Moz-dependent Hox expression controls segment-specific fate maps of skeletal precursors in the face

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Development of the facial skeleton depends on interactions between intrinsic factors in the skeletal precursors and extrinsic signals in the facial environment. Hox genes have been proposed to act cell-intrinsically in skeletogenic cranial neural crest cells (CNC) for skeletal pattern. However, Hox genes are also expressed in other facial tissues, such as the ectoderm and endoderm, suggesting that Hox genes could also regulate extrinsic signalling from non-CNC tissues. Here we study moz mutant zebrafish in which hoxa2b and hoxb2a expression is lost and the support skeleton of the second pharyngeal segment is transformed into a duplicate of the first-segment-derived jaw skeleton. By performing tissue mosaic experiments between moz and wild-type embryos, we show that Moz and Hox genes function in CNC, but not in the ectoderm or endoderm, to specify the support skeleton. How then does Hox expression within CNC specify a support skeleton at the cellular level? Our fate map analysis of skeletal precursors reveals that Moz specifies a second-segment fate map in part by regulating the interaction of CNC with the first endodermal pouch (p1). Removal of p1, either by laser ablation or in the itga5b926 mutant, reveals that p1 epithelium is required for development of the wild-type support but not the moz duplicate jaw-like skeleton. We present a model in which Moz-dependent Hox expression in CNC shapes the normal support skeleton by instructing second-segment CNC to undergo skeletogenesis in response to local extrinsic signals.

KEY WORDS: Craniofacial, Skeleton, Zebrafish, Moz, Hox, Morphogenesis

INTRODUCTION

Hox genes regulate organ identity along the anteroposterior axis (Hombria and Lovegrove, 2003), yet how Hox genes control segment-specific organ shape at the cellular level is poorly understood. The vertebrate facial skeleton is derived from cranial neural crest cells (CNC) arranged into a series of segments, or ‘arches’, of the pharynx (Le Douarin, 1982). In zebrafish, CNC of the second (hyoid) segment express hoxa2b and hoxb2a and generate the support skeleton for the jaw and gill cover, whereas first (mandibular) segment CNC do not express Hox genes and generate the jaw skeleton (Hunt et al., 1991; Schilling and Kimmel, 1994). In diverse vertebrates, loss of the Hox2 genes, Hoxa2 or hoxa2b/hoxb2a, causes a mirror-image duplication of the jaw skeleton to form in the second segment (Gendron-Maguire et al., 1993; Hunter and Prince, 2002; Miller et al., 2004; Rijli et al., 1993). In a reciprocal manner, forced expression of Hoxa2 throughout the first segment causes partial transformation of the jaw skeleton toward a second-segment skeletal morphology (Grammatopoulos et al., 2000; Hunter and Prince, 2002; Pasqualetti et al., 2000).

moz encodes a histone acetyltransferase required to maintain Hox expression in the hindbrain and in postmigratory CNC and epithelia of the second segment (Fig. 1) (Miller et al., 2004). In wild-type larvae, the first-segment-derived skeleton includes Meckel’s (M) and palaatoquadrate (Pq) cartilages, which form the primary lower and upper jaw elements, respectively. The second-segment-derived support skeleton includes the ceratohyal (Ch) cartilage, the hyosymplectic (Hs) cartilage [composed of hyomandibular (Hm) and symplectic (Sy) elements], which bridges the upper jaw to the ear, and opercular (Op) and branchiostegal ray (Br) dermal bones, which support the gill cover (Fig. 2A). In moz mutants, second-segment support cartilages are replaced by an extra set of jaw-like cartilages, M-like (M’) and Pq-like (Pq’), and second-segment dermal bones are lost (Fig. 2B) (Miller et al., 2004). This ‘homeotic’ phenotype resembles that seen in zebrafish with hoxa2b and hoxb2a function reduced by morpholinos [Fig. 2C and Hunter and Price (Hunter and Prince, 2002); hereafter referred to as hox2-MO animals]. We chose to focus our study on the moz mutant for several reasons. First, the moz mutant phenotype is more consistently expressive than the phenotype seen in hox2-MO larvae. Second, whereas Moz is required for the maintenance of Hox expression in postmigratory CNC, Moz is not required for earlier Hox expression at premigratory stages (Miller et al., 2004). Because early Hox expression and neural crest induction and migration are normal in moz mutants, studying moz allows us to selectively probe the postmigratory functions of Hox genes in skeletal patterning.

