

Have gene knockouts caused evolutionary reversals in the mammalian first arch?

Kathleen K. Smith and Richard A. Schneider

Summary

Many recent gene knockout experiments cause anatomical changes to the jaw region of mice that several investigators claim are evolutionary reversals. Here we evaluate these mutant phenotypes and the assertions of atavism. We argue that following the knockout of *Hoxa-2*, *Dlx-2*, *MHox*, *Otx2*, and *RAR* genes, ectopic cartilages arise as secondary consequences of disruptions in normal processes of cell specification, migration, or differentiation. These disruptions cause an excess of mesenchyme to accumulate in a region through which skeletal progenitor cells usually migrate, and at a site of condensation that is normally present in mammals but that is too small to chondrify. We find little evidence that these genes, when disrupted, cause a reversion to any primitive condition and although changes in their expression may have played a role in the evolution of the mammalian jaw, their function during morphogenesis is not sufficiently understood to confirm such hypotheses. *BioEssays* **20**:245–255, 1998. © 1998 John Wiley & Sons, Inc.

INTRODUCTION

The development and evolution of the visceral arches is one of the oldest and best studied topics in vertebrate morphology. This area has recently been re-examined in light of the discovery of numerous genes that may play a role in the origin and patterning of these structures. Techniques of gene targeting in mice that knockout the function of specific genes^{1–3} have allowed developmental biologists to probe the morphological consequences of the deletion of gene products. Many of these deletions affect the first visceral arch of mammals; an overview of this work provides an excellent opportunity to evaluate the specific ways in which data derived from molecular genetic studies can be integrated with classic problems and data in morphology, homology, and evolution.

The first visceral arch includes all the cartilages and bones associated with the jaws. Virtually all aspects of the primitive condition were transformed during the 200,000,000-year evolution of the lineage leading to mammals. Many bones were lost or reduced, two elements that formed part of the ancestral jaw joint became incorporated into the middle ear as ossicles involved in sound transmission, one component expanded to close off the lateral braincase wall, and a new articulation point developed between the jaw and skull. Details of these evolutionary transitions come from embryological and paleontological evidence collected during the nineteenth and twentieth centuries.^{4–7} A remarkably complete fossil record has permitted detailed study of intermediate stages between reptiles and mammals and analyses of these fossils have provided an ever increasing understanding of the evolutionary basis of this transformation.^{8–13}

More recently, many genes have been identified that affect patterning of the first arch.^{14–17} In particular, gene knockouts of *Hoxa-2*, *Dlx-2*, *MHox*, and *Otx2*, as well as double null mutations in retinoic acid receptor (*RAR*) genes, produce ectopic cartilages. These cartilages have been interpreted as being homologous to one of the jaw elements in primitive vertebrates.^{18–24} Such results have led many workers to hypothesize that the phenotype produced by the null mutations is an evolutionary reversal or atavism and to

Departments of Biological Anthropology and Anatomy and Zoology,
Duke University Medical Center, Durham, NC.
Department of Zoology, Duke University, Durham, NC.
Contract grant sponsor: National Science Foundation; Contract grant
number: IBN 9407616.

*Correspondence to: Kathleen K. Smith, Box 3170 Duke University
Medical Center, Durham, NC 27710; E-mail: kksmith@acpub.duke.edu

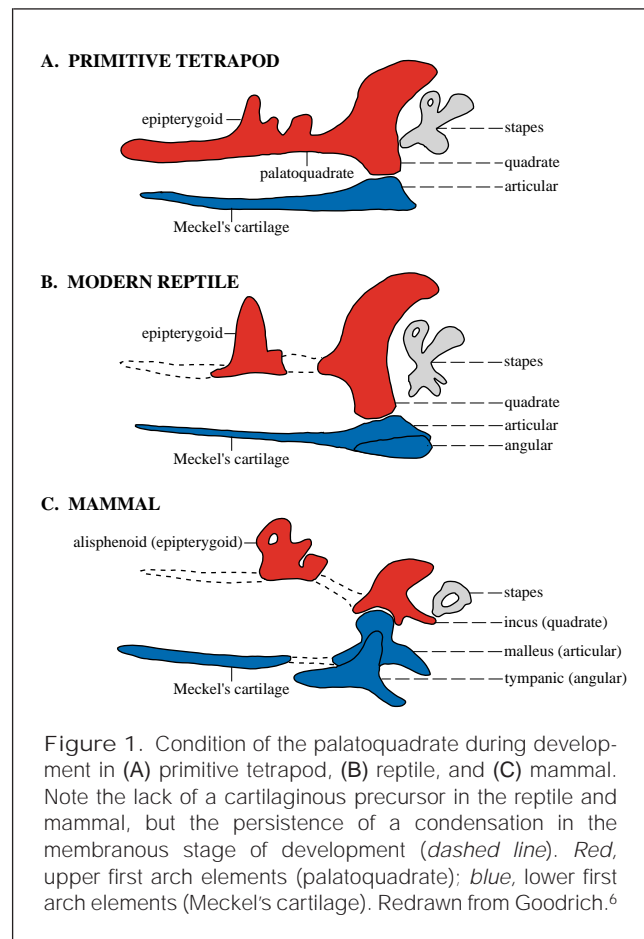
conclude that these genes played an important role in the evolution of the first arch in mammals.

This paper reanalyzes the mutant phenotypes produced by the knockout of several genes and evaluates whether the claims of evolutionary reversals are supported by the data. We review the anatomy and evolution of the first arch in mammals and their ancestors, and provide a foundation for the assessment of atavisms and homology. In this context, we argue that the conclusions of several investigators are based on inaccurate identification of skeletal elements in the mutant animals, a misunderstanding of the morphological condition in mammalian ancestors, or inappropriate assessments of homology and atavism. We therefore reject the hypotheses of evolutionary reversal. Instead, we offer an alternative hypothesis to explain the common appearance of ectopic cartilages in the null mutants based on the mechanisms of cartilage formation in the skull and the conditions already present during development of mammalian embryos. We hope that this case study will allow synthesis of the concepts and data of classic evolutionary morphology with current molecular genetic studies, encourage a closer examination of how genes exert their effects during morphogenesis, and facilitate a more rigorous discussion of the relation between mutant phenotypes and evolutionary transformations.

FIRST ARCH ANATOMY IN MAMMALS AND OTHER VERTEBRATES

Primitively in jawed vertebrates a series of visceral arches supports the gill apparatus. Each arch contains skeletal, muscular, nervous, and circulatory elements. Neural crest cells provide the mesenchyme for all skeletal and connective tissues in the arches and pattern the muscles.^{25,26} In the jawed vertebrates the first of these arches contains upper and lower skeletal portions that form the jaws⁶; in the primitive condition the upper component is the palatoquadrate cartilage (in the papers reviewed here often called the pterygoquadrate) and the lower element is Meckel's cartilage (Fig. 1). During vertebrate evolution, these two cartilages were reduced in multiple lineages and no longer form functional jaws in adults. Instead, the functional jaws are formed by a series of dermal bones.

