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Neural Crest Induction and Differentiation

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Neural Crest Induction and Differentiation

Edited by

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DEDICATION

à mes parents

à Victoire, Louis et Fanny

INTRODUCTION

The neural crest was first described in 1868 by Wilhelm His, as "the cord in between" (Zwischenstrang), a group of cells located between the developing neural plate and the future epidermal ectoderm. These cells were seen leaving the dorsal neural tube and aggregating lateral to the neuraxis to give rise to spinal ganglia. Ever since the neural crest has fascinated generations of developmental and evolutionary biologists. My interest in the neural crest does not go back quite as far; it started in April 1986 when as a first year graduate student I attended my first Neuroscience Conference in Bordeaux. The keynote speaker that year was Dr. Nicole Le Douarin, whose lecture sparked my interest in neural crest. A few years later as a postdoctoral research in the laboratory of Dr. Igor Dawid at the NIH, I was able to pursue that interest by initiating studies on neural crest specification in Xenopus. This work eventually established the fundamental role of canonical Wnt signaling in the induction of the neural crest. Understanding the molecular basis of neural crest specification and diversification has remained a major research interest of mine since I set up my laboratory in 1998 at the University of Pennsylvania.

In this book I have tried to compile contributions by recognized leaders in the neural crest field who discuss all aspects of modern neural crest biology. From its evolutionary significance, to its specification, migration, plasticity and contribution to multiple lineages of the vertebrate body, to the pathologies associated with abnormal neural crest development and function. Each chapter is intended to be a source of information on the most current advances in the field, highlighting areas of emerging importance as well as some of the challenges that are facing the scientific community working with this cell population.

The first chapter of this book by Noden and Schneider provides an historical overview of some of the debates surrounding the neural crest: from the early days when the neural crest threatened the germ layer theory, to more recent times with the re-evaluation of the concepts underlying the mechanisms of craniofacial patterning. The next two chapters deal with the specification of the neural crest. In Chapter 2, Basch and Bronner-Fraser describe how several signaling pathways, including BMP, Wnt, FGF and Notch signaling, converge at the neural plate border to generate the neural crest and how their concerted action is essential to proper neural crest development. One of the immediate consequences of the induction of the neural crest is the activation of a number of neural crest-specific genes that define the identity of this cell type. In Chapter 3, Sargent describes more specifically the

importance of two sets of transcription factors implicated in the earliest stages of neural crest induction, the Msx/Dlx and TFAP2 families.

One of the key characteristics of the neural crest is its ability to delaminate and migrate away from the dorsal neural tube. In the following chapter (Chapter 4), Duband describes this important step in the ontogeny of the neural crest and summarizes recent advances and trends that have changed the way we look at the delamination and migration of the neural crest. The developmental program that regulates neural crest cell fate as they migrate into the periphery is believed to be both plastic and fixed. In their chapter, Sandell and Trainor (Chapter 5), describe some of the factors and contexts that influence the plasticity and determination of the neural crest, more specifically focusing on the complexity of the tissue interactions that regulate craniofacial morphogenesis. Chapter 6, by Dupin, Creuzet and Le Douarin, reviews a number of in vivo cell tracing and in vitro single cell culture experiments, which provided important insights into the biology of the neural crest. This chapter also emphasizes the role of Hox genes, morphogens and tissues interactions in the development of the skeletogenic neural crest cells.

One of the most remarkable aspects of the neural crest is its amazing multipotency and for this reason five chapters (Chapters 7 to 11) analyze the contribution of the neural crest to several lineages of the vertebrate body. With regard to the neural crest participation to the head skeleton, Knight and Schilling (Chapter 7) discuss recent genetic studies of craniofacial development in zebrafish that have revealed new tissue interactions important to the process of cranial neural crest development. Brown and Baldwin (Chapter 8) focus their review on the derivatives of the cardiac neural crest and discuss the influence of a subset of genes on neural crest-mediated cardiovascular remodeling. Chapter 9 presents an overview of the genetic regulation of pigment cell development. In this chapter, Silver, Hou and Pavan, provide a comparative analysis of studies in different organisms revealing the high level of conservation of this genetic pathway. Other important neural crest deriviants are the sensory neurons residing in the trigeminal and epibranchial ganglia in the head and forming spinal and dorsal root ganglia in the trunk. Raible and Ungos, in Chapter 10, review some of the signals involved in the specification and diversification of the dorsal root ganglia neurons. The enteric nervous system is entirely derived from the vagal and sacral neural crest cells. These cells have to migrate further than any other neural crest cell population in order to colonize the gastrointestinal tract. In Chapter 11, Anderson, Newgreen and Young, describe recent genetic and biological studies that have implicated a variety of signaling pathways in the survival, proliferation, migration and differentiation of the neural crest-derived enteric nervous system.

In the developing embryo, multipotent neural crest progenitors need to integrate cues from the extracellular microenvironment to ensure the generation of appropriate numbers of differentiating progeny. In Chapter 12 Sommer discusses a number of in vitro and in vivo studies that revealed the importance of growth factor signaling in eliciting diverse biological responses in neural crest cells.

The discovery that a pool of neural crest stem cells persists in several neural crest-derived tissues in the adult has important therapeutic implications. In Chapter 13, Teng and Labosky speculate on future possibilities that will combine our current knowledge of neural crest gene function in the embryo and the manipulation of adult neural crest stem cells in vitro and eventually in vivo. Abnormal survival, proliferation, migration and differentiation of neural crest cells leads to organ and tissue dysplasias with highly diverse clinical and pathological features. The concept of neurocristopathy was introduced three decades ago to unify the diseases arising from aberrant neural crest development. In Chapter 14, Etchevers, Amiel and Lyonnet describe a number of recent advances in basic and clinical research that shed light on some of these pathologies. As a vertebrate innovation the neural crest is considered one of the important steps in the evolution of chordates, and in the last chapter (Chapter 15) Barrallo-Gimeno and Nieto review evidence that have emerged in the last few years that may help explain the steps in the development and appearance of the neural crest during evolution.

In the last several decades a great deal has been learned about the biology of the neural crest. Still a number of important questions remain unresolved. We hope this volume will stimulate activities and efforts to further our understanding of the key processes implicated in the development of this remarkable cell population.

Jean-Pierre Saint-Jeannet, Ph.D.
Philadelphia, March 2006

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Jean-Pierre Saint-Jeannet, Ph.D.

CHAPTER 1

Neural Crest Cells and the Community of Plan for Craniofacial Development: Historical Debates and Current Perspectives

Drew M. Noden and Richard A. Schneider*

Abstract

After their initial discovery in the mid 1800s, neural crest cells transitioned from the category of renegade intra-embryonic wanderers to achieve rebel status, provoked especially by the outrageous claim that they participate in skeletogenesis, an embryonic event theretofore reserved exclusively for mesoderm. Much of the 20th century found neural crest cells increasingly viewed as a unique population set apart from other embryonic populations and more often treated as orphans rather than fully embraced by mainstream developmental biology. Now frequently touted as a fourth germ layer, the neural crest has become a fundamental character for distinguishing craniates from other metazoans, and has radically redefined perceptions about the organization and evolution of the vertebrate jaws and head. In this chapter we provide an historical overview of four main research areas in which the neural crest have incited fervent discord among workers past and present. Specifically, we describe how discussions surrounding the neural crest threatened the germ layer theory, upended traditional schemes of vertebrate head organization, challenged assumptions about morphological conservation and homology, and redefined concepts on mechanisms of craniofacial patterning. In each case we frame these debates in the context of recent data on the developmental fate and roles of the neural crest.

Introduction

> *"The biological science of the last half-century is honourably distinguished from that of preceding epochs, by the constantly increasing prominence of the idea, that a community of plan is discernable amidst the manifold diversities of organic structure."*
>
> —T.H. Huxley, 1858[1]

During the past 125 years, the neural crest has featured prominently as a provocateur for many great debates in vertebrate biology. Initially described by His in 1868 as a novel longitudinal band of cells dorsal to the spinal cord, this mesenchymal population was subsequently named the neural crest by Marshall in 1879[2] and immediately piqued the interest of embryologists and morphologists for a variety of reasons. In particular, the neural crest appeared at an embryonic stage that was surprisingly later than what had been observed for other progenitor populations, they displayed an unusually high degree of motility and dispersion throughout

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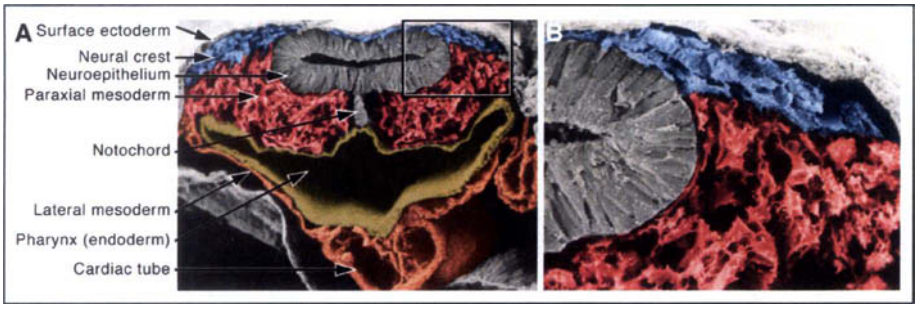


Figure 1. Embryonic tissues of the head. A) Pseudocolored scanning electron micrographs showing the major embryonic progenitor tissues of the head in a transverse section cut at the level of the caudal midbrain from a stage 10- (9-somite) chick embryo. B) At higher magnification (inset box), neural crest cells can be observed emigrating from their origin along the dorsal midline and are partially overlying paraxial mesoderm. These neural crest cells will continue their translocations until they fully underlie the pharynx. Modified from reference 173; original micrographs by K. Reiss.

the body, and they seemed to diversify into a range of cell types that was unexpected given their ancestry from neural ectoderm. These striking attributes, as well as those that became apparent from subsequent experimental analyses, sparked controversy on at least four intellectual fronts.