Moz is required not only for hoxa2b and hoxb2a expression in second-segment CNC but also for hoxb2a expression in a subset of ectoderm and endoderm that surrounds second-segment CNC (this work) (Miller et al., 2004). It is clear from studies in chicken, zebrafish and mouse that signals from the ectoderm and endoderm pattern the CNC-derived facial skeleton (Couly et al., 2002; Crump et al., 2004a; Crump et al., 2004b; Eberhart et al., 2006; Helms and Schneider, 2003; Piotrowski and Nusslein-Volhard, 2000; Ruhn et al., 2003). Although Hox2 genes are expressed in the ectoderm and endoderm, the skeletal patterning function of Hox2 expression in these tissues has not been directly tested. Using tissue mosaic experiments in zebrafish, in which we selectively restored or removed Moz or Hoxa2b/Hoxb2a function from specific facial tissues, we demonstrate that Moz and Hox2 genes function in CNC, but not in the ectoderm or endoderm, for patterning of the second-segment-derived skeleton. Next, we used single cell labelling and time-lapse analysis to show that Moz specifies the fate map of second-segment skeletal precursors. In particular, dorsal second-segment CNC, which form part of the Hs cartilage and Op dermal bone in wild type, fail to undergo skeletal differentiation in moz mutants, effectively positioning the duplicate jaw-like skeleton more
ventrally. Additionally, whereas p1 endoderm is crucial for normal Hs cartilage development (Crumpl et al., 2004b), the moz jaw-like cartilage (Pq) that replaces Hs does not require p1. We conclude that an important CNC-intrinsic function of Moz is to confer competence on skeletogenic CNC to respond to spatially restricted endodermal, and perhaps ectodermal, signals. Hence, normal Moz-dependent Hox expression determines where in the second segment skeletal differentiation occurs, thus establishing the spatial geometry of a support skeleton.

MATERIALS AND METHODS

Zebrasfish strains

Zebrasfish (Danio rerio) were raised at 28.5°C. fli1:GFP, moz, and tiga strains are as described (Crumpl et al., 2004b; Lawson and Weinstein, 2002; Miller et al., 2004). In the construction of the moz: fli1:GFP strain we discovered that fli1:GFP and moz were tightly linked on LG5 (2/100 recombinants from a moz × fli1:GFP cross). Once constructed, the brighter fluorescence of homozygous moz: fli1:GFP embryos allowed us to greatly enrich for moz mutants at 24 hours postfertilization (hpf).

Phenotypic analysis

Facial skeletons were stained with Alcian Green, and flat mount dissections were performed as described (Crumpl et al., 2004a; Crumpl et al., 2004b). Whole-mount in-situ hybridization and the hoxa2b probe are as described (Miller et al., 2004). As the previously described hoxa2b probe (Miller et al., 2004) was found to cross-react with fli1:GFP, we used gene-specific primers containing the T3 promoter to amplify and synthesize the probe. Primers used were 5′: ACCCTGGTCCACTATACTTC and 3′: CGCGCAATTAACCCTCACTAAAGGGGAATCAAAGGCCTCCGTA-G. For frozen sections, larvae were embedded in a mixture of 0.9% agar, 1% low melt agarose and 5% sucrose, frozen by suspension above liquid nitrogen, and cut at 16-μm intervals with a Leica CM3050S cryotome. In-situ hybridization was then performed on frozen sections as described (Rodriguez-Mari et al., 2005). hoxa2b-MO and hoxb2a-MO were injected together into 1-cell embryos at 5 ng/ml each in a 3-nl volume (Miller et al., 2004).

Transplants and microelectroporation

Alexa-568-labelled CNC, endoderm and neural precursors were transplanted at shield stages as described (Crumpl et al., 2004a; Crumpl et al., 2004b). The transplant technique for facial surface ectoderm precursors was similar to that described for CNC transplants (Crumpl et al., 2004b), except that donor ectoderm was placed 120° from dorsal and midway between the margin and animal pole of host embryos at shield stage. All transplants were unilateral, and, except where indicated otherwise, both donors and hosts were selected for analysis if donor tissue constituted at least half of second-segment CNC, ectoderm or endoderm based on inspection in a fluorescence dissecting microscope. Microelectroporations were performed as described (Crumpl et al., 2004b; Lyons et al., 2003). Each point represents a cell or the centroid of a pair of cells in an individual. Lateral and dorsal cross-sectional views were made at the level of labelled cells using Zeiss LSM software and plotted onto schematics of generalized pharyngeal segments.