In adult, nonmammalian amniotes (living reptiles and birds) the palatoquadrate or upper jaw element is represented by two distinct cartilage-replacement bones, the epipterygoid anteriorly and the quadrate posteriorly. In reptiles such as turtles and crocodiles the epipterygoid and quadrate bones are connected by a cartilage during development, but in lizards, snakes, and birds there is no connection between these elements even in embryos (Fig. 1). In adult reptiles, the epipterygoid forms a vertical strut on the side of the braincase and the quadrate is the site of connection between the upper jaw and the skull. The



functional upper jaw is composed of a number of dermal bones, including the premaxilla, maxilla, quadratojugal, palatine, pterygoid, and ectopterygoid. This condition characterizes not only living reptiles and birds, but also most fossil amniote taxa, including the extinct therapsid ancestors of mammals.²⁷ The reptilian lower jaw is also made up of a number of separate bones. Most of these bones are dermal bones, including the main bone that bears the teeth, the dentary. Other bones, such as the articular, ossify from part of Meckel's cartilage. The articular contacts the quadrate to form the jaw joint. Therefore, although the jaws themselves are primarily formed from dermal bones, the actual connection between the upper and lower jaw is formed from two ossified remnants of the primitive cartilaginous upper and lower portions of the first arch, the quadrate and the articular. This condition is seen in living reptiles, birds, and also in most fossil amniotes, including the therapsids.

The first arch in mammals has undergone significant evolution from the condition described above. First, the homologue of the epipterygoid bone contributes to part of the mammalian alisphenoid, which fuses with other bones of the skull to produce a solid box around the brain. The

quadrate, or upper part of the primitive jaw articulation, is reduced in size and no longer participates in the jaw joint. Instead the quadrate becomes one of the middle ear ossicles, the incus. The lower jaw, which in early therapsids is generally made up of six different bones, is reduced to a single bone, the dentary. Most of the remaining lower jaw bones are lost, although the articular, like the quadrate, is reduced and forms one of the middle ear ossicles, in this case the malleus. Another lower jaw bone called the angular in therapsids becomes the tympanic bone in mammals and supports the tympanic membrane. As the primitive jaw joint has been reduced to form ear ossicles, a new process of the dentary, the condyle, articulates with the dermal squamosal bone of the skull to form a new jaw joint in mammals, the dentary-squamosal joint. Figure 2 summarizes the first arch structural changes during evolution, and Table 1 outlines the homologies between elements seen in reptiles and mammals.

THE EFFECTS OF GENE KNOCKOUTS ON THE FIRST ARCH OF MICE

Gene-targeting experiments replace wild-type alleles with mutant ones to produce heterozygous embryos with one defective (null) copy or homozygotes with two defective copies of a specific gene. Numerous genes have been targeted for deletion in mice and many have proven to be necessary for normal embryonic differentiation and morphogenesis. Several null mutations affect patterning of the first arch, and major phenotypic defects in the facial and particularly first arch region have been reported. For example in the null mutations of *Hoxa-1*, otic structures are affected, including in some cases the middle ear bones.¹⁴ Animals homozygous for the null mutations of *Msx-1* have a cleft palate and are missing most of their dentition.²⁸ Null mutants of the *gooseoid* gene have malformed bones of the facial and dentary region, and also exhibit significant defects in the alignment of musculature of the first and second arches.^{15,16} Here, we focus on five specific mutations. While a variety of defects are present in these mutants, each has one element in common: an ectopic cartilage that is reminiscent of the primitive palatoquadrate. Some of the phenotypic effects of the null mutations are summarized in Table 2 and are described briefly in the following section.

Hoxa-2

Hoxa-2 is related to the *proboscipedia* gene of *Drosophila* and is normally expressed in the second, third, and fourth rhombomeres (r2–r4) of the mouse hindbrain.^{29,30} Expression of *Hoxa-2* is carried by the neural crest cells from r3 and r4 to the tissues of the second visceral arch. The expression of *Hoxa-2* is down-regulated in the neural crest cells from r2 (which give rise to tissues of the first visceral arch), so that

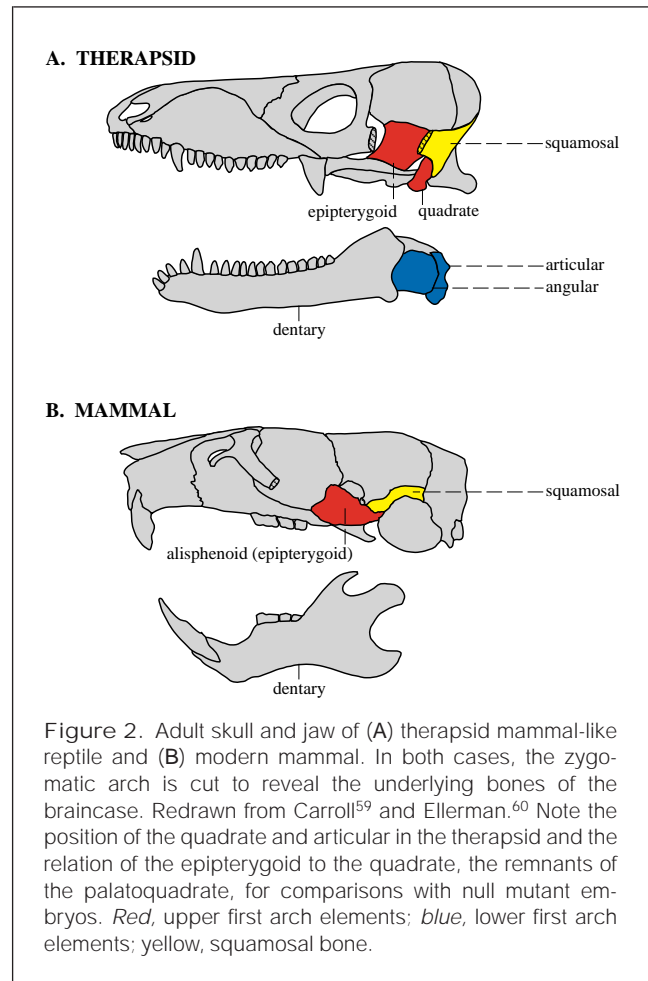


Figure 2. Adult skull and jaw of (A) therapsid mammal-like reptile and (B) modern mammal. In both cases, the zygomatic arch is cut to reveal the underlying bones of the braincase. Redrawn from Carroll⁵⁹ and Ellerman.⁶⁰ Note the position of the quadrate and articular in the therapsid and the relation of the epipterygoid to the quadrate, the remnants of the palatoquadrate, for comparisons with null mutant embryos. Red, upper first arch elements; blue, lower first arch elements; yellow, squamosal bone.

tissues of the first arch do not normally express this gene.^{30,31} Null mutations of *Hoxa-2* have been independently produced and discussed by two laboratories.^{18,19,32} *Hoxa-2* null mutations appear to cause homeotic transformation of the second arch-derived tissues into first arch elements. In homozygous null mutants, first arch elements are duplicated as a mirror image in the normal position of second arch elements; no neural crest-derived second arch elements are found (Fig. 3). The duplicated elements include the malleus, incus, and tympanic bones, the proximal portion of Meckel's cartilage, and portions of the squamosal bone. In addition, there is a long narrow ectopic element connecting the duplicated incus and a region near the alisphenoid that Rijli et al.^{19,32} suggest is homologous to the primitive palatoquadrate (=“pterygoquadrate”). Rijli et al.¹⁹ conclude that “the presence in the *Hoxa-2* mutants of both a reptilian pterygoquadrate remnant and an incus among the duplicated elements indicates that the present-day ground pattern may correspond to an evolutionary state intermediate between therapsid reptiles and mammals in which the quadrate had

