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Developmental Biology 276 (2004) 225–236

DEVELOPMENTAL BIOLOGY

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Developmental origins and evolution of jaws: new interpretation of "maxillary" and "mandibular"

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Received for publication 6 April 2004, revised 6 August 2004, accepted 31 August 2004
Available online 6 October 2004

Abstract

Cartilage of the vertebrate jaw is derived from cranial neural crest cells that migrate to the first pharyngeal arch and form a dorsal "maxillary" and a ventral "mandibular" condensation. It has been assumed that the former gives rise to palatoquadrate and the latter to Meckel's (mandibular) cartilage. In anamniotes, these condensations were thought to form the framework for the bones of the adult jaw and, in amniotes, appear to prefigure the maxillary and mandibular facial prominences. Here, we directly test the contributions of these neural crest condensations in axolotl and chick embryos, as representatives of anamniote and amniote vertebrate groups, using molecular and morphological markers in combination with vital dye labeling of late-migrating cranial neural crest cells. Surprisingly, we find that both palatoquadrate and Meckel's cartilage derive solely from the ventral "mandibular" condensation. In contrast, the dorsal "maxillary" condensation contributes to trabecular cartilage of the neurocranium and forms part of the frontonasal process but does not contribute to jaw joints as previously assumed. These studies reveal the morphogenetic processes by which cranial neural crest cells within the first arch build the primordia for jaw cartilages and anterior cranium.

Keywords: Jaw cartilages; Head; Arches; Maxillary; Mandibular; Axolotl; Chick; Neural crest; Evolution

Introduction

Jawed vertebrates, or gnathostomes, represent the majority of extant vertebrate species. The evolution of jaws allowed gnathostomes to become effective predators and probably accounted for much of their subsequent success (Mallatt, 1996). The classical view is that jaws evolved via modifications of ancient gill arch cartilages (viscerocranial elements), but little is known about the underlying mechanisms (for review, see Hall, 1999a; Janvier, 1996; Mallatt, 1996). In the embryo, the jaw cartilage supports the mouth and is derived from the first pharyngeal (mandibular) arch, which consists of the palatoquadrate cartilage (contributing to the upper jaw) and Meckel's cartilage (forming the presumptive lower jaw) (de Beer, 1937; Depew et al., 2002; Hall, 1999a; Köntges and Lumsden, 1996; Liem et al., 2001). These endoskeletal jaw cartilages form a developmental and evolutionary framework for adult vertebrate jaws (Kardong, 1995; Liem et al., 2001; Smith and Schneider, 1998). Because Hox genes have marked inhibitory effects on jaw formation (Grammatopoulos et al., 2000; Pasqualetti et al., 2000), it has been proposed that the origin of jaws was facilitated by a loss of Hox (homebox transcription factor) expression from the first pharyngeal arch (Cohn, 2002; but see Takio et al., 2004). The jaw and visceral skeleton owe their embryonic origins entirely to the neural crest (Hall, 1999b; Le Douarin and

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doi:10.1016/j.ydbio.2004.08.046

Kalcheim, 1999; Santagati and Rijli, 2003), a migratory and multipotent cell population unique to vertebrates (Le Douarin and Dupin, 2003).

Cranial neural crest cells emerge from the dorsal neural tube and migrate extensively to differentiate into the craniofacial skeleton and the neurons and glial cells of the peripheral nervous system, among other derivatives (Le Douarin and Kalcheim, 1999). In all vertebrates, cranial neural crest cells migrate in a conserved and characteristic pattern of three distinct streams termed trigeminal (or mandibular), hyoid (or preotic), and common branchial (or postotic) (Epperlein et al., 2000; Lumsden et al., 1991; Meulemans and Bronner-Fraser, 2002; Trainor et al., 2002). The most rostral (trigeminal) neural crest stream populates the entire anterior head. These cells emerge from the neural tube and eventually contribute to the trigeminal ganglion and to all mesenchymal derivatives of the first pharyngeal arch (mandibular arch sensu lato). Within the first arch, neural crest cells condense to form a dorsal primordium (termed maxillary) below the eye, and a ventral primordium (termed mandibular) below the oral cavity (Chai et al., 2000; Francis-West et al., 1998; Meulemans and Bronner-Fraser, 2002). Interestingly, this pattern represents a common trait of those vertebrate embryos examined to date. In most tetrapods, the maxillary and mandibular condensations appear to progressively proliferate to fill facial prominences, swellings of mesenchyme encased in epithelium termed maxillary and mandibulary prominences (Richman and Lee, 2003). It has been assumed that the embryonic skeleton of the lower jaw (Meckel's cartilage) is formed from the ventral, mandibular component of the first pharyngeal arch whereas the palatoquadrate cartilage is formed from the upper, maxillary component of the arch (Depew et al., 2002; Francis-West et al., 1998; Larsen, 1993; Mina, 2001). Palatoquadrate is therefore often referred to as the "maxillary cartilage" and Meckel's element as the "mandibular cartilage" (Depew et al., 2002; Larsen, 1993). However, there has been no direct evidence that supports or refutes this prevailing assumption.

In the present study, we utilize direct lineage labeling and careful morphological observations in an anamniote (axolotl) and amniote (chick) to observe and compare the patterns of late neural crest migration as they form these distinct cartilages. Contrary to the generally held notion, our data suggest that both palatoquadrate and Meckel's cartilage, as fundamental cartilaginous structures of jawed vertebrates, derive from a common ventral condensation within the first pharyngeal arch that is here referred to as maxillomandibular. The dorsal condensation (here termed "trabecular") of the first pharyngeal arch was found to give rise to anterior neurocranial structures, predominantly to the trabecula cranii. The data show that the so-called first arch of vertebrates is a much more complex structure than previously assumed, emphasizing the necessity for precise fate mapping of this region of the vertebrate embryo.

Materials and methods

Embryos

Embryos of the Mexican axolotl (*Ambystoma mexicanum*) were obtained, reared, and staged as previously described (Epperlein et al., 2000). Before operating on the embryos, they were decapsulated manually. Fertilized chicken eggs were obtained from commercial sources, incubated at 38°C as indicated elsewhere (Sechrist et al., 1993), and staged according to Hamburger and Hamilton (1951).

Injections of DiI

A fixable form of DiI, Cell Tracer CM-DiI (Molecular Probes), was dissolved in absolute ethanol and diluted to a working concentration of about 0.1 mg/ml in 0.3 M sucrose. To visualize continuous migration of neural crest streams, DiI was injected into anterior cranial neural folds of axolotl embryos between stages 19 and 22 using an Inject + Matic microinjector.