Laser ablation

The first endodermal pouch was ablated using high-power laser irradiation. In order to visualize laser irradiation, fli1:GFP or moz; fli1:GFP embryos were injected with RNA encoding for the Kaede photoclonable fluorescent protein (Ando et al., 2002) at the one-cell stage. At 28-32 hpf, embryos were mounted in 0.2% agarose and, using a Biorad Radiance 2100 multi-photon microscope, a preliminary scan was performed to localize p1. Then, using the ROI tool of the Biorad Lasersharp software to select only p1, we irradiated p1 for 5 minutes with a 10 W Milenia/Tsunami (Spectraphysics) laser tuned to 780 nm and at 100% power. Scans taken immediately after irradiation showed that the Kaede protein was efficiently converted to red emission specifically in p1 (data not shown). Embryos were re-imaged 4 hours post-irradiation using Nomarski polarization and fixed at 5 days postfertilization (dpf) for Alcian staining. In approximately half of irradiated animals, Nomarski imaging revealed that p1 cells were visibly damaged compared with neighbouring unirradiated cells. In a fraction of animals, p1 cells did not appear damaged and no cartilage defects were observed. Thus, we only used animals in which p1 was visibly damaged for our skeletal analysis.

Microscopy

Digital images of facial skeletons and in situ hybridization were obtained on a Zeiss Axiohot 2 microscope using Axiocam software. Levels were uniformly adjusted using Adobe Photoshop CS2 software. Fluorescent imaging and time-lapse recordings were done on a Zeiss LSM5 Pascal confocal microscope as described (Crumpl et al., 2004b). Except when indicated otherwise, anterior is to the left and ventral is down in all panels.

RESULTS

Limited expression of second-segment Hox genes outside CNC

In order to address how Hox2 genes specify second-segment skeletal morphology, a detailed understanding of second-segment Hox2 expression is essential. However, the facial expression of Hoxa2 in mouse and chicken (Coully et al., 1998; Hunt et al., 1991; Maconochie et al., 1999) and hoxa2b and hoxb2a in zebrasfish (Hunter and Prince, 2002; Miller et al., 2004) are less well characterized outside the CNC. Hence, we examined hoxa2b and
hoxb2a expression in horizontal sections of zebrafish larvae at 34 hpf, a time after CNC migration (11-18 hpf) but before the first skeletogenesis (52 hpf) (Fig. 1A,C). In accord with previous whole-mount analysis (Hunter and Prince, 2002; Miller et al., 2004), sections show that both hoxa2b and hoxb2a are expressed in postmigratory CNC. In addition, hoxa2b, but not hoxb2a, is expressed in a small region of ventral ectodermal extending from the anterior portion of the second pharyngeal segment into the first segment. This ectodermal expression appears similar to that described for mouse Hoxa2 (Maconochie et al., 1999). However, we did not detect widespread expression of hoxa2b or hoxb2a in second-segment ectoderm. In the endoderm, hoxa2b, but not hoxb2a, was expressed in the second pouch but not in the first pouch or endoderm medial to second-segment CNC. Thus, as reported for Hoxa2 in other vertebrates, zebrafish hoxa2b is expressed in a very limited pattern in the pharyngeal ectoderm and endoderm, and hoxb2a pharyngeal expression is restricted to CNC. Lastly, we found hoxa2b and hoxb2a expression to be either absent or very reduced in the CNC, ectoderm, and endoderm of moz– larvae (Fig. 1B,D).

