected in mutant embryos. (FQ) Fusion of hypoplastic mandible and maxilla is seen in the Msx1/2 mutant (Q). Note that tympanic ring was also hypoplastic in the mutant. (R,S) Lateral views of Alcian Blue-stained control (R) and Msx1/2 mutant (S) heads at E15.5. Note the parietal, supraoccipital and caudal processes of chondrocranium were absent in mutant. bo, basioccipital; bs, basisphenoid; c1, first cervical vertebra; e, eye; eo, exoccipital; fb, frontal bone; ip, interparietal bone; jg, jugal bone; ln, lateral nasal prominence; m, Meckel’s cartilage; md, mandible; mn, median nasal prominence; mx, maxilla; na/nb, nasal bone; ns, nasal septum; oc, otic capsule; oe, outer ear; pb, parietal bone; pl, palatine; pmx, premaxilla; ps, palatal shelf; so, supraoccipital bone; sq, squamosal bone; t, tongue; tr, tympanic ring; ty, thyroid cartilage. Scale bars: 1 mm.
Defective development of structures derived from the cranial and cardiac neural crest in Msx1/2 mutant embryos

In Msx1/2 mutants, the bones of the skull vault failed to develop (Satokata et al., 2000) (Fig. 1J-O). Because agenesis of the skull vault occurred even in embryos that did not have exencephaly, the skull vault defect was not a consequence of exencephaly. In E18.5 embryos, the tympanic ring was hypoplastic, and the maxilla and mandible were fused (Fig. 1P,Q). The parietal, supraoccpital and caudal processes of the chondrocranium were also deficient (Fig. 1R,S). Homeotic transformations were not evident in any skeletal elements (Fig. 1P-S, data not shown).

Afferent neurons and glial cells of the cranial and dorsal root ganglia are derived from the neural crest (Le Douarin and Kalcheim, 1999; Barlow, 2002). To examine the development of the cranial and dorsal root ganglia in Msx1/2 null embryos, we performed whole-mount immunohistochemistry on E10.5 embryos using an anti-neurofilament antibody (Fig. 2). In all mutant embryos examined (n=4), the oculomotor nerve (III) was absent or disrupted, and the trigeminal ganglion (V) was significantly reduced in size (Fig. 2B-D). These data show that loss of Msx1/2 resulted in hypoplasia and mispatterning of the cranial ganglia. The dorsal root ganglia, by contrast, were indistinguishable from wild type (data not shown). The proximal part of the IXth nerve was missing, and the distal portion of the IXth nerve was fused with the Xth nerve. An abnormal connection between the trigeminal and facial-acoustic nerves (VII-VIII) was evident in two embryos (Fig. 2B,C). The fusion of the IXth and Xth nerve was less dramatic in Msx1+/−; Msx2+/− and in Msx1+/−; Msx2+/− embryos than in Msx1+/−; Msx2+/− embryos (Fig. 2E,F, data not shown), suggesting that patterning defects of the cranial nerve are Msx gene-dosage dependent.

Cardiac neural crest cells contribute to the cardiac outflow septum, and are required for the proper alignment of the aorta and pulmonary trunk (Kirby and Waldo, 1995). Although neither Msx1 nor Msx2 individual mutant mice exhibit defects in the development of the cardiac outflow tract (Kwang et al., 2002), all Msx1/2 null mice that we examined (n=4) had conotruncal abnormalities, including double outlet right ventricle (DORV), Tetralogy of Fallot and persistent truncus arteriosus (PTA) (Fig. 3, data not shown). Each of these defects is attributable to defective neural crest development (Kirby and Waldo, 1995). In addition, double-mutant mice exhibited ventricular-septal defects (VSDs), hypoplastic valves, and dysmorphogenesis of the ventricular wall and myocardium (these defects will be described in detail elsewhere). Mutant hearts also contracted irregularly (data not shown), which, together with the finding of generalized edema, suggested that Msx1/2 null mutants had cardiac insufficiency. We did not detect conotruncal abnormalities in Msx1+/−; Msx2+/− or Msx1+/−; Msx2+/− embryos (data not shown).