For injections at a pharyngula stage, a small amount of DiI was focally injected into either dorsal or ventral aspects of the mandibular arch of axolotl (dorsal or ventral neural crest cell condensations, respectively; Figs. 3A and D) using an IM 300 microinjector and fine glass micropipettes attached to a needle holder (Oxford instruments). Those embryos in which the DiI stained neural crest cells stayed precisely at the same place and did not spread out within 5 min ($n = 10$ for dorsally, and $n = 27$ for ventrally injected ones) were developed to stage 40, sectioned and analyzed. In chick, small focal injections of DiI were made into either the dorsal "maxillary" ($n = 8$) or ventral "mandibular" ($n = 9$) facial processes (Figs. 3G and J) in stage 13–14 chick embryos and analyzed at E7.

Injections of FITC-dextran and GFP followed by cranial tube transplantations

For expression of FITC-dextran or green fluorescent protein (GFP), about 1–5 ng GFP mRNA (in 20-nl solution) was injected using an IM 300 microinjector into one blastomere of a two- to four-cell axolotl embryo. For injection, the embryos were placed in an agar dish containing 5% Ficoll in 1/10 Steinberg solution and antibiotics (Gibco). After 1 day, the embryos were transferred into normal strength saline. Embryos were then allowed to develop to the neurula (stages 15–17) and used for grafting. Using tungsten needles, dextran- or GFP-stained head neural folds were grafted orthotopically into uninjected host sibling embryos in which the corresponding part of neural fold had been excised before.

Sectioning and immunostaining

Axolotl embryos were anaesthetized in a solution of tricaine methane-sulfonate (MS-222, Sandoz) and fixed in

4% paraformaldehyde in 0.1 M phosphate-buffered saline (PFA/PBS) at least overnight. After washing in PBS, specimens were dehydrated through a graded series of ethanol and embedded in JB4 (Polysciences, Inc.). Sections (5 μm) were cut with a Reichert-Jung microtome (Biocut 2035), stained with Azure B-Eosin (SERVA), and mounted in DePeX (SERVA).

Embryos processed through in situ hybridization were stored in 100% methanol and cut (100 μm) using a Vibratome Series 1000 sectioning system (Ted Pella, Inc.). Axolotl sections were counterstained with a primary polyclonal anti-fibronectin antibody (1:100, Dako) followed by a goat anti-rabbit Cy3 secondary antibody (1:100, Dako) in order to visualize tissue borders. Afterwards, these sections were stained with DAPI (0.1–1 μg/ml PBS) to mark cell nuclei. Some sections were stained with the skeletal muscle marker 12/101 (Developmental Studies Hybridoma Bank).

Chick embryos fixed in PFA/PBS were sectioned (25 μm) using a Leica cryostat. Rabbit anti-collagen II (IgG, BABCO) antibody was used diluted at 1:200 to detect chondrocytes. As a secondary antibody, Alexa-Fluor 488 anti-rabbit IgG1 (Molecular Probes) was used at 1:200 dilution. Immunostained sections were counterstained with DAPI, rinsed in PBS, and mounted on slides using Perma Fluor (Immunon). Separate brightfield and fluorescence images were captured with a Zeiss Axiocam or SPOT RT camera, merged, and optimized using Spot, MetaView, and Adobe Photoshop software.

In situ hybridization

In situ hybridization was performed on axolotl albino embryos as described by Henrique et al. (1995) with the addition of an extra wash in MAB-T (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween 20) overnight at 4°C (Meulemans and Bronner-Fraser, 2002). Hybridization was performed at 65°C. Preparation of riboprobes was done as previously described (Cerny et al., 2004).

Scanning electron microscopy and cartilage staining

Scanning electron microscopy (SEM) and staining of cartilage using Alcian Blue followed our protocols as described in detail elsewhere (Cerny et al., 2004).

Results

We examined the developmental origins of jaw cartilages from neural crest condensations in axolotl and chick embryos as representatives of early and late tetrapods. From an embryological standpoint, axolotl embryos represent an advantageous model system for studying tissue morphogenesis (Beetschen, 1996). Their relatively large size and slow development (Cerny et al., 2004; Epperlein et al., 2000) allow accurate tracing of neural crest cells during condensation into cartilage and early jaw development. In contrast, chick embryos have smaller cells and large cell numbers but represent the best-studied and best-understood model for amniote neural crest development (Le Douarin and Kalcheim, 1999), and are used here for comparison to axolotl.

The postoptic portion of the trigeminal neural crest stream covers the surface of the first (mandibular) arch

As in other vertebrates, the trigeminal neural crest stream in axolotl originates from the posterior prosencephalon, mesencephalon, and anterior rhombencephalon (Cerny et al., 2004; Epperlein et al., 2000). These anterior neural crest cells are further distinguished from posterior cranial neural crest cells by their lack of Hox gene expression. These cells migrate as a broad sheet that first uniformly populates the anterior head but soon becomes mechanically subdivided by the optic vesicle into neural crest cells situated preoptically and mandibular arch neural crest cells situated postoptically, which cover the first pharyngeal (mandibular) arch mesoderm (Cerny et al., 2004) (Figs. 1A–C).

Dorsal "maxillary" and ventral "mandibular" neural crest condensations of the first arch appear to be a conserved feature of vertebrates

Mandibular arch neural crest cell migration ceases around the pharyngula stage (axolotl stage 34; Figs. 1B and C) with neural crest cells distributed homogeneously over the surface of the central mandibular arch mesoderm (Cerny et al., 2004). Subsequently, neural crest cells become depleted from the middle of the first arch by an as yet unknown mechanism and accumulate into two condensations (Fig. 1D), one dorsally, closer to the optic vesicle and nose, and another at the ventralmost aspect of the mandibular arch (Fig. 1D). Although a thin layer of neural crest cells remains over the surface of the arch mesoderm at this time, the majority of neural crest cells appear in two aggregated condensations (not shown). The dorsal (often referred to as "maxillary") and ventral (termed "mandibular") condensations of the first arch neural crest cells are morphologically visible in all vertebrate embryos examined, including jawless lampreys (Meulemans and Bronner-Fraser, 2002).

At the end of the pharyngula stage, the basic vertebrate body plan represents a vertebrate phylotype, a conserved developmental stage shared by both gnathostomes and agnathans (Kuratani et al., 2001, 2002). From this point in development onwards, body plans among vertebrates diverge. In agnathans, neural crest cell condensations develop into cartilages of the larval oral apparatus. In lower gnathostomes, the dorsal condensation has been thought to develop into the upper jaw cartilage and the ventral one into lower jaw (Kardong, 1995; Larsen, 1993; Liem et al., 2001).