First, discovery of the origins and derivatives of the neural crest challenged fundamental notions about the basic building blocks of vertebrate embryos, especially the prevailing germ layer doctrine, which imparted exclusive and unique potentials to ectoderm, mesoderm, and endoderm (Fig. 1). Second, data regarding the underlying developmental organization of the neural crest necessitated that paradigmatic evolutionary scenarios for the plan of the vertebrate head be rejected, revamped, or reinvented. Third, analyses of the embryonic distribution and contributions of cranial neural crest cells defied concepts on the conservation of morphological boundaries across vertebrate taxa, and revealed problems in assigning musculoskeletal homologies. Fourth, experiments designed to examine the mechanistic potential of the neural crest created conflicting interpretations on the sources of patterning information that underlie craniofacial morphogenesis.

In this chapter we render the histories for each of the above controversies in light of current perspectives on the fate of cranial neural crest cells across many diverse species. Our goal is to provide resolution to past debates surrounding the neural crest by conveying an expanded, though far from complete, contemporary portrayal of the communal roles that these cells play during craniofacial development.

Primary Germ Layers and Neural Crest Cell Lineages

> *"Each of these three layers hurries toward its goal; although each is not yet independent enough to indicate what it truly is; it still needs the help of its sister travelers, and therefore, although already designated for different ends, all three influence each other collectively until each has reached an appropriate level."*
>
> —C. Pander, 1817[3]

The above insightful description is based on observations of early chick embryo development, and laid the foundation for the precept that many animals establish three stacked or concentric germinal layers from which all intra- and extra-embryonic structures subsequently arise.[4] During the ensuing decades of the 19th century, an apparently equivalent trilaminar organization was found among many diverse taxa.[5,6] However, driven by a post-Darwinian fervor to integrate evolutionary and developmental processes, the model itself became much more rigid and in fact restrictive, projecting the requirement that each germ layer represent an exclusive and autonomous source of particular cell types.

Observational data consistent with this notion were available for some ectodermal and endodermal populations. Mesodermal populations, however, with their frequent transformations between epithelial and mesenchymal states, were generally less well defined but nonetheless were assumed to follow equivalent rules. Further constraints upon the model were imposed by an unbridled zeal to unify developmental and evolutionary events across a wide spectrum of animal groups. Often, putative similarities of germ layers, which were considered shared among many taxa, were given primacy over features found only in a few species.

Within this dogmatic intellectual climate, extant during the last decade of the 19th century, several investigators including Kastschenko, Goronowitsch, and Platt described a dual origin of head mesenchyme.[2,7-9] Exploiting intrinsic cytological distinctions between mesodermal and ectodermal cells in the shark, *Acanthias*, and the mud puppy *Necturus*, Platt[10,11] thoroughly documented movements of neural crest cells into the pharyngeal region and their subsequent differentiation as cartilage and odontoblasts. A few vertebrate biologists welcomed her discovery, which seemed to unite features common to all jawed vertebrates. However, the apparent violation of the germ layer doctrine was soundly rejected by a majority of the scientific community, and some confirmatory findings such as those of Watson (1911) in marsupials,[12] were denied publication for several decades.[2,13]

While convincing evidence for neural crest contributions to the pharyngeal skeleton accumulated from many descriptive and experimental studies primarily in amphibian species,[8] the full extent to which neural crest cells participate in mid-facial as well as jaw and pharyngeal development did not emerge until stable cell labeling methods became available, first in avian species[14-18] and more recently in mice[19-25] and frogs.[26,27] Along with cartilage, a broad assortment of dense and loose connective tissues, smooth muscles, and secretory cells were added to the catalog of cranial neural crest derivatives.

In addition to the extensive array of sensory and autonomic neuronal and supportive cells, and also peripheral pigment cells, the diversity of cell types formed by the neural crest rivals that of mesoderm, prompting some to elevate the neural crest to germ layer status.[28] This comes, ironically, at a time when support for the autonomy of germ layers in vertebrate development is waning.[29] Processes leading to the emergence of mature cell phenotypes are progressive, with each stage building upon prior modifications. For some lineages, important programmatic events commence concomitant with or shortly following the formation of germ layers. For others, commitment seems to be initiated earlier such as delineation of early angioblasts,[30] which occurs in epiblast populations, with germ layers subsequently serving as convenient staging arenas.

An often-asked question is why does cephalic paraxial mesoderm not form the complete assembly of connective tissue lineages within the head as it does in the trunk? With few exceptions, such as transient preotic epithelial condensations found in the frog, *Xenopus*, head mesoderm likely lost the ability to form somites before the emergence of amphibians as well as in most fish groups. Suggestions that a loss of epithelialization may have stripped head mesoderm of certain connective tissue competencies are negated by lineage mapping studies, which demonstrate that head paraxial mesoderm normally forms the same diversity of connective tissues as do somites and neural crest cells.[31,32] Therefore, the answer does not appear related to restricted ability to form certain cell types.

The germ layer doctrine was tissue-centric, focusing on emerging cell phenotypes, and in this context the redundancy between ectodermally-derived neural crest cells and paraxial mesoderm remains perplexing. However, consideration of another developmental parameter, *morphogenesis*, provides a very different perspective. In this process, the history of mesenchymal populations in large part determines their ability to respond collectively to inductive stimuli. As discussed below, many of the skeletogenic signals impinging upon the neural crest and paraxial mesoderm are similar, but the spatial organization of their responses is dissimilar. Thus, the formation of an iterative, multi-element pharyngeal musculoskeletal system, which encompasses a number of adaptations characterizing the rise of gnathostomes (i.e., jawed vertebrates),[33,34] was apparently not within the programming competence of paraxial (or lateral) mesodermal populations.

Neural Crest and the Cranial Bauplan

"At the present day, the very questions regarding the composition of the skull, which were mooted and discussed so long ago by the ablest anatomists of the time, are still unsettled."

—T.H. Huxley, 1858[1]

Almost 150 years ago, Huxley delivered a pivotal lecture to the Royal Society of London in which he woefully made the above observation. Despite much subsequent attention and countless advances in scientific methods of analysis, in many regards Huxley's remark still applies to current disagreement about the basic plan of the vertebrate head. Generally, there are two perspectives, each of which incorporate data on the cranial neural crest to bolster their arguments. The first school of thought views the head as a modified extension of the trunk and emphasizes the segmented nature of its components. The second regards much of the head as a novel appendage to the body and focuses on lack of correspondence among its many parts, especially those derived from the neural crest.

Early segmental theories on origins of the vertebrate head were developed with the transcendental trappings of philosophical anatomy and idealistic morphology. During the 18th and 19th centuries, patterns of repetition and unity of form were believed to underlie the organization of all structures within the body. Correspondingly, the skull was seen as being constructed by a series of discrete units homologous to vertebrae. Goethe is credited with first proposing in 1790 that the skull is composed of several vertebrae, and his idea gained widespread acceptance and elaboration by leading zoologists such as Oken, Spix, Bojanus, Dumerfl, Blainville, Geoffroy, and Owen.[6] However, the theory was not without dissenters. For example, Cuvier argued in 1837 that similarity between the skull and vertebrae could only be found in the most caudal regions of the head, and even this, he believed, was due to equivalent functional requirements and not a unity of plan.[35] Likewise, works of Agassiz and Remak were critical of the theory on embryological grounds.[36]

Opposition gained momentum when Huxley (1858) attacked the extremes to which the vertebral theory had been taken.[1] He argued, "it is no more true that the adult skull is a modified vertebral column, than it would be to affirm that the vertebral column is a modified skull" (p.433). Huxley had made detailed developmental dissections among all major classes of vertebrates and observed that the basic organization of the skull is inherently different from that of the spinal column in both pattern and process of ossification. Though he concluded that vertebral organization does not extend across the entire skull, Huxley did concede that the occipital bones around the notochord may have been derived from vertebrae, and that a segmental plan, albeit different from that of the trunk, could exist in the head.

By allowing for these possibilities, Huxley actuated two opposing viewpoints that were to follow for nearly a century. One viewpoint emphasized the incorporation of vertebrae solely into caudal regions of the head. Workers including Gegenbaur, Stöhr, Rosenberg, and Sagemehl divided the skull into rostral nonvertebral and caudal vertebral portions.[36,37] Most often the boundary was placed at the level of the notochord tip, suggesting that important distinctions were to be made between chordal and prechordal regions of the head.[38] This put the basisphenoid within the caudal, chordal domain, but generated considerable uncertainty about the positions of remaining sphenoid elements in different species. Other researchers emphasized the presence in some taxa of a cranio-occipital joint, which aligned with the exit of the vagus nerve from the skull. This observation led to theories stressing prespinal and spinal portions of the head. In 1875, Fürbringer attempted to integrate these views by proposing that changes in the prespinal part of the skull underwent successive steps throughout the history of gnathostomes.[39] He categorized the prespinal head of different taxa as a palaeocranium (e.g., cyclostomes), a protometameric neocranium (e.g., elasmobranchs and Amphibia), and an auximetameric neocranium (e.g., Amniota). More than half a century later workers such as Augier used similar ideas to explain the progressive enlargement of rostral portions of the head during vertebrate evolution.[31]

The other, and more widely accepted viewpoint that followed Huxley's lecture championed the concept of segmentation as manifest in neural and peripheral musculoskeletal structures. These ideas gained particular prominence due in part to the 1875 works of Dohrn and Semper, who proposed an annelid origin for vertebrates.[6] Additionally, Balfour's seminal descriptions in 1877 of a series of mesodermal "head cavities" in cartilaginous fishes provided unassailable evidence for a set of iterative structures that he believed were distinct from gill structures yet established serial relations with pharyngeal clefts and cranial nerves.[40] Further research on the arrangements of cephalic neuromeres (i.e., segmental swellings in the brain), cranial nerves, and pharyngeal arches in other organisms seemed to support the theory that the preoccipital part of the head in vertebrate embryos was essentially organized as a series of repeated units.[10,41-43]

