**Hox genes, neural crest cells and branchial arch patterning**

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Proper craniofacial development requires the orchestrated integration of multiple specialized tissue interactions. Recent analyses suggest that craniofacial development is not dependent upon neural crest pre-programming as previously thought but is regulated by a more complex integration of cell and tissue interactions. In the absence of neural crest cells it is still possible to obtain normal arch patterning indicating that neural crest is not responsible for patterning all of arch development. The mesoderm, endoderm and surface ectoderm tissues play a role in the patterning of the branchial arches, and there is now strong evidence that Hoxa2 acts as a selector gene for the pathways that govern second arch structures.

**Introduction**

The classic models for craniofacial patterning argue that the morphogenetic fate and the Hox gene identity of the neural crest is pre-programmed carrying positional information acquired in the hindbrain to the peripheral nervous system and branchial arches. This is a very topical issue due to the high degree of interest in the development of the hindbrain and cranial neural crest and the roles they play in craniofacial patterning. Although the vertebrate head is composed principally of neural crest cells, it also relies on contributions from paraxial mesoderm, ectoderm and endoderm. In this review we discuss the recent analyses suggesting that craniofacial development is not dependent upon neural crest pre-programming but is regulated by a more complex integration of cell and tissue interactions.

**Hindbrain segmentation and its influence on craniofacial development**

The vertebrate head is a complex assemblage of the central and peripheral nervous systems, axial skeleton, musculature and connective tissues. Hence, proper craniofacial development requires the orchestrated integration of multiple specialized tissue interactions. How then do the facial structures form in the correct location with the appropriate shape and size? The patterning information could be intrinsic to each tissue precursor or alternatively, the program for patterning could depend upon interactions between the mesenchymal and epithelial tissues surrounding each cell type.

One key source of patterning information in the developing head is the vertebrate hindbrain, which exerts a profound influence on craniofacial morphogenesis in part through its ability to generate cranial neural crest. During early embryo development the hindbrain is transiently subdivided into seven contiguous segments called rhombomeres (r) [1]. Each rhombomere has a unique identity based on segment-restricted domains of Hox gene expression that are ordered and partially overlapping and gives rise to a well-defined region of the adult brain [2–4]. This segmental organization is critical for establishing the proper spatial organization of the cranial ganglia, branchiomotor nerves and pathways of cranial neural crest migration (Figure 1). The first subsets of neurons form in the even numbered rhombomeres [5]. The motor nerves that innervate the first three branchial arches (trigeminal, facio-acoustic, glossopharyngeal) arise in a two-segment periodicity [6,7]. Hindbrain-derived neural crest cells migrate in three segmental streams adjacent to r2, r4 and r6, which populate the first, second and third branchial arches respectively [8–11]. Hence hindbrain segmentation is a conserved strategy used by vertebrates for organizing the diverse craniofacial features.

**The neural crest and pre-patterning model**

The cranial neural crest is a pluripotent, mesenchymal population that plays a critical role in construction of the vertebrate head. Arising at the junction between the neural plate and surface ectoderm, cranial neural crest cells form nerve, ganglia, cartilage, bone and connective tissue. Many craniofacial malformations are therefore largely attributable to defects in the proliferation, migration, or differentiation of this cell population. Transpositions of neural folds in a number of species [12–16] led to the concept that regional diversity in the vertebrate head was a consequence of patterning information provided by the neural crest. When presumptive first arch (mandibular) neural crest primordia were transplanted more posteriorly in the neural tube in place of presumptive second (hyoid) or third (visceral) arch neural crest, the transplanted neural crest cells migrated into the nearest arch but therein formed ectopic proximal first arch skeletal elements such as the quadrate and Meckel’s cartilage [16]. Not only were these crest-derived structures inappropriate for their new location but the muscle cell types and attachments associated with the ectopic structures were also characteristic of a first arch pattern. This suggested that myogenic populations and other cell types receive spatial cues from the invading neural crest derived connective tissue. Molecular evidence supporting this scenario was provided.
by the observation that the same domains of Hox gene expression that are restricted in the hindbrain were emulated in the ganglia and branchial arches, reflecting the origins of the neural crest cells contributing to these tissues [17,18].