Table 1. Frequency of Msx1/2 compound mutants

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total*</th>
<th>Msx1+/−</th>
<th>Msx2+/−</th>
<th>Msx1+/−;Msx2+/−</th>
<th>Msx1+/−;Msx2+/− (%)</th>
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</thead>
<tbody>
<tr>
<td>E8.5</td>
<td>110</td>
<td>9 (8.2)</td>
<td>7 (6.4)</td>
<td>9 (8.2)</td>
<td>11 (10.0)</td>
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<tr>
<td>E9.5</td>
<td>815</td>
<td>48 (5.9)</td>
<td>62 (7.6)</td>
<td>103 (12.6)</td>
<td>91 (11.2)</td>
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<tr>
<td>E10.5</td>
<td>212</td>
<td>9 (4.2)</td>
<td>6 (2.8)</td>
<td>27 (12.7)</td>
<td>24 (11.3)</td>
</tr>
<tr>
<td>E12.5</td>
<td>237</td>
<td>10 (4.2)</td>
<td>18 (7.6)</td>
<td>26 (11.0)</td>
<td>24 (10.1)</td>
</tr>
<tr>
<td>E15.5</td>
<td>110</td>
<td>4 (3.6)</td>
<td>10 (9.1)</td>
<td>10 (9.1)</td>
<td>19 (17.3)</td>
</tr>
<tr>
<td>E16.5</td>
<td>81</td>
<td>6 (7.4)</td>
<td>2 (2.5)</td>
<td>18 (22.2)</td>
<td>14 (17.3)</td>
</tr>
<tr>
<td>E18.5</td>
<td>87</td>
<td>3 (3.4)</td>
<td>9 (10.3)</td>
<td>7 (8.0)</td>
<td>9 (10.3)</td>
</tr>
<tr>
<td>NB</td>
<td>56</td>
<td>3 (5.4)</td>
<td>8 (14.3)</td>
<td>3 (5.4)</td>
<td>11 (19.6)</td>
</tr>
</tbody>
</table>

*Total of all genotypes, including wild type, Msx1+/−; Msx2+/−, Msx1+/−; Msx2+/−, Msx1+/−; Msx2+/−, Msx1+/−; Msx2+/−, Msx1+/−; Msx2+/−, Msx1+/−; Msx2+/−. NB, newborn.

Fig. 2. Defects in neural crest-derived cranial ganglia in Msx1/2 mutant embryos. (A-F) Whole-mount immunohistochemistry of neurofilament at E10.5. Control (A), Msx1/2 double homozygous (B,C,D), Msx1+/−; Msx2+/− (E), and Msx1+/−; Msx2+/− (F) embryos. (B-D) Double homozygous mutant embryos show a hypoplastic trigeminal nerve (V; arrows), and fused glossohypopharyngeal (IX) and vagus (X) nerves in the distal portion (asterisk). An abnormal connection between the trigeminal and facial (VII) nerves can be observed in Msx1/2 mutant embryos (arrowheads, B,C). Note the double-mutant embryo shown in D has impaired peripheral nerve growth and an interruption of the projection of the vagus nerve. The glossohypopharyngeal and vagus nerves were also fused in Msx1+/−; Msx2+/− and Msx1+/−; Msx2+/− embryos (asterisk, E,F). III, oculomotor nerve. Scale bar: 0.5 mm.
Normal expression of marker genes in pharyngeal arches

The array of defects in Msx1/2 mutant embryos suggested that the function of Msx1 and Msx2 is crucial for neural crest development. A simple hypothesis that would explain neural crest defects in Msx1/2 mutants is that neural crest cells fail to populate target sites in the craniofacial region (Fig. 4). We used whole-mount hybridization to assess the expression of the neural crest markers Ap-2α (Tcfap2a – Mouse Genome Informatics), Dlx5 and Twist in E9.0 and E9.5 embryos. Ap-2α is expressed in migratory cranial neural crest and in postmigratory crest in the pharyngeal arches (Mitchell et al., 1991). Dlx5 and Twist are expressed in the first and second pharyngeal arches (Acampora et al., 1999; Depew et al., 1999; Wolf et al., 1991).

