

Cranial skeletal biology

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To artists, the face is a mirror of the soul. To biologists, the face reflects remarkable structural diversity — think of bulldogs and wolfhounds or galapagos finches. How do such variations in skeletal form arise? Do the same mechanisms control skeletogenesis elsewhere in the body? The answers lie in the molecular machinery that generates neural crest cells, controls their migration, and guides their differentiation to cartilage and bone.

“What is a face, really? Its own photo? Its make-up? Or is it a face as painted by such or such painter? ... Doesn't everyone look at himself in his own particular way? Deformations simply do not exist.” Pablo Picasso¹

Despite its remarkable topological complexity, one might legitimately wonder whether studying skeletal development in the head is a little superfluous. Is there any reason to suspect that factors controlling chondrogenesis and osteogenesis in the head are distinguishable from those operating in the limbs or the spine? If skeletal development in the head simply parallels skeletogenesis elsewhere in the body, then studying this process in such a complicated structure would seem to be a gratuitous endeavour. As it turns out, the molecular mechanisms inducing chondrogenesis and osteogenesis in cranial neural crest cells, which produce the facial and jaw skeleton, are distinct from those operating in mesodermal cells, which produce the remainder of the skeleton. Our ability to prevent or at least mitigate cranial skeletal anomalies, and to enhance cranial skeletal repair, depends upon understanding these cranial-specific pathways.

Organization of the cranial skeleton

For those interested in skeletal biology, the appendicular and axial skeletons hold a distinct appeal for analysis, as their elements exhibit an uncomplicated anatomy. Regardless of whether an animal uses its forelimbs for flying or playing flamenco guitar, the basic morphology is preserved. Likewise, axial organization is highly conserved: even mammals as dissimilar as a giraffe and a porpoise possess the same number of cervical vertebrae. The cranial skeleton, on the other hand, is composed of an often-bewildering assortment of neural crest- and mesoderm-derived cartilages and bones that have been highly modified during evolution. This makes comparisons among divergent species a challenge.

Such anatomical complexity and embryonic amalgamations may initially diminish one's enthusiasm for understanding how the cranial skeleton is generated and undergoes repair. Nonetheless, studies in cranial skeletogenesis have provided unparalleled insights into the cellular and molecular mechanisms that generate and shape cartilage and bone. Our growing realization that skeletogenesis in the head is a unique and separable process from that occurring elsewhere in the body makes this a difficult, but worthwhile, road to take.

Genesis and exodus of the cranial neural crest

Most of the head skeleton is derived from the cranial neural crest, which arises from the dorsal margins of the