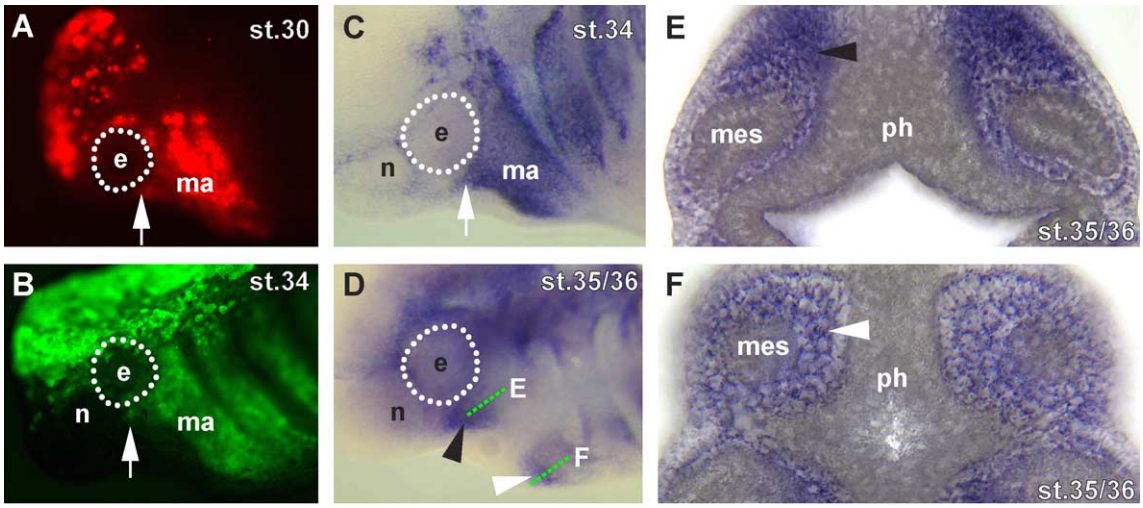

Fig. 1. Axolotl cranial neural crest streams and early development of facial condensations. Neural crest cells are visualized by DiI (A) or GFP (B) labeling, or after in situ hybridization using the *snail* riboprobe (C–F). (A) The first neural crest stream covers the entire anterior head; the arrow points to the "maxillary" part situated close to the eye (e, dotted circle). (B) GFP labeling of all neural crest cells shows cranial streams. The arrow points again to the "maxillary" part. (C) At the pharyngula stage, neural crest cells populate the first arch homogeneously. (D) Shortly thereafter, neural crest cells appeared reduced in the middle of the arch. Black and white arrowheads point to dorsal and ventral accumulations of neural crest cells, respectively. Green dotted lines show the position of horizontal sections through the dorsal (E) and ventral (F) neural crest condensations within the same mandibular arch. The dorsal center develops anteromedially whereas the ventral one develops close to the central mesodermal core (mes) of the first branchial arch. ma, mandibular arch; n, nose; ph, pharynx.

In amniotes, these two neural crest condensations contribute to the corresponding facial prominences that are also referred to as maxillary and mandibular. It has been predicted that these prominences give rise to the upper and lower jaw structures, respectively. However, direct evidence for such contributions, particularly into skeletal derivatives of the jaw and face, has been lacking (Richman and Lee, 2003).

Both palatoquadrate and Meckel's cartilages originate solely from the ventral condensation

In axolotl, obvious morphological differences between the dorsal versus ventral condensation become apparent around stage 35, as documented in longitudinal sections (Figs. 1D–F). Cranial neural crest cells in the "maxillary" area appear as a dorsally enlarged accumulation of cells, predominantly along the anterior portion of the arch (Fig. 1E), as visualized by *snail* expression. In contrast, the ventrally localized "mandibular" condensation (Fig. 1F) forms a homogeneous tube that extends slightly toward the medial portion of the arch.

In order to better visualize the changes that occur within condensations of neural crest mesenchyme as a function of time, we transplanted neural folds labeled with either FITC-dextran or GFP into unlabeled host embryos. This allowed distinction of neural crest cells from the tissues through which they migrate and differentiate. As an alternative labeling approach that involved no microsurgery, we labeled neural folds in some embryos with focal injections of the lipophilic dye DiI. We then carefully analyzed the morphogenetic changes of labeled neural crest cells at consecutive stages in order to understand how mesenchymal condensations differentiate into jaw cartilages and build facial primordia. Both labeling methods yielded identical results.

Our analysis of sectioned embryos revealed that neural crest cells from the ventral, commonly designated mandibular, condensation contribute to both Meckel's (Fig. 2D) and palatoquadrate cartilage (Fig. 2H). Neural crest cells at the ventralmost aspect of the mandibular condensation (Fig. 2A) subsequently develop into Meckel's cartilage (Figs. 2B–D). Careful analysis of sections reveals that the palatoquadrate cartilage (Fig. 2H) derives from a more dorsal part of this same ventral, "mandibular" condensation (Fig. 2E). The palatoquadrate arises from a compact cell mass (Figs. 2E and F) that is initially localized in the medial part of the mandibular condensation. With time, this rearranges to a more posterior position within the arch (compare Figs. 2E, F, G and H). The identity of the cartilage was confirmed by the presence of a group of mandibular levator muscles (Fig. 2H, m.l.m.) that are always associated with the palatoquadrate (Ericsson and Olsson, 2004; Piatt, 1938). Progressive development of this muscle from an initially undifferentiated central mesodermal mass within the mandibular arch can be visualized by comparing Fig. 2F (undifferentiated central mesodermal mass indicated by white asterisk) with Fig. 2G (condensation now splits into a lateral and medial mass, indicated by two white asterisks) and finally with Fig. 2H (group of three levator mandibulae muscles, m.l.m., recognized by staining with the skeletal muscle marker 12/101 in red).

These results suggest that the cranial neural crest mass of the ventral ("mandibular") condensation becomes sub-