TABLE 1. Homologies of First Arch Skeletal Elements in Reptiles and Mammals

Reptiles	Mammals
Upper portion of first arch	
Cartilage replacement bones	Cartilage replacement bones
Palatoquadrate	Lost
Epipterygoid	Alisphenoid (in part)
Quadrate	Incus
Dermal bones	Dermal bones
Premaxilla	Premaxilla
Maxilla	Maxilla
Quadratojugal	Jugal
Pterygoid	Pterygoid
Palatine	Palatine
Lower portion of first arch	
Cartilage replacement bones	Cartilage replacement bones
Meckel's cartilage	Meckel's cartilage
Articular	Malleus
Dermal bones	Dermal bones
Angular	Tympanic
Surangular	Lost
Dentary	Dentary
Prearticular	Goniale
Coronoid	Lost
Splenial	Lost

From references 6, 8, 27, and 41.

not yet disappeared, while the incus was already individualized" (p. 1347).

Dlx-2

Dlx-2 is one of a family of at least six *Dlx* genes in mammals that are similar to the *Distal-less* gene in *Drosophila*.³³ *Dlx-2* is normally expressed in neurogenic and chondrogenic neural crest cells of the visceral arches,³⁴ as well as in the forebrain and other regions of the body.³⁵ Qui et al.²¹ produced a null mutation in the mouse *Dlx-2* gene. In the null mutants, numerous gross craniofacial defects exist, mostly involving structures of the upper part of the first arch. Most of the alisphenoid is missing, the incus is abnormal, and is fused to a large irregularly shaped ectopic cartilage (Fig. 4). The authors identify this ectopic cartilage as the "pterygoquadrate" cartilage because it appears to connect the incus to anterior braincase elements. In addition, there are a number of abnormalities of dermal bones, including several ectopic bones in the region of the squamosal. Qui et al.²¹ claim that "most of the proximal first-arch skeletal abnormalities found in the *Dlx-2* mutants can be conceived as a transformation of this region of the skull toward a reptilian form. If this hypothesis is correct, it predicts that *Dlx-2* has an

important role in the evolution of the proximal first arch." (p. 2535).

MHox

MHox is a novel mouse gene related to the *paired* class of homeoboxes,³³ normally expressed in the mesoderm of facial, limb, and vertebral skeletal precursors. This gene may regulate epithelial—mesenchymal interactions during development.³⁶ *MHox*-null mutants have diverse chondrogenic and osteogenic defects in both the head and limbs.²² In the cranium, the predominant result is an overall decrease in dermal bone in the skull with particular reduction of the squamosal and alisphenoid bones, two elements associated with the first arch. A very large ectopic cartilage appears rostral to the malleus that is fused with the incus, stapes and tympanic and occupies much of the region of the side of the braincase (Fig. 5). Martin et al.²² homologize the ectopic cartilage seen in the *MHox* mutant to the palatoquadrate. They state that "in *MHox* mutants, the incus fails to demarcate and the palatoquadrate is partially retained, forming an articulation with the malleus. Thus, *MHox* mutations result in cranial skeletal components that are morphologically similar to those seen in phylogenetically more primitive animals such as reptiles" (p. 1246).

Otx2

Otx2 is similar to the *orthodenticle* gene of *Drosophila* and is normally expressed in the entire epiblast at the pre-streak stage, becoming localized to the anterior region of the embryo in mid-streak stages.³⁷ Expression is found in all germ layers and *Otx2* is thought to function in specifying territories in the rostral brain and possibly the entire rostral head of vertebrates. In homozygous null mutants, all structures of the rostral head generally are missing, and the embryos never survive beyond embryonic day 10.^{23,24} Heterozygotes exhibit a wide range of phenotypes from complete acephaly to relatively minor skeletal and neural defects. In general, the dentary bone exhibits major defects, and a variety of elements show lesser degrees of malformation. These latter elements include Meckel's cartilage, the malleus, incus, tympanic, squamosal, alisphenoid, and the dermal bones of the face and mouth (e.g., maxilla, premaxilla, palatine, pterygoid, and jugal, among others). Some embryos have a cartilaginous connection between the incus and alisphenoid, interpreted by Matsuo et al.,²³ as representing an atavistic palatoquadrate. They suggest that the pattern of defects observed "might implicate this gene having an evolutionary significance that would have played a role in the establishment of the masticatory apparatus and neurocranium" (p. 2656).²³

TABLE 2. Summary of Defects Associated with the Upper and Lower Portions of the First Arch and Selected Braincase Regions in Gene Knockout Experiments

	<i>Hoxa-2</i> ^{18,19,32}	<i>Dlx-2</i> ²	<i>MHox</i> ²²	<i>Otx-2</i> ^{22,24}	<i>RAR</i> ²⁰
Upper portion of first arch					
"Palatoquadrate" ^a	D	D	D	D	D
Alisphenoid	D	D	D	D	D
Incus	D	D	D	D	D
Maxillary	D	D	D	D	D
Jugal	UNK	D	D	D	UNK
Pterygoid	UNK	D	D	D	D
Palatine	D	D	D	D	D
Lower portion of first arch					
Meckel's cartilage	D	ND	D	D	ND
Malleus	D	ND	D	D	ND
Tympanic	D	ND	D	D	ND
Dentary	D	ND	D	D	ND
Braincase					
Squamosal	D	D	D	D	ND
Frontal	UNK	ND	ND	D	D
Parietal	UNK	ND	ND	ND	D
Basisphenoid	D	D	UNK	D	D

D, structures in which a defect appeared in the null mutant; ND, structures in which no defect was reported; UNK, structures not discussed in the original paper, so it is unclear whether defects were absent, or just not reported (unknown).

^aPalatoquadrate is in quotes, as we believe this is an ectopic cartilage independently derived in each mutant; its homology with the primitive condition is uncertain.