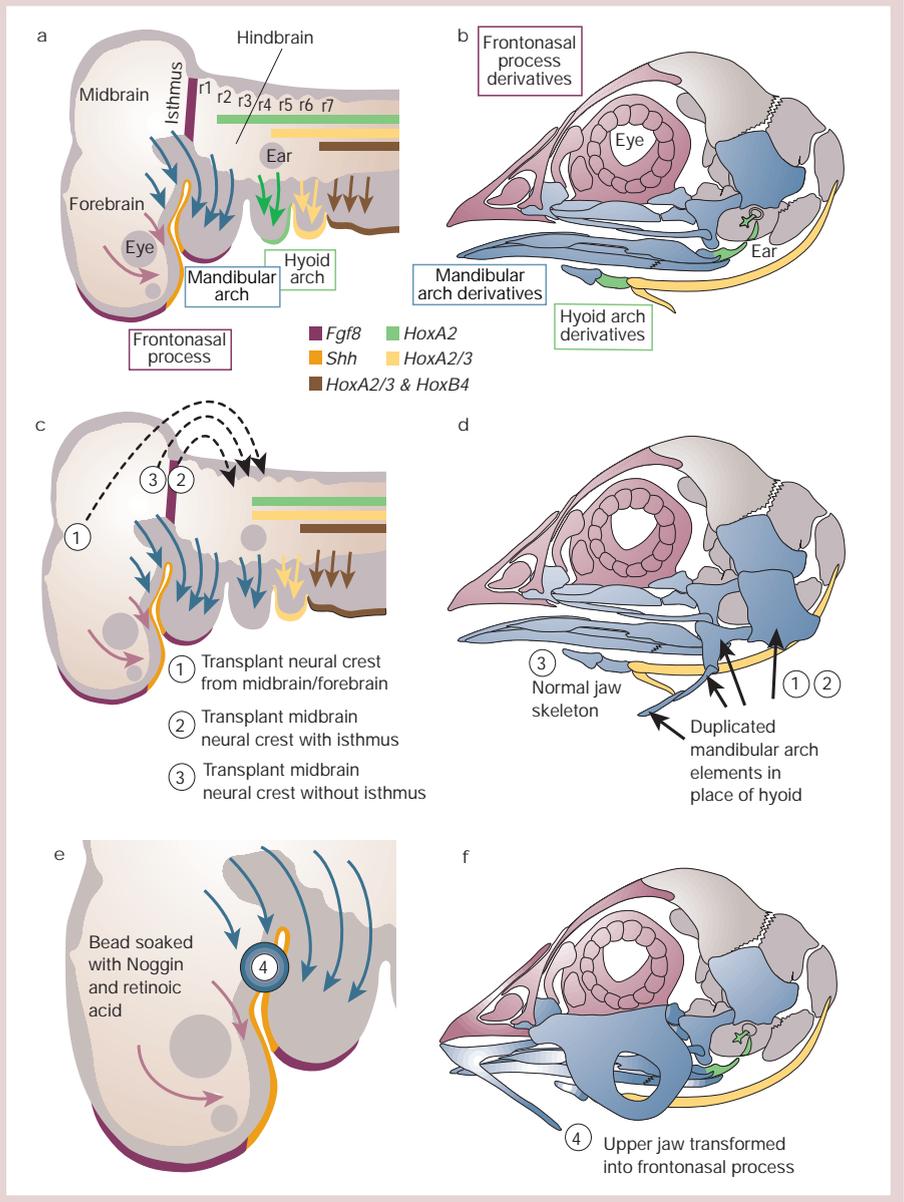
neural folds. Bronner-Fraser and colleagues showed that a Wnt protein is necessary and sufficient for neural crest induction². In the absence of Wnt signalling, neural crest cells were not generated. Exogenous Wnt protein was sufficient to regenerate the missing crest, and could induce neural crest from naive neural ectoderm. New data indicate that Snail family members are also involved in the generation and early migration of neural crest³, but how they interact with Wnts and bone morphogenetic proteins (BMPs) remains a mystery.

The last century has brought an appreciation for how cranial neural crest cells get from 'here', the dorsal part of the neural tube, to 'there', the facial primordia. Whereas coal dust was once sprinkled over avian embryos to label migrating neural crest cells, innovative visualization techniques now afford us with a bird's eye view of migratory routes and cellular events that occur during neural crest emigration from the neural tube⁴. Why was there such interest in the movement of neural crest cells? The consequences of inaccurate or interrupted migration cannot be over-emphasized, as a plethora of craniofacial malformations have as their primary aetiology a perturbation in this cellular exodus. For example, pharyngeal arch syndromes and neurocristopathies are associated with aberrant neural crest migration^{5,6}.

Intravital imaging movies now allow investigators to follow neural cell migration in real time and, in so doing, have revealed unexpected cell behaviours⁷. Neural crest cells do not meander on their journey to the craniofacial primordia. Instead, they display highly stereotyped migratory patterns, passing between neural and facial epithelia, and around paraxial mesoderm until they reach their locations in the pharyngeal arches and frontonasal process. The course is not hard-wired within a given neural crest population. Cells transplanted to ectopic locations take their cues from the local environment and arrive in the position that corresponds to their new level of origin^{4,8}. These studies offer fresh insights into aetiologies of neurocristopathies affecting the facial skeleton.