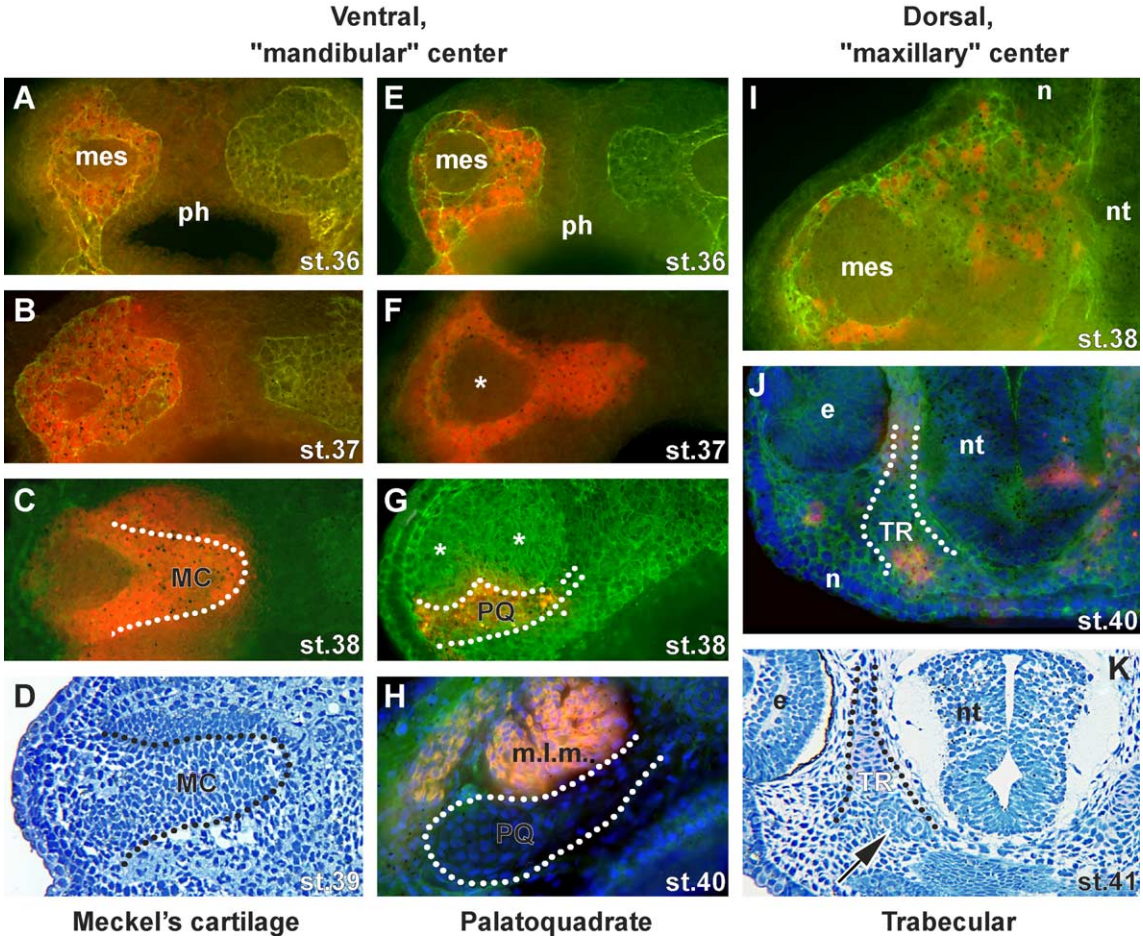

Fig. 2. Both Meckel's (MC) and palatoquadrate (PQ) cartilages develop from a single ventral ("mandibular") center, whereas the dorsal ("maxillary") condensation gives rise to trabecular cartilage (TR). Development of the ventral ("mandibular") center (first two columns; A–H) and dorsal ("maxillary") center (right column; I–K) of the first arch is shown in horizontal (A–I) or transverse (J and K) sections, primarily of the left side of each section. Neural crest cells are visualized by DiI (G and J), fluorescent dextran (A–C, E, and F), or GFP (I) labeling. Neural crest cells stained by DiI, GFP, or fluorescent dextran are red; anti-fibronectin staining reveals tissue borders (green) and DAPI shows cell nuclei (blue) (H and J). For detailed explanation, see the text. mes, arch mesoderm; n, nose; ph, pharynx; nt, neural tube.

divided, with a portion ultimately contributing to the dorsal palatoquadrate cartilage and the remainder to the ventral Meckel's cartilage in the mandibular process. This finding was unexpected as it contradicts the previously held assumption that the upper jaw (palatoquadrate) cartilage arises from the "maxillary" condensation of the first arch in all vertebrates (Depew et al., 2002; Hall, 1999a; Kardong, 1995; Larsen, 1993). However, this is not entirely surprising since, even at early stages, two prechondrogenic cell masses within the single ventral (mandibular) condensation resemble by their shape and position the mature palatoquadrate and Meckel's cartilage of axolotl, similar to the situation observed during formation of early hyoid cartilage in zebrafish (Kimmel et al., 1998). Therefore, based on careful morphological analysis, these results suggest that both jaw cartilages that were previously thought to be derived from "maxillary" and "mandibular" condensation arise from a single ventral neural crest condensation.

The dorsal neural crest cell condensation gives rise to trabecula cranii, a neurocranial element

The above findings raise the intriguing question of what forms from the dorsal "maxillary" neural crest mass. To address this, we examined the morphogenetic movements and differentiation of the dorsal condensation by following labeled neural crest (Figs. 2I–K). Some cells derived from the dorsal condensation contributed to dermis and head mesenchyme (not shown). However, most of labeled cells contributed to a cranial element that was clearly identified as the trabecula cranii (Figs. 2J and K), with no contribution to the palatoquadrate as had been previously suggested. The trabeculae of the neurocranium were discovered first by Rathke in the grass-snake (de Beer, 1931) and later recognized as the essential element of the cartilaginous anterior cranial base (skull) in all vertebrates (Bertmar, 1959; de Beer, 1937; Kuratani et al., 1997). Our results suggest that the dorsal condensation gives rise to trabecular cartilage and

some other neural crest derivatives, but does not contribute to either the palatoquadrate or Meckel's cartilage.

Interestingly, the differentiation of the dorsal neural crest cell mass is about 1–2 days delayed compared to the ventral one. The first signs of chondrocyte differentiation as visualized by collagen type II staining in both palatoquadrate and Meckel's cartilage are detectable from stage 38/39 onwards (not shown), whereas those neural crest cells in the dorsal center are still present as an undifferentiated condensation at the same time.

Direct lineage labeling in axolotl demonstrates that both palatoquadrate and Meckel's cartilage arise from the ventral condensation

The above results strongly suggest that the dorsal condensation gives rise to trabecula of the neurocranium and the ventral condensation gives rise to cartilaginous lower (Meckel's) and upper (palatoquadrate) jaw elements. Because neural crest cells were labeled prior to migration, however, we cannot rule out the possibility that some mixing between these populations may have occurred but was missed in our analysis of sectioned material.

We next turned to direct lineage labeling of these cranial neural crest cell masses using focal injections with the vital dye DiI. Direct labeling allowed us to experimentally confirm the nature of the structures that arose specifically from the "dorsal" and "ventral" condensations within the first arch in axolotl. Embryos received a single injection of DiI into either the dorsal or ventral condensation. Only those embryos with relatively small and focal injections were selected for subsequent analysis in stages following chondrogenesis. When DiI injections were made into the dorsal condensation at stages 34–36 (n = 10, Fig. 3A), labeled neural crest cells were clearly observed several days after injections in the trabecular cartilage between the eye and brain (as best viewed in transverse sections, Figs. 3B and C). However, no staining was seen in the palatoquadrate (Fig. 3C) or Meckel's cartilage. In contrast, focal DiI injections to the "ventral" cranial neural crest cell mass (n = 27, Fig. 3D) revealed a contribution from this portion of the arch to both the palatoquadrate and Meckel's cartilage, but not to the trabecular cartilage (Figs. 3E and F). These results validate our morphological analysis by using direct neural crest lineage labeling and confirm that dorsal condensation contributes to trabecular cartilage of the neurocranium and ventral condensation contributes to both the palatoquadrate and Meckel's cartilage. Although these injections undoubtedly labeled some adjacent cranial mesoderm as well, this tissue makes no contribution to cartilage.