After the turn of the century most workers agreed with the sentiments of Goodrich (1930) "that the head region of the Craniate is truly segmented, that it is composed of a number of segments essentially similar to those of the trunk, and that segmentation originally extended to the anterior end of the body" (p.213).[39] Each head segment contained a somite or equivalent population of mesoderm that formed initially autonomous sclerotomic, myotomic, and dermatomic structures; both dorsal (sensory) and ventral (motor) cranial nerve roots; a discrete endodermal outpocketing; and the musculoskeletal and aortic components of an oropharyngeal arch (Fig. 2; also see Box 1 for discussion of arch terminology). According to de Beer (1937), once these "facts" had been assembled, the race was on to determine precisely "how many segments of the body are involved in the formation of the skull" (p. 15).[36] Indeed, some subsequent metameric models of craniate head organization included additional rostral segments,[44-46] stretching "the creative imagination of most readers to force all anatomical structures into a rigid segmental framework" (p. 133).[47] Nonetheless, due primarily to the preeminence of workers such as Goodrich and de Beer,[36,48,49] as well as their students, the metameric scheme of head segmentation became standard in 20th century comparative anatomy textbooks.[50]

Despite what seemed to be solid conceptual grounds for an archetypal plan of head organization, there were some nonconformists who noted problems with segmental interpretations of both post-otic and preotic regions of the head. The contributions of the somites to the post-otic area seemed to vary among vertebrates. Moreover, evidence from studies of neural crest distribution suggested that patterns of segmentation found in the pharyngeal arches and nervous system did not correspond to that present in occipital somites.[51] This objection was recently confirmed by fate mapping studies (see ref. 159). Along similar lines, several workers opposed plans of segmentation that encompassed all regions of the head. For example, Neal (1918), who was highly skeptical of single organism-based schemes, was the first to note the apparent inverse phylogenetic relationship between overt segmentation in the brain and in head mesoderm.[52] Epithelial mesodermal segments extending rostral to the ear are prominent in many fishes, occasional in amphibians, and lacking in amniotes, whereas the opposite appeared true of hindbrain rhombomeres. Neal argued that rhombomeres (Fig. 3) evolved in conjunction with pharyngeal segmentation and not mesomeric segmentation, particularly since nerve nuclei traverse rhombomeric divisions and correlate with the pharyngeal arches, clefts, and pouches instead of cephalic somites.

Kingsbury and Adelmann (1924) also raised concerns that the segmentation model was driving interpretations, which were often based on scant and purely descriptive biological observations.[53] Kingsbury (1926) maintained that, "if the head is really segmental in its composition, each segment must at least embody a neuromere, a nerve, and a mesodermal somite. Even the most adequate plan of segmentation, such as that of Goodrich, fails fully to meet the requirements" (p. 84).[54] In his work, Kingsbury emphasized the inconsistent and generally inadequate descriptions of head mesoderm and noted the failure by morphologists to link together pharyngeal arches, head somites, and the unsegmented arrangements of several cranial nerves.

Neural crest cells, by arising from a dorsal tissue and moving to form ventral structures, were a constant source of aggravation equally to the most ardent supporters as well as vociferous opponents of segmentation. For example, by the time Goodrich wrote his classic tome in

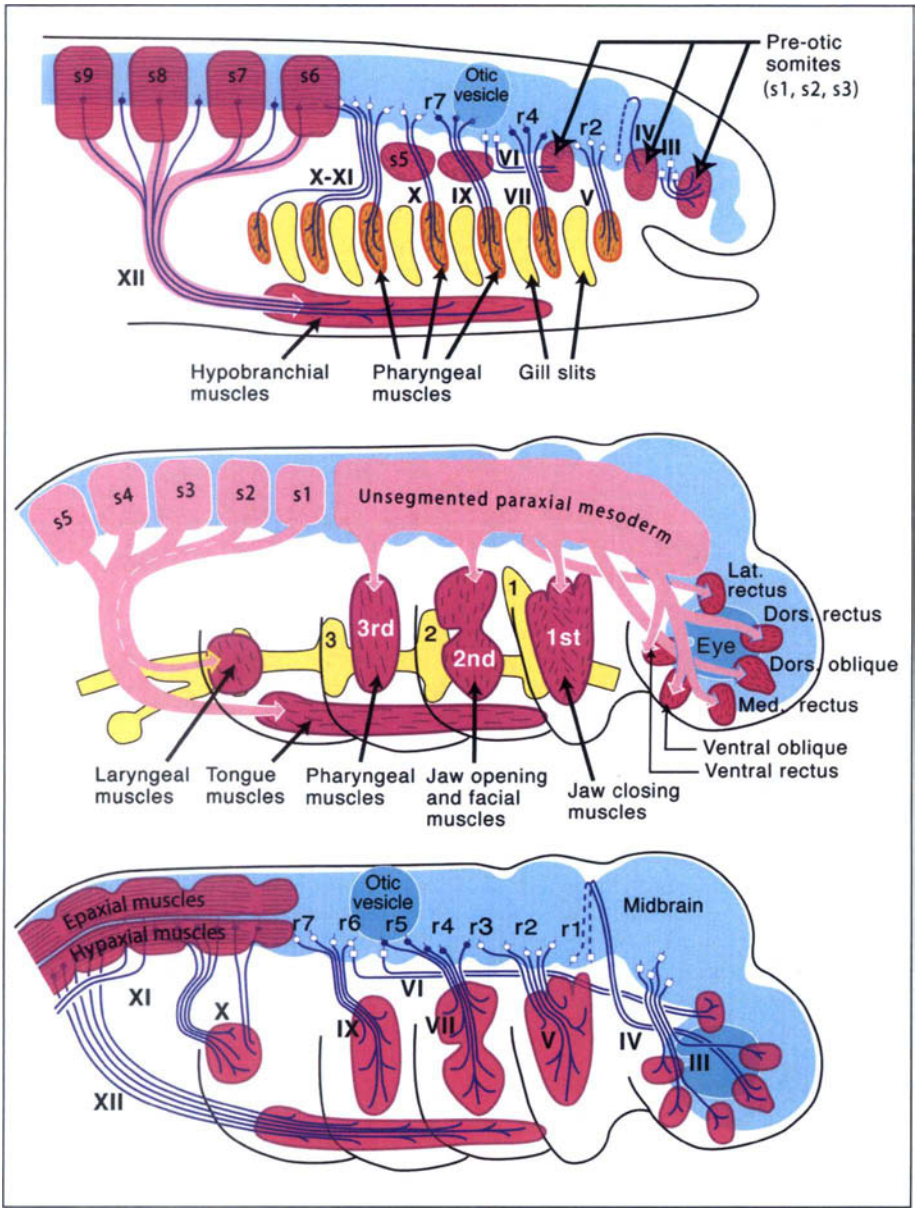


Figure 2. The vertebrate cranial Bauplan. Schematic representations of the classic segmental model for neuromuscular organization of the vertebrate head (top), as well as a contemporary scheme based on recent mapping analyses (middle and lower). In the segmental model, pharyngeal muscles arise from lateral mesoderm between gill slits. Paraxial mesoderm forms somites along the full length of the head, with three preotic somites forming extra-ocular muscles. Additional somites are found adjacent to the otic vesicle, but their homologues—if any—in extant vertebrates are unknown. In the middle sketch, the movements of myogenic myoblasts from paraxial mesoderm into the periphery are illustrated, and the lower drawing adds the distribution of cranial somatic motor nerves. Based on a variety of sources including Goodrich (1918), Noden (1991), and Northcutt (1993).[49,84,174]

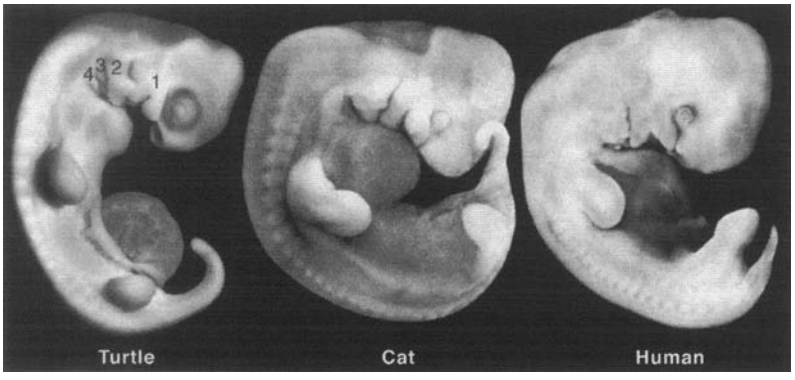
Box 1. Who's the real arch?

Historically, numerous terms have been used to describe the same serially arranged pairs of swellings that appear along the sides of the head in most vertebrates. This superfluity of jargon has caused much confusion and consternation. At some stage in its development, each swelling contains progenitors of cartilage and bone, skeletal and smooth muscle, an aortic arch, a pharyngeal pouch, and a cranial motor nerve. Conventionally in jawed vertebrates (i.e., gnathostomes), the first arch is named "mandibular" and the second arch is named "hyoid".

Branchial Arch (*G. brankhia*, "gills")
Associated with the aquatic respiration of fish and amphibians (anamniotes). Implies a scenario of evolutionary homology to the gills when used in amniotes since amniotes do not possess gills. This term is probably not appropriate for describing the mandibular arch (i.e., the jaws) of gnathostomes. Other related terms: branchial cleft or groove, which refer to indentations on the surface epithelium that separate each successive swelling.

Visceral Arch (*L. viscus*, "internal organ")
Pertaining to a viscus or an organ inside the vertebrate body. Conveys functional and developmental biases particularly with regard to early theories, now proven inaccurate, that the muscles associated with the visceral arch skeleton are un-striated and derived from either endoderm or lateral plate mesoderm. These muscles are in fact striated and derived from paraxial mesoderm like all other skeletal (i.e., voluntary) muscles. Related term: viscerocranium, which refers to all structures associated with the jaws and gills and their homologues.

Pharyngeal Arch (*G. pharunx*, "windpipe" or "throat")
Related to the region between the oral cavity and esophagus of all vertebrates. Has both anatomical and embryological connotations being specifically associated with the endodermally-derived pharynx. Could accurately encompass the mandibular arch, which includes the ectodermally-derived stomodeum, if amended as "oropharyngeal arches," *which is what we prefer.* Related terms: pharyngeal cleft, pharyngeal pouch.