Signals encountered by neural crest cells during their migration to the facial primordia can alter their fate. Some guidance cues have been identified and a common feature among them is that they already have well-documented roles in axon path finding^{9–11}. Experiments conducted by Golding, Gassmann and colleagues demonstrate this point. The receptor tyrosine kinase ErbB4 is involved in controlling neural crest cell migration^{12,13}, but ErbB4 is not expressed on

Figure 1 Experimental approaches to skeletal patterning. **a**, Cellular and molecular organization of a developing avian head shown as a schematized composite of several embryonic stages that illustrates (in lateral view) anatomical relations among the developing brain, migrating neural crest cells (arrows), sensory structures and craniofacial primordia (frontonasal process, mandibular arch and hyoid arch). *Hox* genes show periodic expression in hindbrain rhombomeres (*r*). *Fgf8* and *Shh* are expressed at the midbrain/hindbrain boundary (isthmus) and in epithelia surrounding the craniofacial primordia. Drawing modified from ref. 37. **b**, The facial skeleton arises from neural crest cells that migrate into the frontonasal process, mandibular arch and hyoid arch. Drawing modified from ref. 78. **c**, Several experimental approaches have been taken to test whether neural crest cells are pre-patterned. These include, replacement of hyoid arch neural crest with cells from the midbrain/forebrain boundary (1) and the midbrain/hindbrain boundary (including the isthmus) (2)²⁹, and from the midbrain/hindbrain boundary (without the isthmus) (3)³¹. **d**, Experimental results (1 and 2) include duplication of some elements of the mandibular arch as well as formation of a normal jaw skeleton (3). **e**, Another experimental approach (4) uses beads soaked in specific molecules (such as Noggin and retinoic acid), which alter the neural crest identity⁷⁹. **f**, Results include the transformation of maxillary elements into frontonasal process structures.



neural crest cells. Rather, the protein is abundantly expressed in neural ectoderm¹⁴ and thus constitutes a kind of 'roadside cue' to migrating cells. These investigators surmised that the misplaced axons they observed in *ErbB4*^{-/-} mice might be harbingers of a more global disorganization in cell migration, which occurs in the absence of *ErbB4*. By culturing embryos *in vitro*, the group showed that *ErbB4*^{-/-} cells transplanted into a wild-type host embryo migrated normally, whereas wild-type cells placed into a mutant host deviated from their prescribed route. These data are compelling because they provide direct evidence that epithelia can supervise and instruct migration of neural crest cells, and that there is a shared mechanism for guiding axons and the migrating neural crest.

Skeletogenic capacity of the cranial neural crest

Cranial neural crest cells possess an ability to form cartilage and bone, whereas trunk neural crest cells have minimal skeletogenic capacity¹⁵. Precisely why trunk neural crest cells do not contribute to the axial or appendicular skeleton is somewhat of a conundrum. Is it because trunk neural crest cells are not exposed to appropriate 'skeletogenic' cues in their environment, or because they are

somehow restricted in their competency to form skeletal tissue? If the cranial environment is important for skeletogenic differentiation, then transplanting trunk neural crest to the head should allow trunk cells to form cartilage or bone. But regardless of the axial level to which they were transplanted, trunk neural crest cells failed to make skeletal tissues¹⁶. Even when trunk neural crest cells were explanted and treated with BMPs^{17,18}, which promote skeletogenesis in other tissues, the cells failed to differentiate like their cranial counterparts^{19,20}. Other investigators showed that trunk neural crest cells, if grown for extended periods of time *in vitro*, could differentiate into chondrocytes¹⁵. Likewise, when small grafts of trunk neural crest were transplanted into the head region, a few cells migrated to the appropriate axial level and contributed to cranial cartilages²¹.