Direct lineage labeling of dorsal and ventral neural crest cell condensations in the chick

To further test whether the contribution of the ventral condensation to both Meckel's cartilage and the palatoquadrate is a general feature of tetrapods, we turned to the chick that is currently the best-studied amniote model for neural crest development (Le Douarin and Kalcheim, 1999). Parallel to the DiI injections performed in axolotl embryos, focal injections of DiI were made into either the dorsal "maxillary" (n = 8) or ventral "mandibular" (n = 9) facial processes in stage 13–14 chick embryos and analyzed at E7. Analogous to our findings in axolotl, DiI-labeled neural crest cells from dorsal injections (Fig. 3G) contributed to trabecular cartilage (also called interorbital) with generally no contribution to palatoquadrate or Meckel's cartilage (Figs. 3H and I). In contrast, ventral injections (Fig. 3J) contributed labeled neural crest cells to both the palatoquadrate and Meckel's cartilage (Figs. 3K and L). Thus, similar findings were observed for representatives of two groups of gnathostomes, anamniote (axolotl), and amniote (chick). Surprisingly, in chick, we found that ventral injections contributed a few neural crest cells to the trabecular cartilage as well; suggesting that there must be at least some very late neural crest cell movements or some intermixing within the first arch.

FGF8 expression prefigures the maxillomandibular region and in axolotl is restricted to the ventral portion of the arch

What might account for the specification of the maxillary and mandibular region within the first neural crest stream? In many vertebrate embryos, the early distribution of FGF8 in the ectoderm correlates with the presence of the nascent maxillomandibular region (Crossley and Martin, 1995; Ferguson et al., 2000; Trumpp et al., 1999) and has been proposed to play a role in its determination (Shigetani et al., 2000; Tucker et al., 1999). Interestingly, FGF8 expression precedes the influx of cranial neural crest cells into the first arch and their condensation into dorsal and ventral centers (Richman and Lee, 2003; Shigetani et al., 2002). Therefore, these embryonic craniofacial regions may be specified early in development and be independent of patterning by cranial neural crest streams. In the axolotl, surprisingly, we observe that FGF8 expression in the ectoderm is present only adjacent to the ventral but not the dorsal neural crest condensation (Fig. 4, sections not shown), consistent with a possible role in influencing the neural crest contribution to both palatoquadrate and Meckel's cartilage.

Discussion

By combining lineage analysis with careful morphological observations, our results have established the descendants of the dorsal, previously called "maxillary", and ventral, "mandibular", neural crest condensations that derive from migrating cranial neural crest cells at the trigeminal level. Based on these observations, we propose the following model of first arch neural crest cell regionalization and subsequent jaw cartilage morphogenesis (Fig. 5). Initially,