Arch terminology. Snapping turtle, cat, and human embryos in lateral view at comparable stages. Numbers indicate oropharyngeal arches. Turtle embryo courtesy of D. Packard and mammalian embryos from the Cornell Embryology Collection.

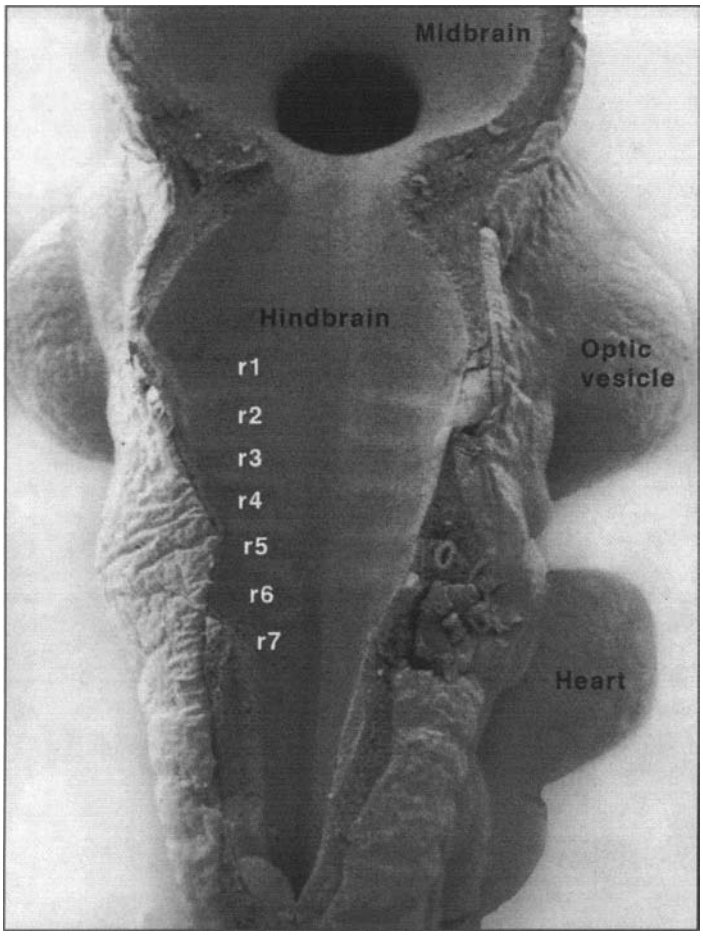


Figure 3. Hindbrain segmentation. SEM, dorsal view of a ferret embryo at embryonic day 14 in which the roof of the hind- and mid-brain regions was removed prior to processing. The segmental organization of the hindbrain is structurally evident by the presence of transverse inter-rhombomeric sulci. Successive rhombomeres are indicated as r1, r2, r3, etc. Courtesy of C. Wahl.

1930, there were numerous studies on the contributions of neural crest to the viscerocranium[2,55,56] and yet he ignored these and argued unequivocally that the visceral arches "are derived from splanchnic mesoblast" (p. 396).[39] Even de Beer (1937), a student of Goodrich, reluctantly accepted the true role of the neural crest in the head and stated, "it is difficult to resist the conclusion that the cartilage of the...visceral arches is derived from the neural crest, strange as it may seem" (p. 476).[13] Notwithstanding this admission, de Beer relegated the neural crest to subordinate status throughout his definitive work on the vertebrate head[9] and did not publish his own study on their skeletal contributions until a decade later.[7]

Towards the end of his illustrious career, Romer attempted to meld these divergent views of cranial organization via his "dual animal" hypothesis.[13] Herein he emphasized the independence of the oropharyngeal and axial regions during vertebrate ontogeny and phylogeny. Romer argued that rather than being fully segmented, the vertebrate head is organized into two functionally and structurally distinct compartments, one essentially external and "somatic" and the

other internal and "visceral," analogous to structural relations evident in thoracic and abdominal regions. Romer based these divisions on muscle types (striated versus smooth), skeletal origins (mesodermal versus neural crest), and nerve components. Of particular importance to Romer's argument was the ancient skeleton that supports the gill apparatus in vertebrate relatives such as Amphioxus, tunicates, and acorn worms, and that phylogenetically predates the skeletal elements of the somatic animal.

In rejecting previous theories of segmentation Romer stated, "it may happen by chance that during development some one gill bar and its musculature may lie below some specific myotome and its derived musculature. But there is no a priori reason to think that the two segmental systems—one basically mesodermal and related to the "somatic" animal, the other basically endodermal, "visceral" in origin-have any necessary relationship to one another" (p. 141).[13] Romer believed his major contribution was to rectify older theories of metamerism with a more accurate picture of vertebrate evolution, beginning with an entirely visceral, sessile, and nonsegmented ancestor. Embryology did not weigh prominently in his model.

Such an emphasis on the role of evolutionary adaptations in shaping the vertebrate head was expanded upon by Gans and Northcutt (1983).[57,58] Noting fundamental differences between vertebrates and protochordates, specifically roles played by cells derived from the neural crest and ectodermal placodes, Gans and Northcutt incorporated the well established fact that all skeletal and connective tissues located rostral to the notochord tip and also lateral and ventral to the pharynx were of neural crest rather than mesodermal origin. They concluded that the vertebrate head "may be conceived as an addition to the existing body of protochordates," and as such "does not represent a modified portion of the existing trunk" (p. 272).[57] Likewise, they rejected classic schemes of segmentation and asserted that the preotic region of the vertebrate head "is intrinsically unsegmented" (p. 271).[57] Yet, a shortcoming of the "new head" theory, and other hypotheses that seemed to invalidate traditional views of segmentation, was that most of the conclusions were based largely on classical descriptive studies. Gans and Northcutt did not incorporate contemporaneous experimental data, which included detailed fate maps of neural crest and paraxial mesoderm populations, as well as revelations about the mechanistic roles that each of these mesenchymal populations play during craniofacial development. More recently, Northcutt (2005) has revisited his "new head" hypothesis and thoroughly addressed these issues.[59]

Two discoveries about cranial paraxial mesoderm forced additional reconsideration of traditional schemes of head organization. The first came from Meier and his collaborators, who found a nascent pattern within paraxial mesoderm in chick,[60,61] mouse,[62,63] snapping turtle,[64,65] shark, newt[66] and medaka.[67] In their work they describe an iterative series of concentrically arranged mesenchymal cells on the dorsal (superficial) and ventral surfaces of paraxial mesoderm, first evident at the neural plate stage and remaining during the stages when neural crest cells move onto the surface of paraxial mesoderm. These domains, which Meier called somitomeres, number seven in amniotes and fish, and fewer in amphibians.[68]

Meier's observations on cranial somitomeres engendered euphoria among the segmentalists, for at long last a key component of metamerism was found. However, to this day, the existence and especially the developmental significance of somitomeres remain controversial.[69,70] Subsequent efforts to observe somitomeres have been unsuccessful,[71,72] and discrepancies in somitomeric boundaries and numbers have emerged depending on the manner in which overlying ectoderm and extracellular matrix are removed.[73] Also, cell labeling experiments using fluorescent dyes indicate that unlike somites, somitomeres lack segmental identity, are not units of lineage restriction, and do not form compartments of cells with discrete spatial properties.[74] Moreover, the cryptic demarcations between somitomeres do not correspond to any identifiable boundary separating individual myogenic or skeletogenic precursor populations.[32]

Once neural crest cells embark upon their translocations to the oropharyngeal region, they establish close contacts with underlying myogenic cells residing in paraxial mesoderm, and these two populations remain in registration during the formation of the arches.[70,75] Anderson

and Meier suggested that the somitomeric pattern might provide specific guidance cues for emigrating neural crest cells.[76] However, neural crest lesion experiments indicate that interactions within neural crest populations may be more essential for establishing the pathways of cell movement.[77] In contrast, somites provide both impediments and conduits for the passage of neural crest cells within and over their surface.[78,79] Moreover, replacing head mesoderm with somites creates a local barricade to the dispersal of cranial neural crest.[80] Thus, the relevance of somitomeres to the developmental organization of the vertebrate head remains uncertain.

A second significant contribution that caused a reconsideration of previous schemes of cranial organization was the discovery that paraxial mesoderm is the exclusive source of all skeletal muscles in the amniote head.[81,82] Myogenic precursors secondarily move into corresponding oropharyngeal arches in concert with neural crest cells, and also in registration with the appropriate cranial motor nerves. This fact, along with extended analyses of the contributions by cranial neural crest cells to skeletal and other connective tissues, led to a formal revision of vertebrate craniofacial organization, in which paraxial mesoderm of the head was portrayed no differently than that of the trunk with respect to the generation of types of cells.[75,83,84] Such ideas focused attention upon the existence of an interface between neural crest and paraxial mesoderm, and suggested that the location of this boundary might be a constant and defining landmark among vertebrate taxa.

Topology and Homology along the Neural Crest-Mesoderm Interface

> *"Sometimes, however, the nonconcordance of morphological relations present problems of special difficulty, for the answer in such cases does not appear to be as simple as mere nonhomology of the structure in question with other structures."*
>
> —G.R. de Beer, 1937[36]

Gans and Northcutt (1983)[57] and others[31,85] postulated that the boundary between neural crest and mesodermal mesenchyme represents a fundamental division between the rostral "new" head and caudal "old" head of vertebrates. Such a hypothesis evokes several questions. Is the location of this interface constant at different stages of development and in various species? Is this interface a permanent barrier that maintains complete separation between the two mesenchymal populations? And is there evidence for cooperation across this interface?

In most species the interface between neural crest and mesodermal mesenchyme is cytologically cryptic, identifiable only when a lineage-specific label is utilized. In avian embryos, the interface is located at the mesencephalic-prosencephalic junction, beside the adenohypophyseal diverticulum (anterior pituitary), and extends caudally along the dorsolateral margins of the pharynx and pharyngeal pouches to the laryngotracheal diverticulum (Fig. 4). This boundary separates dorsal and caudal mesodermal mesenchyme from rostral and ventral neural crest-derived populations. The location of the interface in lampreys, zebrafish, several amphibians, birds, and mammals (mice) has been defined through cell-lineage analyses using vital dyes, radioactive and fluorescent labels, interspecific transplantations, or reporter gene constructs.[2,9,32,34,83,86,87] According to Thorogood (1993), "the broad distribution of the mesenchyme from the two lineages is surprisingly regular and consistent" (p. 113).[88] However, most analyses have been limited to early developmental stages, and in far fewer species has the location of the interface been defined at later stages, when all skeletal elements are in place. This is especially problematic for vertebrates that undergo metamorphosis.