How does one reconcile this experimental paradox? One possibility is that a 'community effect' influences the skeletogenic capacity of trunk neural crest. When trunk cells are sufficiently dispersed, inhibitory effects from other trunk cells may be mitigated and a few cells may then respond to skeletogenic cues in their environment. Although a small group of cells might hear these signals, a large

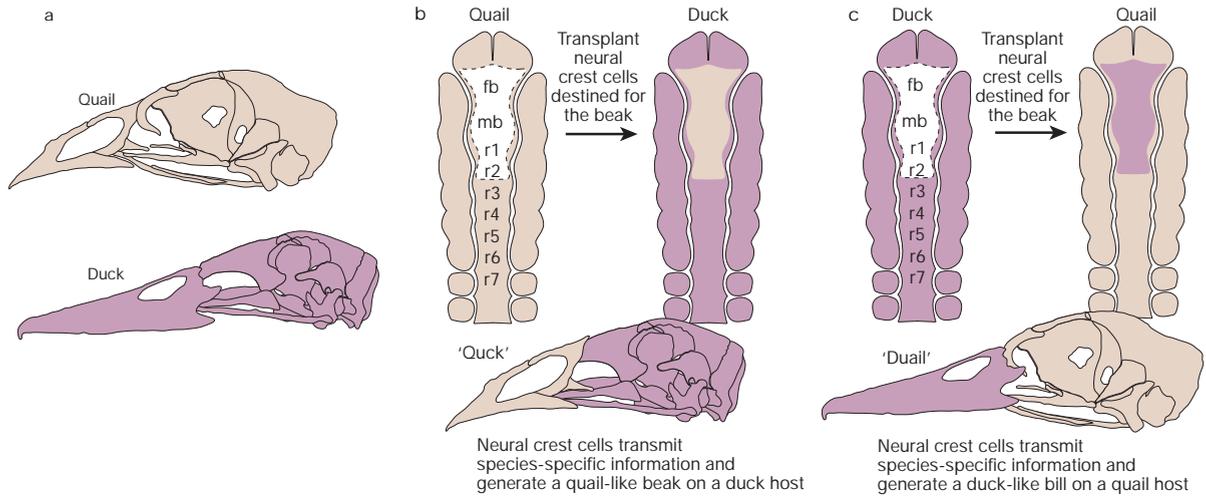


Figure 2 Contributions of neural crest to beak morphology. **a**, Quail and duck embryos are anatomically distinct and thus are a powerful system for studying craniofacial patterning. **b**, When quail neural crest cells from forebrain (fb), midbrain (mb) and first

two rhombomeres (r) are transplanted into a duck host, a quail-like beak forms instead of a duck's bill³⁵. **c**, Likewise, duck neural crest cells make a duck-like bill when transplanted into a quail host. Drawings modified from refs 80,81.

population of trunk neural crest may be impervious. What actually constitutes this inhibitory signal is still unknown. In any case, gauging the skeletogenic capacity of different neural crest populations is of vital interest to those who seek to stimulate regeneration of cranial skeletal tissues.

A dual origin of the cranial skeleton

Twenty years ago, the 'new head' hypothesis proposed that much of the cranial skeleton was formed by expansion of a rostral neural crest cell population, and that the dividing line between mesoderm and neural crest had significance for the development and evolution of the skeleton²². A genetic approach provides convincing new data about the relative contributions of neural crest and mesoderm to the cranial skeleton. *Wnt1* is expressed in the dorsal neural tube, around the time of neural crest induction. By placing the *Wnt1* promoter upstream of the *Cre* gene and crossing mice carrying this transgene with a reporter line, indelibly labelled neural crest cells were generated whose ultimate fate could be followed throughout the lifetime of the animal²³.

These data supported earlier avian fate maps showing the dual neural crest and mesodermal origin of the cranial vault²⁴, and underscored previous observations²⁵ that the neural crest cells contribute to another cranial skeletal tissue, the teeth. Dentin, dental pulp, alveolar bone and the periodontal ligament, which anchors the teeth to the bone of the jaws, are all derived from the neural crest²⁶. These studies are a necessary first step towards using undifferentiated adult neural crest cells from the pulp and marrow cavity for the repair of dental and skeletal defects. Patients cursed with cavities and gum disease will undoubtedly take great comfort from the fact that cellular and molecular therapies, rather than drills and scalpels, may one day be used by dentists to treat their afflictions^{27,28}.

Establishment of cranial skeletal architecture

After more than a century of exploring mechanisms that generate the cranial skeleton, two prevailing theories have emerged. The first presupposes that cranial neural crest cells carry out autonomous programs for patterning, while the second theory posits that neural crest cells are naive and acquire patterning information through interactions with the local environment.