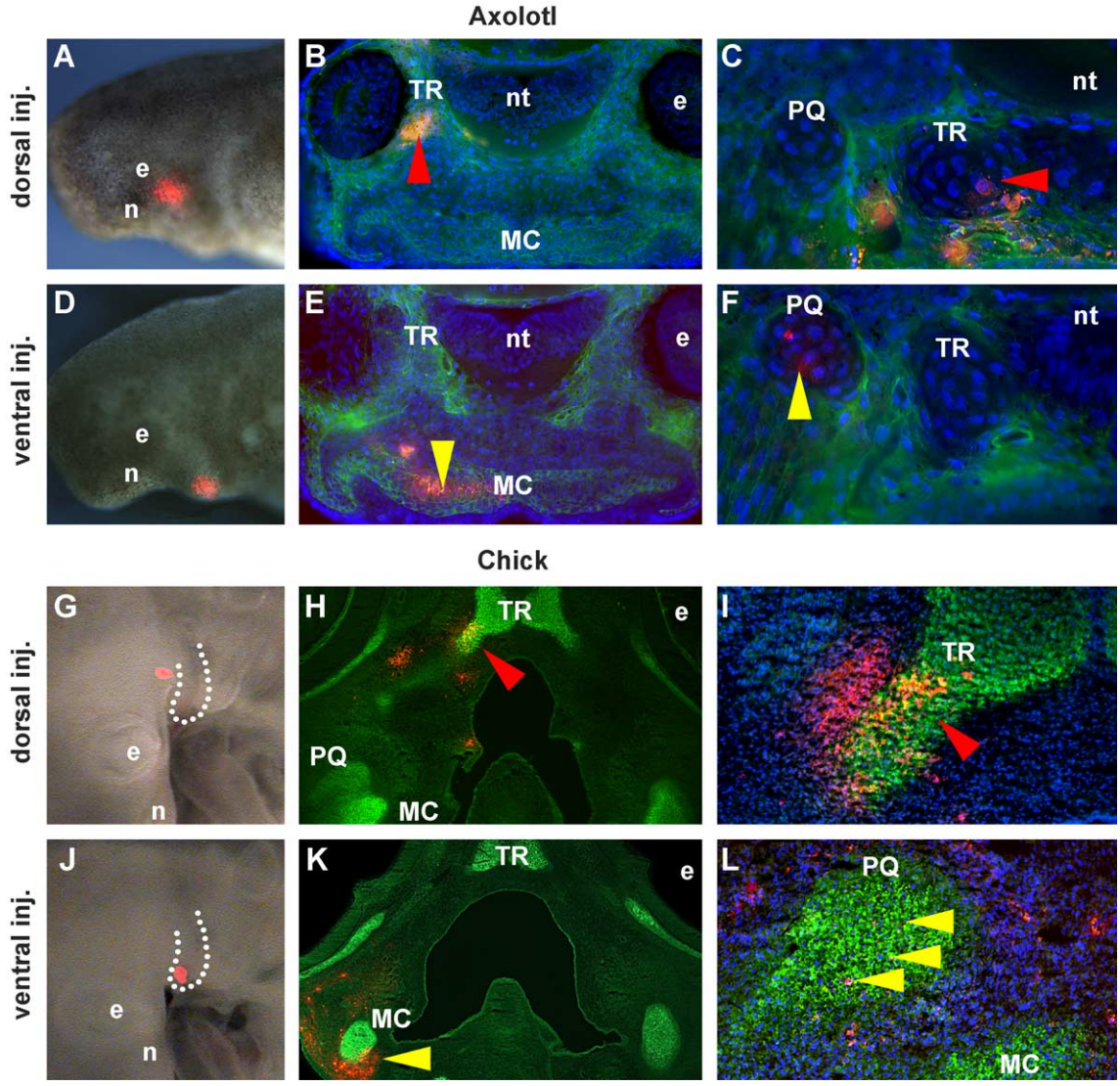

Fig. 3. Focal DiI injections into either dorsal (A and G) or ventral (D and J) neural crest condensation in both axolotl (A–F) and chick (G–L) confirm that both Meckel's (MC) and palatoquadrate (PQ) cartilages arise from a single ventral condensation, whereas the dorsal condensation develops into trabecular cartilage (TR). Transverse sections show the position of stained cells several days after the injection; anti-fibronectin counterstaining (green in B, C, E, and F) reveals tissue borders in axolotl and anti-collagen type II (green in H, I, K, and L) stains cartilage in the chick. DAPI stains cell nuclei (blue). Red arrowheads point to stained cells after the dorsal injection, yellow arrowheads point to stained cells after the ventral injection. e, eye; n, nose, nt, neural tube.

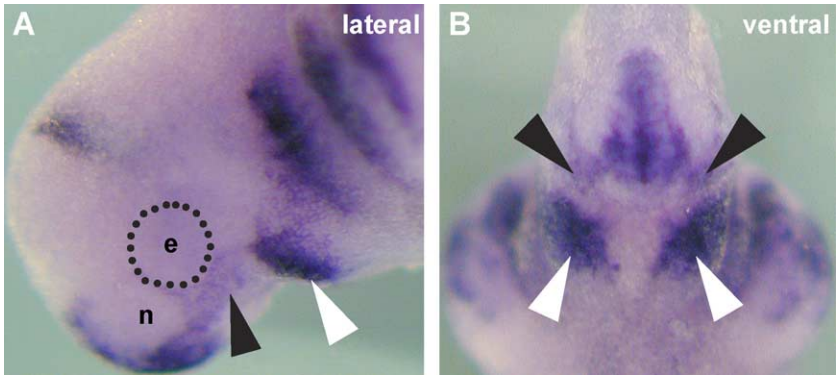

Fig. 4. FGF8 expression in the first arch of axolotl (stage 30). Staining is visible in the ventral portion of the first arch only (white arrowhead), which develops into both the palatoquadrate ("maxillary") and Meckel's ("mandibular") cartilage. Black arrowheads point to the upper part, incorrectly named as maxillary. e, eye; n, nose.

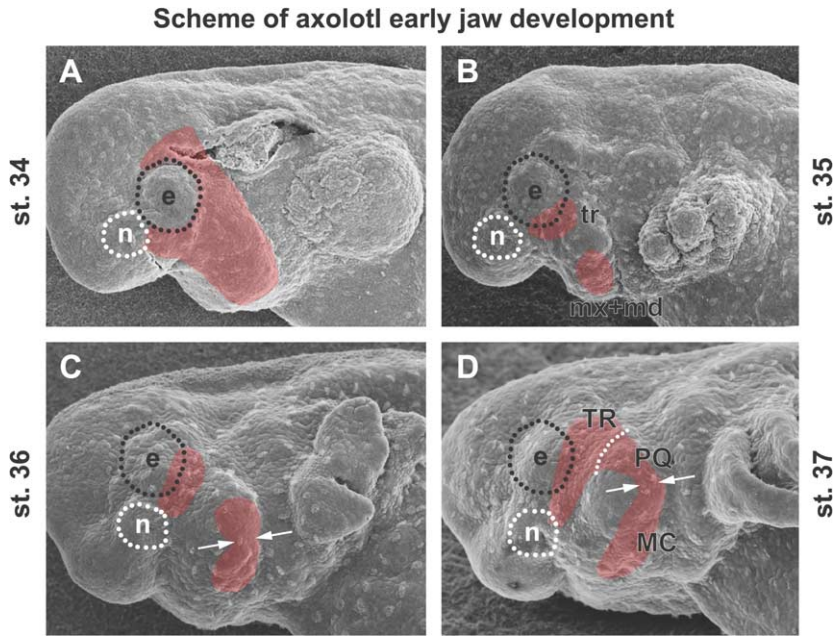

Fig. 5. Early steps in jaw morphogenesis in axolotl. SEM images at stages 34–37, head to the left. Development of neural crest cells (red) in relation to the first pharyngeal arch, nose (n), and eye (e) is schematically outlined. (A) After extensive migration, neural crest cells homogeneously cover the entire surface of the first arch mesoderm. Soon after (B), they condense into a dorsal trabecular (tr) center and a ventral, maxillomandibular (mx + md) center. Subsequently (C and D), the ventral center extends more dorsally and differentiates into palatoquadrate (PQ) and Meckel's (MC) cartilage. The dorsal center gives rise into trabecular cartilage (TR) instead. White arrows point to the position of the jaw-joint (in C and D), white dotted line in D indicates schematically the border between trabecula and palatoquadrate cartilage.

cranial neural crest cells populate the entire surface of the mandibular arch mesoderm (Fig. 5A). They establish a sheath of neural crest around the central mesodermal rod. Later, neural crest cells split into two distinct condensations: (1) a dorsal trabecular condensation in the vicinity of the eye and nose that probably prefigures the maxillary prominence and (2) a ventral "maxillomandibular" condensation within the mandibular arch (Fig. 5B). The dorsal center contributes to trabecular cartilage of the neurocranium and probably to portions of the frontonasal process as well, comprising fundamental elements of the anterior cartilaginous skull of all vertebrates. The ventral center becomes subdivided such that its ventralmost portion elongates medially to develop into Meckeĺs cartilage, the endoskeletal framework for the adult lower jaw. The more dorsal portion extends further dorsally and develops into the palatoquadrate cartilage (the proximal part of which later ossifies to become the quadrate). This then connects Meckeĺs cartilage of the lower jaw with the trabecular cartilage and neurocranium, and forms the primary jaw-joint in all vertebrates (Figs. 5C,D and 6A,B).

Early morphogenesis of jaw cartilages in the evolutionary context of anterior pharyngeal arches

During the course of evolution, the vertebrate cranium and jaw have served the common functions of protecting the brain/sensory organs and capturing or biting food, respectively. However, the way in which individual cranial components participate in these functions has been extensively modified between species. Thus, function has remained constant while form has changed. A well-known example of such structural remodeling during evolution is the change of jaw structure in mammals that culminated in a new jaw joint and two additional auditory ossicles in the middle ear (Janvier, 1996; Liem et al., 2001; Smith and Schneider, 1998).