Detailed mapping experiments using quail-chick transplantations have identified neural crest cells as the exclusive source of skeletal and other connective tissues in the midfacial, oral, and pharyngeal regions of the head.[16-18,31,81,82,89,90] For the most part, these mapping studies reveal that each skeletal element originates fully on either side of the interface. More recently, transgenic mice carrying reporter constructs activated in neural crest or mesodermal progenitors have been used to provide a robust examination of skeletal origins in mammals.[21,23,24,91] Again, most elements fall into the categories predicted based on homologies between birds and mammals.

One region of the skull for which there has been much contention is the calvaria. In mice the interface between skeletogenic neural crest and mesodermal populations corresponds roughly to the site of the coronal suture, between the frontal and parietal bones.[21,25,92] Some neural crest cells do move further caudally where they participate in suture formation but do not contribute directly to the intramembranous ossification associated with the parietal bones.[21] The interface appears to be located more caudally in post-metamorphic *Xenopus*, between the large frontoparietal and occipital bones.[26,27]

Transplantation approaches in avians have produced conflicting results. Some investigators found the boundary within the frontal bone, at the junction of the supraorbital (rostral) and calvarial (caudal) parts of this bone.[17,18,89,90] These regions arise from separate ossification centers that fuse together in most avian species. Other investigators concluded that the boundary for neural crest contributions to the roof of the avian skull was located further caudally, at the junction between parietal and occipital bones.[31] While these contradictory findings could have arisen for a variety of reasons,[70] a more recent study using a replication-incompetent retrovirus, which contains a stable reporter construct,[93] has confirmed that the interface in chick embryos is located within the frontal bone, with the parietal being derived exclusively from mesoderm.[32] Thus, in both birds and mammals, the calvaria is of dual mesenchymal origin.

The apparent difference in the precise location of the neural crest-mesoderm interface along the roofing bones of chick and mouse is problematical and prompts several questions. Has the location of the interface indeed shifted during the evolution of one or both of these species, and, if so, what was the location in the common ancestor of birds and mammals? Or, alternatively, has the site of the interface remained constant while specific patterns of fusion or separation of ossification centers changed? Overriding both of these questions is the issue of whether or not the site of the interface is at all pertinent to subsequent patterns of ossification.

In the absence of mapping data from multiple species, assessing the evolutionary stability of the interface between neural crest and mesoderm is filled with uncertainty. The common tetrapod ancestor had a nearly solid roof within which putative frontal and parietal bones are recognized.[94-96] But also present are prefrontal, postfrontal, postparietal, and additional more caudal elements. The phylogenetic fates of these roofing bones have been disputed for decades as they have undergone variable amounts of loss, reduction, expansion, and/or fusion. Most of these changes appear in association with the large openings that evolved in the temporal region of the skull.[97] In the fossil record, archosaurs (diapsids) underwent a reduction of the postfrontal and of the postparietal in their skulls, and of the dermal roof.[98,99] Birds eventually lost the postfrontal. In the mammal-like reptiles (synapsids), the skulls contain a greatly enlarged lateral fenestra that is accompanied by reduction in the size of the calvaria.[100,101] Mammals subsequently lost their postfrontal and reexpanded their frontal and parietal bones.

While considerable attention has been given to identifying homologies among bones in the oropharyngeal arches[102,103] and in the floor and lateral wall of the braincase,[38,104-112] comparable verifications are not available for roofing elements.[29,46,101,113] Comparative analyses of the locations and regulatory mechanisms associated with mammalian sutures are valuable,[114] especially because of their significance in the genesis of craniosynostoses,[115] but they are not informative regarding ancestral patterns. Given the complexities in following the evolution of specific roofing elements, the nomenclature used to label bones in the avian and mammalian calvariae are based on proposed rather than proven homologies. In the chick, the frontal-parietal junction overlies the otic capsule, whereas in mice this boundary occurs over the orbit. Thus, on both embryological (i.e., dual mesenchymal origin), and topographical (i.e., anatomical) grounds, labeling this element in the chick as the "frontoparietal" bone, and calling the parietal a postparietal or interparietal, may be more apropos. If this is the case, then the interface between neural crest and mesoderm can be considered constant among these avian and mammalian species.

Mechanisms that pattern the skull roof, like those operating in the pharyngeal arch skeleton, involve multiple hierarchical and reciprocal interactions whereby individual tissues participate

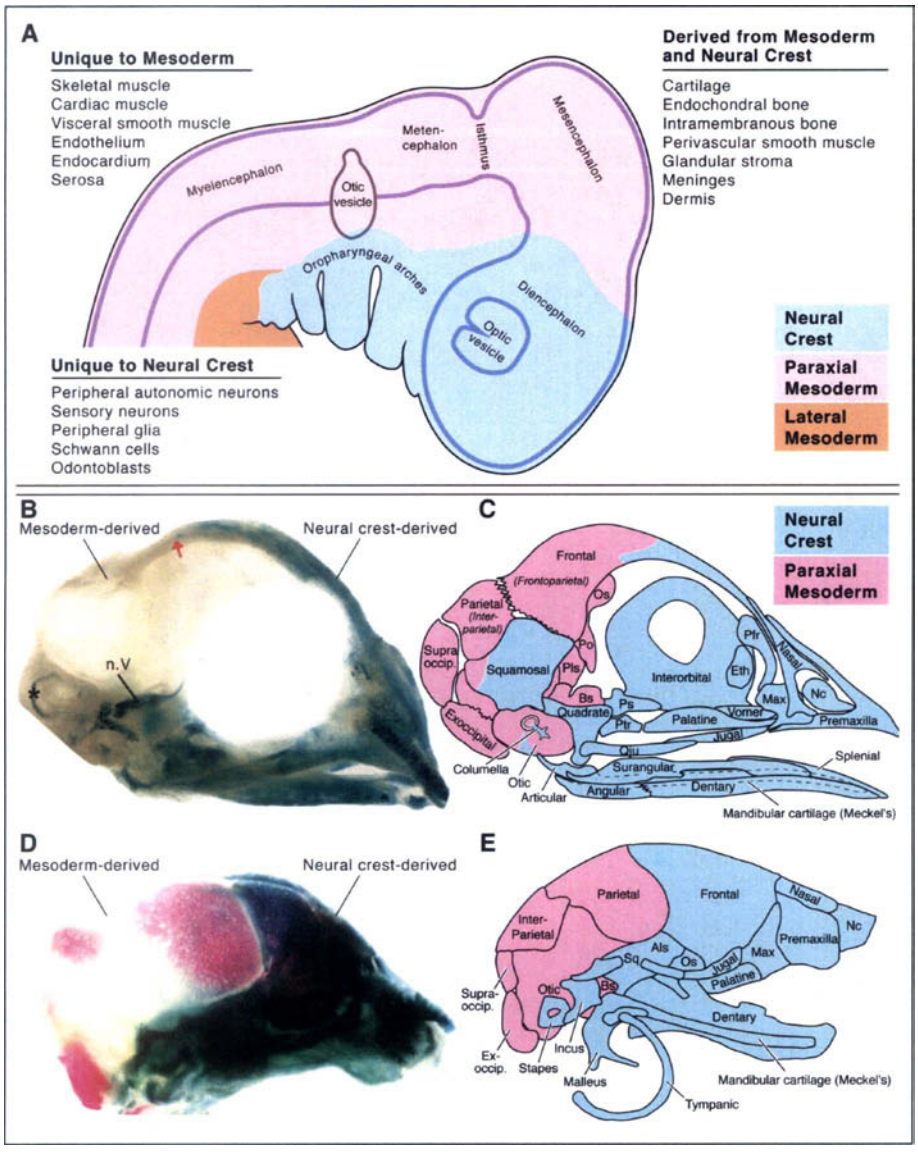


Figure 4. Neural crest-mesoderm boundary in the head. A) The location of the neural crest-mesoderm interface is shown at a stage following the initial translocation of neural crest cells but before the secondary movements associated with muscle morphogenesis. Listed are tissue types derived in amniotes from either neural crest or mesoderm exclusively, or from both of these mesenchymal populations. B) The extent and boundaries of neural crest contributions to the avian skull are shown in a bisected head from a 14-day chick embryo whose neural plate, including neural crest precursors, and surface ectoderm were washed with a replication-incompetent retrovirus containing the *LacZ* (β galactosidase) reporter gene. Note the complete labeling (blue stain) of frontonasal, maxillary, mandibular, and other pharyngeal arch skeletal structures in addition to sensory ganglia such as the trigeminal (n.V). The red arrow points to the site of the neural crest-mesoderm boundary between rostral and calvarial parts of the frontal bone. Asterisk (*) denotes labeled cells within a semicircular duct, which is derived from the otic placode. Figure legend continued on next page.

Figure 4, continued. C) Schematic showing the contributions of mesodermal and neural crest mesenchyme to the cartilages and bones in the avian head skeleton based on data from quail-chick chimeras and retroviral labeling. D) Neural crest-derived tissues in a *Wnt1-Cre/R26R* transgenic mouse embryo appear blue following X-Gal staining. Bones are also stained with Alizarin Red. Image courtesy of G. Morriss-Kay. E) Schematic of neural crest contributions to the mouse head skeleton as extrapolated from transgenic data. Redrawn from reference 175. Abbreviations are as follows: Als = Alisphenoid; Bs = Basisphenoid; Eth = Ethmoid; Nc = Nasal capsule; Os = Orbitosphenoid; Ps = Parasphenoid; Pls = Pleurosphenoid; Po = Postorbital; Prf = Prefrontal; Ptr = Pterygoid; Qju = Quadratojugal; Sq = Squamosal; Supraoccip = Supraoccipital; n.V = Trigeminal Nerve.

according to their unique history and responsiveness.[70,113] In oropharyngeal regions, neural crest populations bring essential pattern generating properties based on their sites of origin,[8,89] whereas in the frontonasal region neural crest populations appear more dependent upon signals emanating from surrounding tissues for their morphogenesis.[116-120] The meager data available relative to the roof of the skull suggest that the brain plays an important inductive role.[121-123] If skeletal patterning of the calvaria is indeed established through the actions of extrinsic signals, then evolutionary changes in the distribution of neural crest and mesodermal mesenchymal populations lateral and dorsal to the brain may be irrelevant.