Each of these markers was expressed normally in the first and second arches (Fig. 4A-F), suggesting that the anomalies in neural crest-derived structures in the pharyngeal arches of Msx1/2 mutants are not a result of large-scale deficiencies in the distribution of neural crest. Use of the Wnt1-Cre/R26R system (Chai et al., 2000; Jiang et al., 2000) to mark neural crest in Msx1/2 mutants confirmed these findings (Fig. 4G,H).

Delayed migration, mispatterning and inappropriate mixing of subpopulations of neural crest in Msx1/2 mutant embryos

To determine whether more subtle changes in the specification or distribution of subpopulations of neural crest are responsible for the observed morphological defects, we examined the expression of marker genes at several stages of neural crest development (Figs 4-8). We used Ap-2α to assess neural crest development at E8.5. Ap-2α is normally expressed in crest cells in the neural folds and in migratory neural crest cells (Mitchell et al., 1991) (Fig. 4L,M).

At E8.5, the intensity of Ap-2α staining was reduced in the neural folds of pro-rhombomere B in the mutant (two out of two embryos; Fig. 4L,P). As demonstrated previously, Msx1 and Msx2 were both expressed in the neural folds and migratory neural crest in patterns that overlap with Ap-2α (Fig. 4Q-T). Although E8.5 Msx1/2 mutant embryos had no detectable morphological abnormalities, these data suggest that loss of Msx1/2 resulted in defects in the specification or distribution of subpopulations of cranial neural crest cells.

To assess neural crest development in embryos subsequent to E8.5, we continued to use Ap-2α (Figs 5, 6). We also used cdh6 and Sox10, which are expressed in neural crest cells as they emigrate from the neural tube (Inoue et al., 1997; Southard-Smith et al., 1998).

At E9.5, Msx1 and Msx2 are expressed in the dorsal neural tube (Davidson, 1995) (Fig. 5A,B). Consistent with previous descriptions, Ap-2α, cdh6 and Sox10 were expressed in streams of neural crest migrating from r2 and r4 in the preotic hindbrain, and from r6-r8 in the postotic neural tube (Mitchell et al., 1991; Inoue et al., 1997; Southard-Smith et al., 1998) (Fig. 5C,E,G,I,K; Fig. 6A,C,E,G,I,K,M,O). Ap-2α, cdh6 and Sox10 expression was not detectable in r3 or r5, or in adjacent migratory crest cells (Fig. 5M,O, data not shown).

Differences in the expression of each of these three markers were evident in Msx1/2 mutant embryos. Ap-2α, cdh6 and Sox10 were expressed ectopically in strips of cells located in normally neural crest-free areas adjacent to r3 (arrowhead in

![Fig. 3. Cardiac outflow tract defects in Msx1/2 mutant embryos. Histological analysis of E12.5 (A,B) and E15.5 (C-H) control (A,C-E) and Msx1/2 mutant (B,F-H) embryo hearts. (A,B) At E12.5, the septation of the pulmonary trunk (PT) and the ascending aorta (Ao) is evident in the control (A), but not in the mutant (arrow in B). (C-H) Serial transverse sections from caudal to rostral show the aorta and pulmonary trunk arising from the right ventricle in the mutant (double outlet right ventricle). Msx1/2 mutants also exhibit dysmorphogenesis of the ventricular wall and myocardium. The location of the thymus (TH) was abnormal. RV, right ventricle; LV, left ventricle. Scale bars: in B, 0.5 mm for A,B; in E, 0.5 mm for C-H.]

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Development
Fig. 5D,H,J,L; Fig. 6B,F,H,N). Cross sections showed that for all three markers this expression was in mesenchyme (Fig. 5M-P, data not shown). These data suggest that, in Msx1/2 mutants, neural crest cells were located abnormally in areas adjacent to r3.