Moz functions in CNC to specify a support skeleton

As we found Hox2 expression in tissues outside the CNC, we asked whether Moz and Hox2 genes function exclusively in CNC or additionally in other head tissues for skeletal patterning. In order to test if Moz is sufficient in CNC to restore normal skeletal patterning to moz mutants, we transplanted wild-type CNC precursors into moz– hosts. In transplants where donor wild-type CNC populated a large proportion of the second segment, we found that wild-type CNC formed a wild-type support skeleton in otherwise moz– hosts (Fig. 2E-L). Indeed, wild-type CNC restored both normal cartilage (Hm, Sy, Ch) and dermal bone (Op, Br) development to moz– hosts. Strikingly, in transplants with fewer donor cells, moz– CNC tended to sort to the anterior, ventral region of the mosaic second segment (Fig. 2I-L). In these cases, we observed hybrid cartilage patterns in which wild-type CNC formed support cartilage and moz– CNC formed jaw-like cartilage in the same segment. The strict CNC autonomy of the moz– phenotype suggests that individual or small groups of CNC interpret the presence of Moz independently and differentiate accordingly.
The rescue of moz- skeletal transformations by wild-type CNC shows that Moz need only be functional in second-segment CNC for a normal support skeleton to develop. In order to test if Moz is required in CNC for support skeletal development, we transplanted moz- CNC precursors into wild-type hosts to generate animals in which Moz is selectively lost in CNC. When we examined animals in which a large proportion of the second segment consisted of moz- CNC, the support skeleton was largely transformed into a jaw-like character (Fig. 2M-P), similar to that seen in fully moz- animals. Further analysis suggested that the lack of a complete transformation was due to remaining wild-type CNC in the second segment. In order to create animals in which the entire second segment was populated by moz- CNC, we transplanted wild-type CNC into the second segment in order to create animals in which the entire second segment was populated by moz- CNC, we used use of a newly discovered mutant, b1092. Homozygous b1092 mutants lack all CNC-derived skeletal elements due to a defect in CNC generation, and this defect is completely rescued by wild-type CNC transplants (J.G.C., unpublished). When instead of wild-type CNC we transplanted moz- CNC into b1092 mutants, we observed that second segments of normal appearance formed early (Fig. 2Q,R) and hoxa2b expression was lost in second-segment CNC (Fig. 1E). In these ‘rescued’ moz- second segments, we observed transformed jaw-like skeletons, indistinguishable from those seen in fully moz- animals (Fig. 2S,T). Thus, the function of Moz in CNC is both necessary and sufficient to explain all the facial skeleton defects seen in moz mutants.

Our results from CNC transplants suggest that Moz has no skeletal patterning functions in non-CNC tissues. In order to examine functions of Moz directly in the ectoderm, endoderm and neural tube, we tested whether wild-type transplants of each of these tissues could restore normal skeletal development to moz- hosts (Fig. 3). We used high-resolution confocal imaging to confirm that wild-type donor cells were targeted to individual tissues (Fig. 3), and for wild-type ectoderm transplants we verified that hoxa2b was expressed in the ectoderm but not the CNC of moz- hosts (Fig. 1F). However, we found that transplants of wild-type ectoderm, endoderm or neural tube precursors were unable to rescue moz- skeletal transformations. Thus, we do not detect a role for ectodermal and endodermal hoxa2b expression in patterning the support skeleton.

Hox genes are CNC-autonomous effectors of Moz in facial skeletal patterning

Previous analysis of moz mutants strongly suggested that Moz regulates skeletal patterning through the regulation of Hox2 genes: hoxa2b and hoxb2a expression are lost in moz mutants and the knockdown of hoxa2b and hoxb2a largely phenocopies the homeotic transformations seen in moz (Hunter and Prince, 2002; Miller et al., 2004). Thus, we tested whether Hox2 function, like Moz, is also sufficient in CNC to specify a support skeleton. In order to create embryos in which Hox2 function is eliminated in all tissues except CNC, we transplanted wild-type CNC precursors into hox2-MO larvae (Fig. 2C,D). Upon skeletal analysis, we observed that wild-type CNC fully rescued hox2-MO skeletal transformations. Thus, Hox2 genes need only be functional in CNC for support skeleton development.

In addition, we tested whether Moz regulates Hox2 expression cell-autonomously. Using wild type to moz- and moz- to wild type CNC transplants, we created second segment mosaics for wild-type and moz- CNC and then examined these segments for hoxb2a expression (Fig. 4). We found a striking positive correlation between hoxb2a expression and the localization of wild-type CNC in the mosaic segments. Interestingly, we frequently observed a spatial segregation of wild-type and moz- CNC, with moz- CNC occupying more ventral and anterior regions. Thus, whereas in fully moz- animals CNC can occupy the entire second segment, our mosaic experiments suggest that there may be adhesive differences between wild-type and moz- CNC that are revealed by competition.