Collectively, these pivotal studies led to speculation that the spatial organization of cranial structures was determined by the neural crest and that the pattern was irreversibly set before the neural crest emigrates from the neural tube. Under this pre-patterning model, positional information including Hox genes was carried passively from the hindbrain to peripheral tissues and branchial arches by the neural crest, where it was elaborated to form the characteristic head structures.

**Cranial neural crest plasticity**

The neural crest pre-patterning model predicts that experimental alterations to the spatial organization of the hindbrain should result in a re-organization of the patterns of Hox gene expression and neural crest migration, and ultimately craniofacial abnormalities. Owing to the ease of tissue manipulation, the chick embryo has been the primary species for testing this hypothesis via rhombomere transplantations, rotations and ablations. These analyses have yielded conflicting results regarding the degree of autonomy of Hox gene expression [19]. Recently there have been significant advances in our understanding of these developmental issues, which have arisen principally from the development of new techniques for transposing cells within the hindbrains of mouse [20••] and zebrafish [21••] embryos.

In the mouse, cells from r3, r4 or r5 were heterotopically grafted into r2 [20••]. The majority of the transplanted cells remained as a cohort and maintained their Hox gene antero–posterior (A–P) identity (Figure 2). A few transplanted cells, however, became separated from the primary graft and dispersed becoming intermingled with the neighboring populations. These cells displayed plasticity as they failed to maintain their appropriate Hox gene expression patterns and consequently altered their identity in their new location. This implies that single or dispersed rhombomere cells lack the neighboring signals necessary to reinforce their identity. Hence they respond and adapt to their new surrounding environment by altering gene expression [20••].

Further evidence for neural plasticity and an influence of cell community effects has been provided through the formidable task of transplanting single rhombomere cells in zebrafish [21••]. The transposition of single hindbrain cells from r2 into r6 or vice versa resulted in a complete switch in Hox gene expression (Figure 2). This was accompanied by changes in cell fate, which was now characteristic of the new location of the transplanted cells. This degree of plasticity is dependent upon the timing and size of the transplant. At later stages when morphological boundaries are well established, rhombomere cells are more likely to be irreversibly committed and maintain their Hox gene expression characteristics. This implies that cells in the neural tube progressively lose responsiveness to the environmental signals that specify their segmental identities. Together the mouse and fish studies show that cell-community effects and their associated signals are important in maintaining the axial identity of an individual cell in the hindbrain.

These grafting experiments also revealed the absence of pre-programming in the character or fate of cranial neural crest cells. In heterotopic transpositions of cells within the mouse and zebrafish hindbrains, graft-derived neural crest
cells migrated into the nearest branchial arch without any evidence of pathfinding or re-routing to their original axial level (Figure 2). Plasticity in Hox gene expression in mouse neural crest cells was evident by the complete downregulation of Hoxb1, Hoxb2, and Hoxa2 in these cells [20••]. In zebrafish, experimental embryos raised to larval stages revealed that the transplanted cells differentiated and contributed to pharyngeal cartilages appropriate to their new A–P location [21••]. Therefore these results show that the A–P character of cranial neural crest cells are neither fixed nor passively transferred from the hindbrain to the branchial arches. Such experiments reveal a surprising degree of plasticity in cranial neural crest cells, inconsistent with the pre-patterning model. Instead, since transposed neural crest can be reprogrammed it appears crest cells rely on distinctive cues in the branchial arch environment through which they migrate to elaborate their proper regional identity. Furthermore, the size of the cell community is functionally important suggesting that a far more complex balance of genetic and cellular interactions are involved in hindbrain and neural crest patterning that previously thought.