Almost 20 years ago, Noden showed that moving neural crest cells from the midbrain to a more posterior location in the hindbrain resulted in an embryo with a duplicated jaw skeleton²⁹. These data suggested that neural crest cells contained, at the time of their transplantation, intrinsic information for elaborating the complex morphology of the jaw skeleton (Fig. 1c, d). This interpretation was challenged by Le Douarin and Couly³⁰, and more recently by Krumlauf and colleagues, who suspected that the duplicated jaw skeleton arose as a consequence of Noden inadvertently transplanting an adjacent region of the neural tube called the isthmus³¹. The isthmus expresses *fibroblast growth factor 8 (Fgf8)*, a gene encoding a secreted protein that can downregulate the homeobox gene *HoxA2*, whose own expression is required for the formation of second arch structures³²⁻³⁴. If Noden's graft included isthmus tissue then *HoxA2* should be downregulated and the result would be duplicated lower jaw structures. When midbrain/hindbrain crest were transplanted without the isthmus the result was a normal jaw skeleton, and when the transplants included the isthmus the result was a duplicated jaw skeleton.

These data suggested that neural crest cells do not possess inherent patterning information. Or do they? When we exchanged neural crest cells of the presumptive beak region between quail and duck embryos, the facial features of the chimaeras more closely resembled the donor species³⁵. When quail neural crest cells destined to form the beak were transplanted to a duck host, the result was a duck embryo with a quail-like beak ('quack'). Conversely, when duck cells were transplanted into quail hosts, quail embryos with duck-like bills ('duails') were produced (Fig. 2). Molecular and cellular analyses revealed that donor neural crest cells followed their own morphogenetic program and re-patterned host facial ectoderm like that of the donor species. These data demonstrate that neural crest cells can influence surrounding tissues.

To what extent do surrounding tissues regulate neural crest cell fate? Hu, Marcucio and Helms showed that a zone of frontonasal ectoderm stimulated the proliferation and differentiation of underlying neural crest cells (Fig. 3). When transplanted to an ectopic location, the frontonasal ectodermal zone activated a cascade of molecular events that ultimately re-programmed the neural crest-derived mesenchyme and produced a duplication in