While most skeletal structures appear to form on either side of the interface, the cartilaginous otic capsule and several parts of the sphenoid complex incorporate skeletogenic cells from both the neural crest and paraxial mesoderm, in chick[17,18,31,81,82] and mouse.[21,23,24] These exceptions to the principle of segregation likely represent situations where ancestral neural crest-derived pharyngeal arch elements abut mesodermal neurocranial tissues. As occurred with the transformation of the jaw joint and "release" of elements for adaptation as middle ear ossicles, these former pharyngeal chondro-competent cells apparently became recruited into nearby cartilages.

Attempts to shift the location of the neural crest-mesoderm interface experimentally have generated mixed results. Schneider (1999) transplanted quail neural crest cells into locations normally occupied by head mesoderm, and produced cartilages in the lateral sphenoid skeleton and otic capsule that were morphologically indistinguishable from elements normally generated by mesoderm.[108] The implication from these experiments is that neural crest cells can respond to the same cues that both promote skeletogenesis and enable proper patterning in mesoderm. Yet, similar grafts of cranial neural crest populations into trunk mesoderm produce only small foci of cartilage within vertebrae.[124] To date, attempts to replace neural crest-derived skeletal elements with mesoderm have been unsuccessful.[80] Other clues come from in vitro experiments by Chiakulus (1957) and Fyfe and Hall (1979).[125,126] These workers artificially mixed populations of neural crest and mesodermal cells, and showed that cartilages derived from mesodermal mesenchyme fused together readily, as did all cartilages derived from neural crest mesenchyme. However, attempts to join cartilages of neural crest and mesodermal origins were unsuccessful, suggesting that the maintenance of these sharp boundaries during normal development may depend in part upon differences in cell cohesivity.

The rule of exclusivity that occurs on either side of the neural crest-mesoderm interface seems to apply only to connective tissues. Both angioblast and myoblast populations generated within paraxial mesoderm will cross over to the other side. Angioblasts are highly invasive, moving omni-directionally in order to ensure that all parts of the embryo are populated.[127-130] Indeed, these cells penetrate neural crest populations en route to oropharyngeal and periocular locations, allowing the assembly of aortic arches immediately upon the completion of neural crest dispersal. An interesting observation in this context is that human fetal hemangiomas often appear to be constrained by the boundary between neural crest and mesoderm along the temporal region of the head.[131]

Additional disruptions of the interface occur whenever individual muscles leave their original arch of origin. This is especially evident each time pharyngeal arch muscles establish attachments with mesodermal skeletal structures. In most cases the sites of attachment are formed by neural

crest cells that moved along with the myogenic cells from their original arch.[90] Perhaps the most extreme example of this is the trapezius muscle, which is one of several muscles that receives innervation from cranial nerve XI, yet is not associated with head skeletal structures. Lineage tracing analyses in transgenic mice reveal that neural crest cells from a caudal pharyngeal arch travel with these myoblasts and form tendinous and skeletal cells within the spine of the scapula.[23] This excursion seemingly recapitulates movements established ancestrally, when parts of the pectoral girdle abutted caudal portions of the skull.

The interface between neural crest and mesoderm is, not unexpectedly, a site at which signals affecting the differentiation and morphogenesis of both populations are exchanged. Prior to the arrival of neural crest cells, paraxial mesoderm cells are unable to progress along the muscle differentiation pathway due to the actions of inhibitory factors, especially BMPs and WNTs, released from overlying surface ectoderm. Neural crest cells physically separate these two tissues, but more importantly they release the BMP antagonists NOGGIN and GREMLIN plus the WNT antagonist FRZB, thereby allowing myogenesis to proceed.[132]

At later stages the morphogenesis of head muscles is dependent upon positional cues provided by neural crest cells.[87] Manipulation of pharyngeal arch neural crest populations, either by transplantation[89] or genetic alteration[133,134] results in changes in the orientation and attachments of pharyngeal arch muscles. Moreover, even trunk mesodermal cells grafted to the head will move within neural crest-populated areas and subsequently form normal pharyngeal arch or extra-ocular muscles.[135] These results demonstrate the integrated relationship between neural crest and mesoderm populations, physically separated but in close communication across the interface. Moreover, they show that embryonic muscle anatomy is largely a reflection of the patterning imposed by oropharyngeal and periocular neural crest populations.

Neural Crest and the Origins of Craniofacial Pattern

> *"Not even experimental methods have so far been able to unravel the fate of all cells, or to solve all of the problems regarding the role of the neural crest in determination processes. One explanation of the many contradictory statements made seems to be that many investigators have not attacked the problems on sufficiently broad lines...Further investigations with a variety of methods and giving due consideration to possible sources of error will certainly give us a deeper insight into the many problems offered by the developing neural crest."*
>
> —S. Hörstadius, 1950[8]

As evidenced by the above passage from Hörstadius' 1950 monograph on the neural crest, attempts to understand cell differentiation and tissue patterning have historically been volatile and erratic. Cell differentiation has consistently been more accessible using reductionist approaches but often at the expense of information on populations as a whole, whereas tissue patterning has typically been mired in phenomenology that has lacked robust mechanistic analysis. This uneasy relationship is reflected in the preceding overview of debates regarding the roles of neural crest cells in craniofacial development. While much research has provided data on locations and lineages, the most unsettled issues remain those involving multicellular organization and tissue patterning.

With regard to patterning, generally two perspectives have emerged, each reflecting biases prevalent at the time they were proposed. The first imbues cranial neural crest cells with the ability to execute spatial patterning based on properties that they acquire prior to emigrating from the neuroepithelium. The second emphasizes ongoing interactions between neural crest cells and surrounding tissues that are necessary for both the differentiation and morphogenesis of oropharyngeal arch musculoskeletal structures, with the implication that premigratory neural crest cells are relatively naïve. While this conceptual dichotomy has made for lively debates, it is tempered by recently emerging evidence that regulatory processes underlying craniofacial development involve progenitor populations acting in neither fully autonomous nor wholly dependent manners. Rather, each progenitor population carries circumstantial capabilities and restrictions based on its embryonic history. While there may be hierarchical inequities that

drive the outcome of interactions among the various players, proper histogenesis and morphogenesis require communal relationships. Such a phenomenon was revealed by Schotte and Spemann in the early 1930s following transplantations of mouth-forming tissues between frogs and newts.[136-138] In these experiments, local signals directed the regional character of the outcome, but intrinsic constraints imposed species-specific features.

Similarly in the early 1950s, Hörstadius provided remarkable insights into the issues of induction and determination.[8] In his experiments, amphibian neural folds containing neural crest precursors were transplanted from one axial level of the head to another. The resultant larvae developed with abnormal jaw and branchial cartilaginous skeletons. However, these were not random dysmorphologies. Rather, the skeletal structures produced by progeny of grafted neural crest cells closely resembled those they would have formed in their original location. These pioneering experiments defined the importance of prior history in the execution of tissue assembly.

Several decades later, Noden[89,139] demonstrated the same in avian embryos, with the advantages that better cell marking tools were available using the quail-chick chimeric system[15] and embryos could be reared until nearly all bones of the head were ossified. Neural crest progenitors transplanted from one site along the midbrain-hindbrain axis to a different site dispersed appropriate to their new location,[139] but their subsequent patterns of morphogenesis were inappropriate. Transplanting neural crest precursors from midbrain (future mandibular arch) to the hindbrain (future hyoid arch) levels produced embryos with an additional jaw skeleton in the location normally occupied by hyoid elements.[89]

These findings, fully consistent with those of Hörstadius, have been used to support the argument that neural crest cells are "prepatterned" prior to their migration. However, results of these transplantations and several subsequent studies indicate that spatial programming involving cranial neural crest cells is a much more complex process. Among Noden's transplants were many in which donor cells would normally have formed maxillary or frontonasal structures. Yet, the outcome in all these was strikingly similar: formation of a mandibular arch skeleton (squamosal-quadrate-pterygoid-proximal mandible). Clearly then, neural crest cells are bringing a response bias to each peripheral location, but one that is not consistently based on the precise site of their origin.

Additionally complicating the interpretation of Noden's data was the observation that some cells derived from these neural crest transplants adopted patterns of skeletogenesis appropriate for their new locations. In each case these cells were near the perimeter of areas occupied by grafted cells. Critical analysis of controls indicated that cooperativity among neural crest populations in adjacent arches is normal; individual skeletal elements such as the basihyoid cartilage and articular bone are formed by progenitor populations that arise at different axial levels.[32,89,90]

Much work has been done to explore the interactions through which neural crest cells acquire their patterning biases. For example, Le Douarin and coworkers found that contacts with neuroepithelium during the emigration process are important.[140] Not surprisingly, signals involved in establishing regional identities within the developing brain also affect prospective neural crest cells. Neuroepithelial cells at the midbrain-hindbrain boundary (isthmus) produce Fibroblast Growth Factor 8 (FGF8), which activates in neighboring cells a cascade of gene activities that collectively are necessary to specify the formation of the cerebellum, caudally, and the colliculi, rostrally.[141,142] Trainor and his colleagues[143] recently demonstrated that this isthmic signaling center also provides positional cues to nearby neural crest progenitors, which could explain how many of the neural crest progenitor populations grafted by Noden and Hörstadius acquired mandibular arch patterning information.