In addition, Ap-2α was misexpressed in the dorsal neural tube and the adjacent mesenchyme at the levels of r2, r4 and r6-r8 (Fig. 5D,F,N,P; Fig. 6B,D,J). Because in normal embryos neural crest cells have already emigrated from the embryonic hindbrain by the 14-somite stage (Serbedzija, 1992), these data suggest that the combined loss of Msx1 and Msx2 caused a significant retardation in the production, delamination or migration of a subpopulation of neural crest cells. Finally, Ap-2α and Sox10 transcripts were reduced in the region of the trigeminal ganglion (Fig. 6B,F,N); there was also a reduction in the number of streams of postotic crest expressing these transcripts, from three to two (Fig. 6L,P). We detected Ap-2α expression in pharyngeal arches 3, 4 and 6 (Fig. 6D,L), suggesting that cardiac neural crest cells reach their target site in Msx1/2 mutants.

Altered expression of Krox20 and Epha4 but normal expression of Hoxb1 and Hoxd4 in hindbrains of Msx1/2 mutant embryos

Changes in marker gene expression in cranial and cardiac neural crest populations raised the issue of whether loss of Msx1 and Msx2 affected rhombomere identity. We examined the expression of the hindbrain markers, Krox20, Hoxb1, Hoxd4, Epha4 and Crabp1 (Fig. 7). Krox20 is expressed in r3 and r5 (Sham et al., 1993; Swiatek and Gridley, 1993). Hoxb1 is expressed in r4 (Murphy et al., 1989), Hoxd4 in the neural tube from r7 caudally (Morrison et al., 1997), and Epha4 in r3 and r5 (Nieto et al., 1992). Gain- and loss-of-function experiments have shown that Krox20, Hoxb1, Hoxd4 and Epha4 are crucial for the establishment of rhombomere identity (Trainor and Krumlauf, 2000). Crabp1 is expressed in r2 and throughout the hindbrain, from r4 to r6 (Maden et al., 1992).

Whole-mount stains with riboprobes for each of these markers revealed that Krox20 was expressed normally in r3 in E8.5 mutants, but exhibited a restriction in the caudal limit of its expression in r5 (Fig. 7B,D). By E9.5, the r5 expression of
Krox20 was indistinguishable from that of wild type (Fig. 7F). The domains of expression of Hoxb1, Hoxd4 and Crabp1 were not altered in Msx1/2 mutant embryos at E9.0 or E9.5 (Fig. 7H,J,L; data not shown). Intriguingly, however, Epha4 expression was increased significantly in r3, and was expanded anteriorly into r1 and r2 (Fig. 7N,P). That hindbrain marker gene expression was unchanged in r4, r6 and r7 suggests that the defects in neural crest populations derived from these rhombomeres result from events downstream of the establishment of rhombomere identity. The changes in Krox20 and Epha4 expression suggest, however, that loss of Msx1 and Msx2 may at least transiently influence the development of r1, r2, r3 and r5.

**Msx genes are upstream of the extrinsic neural crest regulator Bmp4, but are not upstream of Wnt1, Fgf8 or Tbx1**

We next sought to identify deficiencies in signaling processes that might explain the abnormalities in subpopulations of neural crest. We examined the expression of three markers, Wnt1, Fgf8 and Tbx1 (Fig. 8). Wnt1, expressed in premigratory neural crest cells, is involved in neural crest expansion and subsequent differentiation (Ikeya et al., 1997; Saint-Jeannet et al., 1997). In the dorsal diencephalon of the mouse embryo, Wnt1 expression depends on Msx1 function (Bach et al., 2003). Expression of Fgf8 in the isthmus, median nasal prominence and pharyngeal endoderm is required for the development of both cranial and cardiac neural crest (Abu-Issa et al., 2002; Frank et al., 2002; Trainor et al., 2002; Hu et al., 2003). Expression of Fgf8 in pharyngeal arch ectoderm is important for the formation of the maxilla and mandible (Trumpp et al., 1999). Tbx1, required cell non-autonomously for neural crest cell migration in the third and fourth arch, functions upstream of Fgf8, and is a candidate for the cause of defective pharyngeal arch remodeling in DiGeorge/Velocardiofacial syndrome (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Vitelli et al., 2002; Vitelli and Baldini, 2003). Bmps are expressed broadly in the pharyngeal ectoderm and endoderm, and are thought to influence the development of cranial and cardiac neural crest-lineage cells (Kanzler et al., 2000; Waldo et al., 2001; Ashique et al., 2002; Ohnemus et al., 2002). That Bmp4 both regulates Msx genes and is itself regulated by them is well established (Chen et al., 1996; Bei and Maas, 1998; Zhang et al., 2003).
Fig. 6. Mispatterning and retarded migration of neural crest cells in E9.5-E10.5 Msx1/2 mutant embryos. (A-P) Lateral views of E9.5 (A-H) and E10.5 (I-P) embryos hybridized with digoxigenin-labeled RNA probes for Ap-2α (A-D,I-L), Sox10 (E,F,M-P) and cdh6 (G,H). Note the abnormal expression in mutants of each marker at the level of r3, as indicated by arrowheads (B,F,H,N). Also note the retarded production and migration of neural crest cells from rhombomeres 2, 4, 6 and 7 at E9.5 (B,D). Ap-2α and Sox10 expression in the trigeminal ganglion was significantly reduced (B,F,J,N arrows). In mutant embryos, merging of the migrating neural crest from r6 and r7 at E9.5 (D,F,H) results in the fusion of neural crest streams migrating into third and fourth arch arteries at E10.5 (asterisk in L,P). Boxed areas in I and J are shown at higher magnification in K and L. Scale bars: in A (for A,B), C (for C,D), F (for E,F), H (for G,H), 0.2 mm; in J (for L,J), L (for K,L), N (for M,N), P (for O,P), 0.4 mm.