Cranial elements like trabeculae and anteriormost pharyngeal arch cartilages are another notable example of structural remodeling during evolution. The trabeculae and their direct developmental derivatives (Miyake et al., 1992) have been shown to be of neural crest origin in a variety of vertebrates (Couly et al., 1993; Langille and Hall, 1988a,b; Matsuo et al., 1995; Olsson and Hanken, 1996; Schneider and Helms, 2003). This suggests that these elements are originally viscerocranial (pharyngeal arch) components enlarged during the course of evolution in order to protect anterior brain and sensory organs finally forming a new, prechordal neurocranium (for a review, see de Beer, 1937; Kuratani et al., 1997).

Classical studies based on careful histological observations have suggested that the trabeculae originate within the "maxillary process of the mandibular arch" in a variety of animals (e.g., Allis, 1923, 1924; de Beer, 1931; Haller, 1923; but see others, e.g., Bertmar, 1959; Goodrich, 1930, for a different opinion). Here, we confirm the first view experimentally using a combination of vital dye labeling of late-migrating cranial neural crest cells and morphological

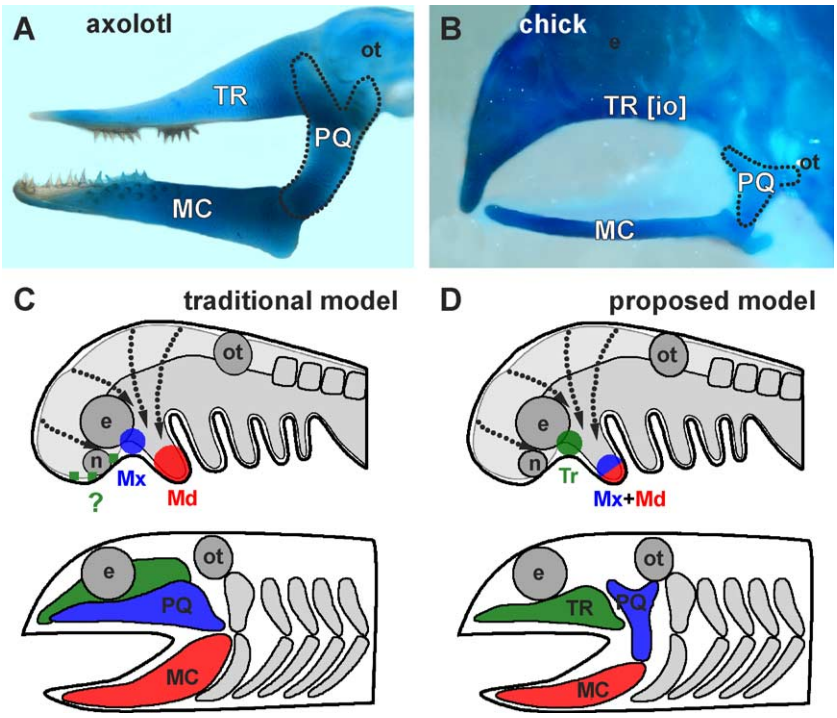

Fig. 6. Alcian Blue staining of axolotl (A) and chick (B) cartilaginous skull. In both, Meckel's cartilage (MC) is a framework for the lower jaw whereas trabeculae (TR) contribute to the upper jaw. Palatoquadrate (PQ, black dotted lines) connects these two elements. In larval axolotl, even teeth develop on trabecular cartilage. (C) A traditional model of developmental origin of jaw cartilages in gnathostomes. The dorsal (maxillary, Mx) neural crest condensation has been proposed to give rise to palatoquadrate cartilage (PQ), which is often described to form the entire upper jaw, whereas the ventral (mandibulary, Md) condensation develops into Meckel's cartilage (MC) that is the lower jaw element. Neurocranium (green) is expected to originate from cranial neural crest cells (green dots and question mark). (D) Our data suggest an alternative interpretation for modern tetrapods, in which the dorsally situated condensation of neural crest cells (here referred to as trabecular, Tr) contributes to trabecular cartilage of the neurocranium (TR). Trabeculae or their developmental derivatives like inter-orbital [io] cartilage and the nasal septum are connected to the anterior palatoquadrate, which forms the hinge of the upper jaw in modern tetrapods. The ventral condensation (probably fused maxillary and mandibular, Mx + Md) gives rise to both Meckel's and palatoquadrate cartilages. Therefore, we conclude that the embryonic mandibular arch of recent animals consists of several fused elements (compare C and D, upper parts). e, eye; n, nose; ot, otic vesicle. Dashed curves represent pathways of neural crest migration.

analysis (Figs. 2 and 3). Moreover, we also show that both jaw cartilages can be traced back to a single, ventral neural crest cell condensation for which we have coined the term "maxillomandibular" (Figs. 2, 3, and 5).

Trabeculae are primarily considered to be bars of a "preoral" or "premandibular" arch based on embryological and topographical homology as well as the supposed metameric organization of cranial nerves (Allis, 1923; de Beer, 1931; Kuratani et al., 1997; Platt, 1897). Following this kind of view, the structure called the mandibular arch of recent vertebrates, in fact, comprises precursors of mandibular arch elements (jaw cartilages) on its ventral aspect and precursors of another cranial arch on its dorsal side (Fig. 5).

Developmental changes in positions of the precursors to these structures are reflected by evolutionary changes of adult jaw structures and probably provide the mechanism through which changes in evolution of the jaws may have arisen. We propose the following model as the ancestral state of the cartilaginous head of basal jawed vertebrate (Fig. 6C, bottom): the anterior braincase is likely to have consisted of a trabecular element (which had enlarged and moved into a horizontal position to protect the brain) to which a large, anteriorly extending palatoquadrate cartilage had been articulated. The palatoquadrate as an upper jaw structure was connected to Meckel's (mandibular) cartilage representing the lower jaw (Fig. 6C, bottom). The embryological position of the condensation of Meckel's cartilage would then be expected in the ventral aspect of the mandibular arch (mandibular prominence), palatoquadrate condensation in the dorsal (maxillary) portion of the mandibular arch (maxillary prominence), and trabecular condensations somewhere in a "premandibular area", closer to the anterior tip of the head (upper part of Fig. 6C).

In modern tetrapods, however, the precursors to both palatoquadrate and Meckel's cartilage apparently have merged into a single "maxillomandibular" condensation in the ventral portion of the first pharyngeal arch (Fig. 6D, upper). The embryonic precursor to the premandibular element, the trabecular cartilage, appears to occupy a dorsal portion of the first arch (Fig. 6D, upper) as conclusively demonstrated by our morphogenetic studies and direct tracing experiments (Figs. 2 and 3).