Retinoic Acid Receptors

Evidence that retinoic acid is a powerful signal transducer in gene regulation comes from numerous sources³⁸ and several genes are independently responsible for different retinoic acid receptors. Lohnes et al.²⁰ produced several combinations of double null mutations for various retinoic acid receptor (*RAR*) genes in which widespread neural and skeletal defects appear. One combination (α/γ) gives rise to substantial cranial defects. In these mutants, most of the neural crest-derived skeletal elements are altered, with severe abnormalities in the mid-face and cranial base. Many craniofacial bones are grossly deficient or absent. The maxilla, palatine, and alisphenoid bones are malformed, and the upper incisors are absent. However, the dentary, malleus, tympanic, jaw joint, lower dentition, and upper molars are all normal. As in many other of the null mutations, a number of ectopic cartilages are observed. There are numerous cartilaginous nodules in the meninges of the brain; in addition, in some null mutants, there is a thin cartilaginous bar connecting the incus and the alisphenoid (Fig. 6). As in the other papers, these authors homologize this bar to the palatoquadrate and claim that "It is likely that this is an atavistic structure corresponding to the therapsid evolutionary state in which the incus had appeared, but was still linked to the newly derived alisphenoid bone through quadrate

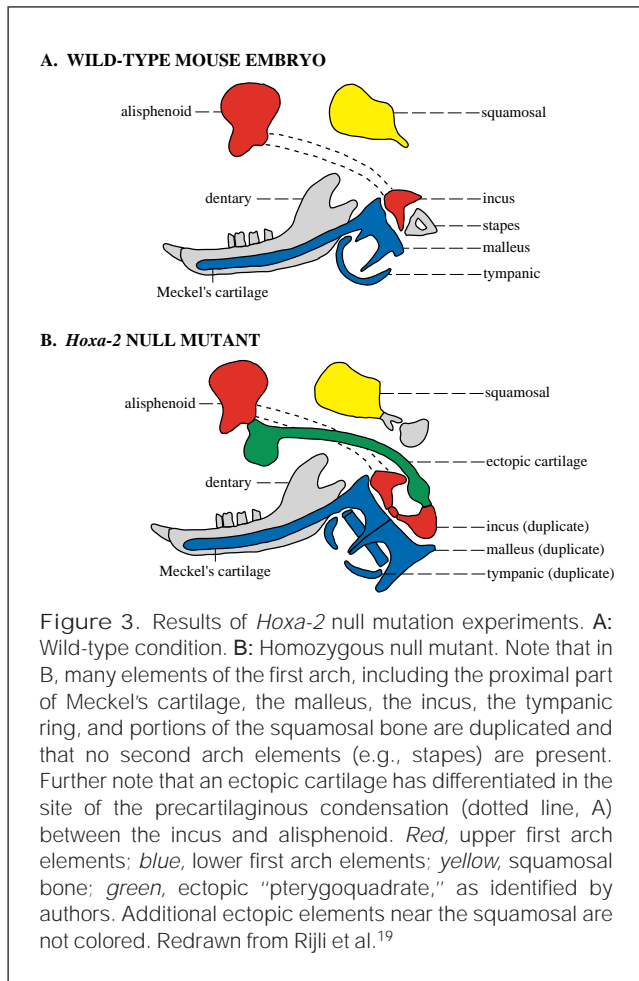
remnants." (p. 2746)²⁰ They conclude that genes for retinoic acid receptors may have been involved in repatterning the first arch during the transition from reptiles to mammals.

DISCUSSION

Identifying Atavisms and Homologies

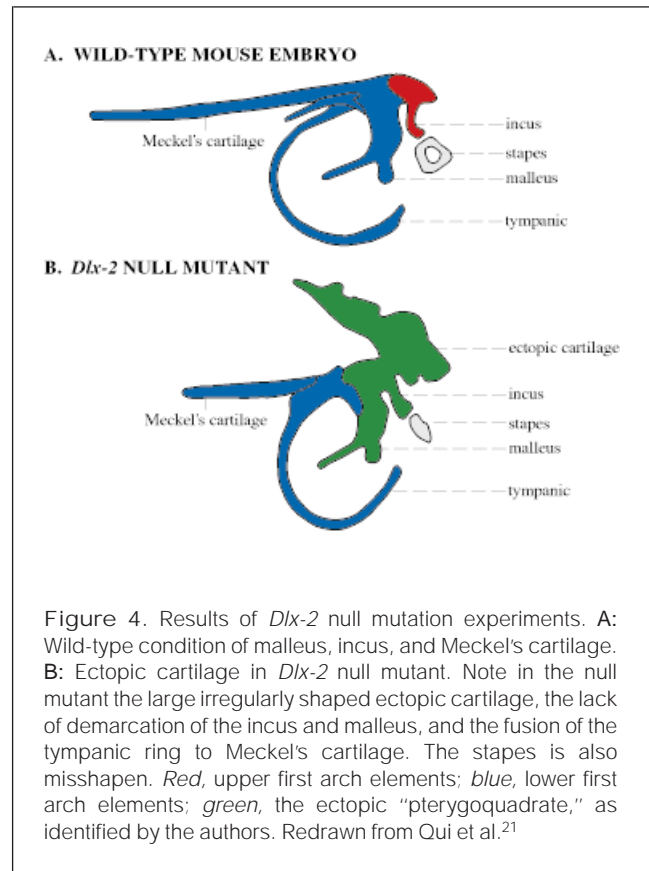
After the knockout of *Hoxa-2*, *Dlx-2*, *MHox*, *Otx2*, and some *RAR* gene combinations, ectopic cartilages of varying sizes and shapes appear between the ear region and the sidewall of the braincase. The authors of several of the studies state that this ectopic cartilage is homologous to the palatoquadrate and is an atavism resembling the condition seen in the immediate ancestors of mammals. These investigators conclude that the gene of interest may have been significant in the evolution of the first arch in mammals. In the following discussion, we define atavisms and provide criteria for their identification. On the basis of these criteria, we argue that the phenotypes produced by these null mutations are not atavisms. We then address problems with the interpretation of homologies of ectopic structures.

In a review of the developmental mechanisms producing evolutionary reversals in vertebrates, Hall³⁹ defines atavisms as "the reappearance of a lost character (morphology or behavior) typical of remote ancestors and not seen in the



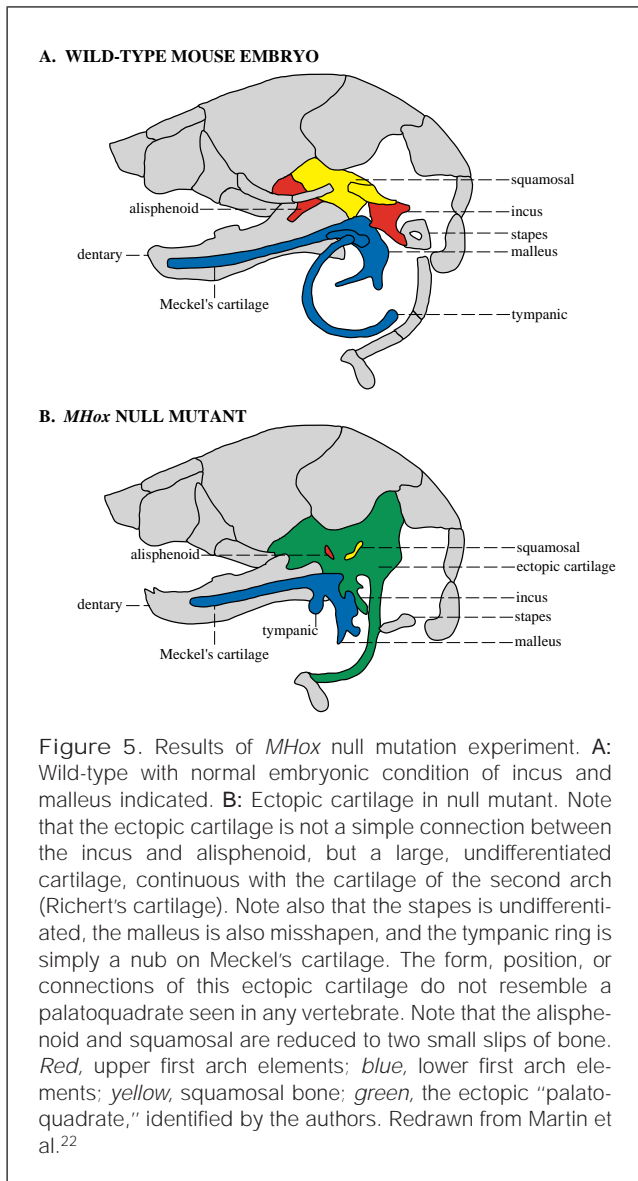
parents or recent ancestors of the organisms displaying the atavistic character" (p. 89). He goes on to outline the "essential features" of an atavism, including: "(1) its persistence into adult life; (2) its absence in the parents or recent ancestors; (3) its presence in only one or a few individuals within a population, or (4) its close resemblance to (identity with ?) the same character possessed by all members of an ancestral population" (p. 89). All the mutants possessing the characters of interest died at birth, so the first criterion cannot be applied. Since these phenotypes were induced by targeted gene mutation, criteria two and three apply almost by definition. Data on the development and evolution of the first arch can be used to evaluate the final criterion, the resemblance of the character to that seen in an ancestral population.