Fig. 7. Altered expression of Krox20 and EphA4 in Msx1/2 mutant hindbrain. Whole-mount in situ hybridization analysis of E8.5 and E9.5 embryos with the indicated probes. (A-F) Expression of Krox20 at E8.5 (A-D) and E9.5 (E,F). The box in A and B demarcates the region shown in C and D. Note the reduction in the level of Krox20 transcripts in the caudal portion of r5 in mutant embryo at E8.5 (B,D), but normal expression at E9.5 (F). (G-L) Expression patterns of Hoxb1 in r4 (G,H), Hoxd4 in the region caudal to r7 (I,J), and Crabp1 from r2 to r6 (K,L) at E9.5 show no apparent change in the mutant hindbrain. (M-P) EphA4 expression at E9.5. Enlarged view of rectangles in M and N is shown in O and P. EphA4 expression was significantly increased in r1, r2 and r3 of the Msx1/2 mutant (arrowheads in P). Scale bars: in B (for A,B), F (for E,F), H (for G,H), J (for L,J), L (for K,L), 0.2 mm; D (for C,D), 0.1 mm.
As is evident from results shown in Fig. 8B, the level of Wnt1 expression in the isthmus and hindbrain of Msx1/2 mutant embryos at E9.5 was comparable to that of control embryos. Similarly, Fgf8 expression did not change in the isthmus, or in the median nasal prominence of Msx1/2 mutant embryos at E9.5 (Fig. 8D,F). Nor did the distribution of Tbx1 transcripts change significantly in pharyngeal endoderm or in the peripharyngeal region (Fig. 8H, data not shown). We note that expression of Fgf8 in the first pharyngeal arch (Fig. 8F) and Tbx1 in the mesodermal core of the pharyngeal arches (Fig. 8H) was slightly reduced in some mutant embryos.

E9.5 Msx1/2 mutant embryos expressed Bmp4 at significantly elevated levels in the cranial mesenchyme (including in migrating neural crest), the maxillary prominence, the distal part of the first pharyngeal arch, and the body wall epithelium (Fig. 8J, data not shown). These data suggest that Msx genes function downstream of, or in parallel to, Wnt1, Fgf8 and Tbx1, but upstream of Bmp4 during neural crest development.