Meckel's and palatoquadrate cartilages are clearly homologous across vertebrate taxa. However, these two elements are reduced in size and importance in multiple vertebrate lineages such that they no longer define the

functional adult jaws. This is particularly clear in the case of the palatoquadrate, which is relegated to a more posterior place (Liem et al., 2001; Smith and Schneider, 1998). This reduction culminates in modern tetrapods, where the palatoquadrate often forms only the jaw-joint (quadrate bone) such that the upper jaw is entirely comprised of dermal bones (predominantly by maxilla and premaxilla), the endoskeletal framework of which represents the trabecula (in axolotl, Fig. 6A) or its developmental derivatives like the interorbital septum (in chick, Fig. 6B). Dermal bones like maxillary and palatine undergo a direct ossification without any contribution from the endoskeletal cartilage components and belong to another likely independent skeletal system—the dermatocranium.

Possible homology between agnathans and gnathostomes

Recent experiments in which cranial neural crest migration in a basal vertebrate, the sea lamprey, was followed by DiI labeling (McCauley and Bronner-Fraser, 2003) suggest that migration patterns in the first pharyngeal arch may be highly conserved between agnathans and gnathostomes (Kuratani et al., 1999, 2001). In lamprey, cranial neural crest cells migrating into the first arch bifurcate ventrally to surround the mouth (McCauley and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2002) in a pattern similar to that observed in gnathostomes. This particular migratory pattern of lamprey resembles the behavior of the ventral condensation in axolotl, suggesting that they may be homologous. Although agnathans possess trabecular cartilage, its developmental origin is not yet known nor is it clear whether this trabecular cartilage is homologous to that of gnathostomes (Damas, 1944; Kuratani et al., 2001).

Our results suggest strong similarities between the general patterns and the processes of neural crest migration that form the jaws of anamniote (axolotl) and amniote (chick) vertebrates. However, some interesting species differences were noted as well. Following injections made into the ventral mandibular condensation, we observed a contribution of a few neural crest cells to the trabecular cartilage in the chick but never in axolotl. This is an interesting difference between species and may result from the fact that there are many more (and smaller) neural crest cells in birds than in axolotl, allowing more regulative development or simply more cell migration and intermixing. Such mixing between dorsal and ventral populations in chick has been observed in later fate mapping studies of facial primordia, suggesting that these regions are not strictly segregated lineage compartments (McGonnell et al., 1998). Clearly, facial regions of chick and axolotl are not identical because the anamniote mandibular arch, in principle, does not display clear maxillary and mandibular prominences. This again points to the fact that there is a strong need for precise fate-mapping experiments to accurately map this region.

Of course, one would expect that DiI injections made directly into the dorsal and ventral condensations would also label some cranial mesoderm in addition to neural crest. Because the mesoderm does not contribute to cartilage, it remains unambiguous that this contribution came from the neural crest.

Patterning of cranial neural crest cells

Patterning of cranial neural crest cells that contribute to the jaws appears to involve a combination of extrinsic and intrinsic factors. Some neural crest populations exhibit plasticity and loss of Hoxa2 gene expression when transplanted from hyoid to mandibular arch (Trainor and Krumlauf, 2001), suggesting an environmental influence on their gene expression. However, there is also some evidence for prepatterning within the neural crest itself; for example, transplantation of quail and duck neural crest into the homologous position in chick embryos results in beak patterning characteristic of the donor tissue (Schneider and Helms, 2003). In terms of molecular mechanisms underlying arch patterning, there is an interesting nested distribution of members of the distalless (Dlx) gene family within the first pharyngeal arch, such that Dlx1/2 are expressed most dorsally, Dlx5/6 genes expressed in the middle of the arch, and Dlx3/7 in the most ventral portion (Depew et al., 2002). Dlx genes appear to be critical for dorsoventral specification of the first arch: such that null mutations in Dlx5/6 result in mirror image duplications of the upper jaw (Beverdam et al., 2002; Depew et al., 2002). The differential expressions of signaling molecules like fibroblast growth factors (FGFs), Sonic hedgehog (SHH), retinoic acid (RA), and bone morphogenetic proteins (BMPs) are also important for patterning of the facial primordial and appear to modulate expression of Hox genes therein (Barlow and Francis-West, 1997; Barlow et al., 1999; Francis-West et al., 1998; Hu and Helms, 1999; Schneider et al., 2001).

The steps between initial molecular specification of the first arch neural crest cells and subsequent jaw formation have yet to be elucidated. Our data provide the first detailed view of the morphogenetic processes by which mesenchymal condensations of facial primordia give rise to the jaws of gnathostomes. There are several comprehensive papers describing the contribution of migrating cranial neural crest cells from different axial levels into facial prominences (e.g., Imai et al., 1996; Köntges and Lumsden, 1996; Osumi-Yamashita et al., 1994; Richman and Lee, 2003), as well as the mesenchymal cell expansion within each primordium (McGonnell et al., 1998). The present study is the first to provide direct cell lineage analysis in vivo relating neural crest cell condensations of the first arch and of developing facial prominences to the cartilagenous structures that prefigure the bones of the jaw and face. In a recent review of face and jaw development in vertebrates, Richman and Lee (2003) proposed possible scenarios

regarding the origin of the maxillary prominence. One possibility, commonly referred to in textbooks, is that the first pharyngeal arch gives rise to both maxillary and mandibular prominences. An alternative possibility is that the mandibular arch contributes only to the posterior portion of the maxillary process (palatoquadrate), but more dorsal postoptic tissues may also make a significant maxillary contribution. By direct lineage analysis of this region, the present study distinguishes between these alternatives for the first time to demonstrate that the latter is the case.

Conclusions

In summary, our findings show that ventral ("mandibular") condensation of neural crest cells within the first pharyngeal arch makes a dual contribution to the palatoquadrate and to Meckel's cartilage. The more dorsally localized ("maxillary") neural crest condensation that appears to presage the maxillary prominence contributes to trabecular cartilage. This suggests that the common terms of "maxillary" and "mandibular" condensations are misleading since the palatoquadrate is a component of the upper jaw and yet derived from ventral, mandibular neural crest. Our data provide the first detailed view on the morphogenetic aspects of the processes in vertebrates by which mesenchymal condensations of facial primordia give rise to jaws.

Acknowledgments

We wish to thank Drs. Andres Collazo, James Hanken, Lennart Olsson, Joy Richman and colleagues in the Bronner-Fraser lab for helpful comments and insights on previous versions of the manuscript. Also comments from several anonymous reviewers that helped us reshape some of our ideas are kindly appreciated. We would like to thank Tanya Moreno for the GFP mRNA, and Torsten Schwalm and Ute Nimtschke for technical advice regarding especially SEM. The skeletal muscle marker 12/101 was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Support from SMWK (Dresden), Academy of Sciences of the Czech Republic (Grant number KJB-5111403); Ministry of Education, Youth and Sport of the Czech Republic (Grant number 1311004); COST OC B-23 and a NATO Science Fellowships Programme to RC; parts of this work were funded by NASA NAG 2-1585 and DE13223 to MBF.

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