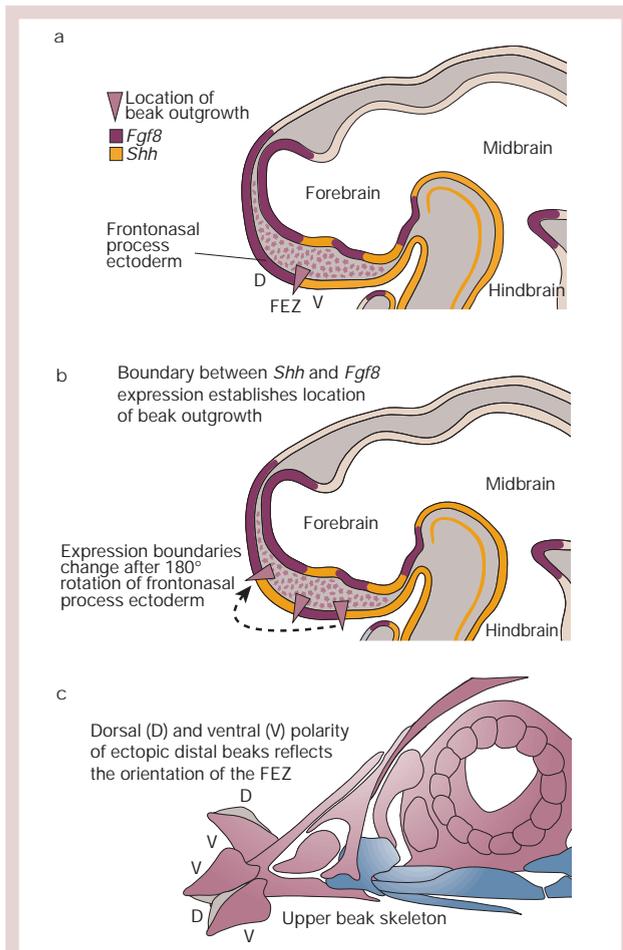


Figure 3 Contributions of epithelia to craniofacial patterning. **a**, A molecular boundary in frontonasal ectoderm, defined by *Fgf8* and *Shh* expression, presages the initial site of outgrowth (arrowhead) and defines dorsoventral polarity of the upper beak³⁶. **b**, Changing the dorsoventral orientation of the frontonasal ectodermal zone (FEZ) alters dorsoventral orientation of the upper beak. A 180° rotation results in three sites where dorsal and ventral ectodermal domains are juxtaposed (arrowheads). **c**, As a result, distal beak structures are duplicated. The first (dorsal-most) beak has a dorsal-ventral (DV) pattern, whereas the second beak has a ventral-dorsal-ventral (VDV) pattern, indicating the ability of epithelium to re-pattern skeletal elements derived from the neural crest.

upper beak structures³⁶. Other epithelia in the head, such as the pharyngeal endoderm, also influence the size, shape and position of some components of the facial skeleton³⁷ (Box 1).

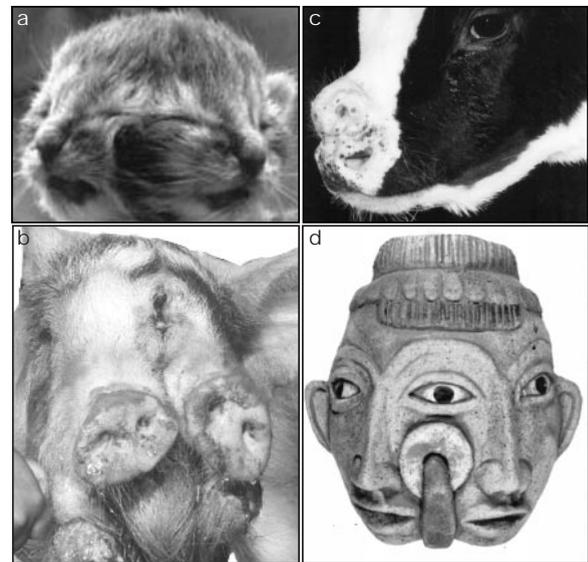
Craniofacial defects provide a window into morphogenesis

Higher vertebrates have evolved specialized neural mechanisms for the recognition of faces³⁸, providing us with an amazing ability to discriminate among hundreds of people. This same exquisite tuning permits us to detect even subtle discrepancies in facial form. Craniofacial malformations compromise not only function (for example, speech and mastication) but also exact a demoralizing toll on the mental well being of the affected individual. Recent advances in human genetics and experimental embryology indicate that we are converging on an understanding of how particular gene perturbations produce cranial skeletal malformations. The zebrafish has become a crucial model system for exploring these questions (reviewed in ref. 39).

Lineage studies have defined the origins of the zebrafish cranial skeleton at the single-cell level⁴⁰, and molecular analyses indicate that the same nested expression patterns of homeobox genes that regulate

Box 1
Facial duplications

We take for granted that faces have two eyes, a nose and a mouth. What happens when nature produces an animal with three or four eyes, two noses and two mouths? These duplications, and those at the opposite end of the spectrum such as cyclopia, stretch our imagination and provoke us to understand the molecular and cellular events that had to have occurred to produce such phenomenal phenotypes. In the figure below, ‘Image’ the cat (panel **a**) and ‘Ditto’ the pig (**b**) are examples not of twinning, but of actual craniofacial duplications. Other duplications can be limited to individual facial structures, such as that seen on the nose of the calf (**c**; from D. Noden). Facial duplications are documented throughout mythology. The ancient mask in panel **d** represents a god who possesses the ability to speak truthfully and deceitfully, as well as see the future, present and past (from Z. Werb).



skeletal patterning in birds⁴¹ and mammals^{42,43} also participate in zebrafish head morphogenesis⁴⁴. As might be suspected from such conserved patterns of homeobox gene expression, mutations in mice and knockdowns in fish produce remarkably similar phenotypes. Disruptions in a Hox binding partner, *lazarus/Pbx4*^{45,46}, and a Hox gene regulator, *valentino/Kreisler*⁴⁷, produce malformations in the pharyngeal cartilages. A knockdown in zebrafish *Hox2* function produces defects in second pharyngeal arch cartilage, where ventral skeletal elements were replaced by duplicated first arch structures⁴⁸, analogous to the murine *HoxA2*^{-/-} phenotype^{32,33}.