Increased apoptosis but unchanged proliferation in subpopulations of cranial neural crest in Msx1−−; Msx2−− embryos

We next assessed apoptosis and proliferation in Msx1/2 double-mutant embryos. Apoptosis was detected by a TUNEL assay, and proliferation by an antibody against 10-phosphorylated histone H3, which marks cells in M phase. These assays were carried out on the same cross sections of embryos. At E9.5, an increase in TUNEL-positive cells relative to controls was evident in the posterior prominence of the optic vesicle, as well as in the maxillary and mandibular prominences of the first pharyngeal arch (Fig. 9B,D,F). The majority of mesenchymal cells at these sites are derived from the cranial neural crest (Chai et al., 2000; Jiang et al., 2000). Whole-mount Nile Blue staining, which marks dying cells, confirmed these results (Fig. 9G-L). Concentrations of Nile Blue-positive cells were evident (1) in the area of the trigeminal ganglion (Fig. 9H, arrow), and (2) in the proximal (open arrowheads in Fig. 9H) and distal portions (arrowhead in Fig. 9H,J) of the first pharyngeal arch. No changes in TUNEL or Nile Blue staining were evident in the hindbrain or in migrating postotic neural crest cells (including cardiac neural crest) of Msx1/2 mutants at E9.5 (Fig. 9J,L, data not shown).

We did not detect significant differences in the percentage of cells stained for phosphorylated histone H3 or BrdU in the pharyngeal arches or trigeminal ganglia (Fig. 9; data not shown). Consistent with the apparent lack of change in cell proliferation, counts of cell densities in the first pharyngeal arch showed no significant differences between Msx1/2 mutants and control embryos (Fig. 9, data not shown). These results suggest that the combined loss of Msx1 and Msx2 influenced the survival but not the proliferation of subpopulations of neural crest-derived mesenchyme in the craniofacial region.

Discussion

The neural crest is specified progressively, beginning at the time of its genesis in the neural tube, and continuing through its migration and later development in target tissues. Although this specification involves interactions between signaling...
pathways and downstream transcription factors, the molecular
details of this process are not clear. The known functions of
the Msx genes as effectors of the Bmp, Wnt and Fgf pathways,
together with the well-documented role of these pathways in
neural crest development, prompted us to undertake a close
examination of neural crest development in
\( \text{Msx1} / \text{Msx2} \) mutants.

We show that \( \text{Msx1} / \text{Msx2} \) have major roles in several aspects of
neural crest development, including the proper segregation of
subpopulations of hindbrain neural crest, the patterning of the
cranial ganglia and the survival of neural crest-derived cells in
the pharyngeal arches.

Msx gene function during the genesis and migration
of neural crest cells

Several lines of evidence suggest that Msx genes are involved
in the early events of neural crest specification. First, in the
chick embryo, \( \text{Msx1} \) is expressed at the lateral edge of the
neural plate, and \( \text{Msx1} \) expression at this site is dependent upon
an interaction between neural and non-neural ectoderm (Streit
and Stern, 1999). Second, the forced expression of \( \text{Msx1} \) in
\( \text{Xenopus} \) embryos induces neural crest cells, and inhibition of
\( \text{Msx1} \) activity by means of a dominant-negative form of \( \text{Msx1} \)
inhbits the production of neural crest cells (Tribulo et al., 2003).
Third, ectopic expression of \( \text{Msx1} \) in the neural tube of
the chick can induce the expression of a neural crest cell
marker (Liu et al., 2004). Despite the suggestion from these
results that Msx genes have a crucial, early role in neural crest
development, our data show that the majority of neural crest
are induced, undergo migration, and populate the
pharyngeal arches in the absence of
\( \text{Msx1} / \text{Msx2} \). We note,
however, that the function of \( \text{Msx3} \), which is expressed in the
dorsal neural tube and in neural crest (Shimeld et al., 1996;
Wang et al., 1996), may be sufficient for these activities. Thus,
a definitive test of the role of Msx genes in murine neural crest
development must await the simultaneous inactivation of
\( \text{Msx1} / \text{Msx2} / \text{Msx3} \).