**Pharyngeal patterning in the absence of neural crest**

The pre-patterning model argues that branchial arch identity is determined by the neural crest. In contrast, the neural crest plasticity described above implies that branchial arch patterning arises due to interactions between the arch components and the neural crest. This raises the question of what happens to the identity of the branchial arches in the absence of contributing neural crest cells. This issue was investigated in chick embryos through rhombomere ablations [22 ••] and in mouse embryos by genetic manipulation of Hoxa1 and Hoxb1, which are required for the generation of neural crest cells in r4 [23••]. In both types of analyses, despite the absence of neural crest cells, the second branchial arch still develops and is properly regionalized. The branchial arch expression patterns of Bmp7 in the posterior endoderm, Fgf8 in the anterior surface ectoderm, Pax1 in the pharyngeal pouch endoderm and Shh in the endoderm were all normal and unchanged. In addition there was no evidence for excessive cell death or loss of proliferation in the arch epithelium, which suggests that the neural crest cells are not the source of any indispensable branchial arch mitogenic or survival signal [23••]. These results clearly demonstrate that the branchial arches are not dependent upon the neural crest for their development and identity. Further support for this idea comes from analyses of Pax gene

![Figure 2](image_url)

Plasticity in the A–P cell fates of transposed hindbrain and cranial neural crest cells. (a) In zebrafish, single r2 cells moved into r6 and adopt an r6 character (green balls with red outline). Similarly single r6 cells grafted into r2 adopt an r2 character (red balls with green outline). This indicates plasticity in fates depending upon the environment. (b) In mouse experiments small groups of r4 cells grafted into r2 maintain their original r4 identity (dark blue balls) if they remain as a group. However, if they disperse and mix with other cells in r2, they lose their r4 character (light red balls in r2). This shows that signals or community effects from neighboring cells can regulate cell fates. Neural crest cells derived from this graft of r4 cells (light red balls in ba1) always lose their r4 or ba2 identity. Reciprocal transplants of r2 cells into r4 display similar cell community and plasticity effects. An r2 character is maintained if the cells remain in a group (red balls) and is lost if they disperse in r4 (light blue balls). Ncc from this graft (light blue balls) that migrate into ba2 do not express ba1 markers. At the bottom of the diagram the shaded balls indicate where evidence of autonomy or plasticity is observed in grafted cells. Note in the hindbrain, plasticity is observed in single dispersed cells and autonomy in cell groups or clusters. This figure is adapted from Figure 2 [19].
expression in *Amphioxus* (the nearest extant vertebrate relative), which show that the pharyngeal pouches are still regionalized despite the absence of neural crest cells [25]. Thus the mechanism for generating pharyngeal pouches predates the evolution of the vertebrate head.

**Patterning roles for the mesoderm, endoderm and ectoderm in branchial arch development**

Since normal branchial arch development can occur independently of a contribution from the neural crest, perhaps the branchial arches rely on the paraxial mesoderm, endoderm and surface ectoderm tissues for their patterning information.

**The mesoderm**

The cranial mesoderm forms the predominantly myogenic cores of each branchial arch, which are enveloped by migrating neural crest cells [26–28]. Until recently, the cranial mesoderm was not thought to play a patterning role during craniofacial development. However, it has now been shown that the cranial mesoderm provides maintenance signals for regulating the identity of second branchial arch neural crest cells [20••]. If the second arch neural crest is transplanted alone into the first arch, it downregulates its expression. If the second arch neural crest is transplanted in combination with second arch mesoderm, then expression is maintained. The cranial mesoderm therefore provides maintenance signals that elaborate the program of *Hox* expression, but cannot initiate *Hox* gene expression in neural crest cells [20••] (Figure 3). This is consistent with the fact that the fate of the cranial mesoderm is primarily myogenic and the musculature is inextricably linked to neural crest derived skeletal and connective tissue patterning. Therefore, one of the roles of the cranial mesoderm may be in maintaining an A–P register between these different primordial tissues, which is essential for subsequent craniofacial morphogenesis [28].