Moz controls the fate map of skeletal precursors

As we determined that Moz functions in CNC, we next examined how Moz functions at the cellular level to specify a particular skeletal arrangement. A prominent difference between the jaw and support skeleton is the dorsoventral location of the upper elements in their respective segments, required because the support but not the jaw skeleton of zebrafish attaches to the otic skeleton above (Fig. 2A; here we define ventral as where CNC contact the yolk, and dorsal as the upper limit of p1). Moz could regulate support versus jaw skeleton morphology either by instructing ‘segmentally equivalent’ skeletogenic CNC in similar regions of their respective...
Fig. 4. Moz controls hoxb2a expression cell-autonomously in CNC.
(A,D) Confocal images at 36 hpf of the first two segments (numbered in A) of fli1:GFP hosts after unilateral transplantation of CNC precursors (red) at shield stage. After live imaging, the same animals were fixed and stained with hoxb2a RNA probe. The transplant recipient sides (B,E) and the contralateral control sides that received no transplant (C,F) are shown. (A-C) Wild-type CNC in a moz\(^{-}\); fli1:GFP host cell-autonomously rescue hoxb2a expression in the second segment. Note the similar zones of red donor and hoxb2a-expressing CNC in A and B; white arrowheads denote a small group of isolated CNC rescued for hoxb2a expression. Second segments of control moz\(^{-}\) sides do not express hoxb2a (C). (D-F) moz\(^{-}\) CNC in a wild-type fli1:GFP host cell-autonomously fail to express hoxb2a in the second segment. Note the reciprocal zones of red donor and hoxb2a-expressing CNC in D and E. Second segments of control wild-type sides express hoxb2a (F). The similarity of hoxb2a expression in B and E shows that non-hoxb2a-expressing cells preferentially sort to the anterior ventral domain of the second segment. Scale bar: 50 \(\mu\)m.

Fig. 5. The fate map of second-segment skeletal derivatives is altered in moz mutants. Summaries of wild-type (A-C) and moz\(^{-}\) (D-F) fate maps and representative examples (G-R). Lateral views (A,D,G,J,M,P) and dorsal cross-sectional views at the level of the labelled cell with anterior to the left and lateral up (B,E,H,K,N,Q) are shown at 24 hpf. The facial skeleton is shown at 4 dpf (C,F,J,L,O,R). Filled circles denote facial cartilage precursors, and open circles denote precursors of neurocranial cartilage that surrounds the otic capsule. Triangles and squares denote precursors of the Op (upper) and Br (lower) dermal bones, respectively. moz\(^{-}\) CNC that made no skeleton are hatched squares. In B and E, areas of surface ectoderm (blue), endoderm (pink), and stomodeal ectoderm (grey) are shown (compare to insets in Fig. 3A,D). In A,B,G,H, first pouch endoderm (P) is labelled. Wild-type examples of anterior Hm cartilage (G-I) and Op osteocyte (J-L) precursors are shown. (M-R) moz\(^{-}\) CNC in similar positions fail to make skeleton. Note that in both wild types and moz mutants the most dorsal CNC in the first and second segment contribute to neurocranial cartilage that surrounds the otic capsule. Scale bars: 50 \(\mu\)m.
between segments. In the moz− second segment, CNC throughout the intermediate zone contributed to Pq′ and more ventral CNC contributed to M′. However, moz− CNC in the dorsal zone, including those along p1, did not contribute to the jaw-like cartilages by 5 dpf. This condition mimics that in the wild-type first segment, in which Hox genes are not normally expressed and dorsal CNC next to p1 do not form jaw cartilage. In addition, CNC in the lateral domain, which give rise to Op and Br bones in wild type, contribute instead to undifferentiated lateral mesenchyme in moz− second segments, consistent with a lack of second-segment dermal bone in moz mutants. Thus, although there is considerable overlap between the moz− and wild-type fate maps, we show that when Hox expression is lost in the moz− second segment, precursors of dorsal facial cartilage and lateral bone fail to undergo skeletal differentiation.

**Moz is required for the development of cartilage precursors adjacent to p1**

As second-segment CNC along dorsal p1 endoderm fail to make cartilage in moz mutants, we made time-lapse recordings of wild-type and moz− skeletal development to further examine the Moz-dependent interaction of dorsal cartilage precursors with p1. In the wild-type example, CNC are doubly labelled by fli1;GFP (Lawson and Weinstein, 2002) and mosaic transplantation of dye-labelled CNC (Fig. 6A; see Movie 1 in the supplementary material). As we reported previously (Crump et al., 2004b), in the wild-type second segment, dorsal CNC adjacent (and posterior) to p1 at 36 hpf form the anterior half of the Hm cartilage by 84 hpf. By contrast, in the first segment, dorsal CNC adjacent (and anterior) to p1 do not contribute to jaw cartilage by 84 hpf. Instead, in agreement with our 24 hpf fate map analysis, dorsoventrally intermediate first-segment CNC contribute to the Pq cartilage.