Our results show that loss of \( \text{Msx1} / \text{Msx2} \) results in the delayed
appearance outside the neural tube of cells expressing neural
crest markers. This is first evident in the expression of \( \text{Ap}-2\alpha \)
at E9.0, and is apparent at later stages in the expression of \( \text{cdh6} \)
and \( \text{Sox10} \). Whether this delay is due to a deficiency in the
production, delamination or migration of neural crest cells
remains unclear. A second intriguing anomaly is the partial
merging or mixing of crest populations. Apparent in the
expression patterns of \( \text{Ap}-2\alpha / \text{H}9251 \), \( \text{cdh6} \) and \( \text{Sox10} \), such mixing
occurred between crest cells emigrating from r2 and those
emigrating from r4, as well as between streams of crest
emerging from the postotic rhombomeres 6, 7 and 8 (Figs 5,

![Fig. 9. Msx1/2 are required for the survival of cranial neural crest cells. (A-F) Double-label immunostaining of apoptotic and proliferating cells. Immunostaining of phosphorylated histone H3 (Rhodamine, red) and TUNEL (FITC, green) were carried out on the same sections to detect cell proliferation and apoptosis in Msx1/2 mutants. Nuclei were counterstained with DAPI. Arrowheads indicate increased apoptotic neural crest-derived cells in the region of the trigeminal ganglion (B), the proximal portion of pharyngeal arch1 (maxillary prominence; D), and the distal
tip of pharyngeal arch1 (mandibular prominence; F) of Msx1/2 mutants at E9.5. Cell proliferation within these sites was not noticeably altered. (G-L) Nile Blue-stained E9.5 control (G,I,K) and Msx1/2 mutant (H,J,L) embryos; lateral (G-J) and dorsal (K,L) views. Increased cell death was detected in neural crest-derived craniofacial structures of the Msx1/2 mutant (arrows, arrowheads and open arrowheads in H and J) consistent with results of the TUNEL assay. We did not detect increased cell death in cardiac neural crest cells in mutant embryos (J). Note the lack of discernable change of cell death in the mutant hindbrain (L). Scale bar in L: 0.2 mm (for G-L).](image)
Development

6. DiI labeling will address the origin and migratory path of aberrant neural crest at the level of r3. Krox20, kreisler (MafB – Mouse Genome Informatics), and the combinatorial actions of Hox family members, establish and maintain boundaries between rhombomeres and between subpopulations of migrating neural crest cells. They do so, at least in part, by controlling the activities of Epha4 and Epha7 (Trainor and Krumlauf, 2000), which control cell-cell affinity. Reduced Krox20 expression in r5 and increased Epha4 from r1 to r3 of Msx1/2 mutants suggests that Msx1/2 may participate in rhombomere development. In normal embryos, neural crest is excluded from r3, whereas in Msx1/2 mutants it is not. Upregulation of Epha4 in Msx1/2 mutants may have some part in abrogating this exclusion. It is intriguing that in Xenopus, forced expression of a dominant-negative form of Epha4 disrupted neural crest segregation (Smith et al., 1997). Overexpression of Epha4 in Msx1/2 mutants may have a dominant-negative effect and, as a consequence, may inhibit neural crest boundary formation between r2 and r4.

We consistently observed aberrant Ap-2α expression in the dorsal midline of Msx1/2 mutant hindbrain from E9.0 through E10.5 (Fig. 5, data not shown). This may reflect a defect in the specification of a subpopulation of neural crest cells. Msx1 and Msx2, as well as Ap-2α can maintain cells in an undifferentiated state (Liu et al., 1999; Odellberg et al., 2000; Hu et al., 2001; Pfisterer et al., 2002). That these three genes have similar functions, and that each is regulated by both Bmp and Wnt signals (Vainio et al., 1993; Willert et al., 2002; Luo et al., 2003), suggest that they may participate in a common molecular cascade in neural crest development. Also supporting this hypothesis is the striking similarity of phenotypes caused by the loss of Msx1/2 and Ap-2α. Both mutants, for example, exhibit exencephaly, craniofacial skeletal defects, hypoplastic cranial ganglia, persistant truncus arteriosus and thoraco-abdominoschisis (open body wall) (Schorle et al., 1996; Zhang et al., 1996; Brewer et al., 2002). In Ap-2α mutants, as in Msx1/2 mutants, increases in the apoptosis of neural crest cells are likely to contribute to at least some defects in neural crest-derived structures (Schorle et al., 1996).