The palatoquadrate is the upper jaw element in primitive vertebrates; however, there is no palatoquadrate in most adult extant reptiles, birds, or mammals, nor in the therapsid ancestors of mammals.^{6,40,41} Instead, only two separate elements, the epipterygoid and quadrate are present, with little or no cartilaginous connection, either in the adult or



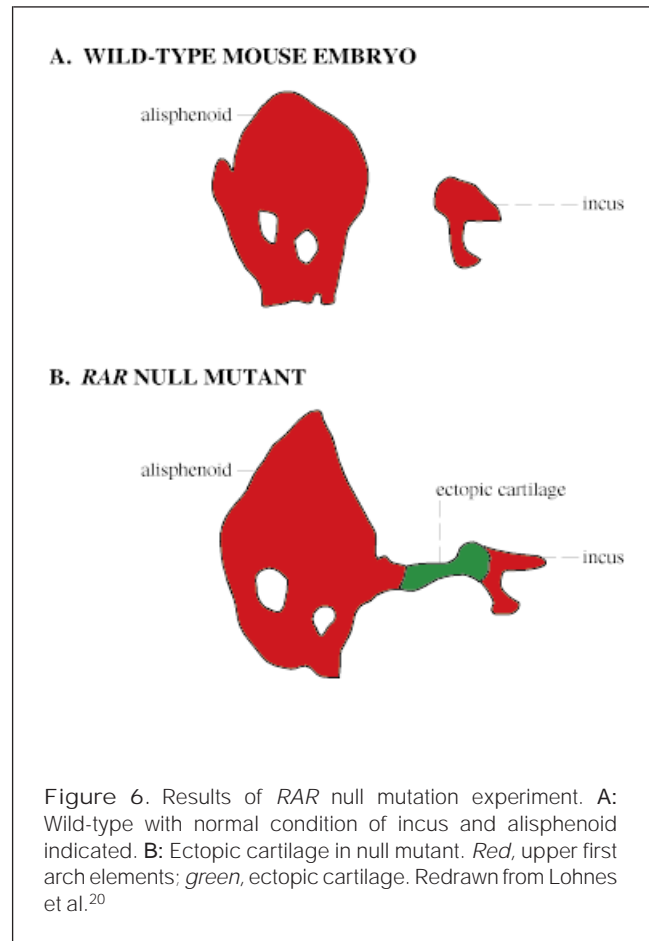
embryo. A palatoquadrate is not a general feature of mammal-like-reptiles. Statements such as "Thus the complex formed by the pterygoquadrate element and caudal incus in *Hoxa-2* mutants might correspond to a mirror image of an atavistic skeletal formation . . . in which the incus had already appeared, while the pterygoquadrate cartilage was still present. Such a skeletal arrangement indeed existed in therapsids, the reptilian ancestors of modern mammals"³² are based on a misconception of the morphology of therapsids as well as living reptiles. In none of the cases reported do the null mutant phenotypes show identity with, or close resemblance, to the character state found in mammalian ancestors or transitional forms.

Misinterpretation of this morphology is due in part to a misunderstanding of the homology of various elements. Homology is the similarity of structures between organisms based on inheritance from a common ancestor and shared developmental pathways.⁴²⁻⁴⁴ While the particular means to identify homologous characters have been debated, there are at least three important criteria.⁴⁵ First, homology between structures is based on overall similarity, including detailed resemblance, positional information, developmental origin, functional anatomy, and spatial relations to surrounding structures. Second, if two structures are found together in



the same organism they cannot be homologous, although a different criterion applies to serial or iterative elements within a single organism.⁴⁶ Third, hypotheses of homology must be in agreement with the distribution of characters and character states on the phylogeny of the organism and its relatives. The homology of two structures is certain when those structures have been derived from a corresponding structure in a common ancestor.

In the papers summarized here, there are many mis-identifications of the homology of various elements. The most critical one, appearing in multiple papers^{19,20,22,32} involves the homology of the incus and quadrate. For example, Martin et al.²² state that "In *MHox* mutants, the incudo-malleolar joint was intact but the incus remained fused to a persistent



quadrate cartilage on the lateral aspect of the skull . . ." (p. 1240). This interpretation suggests that the quadrate and incus are two separate structures and that one was gained while the other was lost. In fact, the quadrate and incus are two different evolutionary states of the same structure; that is, they are homologous, and thus could not both be present simultaneously in a transitional form. These mis-identifications are significant because they have produced misleading interpretations of resemblance between the null mutant phenotype and primitive conditions.

However, the problem with the hypotheses of evolutionary reversal in these papers goes beyond simple mis-identification of elements or misunderstanding of the morphology of ancestral forms. We believe there are two critical issues in assessing the relevance of these null mutation experiments to problems in vertebrate evolution. First, the identification of an atavism requires more than the passing resemblance of a single element to one that might have existed in a similar position in some ancestor. The evolution of the mammalian first arch involved the functional and developmental integration of many characters over hundreds of millions of years. These null mutants do not exhibit any character that bears a

“close resemblance to (identity with?) the same character possessed by all members of an ancestral population.”³⁹ Each null mutation produces not only a differently shaped ectopic element, but also a dissimilar suite of associated disruptions, virtually none of which resembles any aspect of the primitive condition. Further, in none of the null mutants is there a reversal of any of the features that truly characterize the morphology of a mammalian ancestor: existence of a jaw joint between the quadrate/articular (incus/malleus), presence of an epipterygoid bone and an open sidewall of the braincase, appearance of a large number of bones making up the lower jaw, or a reversion of the morphology of the teeth or the first arch musculature. Although many of the null mutants show an open secondary palate, the morphology is obviously pathological and unlike the primitive condition. Thus, the morphology produced by these null mutations does not resemble the condition seen in any ancient ancestor, any mammal-like reptile, or any transitional state.