**The endoderm**

The neurogenic placodes (dorsolateral and epibranchial) form in characteristic positions in all vertebrates suggesting that conserved localized inductive interactions underlie their formation [29]. The epibranchial placodes develop near the branchial clefts in close proximity to the cranial neural crest and the pharyngeal endoderm. Analyses of the nature of the signals, which underlie epibranchial placode formation, have found that the epibranchial placodes do not require cranial neural crest cells for their induction [30]. Rather, it is the pharyngeal endoderm that is the source of the BMP7-inducing signal. The endoderm has also been shown to be responsible for promoting the formation of branchial arch components in amphibians by directing neural crest cells towards a chondrogenic fate [31]. Therefore, the endoderm plays a major role in establishing and patterning the branchial arches.

**The ectoderm**

Similar to the neuroepithelium, it has been suggested that the ectoderm is regionalized into territories, called ectomeres, that contribute to specific regions of the branchial arches [32]. Currently, there is no evidence to support the idea that each ectomere represents a functional developmental unit. In contrast, however, there is evidence that the surface ectoderm plays a major role in the induction of odontogenesis during branchial arch development [33]. The oral ectoderm of the first branchial arch directly instructive or inducing signals is not confined to first arch neural crest cells [34]. Fgf8, which is expressed in the
Anterior surface ectoderm of the first arch, is essential for determining the polarity of the branchial arch [35]. Hence, the surface ectoderm plays an important role in patterning the branchial arch derivatives (Figure 3).

**Independent molecular regulation of Hoxa2 in the hindbrain and neural crest**

The neural plasticity described above correlates with molecular analyses that have identified distinct regulatory elements controlling Hox gene expression in different tissues such as the hindbrain and neural crest. Hoxa2 is expressed in the hindbrain anteriorly up to the r1/2 boundary and in cranial neural crest cells that migrate into the second branchial arch [36]. Transgenic regulatory analyses of Hoxa2 have revealed that multiple cis-acting elements are required independently for hindbrain-specific and neural crest-specific activity [37–39]. In r3 and r5, Hoxa2 expression is directly regulated by the transcription factor Krox20. In contrast, Hoxa2 expression in second branchial arch neural crest cells is tightly controlled by a number of elements, one of which binds to AP-2 family members. Mutation or deletion of this AP-2 site in the Hoxa2 enhancer abrogates expression in cranial neural crest cells but not in the hindbrain. These findings clearly demonstrate that at the molecular level, Hoxa2 is independently regulated in rhombomeres and neural crest cells. This provides a mechanism for how neural crest cells can respond to the environment through which they migrate independently of the neural tube.

**The role of Hoxa2 in branchial arch patterning**

During craniofacial development, neural crest cells migrate into the branchial arches to form the skeletogenic elements [16,40]. In mammals, neural crest of the first arch form Meckel’s cartilage, while neural crest of the second arch form Reichert’s cartilage (Figure 3). The proximal region of Meckel’s cartilage develops into two of the middle ear bones, the malleus and the incus. Reichert’s cartilage forms the stapes (third bone of the middle ear), the styloid process of the temporal bone, the lesser horn and part of the hyoid bone [41]. Both endochondral and intramembranous ossification occur during the first branchial arch differentiation, whereas in the second arch only endochondral ossification takes place.

The targeted inactivation of Hoxa2 results in lethality at birth and homeotic transformations of elements derived from the second arch neural crest into proximal first arch derivatives, including a partial duplication of Meckel’s cartilage and ossification centers of the middle ear bones [42,43]. In these mutants, ectopic intramembranous ossification (dermal bone formation) takes place in the second arch. Therefore, Hoxa2 is essential for proper patterning of structures derived from the neural crest in the second branchial arch, as it inhibits intramembranous ossification and allows only endochondral ossification to occur. Interestingly, only the mesenchymal and not the neurogenic derivatives of the second branchial arch are transformed in Hoxa2-null mutants. The fact that r4 is unaffected in these mutants suggests that the primary role of Hoxa2 is in the neural crest and is independent of the neural tube [42,43]. This provides further support for the idea that neural crest cells are not pre-specified before their migration from the neural tube. Further analyses in Hoxa2-mutant mice involving retinoic acid response show that the segmental identities of the hindbrain and its derived neural crest are not linked and can be altered independently [44]. This suggests that Hoxa2 acts as a positive selector gene in neural crest and branchial arch morphogenesis.