Next, we made time-lapse recordings of moz− second-segment skeletal development (Fig. 6B; see Movie 2 in the supplementary material). Consistent with previous analyses (Miller et al., 2004), we observe that the structure of the CNC segments at the beginning of the recordings is largely normal in moz− animals. By the end of the recordings, we observe that dorsal CNC of the second segment do not form cartilage and instead a more ventral domain of CNC contributes to Pq′. In particular, by contrast to wild-type CNC, moz− CNC along the dorsal portion of p1 fail to chondrify. To test if Moz controls chondrification cell-autonomously in CNC, we made time-lapse recordings of moz− hosts in which a small number of wild-type CNC precursors populated the dorsal second segment (Fig. 6C; see Movie 3 in the supplementary material). The wild-type CNC along the dorsal portion of p1 expanded normally and formed cartilage in an otherwise moz− environment (Fig. 6D).

We conclude that Hox2 expression in zebrafish specifies the dorsal support skeleton by promoting, in a cell-autonomous manner, the expansion and chondrification of CNC adjacent to p1 endoderm.

**Wild-type, but not moz−, second-segment cartilage requires p1 endoderm**

As we saw a difference in cartilage potential of wild-type and moz− CNC adjacent to p1, and as p1 is required for anterior Hm and Sy cartilages in wild types (Crump et al., 2004b), we asked whether p1 is required for the development of the jaw-like Pq′ cartilage that replaces Hm and Sy in moz− animals. In itga5; itga6; itgb26 mutants, in which p1 fails to develop, second-segment-derived anterior Hm and Sy cartilage elements are selectively lost, yet the first-segment-derived Pq and M cartilages form normally (Fig. 7A-D) (Crump et al., 2004b). In our previous study (Crump et al., 2004b), the presence of the fli1;GFP transgene allowed us to select itga5; itga6; itgb26 mutants in which p1 was absent. This live selection was crucial, as only itga5; itgb26 mutants lacking p1 have subsequent cartilage loss. Thus, we used live selection to examine skeletal morphology in moz−; itga5; itga6; itgb26 double mutants in which p1 was completely absent. In moz−; itga5; itgb26; fli1;GFP mutants lacking p1, the second-segment-derived Pq′ and M′ cartilages were similar in morphology to those in moz− single mutants (Fig. 7G-J). However, we did observe increased fusions between Pq′ and Pq in moz−; itga5; itgb26 mutants.
consistent with pouches also acting as barriers between the cartilage-forming CNC of neighbouring segments (Crump et al., 2004a; Piotrowski and Nusslein-Volhard, 2000). We also independently prob the requirement for p1 in wild-type and moz skeletal development by eliminating p1 with laser ablation. As with the genetic loss of p1, the laser ablation resulted in the loss of wild-type, but not moz, second-segment skeletal derivatives (Fig. 7E,F,K,L). In conclusion, we find that, by contrast to normal second-segment derivatives, the duplicate jaw-like cartilages that form from moz second segments do not require p1 endoderm, and, by inference, must be responding to a different source of signals from those that induce the wild-type support skeleton.

FIG. 7. The development of moz second-segment cartilage does not depend on p1 endoderm. CNC segments at 36 hpf (A,C,E,G,I,K) and facial skeletons at 4 dpf (B,D,F,H,J,L) from wild-type fli1::GFP (A,B,E,F), itga5b926; fl1::GFP (C,D), moz; fli1::GFP (G,H,K,L), and moz; itga5b926; fl1::GFP (I,J) animals. In E and K, inclusion of the Normarski channel shows that p1 endoderm has been selectively ablated by laser irradiation, as evinced by the dark pyknotic nuclei. When p1 is eliminated in either itga5b926; fli1::GFP (C) or p1 ablated (–p1) (E) animals, the anterior portion of the Hn cartilage is lost and 5y cartilage is variably reduced (D,F). However, removal of p1 in moz; itga5b926; fli1::GFP (I) or p1 ablated moz (K) animals does not affect the shape of Pq (J,L). For p1 laser ablations, 13/14 wild-type animals had reduced Hm cartilage and 0/5 moz mutants had reduced Pq. Arrows denote p1. Scale bars: 50 μm.