Precisely how these disruptions influence cellular interactions and produce skeletal defects is not clear, but analyses of *sucker/Endothelin-1 (suc/Et-1)* gene function have provided some exciting clues⁴⁹. The zebrafish gene *suc*, and the amniote gene *Et-1*, encode a secreted peptide^{50,51}. In the pharyngeal arches *Et-1* is expressed in a core of mesoderm surrounded by a sheet of *Et-1*-negative neural crest mesenchyme, which in turn is wrapped in *Et-1*-positive epithelia⁴⁹. Loss of *suc/Et-1* disrupts gene expression in the ventral but not the dorsal portion of the pharyngeal arches, which suggests that there is a morphogenetic subdivision of the pharyngeal arch skeleton⁵². Mosaic analyses also indicate that a *suc/Et-1* signal from mesoderm and/or epithelia may be required for a subgroup of neural crest cells to adopt a skeletogenic fate⁵². Thus, interactions between epithelia, mesoderm and neural crest-derived cells are required for the initial steps of skeletogenesis.

Sometimes the clearest view of normal development is attained when embryonic processes go awry. Teratogens offer a window into fetal development, but until recently we had little insight into mechanisms by which these agents exert their untoward effects. Fetal alcohol syndrome is an entirely avoidable suite of physical, mental and neurobehavioural birth defects caused by alcohol consumption during pregnancy. Although it has been appreciated for many years that alcohol-related malformations are associated with excessive cell death, Ahlgren and Bronner-Fraser demonstrated that this cell death occurs via a Sonic hedgehog (Shh)-dependent mechanism⁵³. How are the teratogenic effects of alcohol and the signalling functions of Shh connected? Hu and Helms showed that Shh inhibition in the face caused the downregulation of Patched and Gli1, and induced hypotelorism and facial clefting⁵⁴. Another molecule, retinoic acid (a derivative of vitamin A) is essential for life but, in surplus or shortage, acts as a teratogen. Alcohol and retinol are metabolized by closely related enzymes⁵⁵, which suggests that deficiencies in either molecule could disrupt craniofacial morphogenesis along similar routes. Schneider, Hu and Helms showed that blocking retinoid signalling in the rostral head leads to a loss of Fgf8 and Shh and their downstream effectors⁵⁶, and causes morphological defects reminiscent of those induced by alcohol and Shh inhibition.

Another teratogen, cyclopamine, produces holoprosencephalic defects in mammals⁵⁷ and avians⁵⁸ (reviewed in ref. 59). Cyclopamine acts specifically by inhibiting Shh signalling⁶⁰. The teratogenic consequences of cyclopamine exposure depend upon the gestational age of the fetus. If embryos are exposed during neurulation, cyclopic defects are observed⁶⁰. If embryos are exposed at later developmental stages, the results range from premaxillary agenesis and cleft lip/palate to other midline anomalies⁶¹. A similar spectrum of craniofacial anomalies are seen in *Shh*^{-/-} embryos, which are cyclopic⁶² and *Cdon*^{-/-} mice, which exhibit subtle midline abnormalities such as the loss of a central incisor⁶³.

In some cases the deletion of a gene does not truncate, but instead transforms facial growth. Such is the case for mice lacking Distal-less homeobox gene 5 (*Dlx5*) and *Dlx6*. The mandibular primordia were transformed into ectopic maxillary primordia, complete with epidermal elaborations and skeletal tissue duplications unique to the upper jaw^{64,65}.