Msx1/2 are required for survival of neural crest subpopulations

Our results suggest that apoptosis may contribute to some of the morphological deficiencies of Msx1/2 mutant embryos. The reduction in size of the trigeminal ganglion, as shown by decreased neurofilament expression, is preceded by a reduction of neural crest marker expression and a substantial increase in the number of apoptotic cells relative to control embryos (Fig. 9). Similarly, numbers of apoptotic cells are elevated in the maxillary prominence at E9.5. The mechanisms underlying this increase, including the issue of whether it is cell autonomous, remain unknown.

Previous work has shown that forced expression of Msx2 can cause apoptosis in P19 cells and in the hindbrain of the chick embryo (Marazzi et al., 1997; Takahashi et al., 1998). A prediction of these overexpression experiments is that loss of Msx1/2 should reduce apoptosis, which does not appear to be the case, either in the hindbrain or the pharyngeal arches. Although it is difficult to reconcile these results with ours, it is possible that the forced expression of Msx genes has a dominant-negative effect. Alternatively, the function of Msx genes may differ in cultured cells compared with in embryos, or in the chicken versus the mouse.

Msx genes and pharyngeal arch development

We are intrigued by the finding that loss of Msx1/2 function results in fusion of the maxillary and mandibular prominences, as well as in the reduced growth of these structures. Recent studies have shown that patterning of the craniofacial skeleton is controlled, in part, by non-Hox homeobox genes, including members of the Otx and Dlx families (Matsuo et al., 1995; Kuratani et al., 1997; Depew et al., 2002; Robledo et al., 2002). Because Msx1 and Msx2 are highly expressed in the maxillary prominence and mandibular arch, they could, in principle, function with other non-Hox homeobox genes in the axial patterning of craniofacial structures. In contradistinction to Dlx5/Dlx6 knockouy embryos, which exhibit a homeotic phenotype (Depew et al., 2002; Robledo et al., 2002), Msx1/2 mutant embryos do not show evidence of homeotic transformations of craniofacial features. Although we have not systematically surveyed the expression of non-Hox homeobox genes in Msx1/2 mutants, we have examined the expression of Dlx5, which is unaltered. This is consistent with the view that, in jaw development, the actions of Msx1/2 either parallel that of Dlx5 or are downstream of it. To the extent that a general function for Msx genes can be inferred from our data, such a function seems more likely to include the local control of cell segregation, differentiation and survival, than broad effects on region specification.

Role of Msx1/2 in the developing heart

Neither the loss of Msx1 nor Msx2 individually causes outflow tract defects (Kwang et al., 2002). However, our results show that the combined loss of Msx1 and Msx2 results in major defects in the outflow tract in a high percentage of embryos. We did not detect significant changes in the expression of Fgf8 or Tbx1, which function in the pharyngeal endoderm in signaling processes that influence cardiac crest development (Vitelli and Baldini, 2003). Similarly, the patterning of the caudal hindbrain from which the cardiac neural crest originates appeared to be largely normal in Msx1/2 mutants, as assessed by the expression of Hoxa4 and Crabp1. These results are consistent with our observation that cardiac neural crest cells are present in the pharyngeal arteries at E9.5 (Fig. 6). It will be interesting to determine whether these defects are caused by anomalies in the secondary heart field (Waldo et al., 2001), and whether they are crest-cell autonomous.

Interaction between signaling pathways and Msx genes

Several upstream regulators of Msx genes have been identified. These include Bmps, Wnt/β-catenin and Fgf pathways. Msx1/2 mutant embryos share features with mutants in each of these pathways (Ohnemus et al., 2002; Daeot et al., 2003; Stottmann et al., 2004; Ikeya et al., 1997; Kioussi et al., 2002; Abu-Issa et al., 2002; Frank et al., 2002). The involvement of Msx1/2 in multiple aspects of neural crest cell development implies that, depending on stage and tissue, Msx genes may function to integrate signals from several pathways.

Our results showed that Bmp4 expression is increased in the cranial neural crest and pharyngeal arches of Msx1/2 mutant
embryos. This suggests that Msx genes negatively control Bmp signals in these structures. Although, Msx genes do not appear to control the expression of Wnt1 and Fgf8 in neural crest development, it remains possible that there are additional signaling molecules regulated by Msx genes.

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References


Msx1 and Msx2 in mouse neural crest development