Some of the antecedents to early patterning of neural crest populations have been revealed through analyses of *Hox* genes. Unique combinations of these regulatory genes are expressed among different hindbrain rhombomeres and their associated neural crest cells.[144] The rostral limit of *Hox* gene expression is rhombomere 3, thus neural crest cells that populate the mandibular arch arise in a *Hox*-free zone and do not activate members of this regulatory

network. In transgenic mice partially lacking expression of the hyoid arch-specific *Hox* gene code (i.e., *Hoxa2* mutants), neural crest cells form mandibular skeletal structures in place of hyoid arch elements,[145,146] which is similar to the transplantation-induced phenotypes (with the caveat that these skeletal arrays had reversed rostro-caudal orientation). In converse experiments, forcing expression of *Hoxa2* in mandibular arch neural crest precursors results in the formation of hyoid rather than mandibular skeletal structures.[147,148] Interestingly, expression of *Hoxa2* is downregulated by FGF8, which when expressed ectopically in the hindbrain similarly disrupts development of hyoid arch structures.[149]

A large number of studies have identified signals emanating from tissues encountered by neural crest cells en route to or within the oropharyngeal arches. Interactions with paraxial mesoderm,[144] pharyngeal endoderm,[150-152] and both neural and surface ectoderm[88,153] all modify the execution of prior specifications and are necessary to drive histogenesis and morphogenesis of skeletal tissues. In some cases, paraxial mesoderm acts as an intermediary agent in this signaling.[154] These interactions occur on the way to as well as at the terminal sites of neural crest cell differentiation. Extirpation and transplantation experiments revealed that both pharyngeal endoderm and surface ectoderm provide important skeletogenic signals.[2,150,155] Signals from pharyngeal endoderm, especially FGFs and Sonic Hedgehog, are positive regulators of chondrogenesis in pharyngeal neural crest cells.[156-158] These studies strongly suggest a broader role for epithelia such as pharyngeal endoderm during arch morphogenesis.[151] Graham (2004)[159] has proposed that this deep epithelium is a dominant source of oropharyngeal arch patterning information.

In contrast to the oropharyngeal arches, in which the positional history of neural crest cells is an essential feature of their morphogenesis, the site of origin of frontonasal neural crest populations is not a major morphogenetic influence.[89] Here, local signals emanating from the forebrain and facial ectoderm are essential to patterning of the region.[117,119,120,160,161] This does not in any way lessen the importance of genetic-based responses within the mesenchyme for establishing pattern since other research indicates that differential domains of gene expression in the facial mesenchyme correlate with species-specific variations in the size and shape of beaks among various bird embryos including Darwin's finches.[162,163]

In fact, the extent to which neural crest cells bring essential intrinsic biases to their local developmental environment has been made most apparent by exploiting species-specific differences in surgically-created chimeric embryos. Classical neural crest transplant experiments involving salamanders and frogs showed that patterning the jaws and teeth is largely driven by genetic-based response properties within neural crest populations.[164,165] Similar tactics have been taken with divergent species of birds.[166-170] Schneider and coworkers grafted neural crest progenitors between quail and duck embryos in order to capitalize on three unique features that set these species of birds apart. Quail cells can be detected by using a ubiquitous nuclear marker not present in the duck. Also, quail and duck embryos have morphologically distinct beak and head feather patterns, which permit assays of donor and host contributions to both differentiation and morphogenesis.[171] Finally, quail and duck embryos develop at different rates. Changes to the timing of tissue interactions can therefore be assessed.

These quail-duck transplants revealed that neural crest cells provide species-specific information for patterning the beak and the cranial feathers. When transplanted into duck embryos, quail neural crest cells gave rise to beaks and feathers like those found in quail, specifically by establishing the pattern of their own derivatives as well as those of the host. Reciprocal transplantations of duck neural crest into quail hosts produced analogous alterations. Molecular analyses demonstrated that neural crest mesenchyme is able to bring about species-specific morphology by dominating the initial interactions with overlying epithelium, and in particular by regulating the mesenchymal and epithelial expression of genes known to affect patterning of the beak and feathers.[168,170] Both transcription factors and secreted molecules exhibited temporal shifts in the initiation of their expression consistent with differences in maturation rates between donor and host cells, providing evidence that quail donor neural crest cells created

quail-like morphology on duck hosts by maintaining their own molecular programs and by altering patterns of gene expression in nonneural crest derived host tissues.

What becomes evident after a survey of experimental data on the neural crest and their neighbors is that a continuous and dynamic dialogue exists among multiple embryonic tissues, which in turn mediates craniofacial morphogenesis. Changes to this dialogue on the molecular or cellular level can alter three-dimensional pattern in astounding ways. At least initially, cranial neural crest cells have the potential to form any other structure within their portfolio of derivatives, both histogenetically and morphogenetically. This seems especially true for the most rostral populations, which when rotated 180° to transpose frontonasal and mandibular neural crest progenitors, can still produce normal facial and jaw skeletons.[89] Also confirming the high degree of plasticity and responsiveness inherent within neural crest mesenchyme are loss-of-function experiments in certain murine Dlx genes, which transform the mandibular primordia into the maxillary primordia.[172] Likewise, neural crest cells can be reprogrammed in avian embryos exposed to retinoic acid and the BMP antagonist Noggin, which changes the maxillary primordia into a frontonasal process.[160] Taken together, such results reveal that neural crest cells contain within them intrinsic programmatic modules, which can be activated in a site-specific manner via cues from adjacent tissues and signaling centers.

Conclusion

> *"What role these cells play in the formation of later tissues I do not know, nor do I know what becomes of the "lost" portions of the neural crest which lie between the spinal ganglia, but it has become evident that the whole question of the nature of "mesoderm" in Vertebrates needs revision founded on fact rather than theory."*
>
> —J.B. Platt, 1893[55]

Observational and experimental data on the neural crest gathered during the last two centuries have ignited many heated deliberations about the developmental programs of vertebrates. The discovery of neural crest cells deposed what Hall (1999) called "entrenched notions of germ-layer specificity and the germ-layer theory, a theory that placed a straightjacket around embryology and evolution for almost a century" (p. v).[2] With the luxury of a modern retrospective, the neural crest now seems to be not at all recalcitrant but rather quite a unifying force in the community of plan for craniofacial morphogenesis. Despite the intentions of the original theory, each of the germ layers does not exist in isolation but instead coexists inclusively with neural crest mesenchyme mediating many of their reciprocal interactions. As numerous recent molecular and cellular analyses reveal, the cranial neural crest especially, can be seen as a microcosm for the inherent capabilities and functionalities of ectoderm, mesoderm, and endoderm, by moving so deftly between a totality of histological states and morphological conditions.

In a similar sense, the neural crest serves to coalesce disparate concepts on the basic organization and patterning of the vertebrate head. Through their various movements and dispersions, cranial neural crest cells integrate the community of plan among neural ectoderm, paraxial mesoderm, pharyngeal endoderm, and superficial ectoderm. Patterning of craniofacial connective tissues and all of those structures dependent upon them results from a consortium of influences, with no constituent fully naïve nor totally autocratic. While developmental origins may be critical for guiding oropharyngeal neural crest, patterning of frontonasal and calvarial populations appears predominantly driven by local epithelial-mesenchymal signaling interactions. Many of the molecules that mediate these tissue interactions and regulate differentiation and patterning of the various head regions continue to be elucidated, and tools for experimentally creating quantitative changes to the timing or intensity of signal-response networks are becoming more readily available. Such advances will likely enable direct assaults on heretofore-intractable problems of craniofacial morphogenesis, particularly in mid- and lower facial areas where on the one hand, some of the greatest evolutionary variations have occurred (e.g., quail versus duck or pug versus borzoi), and, on the other hand, minor disruptions can often have the most severe consequences in the form of birth defects that negatively affect human health.

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CHAPTER 2

Neural Crest Inducing Signals

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Abstract

The formation of the neural crest has been traditionally considered a classic example of secondary induction, where signals form one tissue elicit a response in a competent responding tissue. Interactions of the neural plate with paraxial mesoderm or nonneural ectoderm can generate neural crest. Several signaling pathways converge at the border between neural and nonneural ectoderm where the neural crest will form. Among the molecules identified in this process are members of the BMP, Wnt, FGF and Notch signaling pathways. The concerted action of these signals and their downstream targets will define the identity of the neural crest.

Introduction

The neural crest is a transient population of embryonic cells that originate at the border between the neural plate and the prospective epidermis. Around the time of neural tube closure, neural crest cells undergo an epithelial to mesenchymal transition and initiate extensive migrations throughout the embryo. Shortly after migration to their final position, neural crest cells differentiate to form a wealth of derivatives. The mechanisms of migration and differentiation of neural crest have been studied extensively.[1,2] In contrast, little is known about the embryological origins of the neural crest, the signaling events that lead to their formation and the molecular nature of the interactions that generate them.

Formation of neural crest has traditionally been considered a classic example of secondary induction where signals from one tissue elicit differentiation in a competent, responding tissue. This assumption was largely based on the observation that neural crest can be generated de novo by the juxtaposition of epidermis with "naïve" regions of the neural plate or paraxial mesoderm both in vivo and in vitro.[3-6] Interestingly, some of these experiments have shown that both neural plate and epidermis can generate neural crest when combined, suggesting the existence of bidirectional inductive events.

Over the past decade, researchers have been studying the nature of the tissue interactions and molecular signals involved in neural crest induction. Current evidence suggests that both the ectoderm and mesoderm can contribute inductive signals.[5-8] TGF-β, FGF, Wnt and Notch signaling pathways have all been shown to play an essential role in this process. The convergence of these signals at the border between the neural and non neural ectoderm apparently triggers transcriptional events that lead to neural crest specification. The origin and nature of these signals, as well as the evidence for their role in neural crest formation, are described below.