The second issue is perhaps the more significant problem. The simple absence of any of these genes cannot provide much data on the actual mechanisms by which the first arch was modified, because all evidence indicates that these genes are highly conserved among amniotes, if not all jawed vertebrates. All the genes discussed in the above studies were most likely functional, not only in the immediate ancestors of mammals, but in more distant ancestors as well. Any morphological changes must have been produced by changes in a variety of genetic and epigenetic events, such as the timing and regulation of gene expression and interactions among gene products, and not by the gain or loss of function in any of the above genes. We conclude therefore that these experiments provide little information about the evolutionary transformations of the first arch.

Why the Ectopic Cartilages?

The fact that ectopic cartilaginous elements, of variable sizes, shapes, and connections, appear repeatedly in the region somewhere between the alisphenoid bone and the incus is a striking result of the experiments discussed here. An examination of the events leading up to the ectopic cartilage, when integrated with the known morphological processes of skeletogenesis, may unravel the precise effect of the absence of the gene and therefore its normal role in development. The formation of cartilaginous elements has been studied by Hall and his colleagues.⁴⁷⁻⁵¹ This work demonstrates that chondrogenesis is a complex process involving epithelial-mesenchymal induction, condensation, transformation, differentiation, and growth. This process involves a variety of genetic and epigenetic events.⁵¹ In particular, these authors have shown that there is a critical threshold in the formation of cartilage and that the initial size of cellular condensations, and in particular prechondrogenic condensations, is decisive in determining the later form of a

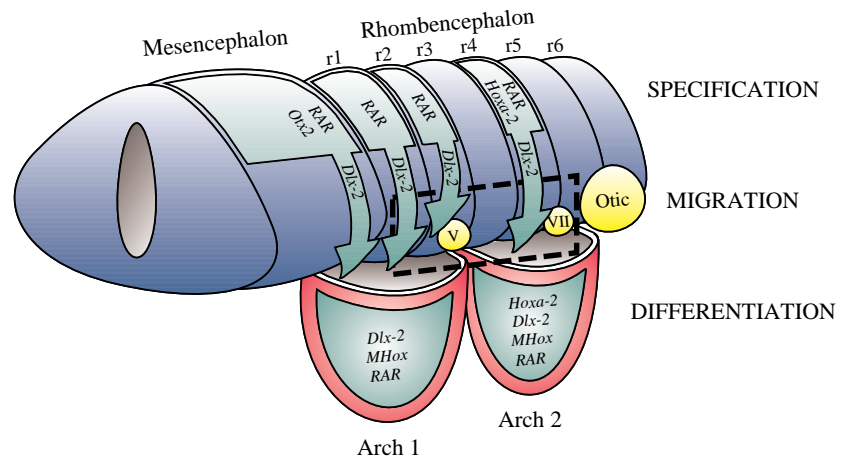
structure. When a cellular condensation is below a minimum size the normal processes of chondrogenesis do not occur. Conversely, abnormally large condensations will result in cartilage where no cartilage normally forms.⁵²

The critical nature of initial condensation size is important in the present context because in some amniotes including mammals, mesenchymal connections or condensations between the epipterygoid and quadrate (alisphenoid and incus in mammals) are present at the blastemal or precartilaginous stage.^{6,27,53,54} Therefore even in the wild-type condition, a condensation exists in the general position of most of these ectopic “palatoquadrate” cartilages. We believe that the repeated appearance of these ectopic cartilages is most likely due to the disruption of a variety of developmental events so that an excess of mesenchyme collects at the normal site of this condensation. Once the critical threshold of cellular condensation is achieved, cartilage formation occurs. Furthermore, based on this property of cartilage formation, we predict that a null mutation in many genes could give rise to an ectopic cartilage at this site, even if these genes have no direct role in producing cartilage in that region during normal development.

As the skeletogenic cells in this region are derived from the neural crest, the most likely proximal cause of an ectopic cartilage would be a disruption of normal processes of neural crest development. The prechondrogenic site and the subsequent ectopic cartilages lie in the vicinity of the normal path of migration of neural crest cells into the first arch region (Fig. 7). An overabundance of mesenchyme in this area would arise from a disruption in the processes of specification (i.e., mis-production or mis-patterning of neural crest cells before migration), migration (so that cells either migrate atypically or accumulate inappropriately at a given site), or differentiation (e.g., loss of normal mechanisms transforming undifferentiated mesenchymal cells into differentiated cells). The specific processes that are disrupted are difficult to evaluate because, overall, the role that each of these genes plays in morphogenesis is not yet understood. Further very few studies present data from more than one stage in development (and the stage discussed is generally very late), which severely limits the information that can be gained about embryogenesis of the skull in an experimentally altered environment. However, comparisons of the phenotypes, combined with preliminary data on the expression patterns and actions of these genes, suggest that in fact different specific events are disrupted by each gene knock-out.

For example in the *Dlx-2* mutant a number of ectopic bony elements are present in several regions of the first arch in addition to the site of the prechondrogenic condensation. This pattern indicates that an excess of skeletogenic mesenchyme has migrated into the region of the first arch. This is consistent with the hypothesis that the *Dlx-2* gene is involved

Figure 7. Schematic diagram illustrating the spatial relations among the posterior brain regions, the trigeminal (V) and facial (VII) sensory ganglia, the otic capsule, and the first two visceral arches of an early embryonic mouse. *Green areas*, expression patterns for the five genes discussed in this paper and the migratory pathways (*arrows*) of neural crest cells between days 8.5 and 11.5 of embryonic development. *Hoxa-2* expression is carried by neural crest cells from rhombomere four (r4) into the second arch.²⁹⁻³¹ Neural crest cells express *Dlx-2* only after they begin to migrate from the neural tube, especially in the region of the primordium of the trigeminal ganglion (V) and in the arches.^{34,35,61} *MHox* is expressed in the neural crest and mesodermally derived mesenchyme of the arches and neurocranium particularly during later embryonic stages but before skeletal differentiation.^{36,62} *Otx2* is detected in the neural tube up to the posterior boundary of the mesencephalon.^{37,63} Various retinoic acid receptor (*RAR*) genes are expressed throughout the neural tube and in the neural crest-derived mesenchyme of the arches.^{38,64,65} Under normal conditions, neural crest cells pass through the region indicated by the black dashed box on their way to the first two visceral arches.^{66,67} In null mutations of *Hoxa-2*, *Dlx-2*, *MHox*, *Otx2*, and some *RAR* genes normal processes may be perturbed at the stages of cell specification, migration, or differentiation. Such disruptions could lead to the accumulation of neural crest cells and the production of ectopic cartilages that connect the area around the trigeminal ganglion (V) to the otic capsule.



in the differential specification of migrating neural crest sub-populations into neurogenic and skeletogenic precursors.^{21,34} By contrast, in the *MHox* mutant there is an absence of dermal bones, particularly in the first arch region (the alisphenoid and squamosal are significantly reduced) probably because normal epithelial-mesenchymal interactions are affected.⁵⁵ In this case neural crest-derived mesenchymal cells usually involved in the formation of the dermal bones of the first arch and surrounding regions appear not to differentiate into osteoblasts. The excess of undifferentiated cells may be accumulating at the site of the prechondrogenic condensation. The size and shape of the ectopic cartilage in the null mutant indicates that the ectopic condensation and normal cartilaginous condensations of the second arch have been fused together.