**DISCUSSION**

**Hox genes are CNC-intrinsic regulators of facial skeletal patterning**

Since the discovery that Hox genes control anteroposterior identity in the facial skeleton (Gendron-Maguire et al., 1993; Rijli et al., 1993), Hox genes have largely been assumed to function within skeletal precursors to confer their distinct characters. Yet Hox genes also have limited expression in the ectoderm and endoderm that surround the skeletonogenic CNC (this study) (Couly et al., 1998; Hunt et al., 1991; Maconochie et al., 1999). Indeed, misexpression studies suggest that Hox2 confers regional identity on the ectoderm and endoderm and that this specification is important for inductive signalling to CNC (Couly et al., 1998; Grammatopoulos et al., 2000). By contrast, our mosaic analyses revealed a Hox2 skeletal patterning role only in CNC in moz and hox2-MO embryos, which lack hoxa2b and hoxb2a expression and function in all facial tissues: specific loss of Moz function in CNC gives homeotic skeletal transformations as severe as those seen in fully moz animals, and restoring Moz function specifically in CNC completely rescues moz support skeleton development. Moz probably acts through Hox2 genes, as we find that Moz controls Hox2 expression cell-autonomously in CNC, and wild-type CNC also completely rescue hox2-MO skeletal defects. Furthermore, as Moz is required for the maintenance of Hox2 expression in postmigratory CNC, but not for Hox expression in premigratory CNC, our moz CNC into wild-type mosaics demonstrate a late skeletal patterning function for Hox2 expression in postmigratory CNC. Recent experiments in mouse, in which Hoxa2 function was eliminated specifically in postmigratory CNC, demonstrate that the late CNC-specific function of Hox2 genes in skeletal patterning is conserved from fish to mammals (Santagati et al., 2005).

We find that Moz-dependent Hox2 expression in CNC, in the absence of Hox2 expression in the endoderm and ectoderm, is entirely sufficient to generate a second-segment-derived support skeleton. The lack of a role for Hox2 genes in the ectoderm might seem to contradict previous experiments in avian and amphibian embryos (Couly et al., 1998; Creuzet et al., 2002; Grammatopoulos et al., 2000), showing that Hoxa2 needs to be misexpressed throughout the embryo, not just in CNC, in order to induce the formation of second-segment skeletal derivatives from first-segment CNC. The results were interpreted to mean that CNC require a signal from Hoxa2-expressing ectoderm. Alternatively, it is known that first and second arch ectoderm differ in more than just Hox expression, e.g. pitx2 expression, normally present in the first segment, does not turn on ectopically in the moz second segment (Miller et al., 2004). Strikingly, in mice, new elements (e.g. a pterygoquadrate-like cartilage) arise in the Hoxa2 second segment that do not form in the wild-type first segment (Rijli et al., 1993), suggesting that first and second arch epithelia (including the ectoderm) have different skeletal-inducing properties even in the absence of Hox2 expression. One possibility is that first, but not second, segment epithelium has an inhibitory influence on the development of Hox-expressing CNC. Our analyses would not detect such an influence, but the misexpression studies would. Nonetheless, our work shows that Hox2 expression in the ectoderm and endoderm is not required for the specification of second-segment skeletal derivatives.

Furthermore, our detailed expression analyses support the same conclusion. In the pharynx, only hoxa2b is expressed outside CNC, and it is expressed in very limited domains of first and second-segment ectoderm and endoderm. We detect no hoxa2b or hoxb2a expression in p1 endoderm, an epithelial domain that we have shown to be crucial for support skeleton development (Crump et al., 2004a; Crump et al., 2004b). Moreover, zebrafish hoxa2b and mouse Hoxa2 are expressed in only a small domain of ectoderm at the first and second segment boundary (this study) (Hunter and Prince, 2002; Maconochie et al., 1999), and avian Hoxa2 ectoderm expression is reported only in a small domain at the second and third segment boundaries (Couly et al., 1998). Thus, the lack of widespread Hox2
CNC-intrinsic factors regulate skeletogenesis in response to spatially restricted extrinsic signals