Clearly then, a dialogue exists among multiple tissues that mediates cranial skeletogenesis. Changes in this 'molecular conversation' can alter craniofacial morphology in astonishing ways, and the combination of genetic techniques with epigenetic approaches will prove to be a valuable tool in unravelling the regulation of craniofacial morphogenesis.

Bone formation gone wrong

During the past decade there has been a dramatic increase in our understanding of the molecular underpinnings of craniosynostoses⁶⁶. We now appreciate that activating mutations in FGF receptors are responsible for a number of craniosynostotic conditions⁶⁷. One puzzling aspect is that FGFs and their receptors are widely expressed during fetal development, yet mutations produce localized skeletal defects. Insight into this issue has come from a recent study addressing molecular differences between fusing and non-fusing (patent) sutures. Signals from the dura mater regulate osteogenesis in overlying skeletogenic mesenchyme⁶⁸, and likely include BMP-family members. In mice, FGFs and BMPs are widely expressed in suture mesenchyme, but only some of the sutures fuse. What regulates the site-specific activity of these growth factors? Longaker, Harland and colleagues showed that the BMP antagonist Noggin was expressed in all sutures, but its expression was downregulated by Fgf2 only in sutures that fuse⁶⁹. Moreover, ectopic Fgf2 expression in a non-fusing suture led to Noggin repression, which caused open sutures to undergo fusion, whereas Noggin mis-expression caused fusing sutures to remain open. The group further demonstrated that activating mutations in Fgf2 diminished

Noggin expression, raising the possibility that premature suture fusion might one day be treated by Noggin.

The sites where skull roofing bones coalesce (that is, the sutures) are presaged by the expression of muscle segment homeobox (*Msx*) transcription factors⁷⁰. As might be imagined, disruptions in *Msx* genes create ossification defects in the skull bones of mice⁷¹ and humans⁷². The severity of these skull defects is inversely proportional to *Msx* gene dosage⁷¹. In an effort to understand how mutations in *Msx2* produce such skeletal phenotypes, Maxson and co-workers created *Msx2* deletions in *Wnt1-Cre/R26R* mice, and showed that the number of *LacZ*-positive cells in the skeletogenic condensations was equivalent between wild-type and mutant embryos. Thus, the skull bone defects were not due to disruptions in the migration or general allocation of neural crest cells to the frontal bone rudiment (R. Maxson, personal communication). Instead, *Sox9* expression and alkaline phosphatase activity were reduced in the mutant mice, indicating that the skeletal defects were caused because of a failure in the specification or proliferation of skeletogenic mesenchyme. This phenotype bears a striking resemblance to the zebrafish mutant *suc/Et-1* described earlier, which fails to develop ventral pharyngeal cartilages because of a defect in specification of skeletogenic mesenchyme. *MsxE* expression is lost in *suc/Et-1* mutants⁵², lending further support for an epistatic relationship between these molecules.

The final frontier for cranial tissue regeneration

The burgeoning field of stem cell biology is an unambiguous indicator of a scientific interest in the regenerative potential of the human body. There are significant gaps in our understanding of stem cell biology that will need to be addressed before therapeutic strategies can be routinely used. For example, the origins of stem cells that form regenerating bone have been surmised but not proven. Influences from the microenvironment in which stem cells reside are also poorly defined. The molecular signals and mechanical stimuli that induce cells to differentiate still remain largely unknown⁷³⁻⁷⁶.

As techniques to diagnose cranial skeletal defects become increasingly reliable, we are confronted with the possibility of treating milder forms of skeletal malformations in the fetus⁷⁷, which would obviate the need for numerous postnatal surgeries. Is our basic science knowledge sufficient to guide surgeons in this endeavour? Most of our information on bone repair comes from analyses of long bone fractures, but accumulating evidence suggests that cranial skeletal repair is a distinctive process. Identifying a source of cranial skeletogenic stem cells, elucidating the molecular signals that drive their differentiation, and developing delivery methods for these cells are worthy objectives. Such research will have direct and profound implications for the treatment of cranial skeletal defects resulting from malformation, disease or injury. □

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