Hox genes appear to be important in imposing unit identity upon neural crest cells prior to migration, perhaps by acting as selectors of particular developmental pathways.^{29,56} These genes are expressed early in development and seem to regulate cascades of genes. The homeotic transformation observed in the *Hoxa-2* null mutant appears to support this hypothesis. However, only a few duplicate first arch structures actually differentiate in the homeotic mutant so that an excess of improperly patterned skeletogenic mesenchymal cells may be accumulating at the site of the ectopic cartilage. Finally, the results of the knockout of the *Otx2* and *RAR* genes are difficult to assess because of the extreme variability of the resulting null mutant embryos. Even within the same null mutation, in some embryos the effect is

extreme (i.e., complete acephaly), and in others minor. The ectopic cartilage is an occasional result of both sets of knockouts. The pattern of variability is consistent with the hypothesis that the null mutations produce an ectopic cartilage through indirect effects, and that the cartilage appears when a threshold level of excess cells is produced.

The most general conclusion of this discussion is that many papers on gene knockout effects focus on the wrong causal level to explain the generation of morphology. The language used to describe gene actions often suggests that genes produce structures directly. Instead, genes supply products that are integrated into morphogenetic pathways.⁵⁷ The correct causal level for the explanation of the loss, gain, or change of a structure lies in the network of interactions among gene products and processes such as mechanical interactions, threshold effects, and other epigenetic phenomena.⁵⁸ Null mutations can be a powerful experimental tool for disassembling the machinery of development. However the means by which the absence of a gene perturbs development cannot be ascertained by examination of structures once they have been formed and patterned. We have presented specific hypotheses about the developmental processes that might be disrupted by the null mutation of several genes and have argued that structures may appear or be changed after a gene knockout even if that gene has no role in patterning that structure during normal development. These hypotheses can be tested in detailed comparisons of ontogenetic processes in mutant and wild-type embryos. Such studies would identify the precise effects of a

gene knockout and indicate where, when, and how that gene functions during morphogenesis, rather than just catalog the end result. Further, these studies would provide critical information on the normal mechanisms of craniofacial development and evolutionary change. At this time we can merely conclude that these genes differ in their signaling pathways and functions, and the cells, tissues, organs, and systems that they influence are diverse. When so little is known about how the skeleton is generated and patterned, and in particular the ways in which these genes actually affect development, we are unable to support any inferences on how these mutant phenotypes relate to the course of morphological evolution.

ACKNOWLEDGMENTS

We thank B.K. Hall, G. Kardon, D. McClay, and the members of his laboratory group, H.F. Nijhout, V.L. Roth, M. Sánchez-Villagra, and A. van Nievelt, for comments on earlier drafts of this paper, and K. Davis for assistance with the illustrations. This work was supported by NSF grant IBN 9407616 (to K.K.S.).

REFERENCES

- 1 Thomas KR, Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51:503–512.
- 2 Capecchi MR (1994) Targeted gene replacement. *Sci Am* 270(3) 52–59.
- 3 Joyner AL (1994) *Gene Targeting*. New York: Oxford University Press.
- 4 Gaupp E (1913) Die Reichertsche Theorie (Hammer-, Amboss, und Kieferfrage). *Arch Anat Physiol Wiss Med Supp.* 5, pp. 1–416.
- 5 Riechert C (1837) Über die Visceralbogen der Wirbeltiere im Allgemeinen und deren Metamorphosen bei den Vögeln und Säugetieren. *Arch Anat Physiol Wiss Med* 1837:120–222.
- 6 Goodrich ES (1930) *Studies on the Structure and Development of Vertebrates*. Chicago: University of Chicago Press.
- 7 Russell ES (1916) *Form and Function*. London: John Murray.
- 8 Allin EF (1975) Evolution of the mammalian middle ear. *J Morphol* 147:403–438.
- 9 Crompton AW (1989) The evolution of mammalian mastication. In *Complex Organismal Functions: Integration and Evolution in Vertebrates*. Wake DB, Roth G (eds): New York: pp 23–40. John Wiley & Sons.
- 10 Crompton AW, Jenkins FA (1979) Origin of mammals. In Lillegraven JA, Kielan-Jaworowska Z, Clemens WA. (eds): *Mesozoic Mammals*. Berkeley: University of California Press, pp 59–73.
- 11 Crompton AW, Hylander WL (1986) Changes in mandibular function following the acquisition of a dentary-squamosal jaw articulation. In Hotton N, MacLean PD, Roth JJ, Roth EC (eds): *The Ecology and Biology of Mammal-like Reptiles*. Washington, DC: Smithsonian Institution Press, pp 263–282.
- 12 Hopson JA, Barghusen HR (1986) An analysis of Therapsid relationships. In Hotton N, MacLean PD, Roth JJ, Roth EC (eds): *The Ecology and Biology of Mammal-like Reptiles*. Washington, DC: Smithsonian Institution Press, pp 83–106.
- 13 Rowe T (1988) Definition, diagnosis and origin of Mammalia. *J Vertebr Paleol* 8:241–264.
- 14 Chisaka O, Musci TS, Capecchi MR (1992) Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene *Hox-1.6*. *Nature* 355:516–520.
- 15 Yamada G, Mansouri A, Torres M, Stuart ET, Blum M, Schultz M, DeRobertis EM, Gruss P (1995) Targeted mutation of the murine *gooseoid* gene results in craniofacial defects and neonatal death. *Development* 121:2917–2922.
- 16 Rivera-Pérez JA, Mallo M, Gendron-Maguire M, Gridley T, Behringer RR (1995) *gooseoid* is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. *Development* 121:3005–3012.
- 17 Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ (1996) Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* 381:235–238.
- 18 Gendron-Maguire M, Mallo M, Zhang M, Gridley T (1993) *Hoxa-2* mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* 75:1317–1331.
- 19 Rijli FM, Mark M, Lakkaraju S, Dierich A, Dollé P, Chambon P (1993) A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. *Cell* 75:1333–1349.