A longstanding debate is the extent to which extrinsic and intrinsic cues instruct facial skeletal patterning. Noden proposed that CNC are intrinsically ‘prepatterned’ (Noden, 1983; Trainor et al., 2002). By contrast, numerous studies suggest that extrinsic signals from the pharyngeal endoderm and oral ectoderm pattern the facial skeleton (Couly et al., 2002; Crump et al., 2004a; Crump et al., 2004b; Eberhart et al., 2006; Helms and Schneider, 2003; Piotrowski and Nusslein-Volhard, 2000; Ruhin et al., 2003). Hox genes determine segment-specific organ structure in diverse animals and have been assumed to pattern distinct ‘identities’ within sets of cells that begin development identically from segment to segment. However, our wild-type and moz fate maps show that the CNC skeletal primordia are positionally non-equivalent in adjacent segments. Thus, Hox genes select which cells in a segment will make skeleton, rather than selecting what sort of skeleton comes from pre-specified cells.

How are these preskeletal CNC subsets selected? We show that Hox2 genes instruct CNC to respond to cues from particular local epithelia, one of which is p1 endoderm. Wild-type second-segment CNC along dorsal p1 form support cartilage, yet positionally equivalent wild-type first-segment, or moz second-segment CNC, do not contribute to jaw-like cartilages. A few wild-type first-segment CNC and moz second-segment CNC near ventral-medial p1 do in fact give rise to jaw and jaw-like cartilages. However, when p1 is genetically removed or laser ablated, jaw and jaw-like cartilages are not affected. Thus, even those non-Hox-expressing CNC close to p1 may not depend on p1 for their development.

In addition, a prominent feature of zebrafish moz mutants and mouse Hoxa2 mutants is the mirror-image symmetry of the skeletal duplications, with the axis of symmetry at the boundary between the first and second segment. p1 endoderm, at this boundary, is clearly not responsible for mirror-image skeletal duplications, because jaw and jaw-like cartilages are unaffected by its absence. Furthermore, both the jaw and moz jaw-like cartilages derive from intermediate and ventral segmental domains, whereas p1 is dorsal. Thus, there are probably additional segmental boundary epithelial signalling centres that account for the mirror-image symmetry. Medial endoderm (Fig. S1A) and bmp4-expressing ventral ectoderm are good candidates.

Wild-type cartilage precursors fate map close to endoderm, whereas dermal bone precursors develop adjacent to surface ectoderm. This segregation suggests a model in which endoderm locally induces cartilage fate and ectoderm induces dermal bone fate. Intriguingly, in moz mutants second-segment Op and Br dermal bones fail to form, yet we know from work on the itga5f026 mutant that dermal bone development does not depend on the presence of p1 endoderm (Crump et al., 2004b). Thus, Moz-dependent Hox expression probably controls the response of CNC to more than just p1 endoderm; signals to dermal bone precursors may come from the ectoderm (Tyler and Hall, 1977). In addition, there is considerable overlap between the wild-type and moz fate maps in the ventral second segment, and thus differences between support and jaw skeletons may also arise by Hox2 genes controlling distinct morphogenetic behaviours of spatially equivalent CNC in the first two segments (O’Gorman, 2005).

In our model, postmigratory CNC encounter endodermal and ectodermal epithelia that have the ability to induce cartilage and bone differentiation in specific locations. Hox genes, functioning intrinsically, then instruct CNC to undergo skeletogenesis in response to a subset of these epithelia, thus positioning cartilage and dermal bone elements in the appropriate geometry. Our findings are consistent with avian grafting experiments showing that distinct domains of head endoderm control the shape and position of facial skeletal elements (Couly et al., 2002; Ruhin et al., 2003). These studies concluded that non-Hox-expressing and Hox-expressing CNC form separate equivalence groups, and that within these equivalence groups the type of skeleton can be reprogrammed by the type of grafted endoderm. However, the interaction might not be direct, as endoderm has a role at premigratory CNC stages, when the avian endoderm grafts were done, in patterning the facial ectoderm (Haworth et al., 2004), which is known to provide signals to skeletogenic CNC. In the future, the generation of p1-specific markers will allow us to test whether p1 endoderm is sufficient at postmigratory CNC stages to induce dorsal support cartilage in Hox-positive but not Hox-negative CNC. At the same time, it will be important to identify the downstream effectors of Hox2, and how this Hox2-dependent ‘set’ regulates the responses of CNC to specific epithelia.

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Supplementary material

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References


of early head ectoderm is regulated by endoderm and prepatterns the orofacial epithelium. Development 131, 4797-4806.