If this is true, then ectopic expression of Hoxa2 in the first arch should result in the development of second arch structures replacing those of the first arch [43,45]. This has been confirmed by recent studies in chick [46••] and Xenopus [47••] embryos that have overexpressed Hoxa2 in the first branchial arches. In both cases overexpression of Hoxa2 resulted in a transformation of first arch structures, such as Meckel’s cartilage and the quadrate, into second arch elements. The duplicated elements are fused to the original elements in a manner similar to that seen in the Hoxa2 knockout mutant. This confirms the role of Hoxa2 as a selector gene specifying second arch fate. In addition, these studies imply that the neural crest is not pre-patterned before its emigration from the neural tube but that it needs to read cues from the arch environment. When first arch crest, before its emigration from the neural tube, is targeted with Hoxa2, these neural crest cells are unable to develop into second arch elements (Figure 3). The upregulation of second arch specific genes in the first arch and the homeotic transformation of cartilage elements only occur after global expression of Hoxa2 in the neural crest and surrounding tissues during formation of the first arch [46••,47••]. These results argue that although neural crest cells are born with some patterning information or identity, the elaboration of their developmental program is achieved through integration with signals from the surrounding tissue environments in which they migrate.

Inroads have been made into the precise mechanisms by which Hoxa2 influences the morphogenesis of second arch elements [41] (Figure 3). During normal development, Hoxa2 is widely expressed in the second arch mesenchyme, but it is excluded from the chondrogenic condensations in the core of the arches. In the absence of Hoxa2, ectopic chondrogenesis coincides with an expansion of Sox9 expression into the normal Hoxa2 expression domain where Sox9 is not normally expressed. Sox9 is a direct regulator of the cartilage-specific gene, Col2a1 [48,49], and using a transgenic approach it has been shown that changes in Sox9 expression are indeed responsible for the ectopic cartilaginous elements found in the second arch of Hoxa2 mutants. This is supported by misexpression of Sox9 in the second arch, which produces a phenotype resembling that of the Hoxa2 mutants [48]. Therefore, Hoxa2 acts very early in the chondrogenic pathway and is upstream of Sox9. In addition, Chafl, an activator of...
Resolution of outstanding interest
the principal co-coordinator.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
** of outstanding interest

An important question concerns the degree to which patterning of branchial arches is responsible for patterning all of arch development. In conjunction with studies of triton and bombinator chimeras, investigations of cephalic neural crest and neural crest migration results in homeosis of jaw elements in normal arch patterning. In amphibian embryos the plasticity of hindbrain and cranial neural crest cells as transposed skeletal, connective, and muscle tissues. These papers address the important question of how and where genetic and model systems transposed. The fate map of cephalic neural primordium indicates that formation of placodes, pouches and epibranchial placodes. The importance of neural crest in amphibian craniofacial skeleton and myogenic mesenchyme: a perspective. In chick embryos shows that by surgically ablating crest it is still possible to obtain normal arch patterning. In conjunction with this indicates that neural crest is not responsible for patterning all of arch development. Genetic mutants specifically lacking both Hoxa1 and Hoxb1 only in the neural tube have uncovered a new role for Hox genes in the regulation of neural crest development. An important question concerns the degree to which patterned arch tissues deviates upon interactions or signals from neural crest. This study in chick embryos shows that by surgically ablating crest it is still possible to obtain normal arch patterning. In conjunction with this indicates that neural crest is not responsible for patterning all of arch development.


52. Irving C, Mason I: Signalling by fgf8 from the isthmus patterns the anterior hindbrain and establishes the anterior limit of Hox gene expression. Development 2000, 127:177-186. This study illustrates how signals from the isthmus can influence patterning of the adjacent neural tube and suggests that Fgf signaling is an important component of the process that patterns the anterior hindbrain.