- 20 Lohnes D, Mark M, Mendelsohn C, Dollé P, Dierich A, Gorry P, Gansmuller A, Chambon P (1994) Function of the retinoic acid receptors (RARs) during development. (I) Craniofacial and skeletal abnormalities in RAR double mutants. *Development* 120:2723–2748.
- 21 Qui M, Bulfone A, Martinez S, Meneses JJ, Shimamura K, Pedersen RA, Rubenstein JLR (1995) Null mutation of *Dlx-2* results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. *Genes Dev* 9:2523–2538.
- 22 Martin JF, Bradley A, Olson EN (1995) The *paired*-like homeobox gene *MHox* is required for early events of skeletogenesis in multiple lineages. *Genes Dev* 9:1237–1249.
- 23 Matsuo I, Kuratani S, Kimura C, Takeda N, Aizawa S (1995) Mouse *Otx2* functions in the formation and patterning of rostral head. *Genes Dev* 9:2646–2658.
- 24 Acampora D, Mazan S, Lallemand Y, Avantaggiato V, Maury M, Simeone A, Brulet P (1995) Forebrain and midbrain regions are deleted in *Otx2-1-* mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* 121:3279–3290.
- 25 Noden DM (1983) The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev Biol* 96:144–165.
- 26 Noden DM (1991) Vertebrate craniofacial development: The relation between ontogenetic process and morphological outcome. *Br Behav Evol* 38:190–225.
- 27 Moore WJ (1981) *The Mammalian Skull*. Cambridge: Cambridge University Press.
- 28 Satokata I, Maas R (1994) *Msx-1* deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nature Genet* 6:348–356.
- 29 Hunt P, Whiting J, Muchamore I, Marshall H, Krumlauf R (1991) Homeobox genes and models for patterning the hindbrain and branchial arches. *Development* 1(suppl):187–196.
- 30 Krumlauf R (1993) *Hox* genes and pattern formation in the branchial region of the vertebrate head. *Trends Genet* 9:106–112.
- 31 Prince V, Lumsden A (1994) *Hoxa-2* expression in normal and transposed rhombomeres: Independent regulation in the neural tube and neural crest. *Development* 120:911–923.
- 32 Mark M, Rijli FM, Chambon P (1995) Alteration of *Hox* gene expression in the branchial region of the head causes homeotic transformations, hindbrain segmentation defects and atavistic changes. *Semin Dev Biol* 6:275–284.
- 33 Duboule D (1994) *Guidebook to the Homeobox Genes*. New York: Oxford University Press.
- 34 Robinson GW, Mahon KA (1994) Differential and overlapping expression domains of *Dlx-2* and *Dlx-3* suggest distinct roles for *Distal-less* homeobox genes in craniofacial development. *Mech Dev* 48:199–215.
- 35 Bulfone A, Kim H-J, Puelles L, Porteus MH, Grippo JF, Rubenstein JLR (1993) The mouse *Dlx-2 (Tes-1)* gene is expressed in spatially restricted domains of the forebrain, face and limbs in midgestation mouse embryos. *Mech Dev* 40:129–140.
- 36 Kuratani S, Martin JF, Wawersik S, Lilly B, Eichele G, Olson EN (1994) The expression pattern of the chick homeobox gene *gMHox* suggests a role in patterning of the limbs and face and in compartmentalization of somites. *Dev Biol* 161:357–369.
- 37 Simeone A, Acampora D, Gulisano M, Stornaiuolo A, Boncinelli E (1992) Nested expression domains of four homeobox genes in developing rostral brain. *Nature* 358:687–690.
- 38 Linney E, LaMantia A-S (1994) Retinoid signaling in mouse embryos. *Adv Dev Biol* 3:73–114.
- 39 Hall BK (1984) Developmental mechanisms underlying the formation of atavisms. *Biol Rev* 59:89–124.
- 40 de Beer GR (1937) *The Development of the Vertebrate Skull*. Chicago: University of Chicago Press.
- 41 Romer AS (1956) *Ostology of the Reptiles*. Chicago: University of Chicago Press.
- 42 Röth VL (1988) The biological basis of homology. In Humphries CJ (ed): *Ontogeny and Systematics*. New York: Columbia University Press, pp 1–26.
- 43 Wagner GP (1989) The origin of morphological characters and the biological basis of homology. *Evolution* 43:1157–1171.
- 44 Hall BK (1994) *Homology—The Hierarchical Basis of Comparative Biology*. San Diego: Academic Press.
- 45 Patterson C (1982) Morphological characters and homology. In Joysey KA, Friday AE (eds): *Problems of Phylogenetic Reconstruction*. San Diego: Academic Press, pp 21–74.
- 46 Roth VL (1994) Within and between organisms: Replicators, lineages, and homologues. In Hall BK (ed): *Homology, The Hierarchical Basis of Comparative Biology*. San Diego: Academic Press, pp 301–337.
- 47 Atchley WR, Hall BK (1991) A model for development and evolution of complex morphological structures. *Biol Rev* 66:101–157.
- 48 Dunlop L-L, Hall BK (1995) Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis. *Int J Dev Biol* 39:357–371.
- 49 Hall BK (1991) Cellular interactions during cartilage and bone development. *J Craniofac Genet Dev Biol* 11:238–250.
- 50 Hall BK, Miyake T (1992) The membranous skeleton: The role of cell condensations in vertebrate skeletogenesis. *Anat Embryol* 186:107–124.
- 51 Hall BK, Miyake T (1995) Divide, accumulate, differentiate: Cell condensation in skeletal development revisited. *Int J Dev Biol* 39:881–893.
- 52 Gruneberg H (1963) *The Pathology of Development. A Study of Inherited Skeletal Disorders in Animals*. New York: John Wiley & Sons.

- 53 Presley R (1993) Preconception of adult structural pattern in the analysis of the developing skull. In Hanken J, Hall BK (eds): The Skull. Vol. 1. Chicago: University of Chicago Press, pp 347–377.
- 54 Presley R, Steel FLD (1976) On the homology of the alisphenoid. *J Anat* 121:441–459.
- 55 Brickell PM (1995) *MHox* and vertebrate skeletogenesis: The long and short of it. *BioEssays* 17:750–753.
- 56 Hunt P, Krumlauf R (1991) Deciphering the *Hox* code: Clues to patterning branchial regions of the head. *Cell* 66:1075–1078.
- 57 Nijhout HF (1990) Metaphors and the role of genes in development. *BioEssays* 12:441–446.
- 58 Ströhman RC (1997) The coming Kuhnian revolution in biology. *Nature Biotechnol* 15:194–200.
- 59 Carroll RL (1988) *Vertebrate Paleontology and Evolution*. New York: WH Freeman.
- 60 Ellerman JR (1941) *The Families and Genera of Living Rodents. II. Family Muridae*. London: British Museum, Natural History.
- 61 Meneses JJ, Christensen L, Sharpe PT, Presley R, Pedersen RA, Rubenstein JL (1997) Role of the *Dlx* homeobox genes in proximodistal patterning of the branchial arches: mutations of *Dlx-1*, *Dlx-2*, and *Dlx-1* and *-2* alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. *Dev Biol* 185:165–184.
- 62 Cserjesi P, Lilly B, Bryson L, Wang Y, Sassoon DA, Olson EN (1992) *MHox*: A mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. *Development* 115:1087–1101.
- 63 Ang SL, Jin O, Rhinn M, Daigle N, Stevenson L, Rossant J (1996) A targeted mouse *Otx2* mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* 122:243–252.
- 64 Dollé P, Ruberte E, Leroy P, Morriss-Kay G, Chambon P (1990) Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* 110:1133–1151.
- 65 Ruberte E, Dollé P, Chambon P, Morriss-Kay G (1991) Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* 111:45–60.
- 66 Osumi-Yamashita N, Ninomiya Y, Doi H, Eto K (1994) The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. *Dev Biol* 164:409–419.
- 67 Trainor PA, Tam PP (1995) Cranial paraxial mesoderm and neural crest cells of the mouse embryo: co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. *Development* 121:2569–2582.