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Signals from the Ectoderm

The localization of neural crest precursors at the border between neural plate and epidermis suggests a potential role for interactions between these two tissues in induction of the neural crest. The grafting experiments of Rollhäuser-ter Horst in amphibians showed that gastrula ectoderm generated both neural and neural crest cells when grafted to the neural folds.[9,10] The juxtaposition of these tissues Axolotl embryos, generated neural crest at the newly formed border.[3] By grafting tissues in from pigmented embryos into albino hosts, they observed that the newly induced neural crest cells originated from both the neural plate and the epidermis. Interestingly, while tissue from the neural plate formed mostly melanocytes, the epidermis tissue formed spinal and cranial ganglia. The role of neural plate and epidermal interactions in neural crest induction was later confirmed in vivo in other organisms by analogous transplantation experiments in chick, fish and *Xenopus* embryos, with essentially similar results.[5,6,11] In vitro cocultures of epidermis and neural plate tissue from both chicken and frog embryos showed that the interactions between these two tissues are sufficient to generate neural crest.[4,6,12] However, the competence of the chick neural plate to respond to signals from the ectoderm is lost by 1.5 days of development (stage 11 HH staging according to Hamburger and Hamilton),[13] suggesting that inductive interactions that lead to neural crest formation may be temporally limited in the chick embryo.[14]

Signals from the Mesoderm

One of the first experiments to address the issue of neural crest induction showed that portions of the archenteron roof of amphibian gastrulae had the capacity to induce neural tissue and neural crest when grafted into the blastocoel of a host embryo. If the grafted tissue was derived from lateral archenteron, only neural crest was induced in the host ectoderm. These experiments led to the proposal that a graded signal from the mesoderm was responsible for neural crest induction.[15] The ability of paraxial mesoderm to induce neural crest was confirmed later by recombination experiments in vitro, both in amphibians[16] and in chick embryos.[17] Amphibian embryos with surgically removed paraxial mesoderm failed to form normal neural crest derivatives, suggesting that signals from the mesoderm are required for neural crest induction.[18] Furthermore, it has been shown that chick paraxial mesoderm can induce expression of Pax-3, an early marker of the neural plate border, when combined with either chick neural plate or 'neuralized' *Xenopus* animal caps (explants of naïve ectodermal tissue that have been exposed to neuralizing molecules).[19] However, recent findings in zebrafish embryos suggest that signals from the mesoderm are dispensable for neural crest formation.[20] Zebrafish mutants in which mesoderm formation was blocked expressed normal neural crest markers and formed neural crest derivatives. The authors proposed that in the absence of mesoderm, the ectoderm is able compensate for the missing vertical signals from the mesoderm.

The neural crest originates adjacent to three embryonic tissues, the non neural ectoderm, the neural plate and the underlying mesoderm. All these tissues are potential sources of inductive signals and their interactions are responsible for neural crest formation. Many of the experiments presented above are based on tissue recombinations and in vitro manipulations. While these experiments have proved extremely useful in testing the necessityof tissue interactions and have helped identify candidate molecules involved in neural crest induction, they may not reflect the complexity of the interactions that take place in vivo.

BMPs

Dorsalin-1, a TGF-β family member, is present in the dorsal neural tube. Purified dorsalin-1 added to explants of intermediate neural plate (portions of the neural plate between the ventral midline and the neural folds) is sufficient to induce migratory neural crest cells.[21] Such intermediate neural plate tissue is considered naïve in the sense that it has not received signals to specify it as dorsal or ventral. Although dorsalin-1 is expressed only transiently after neural tube closure, this observation suggested that other TGF-b family members expressed in the epidermis adjacent to the neural plate may also be involved in the induction of neural crest.

The first candidate molecules identified in the epidermis were BMP-4 and BMP-7. Like dorsalin-1, the addition of these molecules to intermediate neural plate explants could induce neural crest in the absence of epidermis, leading to the proposal that BMPs may be the epidermal signal responsible for neural crest induction.[4] At early stages of development (stages 4 and 5 HH), BMP-4 is expressed in the prospective epidermis of the chick epiblast and it is absent from the future neural plate. This expression pattern is consistent with a role in neural crest induction. However, BMP-4 soaked beads implanted in the prospective neural plate at these stages cannot prevent neural fate.[22] Furthermore, BMP-4 expression is downregulated in the epidermis adjacent to the closing neural folds and, instead, becomes strongly expressed on the neural folds themselves. This expression pattern may suggest a role for BMP-4 in the maintenance rather than the initial induction of neural crest. Consistent with this view, cells expressing Noggin, a BMP antagonist, can prevent expression of neural crest markers when injected in the closing neural tube, but not when implanted next to the open neural folds, at a time when neural crest induction is still taking place.[23] In addition, they block neural crest emigration from the neural tube.[24]

Mutations in different members of the BMP family and their antagonists in mice suggest that these molecules are not absolutely required for neural crest formation. Embryos carrying a homozygous BMP-4 mutation usually die around gastrulation. However, embryos that survive until neural fold stages do have some neural crest derivatives.[25] BMP-7 homozygous null mice present some craniofacial skeletal defects but they are more likely related to bone formation rather than neural crest.[26] In BMP-5 and BMP-7 double mutants, neural crest cells are able to form and migrate normally. In vitro assays culturing neural tubes from these mice yielded neural crest that were indistinguishable from controls.[27] Mice carrying homozygous mutations for the BMP antagonists Noggin[28] or follistatin[29] do not exhibit defects in neural crest formation. While the normal expression pattern of BMPs could not account for the possibility of functional redundancy,[22] it is possible that in these mutants the expression of the other BMP genes is altered leading to ectopic function. However, we cannot rule out the possibility that other unidentified molecules (e.g., other TGF-β family members) stimulate BMP-like signals that could account for the effects described above.

Evidence for the requirement of epidermal BMP signaling in neural crest induction is more compelling in other vertebrates than in amniotes. Inhibition of BMP signaling by injection of a dominant negative BMP receptor, or the antagonists Noggin or chordin into the one cell frog embryo results in expression of neural crest markers analyzed in explanted animal caps.[8,16] The attenuation of BMP signaling elicits the expression of neural crest markers in a dose-dependent fashion. The levels of BMP activity required to induce neural crest are intermediate between those required to specify ectoderm and neural plate. These findings led to the proposal of a model in which the different fates of the ectoderm derivatives are specified by a gradient of BMP activity.[16] Interestingly, over-expression of BMP-4 in *Xenopus* embryos is not sufficient to expand the expression domain of the neural crest marker slug, and while certain concentrations of chordin mRNA injection can induce expression of neural crest markers in animal caps, this expression was found to be weak compared to endogenous levels in the embryo. A much more robust induction occurred when inhibition of BMP signaling was accompanied by exposure to Wnts or FGFs.[8,30] Taken together these data suggest that other signals are required in addition to BMPs in order to induce neural crest.

Genetic analysis of several mutations of the BMP signaling pathway identified in zebrafish embryos also suggests an important role in for these molecules in neural crest induction. *Swirl* (bmp2b), *snailhause* (bmp7) and *somitabun* (Smad5) mutants all display a great reduction in neural crest at trunk levels.[31-33] A direct effect of the BMP pathway on neural crest development is hard to infer from these mutants because the defects associated with these mutations are not exclusively neural crest related, and rather affect the main axis of the embryos, Interestingly, zebrafish bmp2b is functionally more similar to *Xenopus* BMP-4 than

zebrafish bmp4.[34] The neural crest deficiencies observed in these mutants together with bmp2b and bmp7 expression patterns in the fish gastrulae are consistent with the BMP gradient model proposed for neural induction.[31] However, the BMP gradient may simply set the position of the neural plate border rather than directly inducing neural crest cells.

Wnts

Several lines of evidence suggest that members of the Wnt (wingless/INT) family of secreted glycoproteins can act as neural crest inducers.[8,35-37] Both Wnt-1 and Wnt-3a are expressed in the dorsal neural tube. Mice carrying a mutation in both Wnt-1 and Wnt-3a genes exhibit a significant reduction in the number of melanocytes and cranial and spinal sensory neurons as well as deficits in skeletal structures derived from cranial neural crest.[38] However, some neural crest cells form in these animals, suggesting that these molecules may influence later events such as proliferation rather than the initial formation of neural crest. Furthermore, it has been shown that neural crest arise in vitro in the absence of Wnt1 and Wnt3a.[12] Recent evidence suggest that Wnt signals in the dorsal neural tube are critical for neural crest cell differentiation into sensory ganglia.[39] Thus, Wnt signals in the ectoderm other than Wnt1 and Wnt3a may be responsible for early steps in neural crest formation.

Wnt family members are strong inducers of neural crest markers when injected in neuralized animal caps. Over-expression of either Wnt-1 or Wnt-3a in whole embryos leads to an expansion in the neural crest domain and production of supernumerary neural crest cells.[35] Because Wnt signaling can result in cell proliferation,[40] the authors repeated the experiment blocking cell proliferation at gastrula stages and obtained the same results. These data suggest a direct effect of Wnts on neural crest induction, perhaps at the expense of other ectodermal tissues. Similar experiments have shown that Wnt-7B and Wnt-8 can induce neural crest in ectodermal tissue that has been neuralized by noggin or chordin.[8,36,41]

Experiments in chick embryos have shown that Wnt signals are necessary for neural crest formation. Injection of cells expressing a dominant negative Wnt1 construct adjacent to the neural folds blocks expression of the neural crest marker *Slug*. Moreover, addition of soluble Wnts to intermediate neural plate explants generated migratory neural crest cells, suggesting that Wnt signals are sufficient to induce neural crest. The generation of neural crest cells in vitro occurs in a defined minimum medium lacking additives. In contrast, BMP-4 was unable to induce neural crest in these explants in defined medium without additives, suggesting that its effects might be the result of synergistic actions with other signaling molecules. In chick, Wnt6 is expressed in the ectoderm at the correct time and place to be involved in neural crest induction. Taken together, these data suggest that Wnt is an epidermal inducer of neural crest in chick embryos.[37] Similar experiments in zebrafish confirm the requirement for Wnts in neural crest induction.[42] Using a transgenic zebrafish line that expressed an inducible inhibitor of the canonical Wnt pathway, the authors defined a critical period for Wnt signaling in the induction of neural crest. However, this approach globally eliminated Wnt signaling in the whole embryo and although in a stage controlled manner, Wnt signals are important for other developmental processes that may have an indirect effect on neural crest formation. Wnt8 is expressed adjacent to the prospective neural plate, and thus is a good candidate for the neural crest inducer in zebrafish. Consistent with this idea, blocking Wnt8 function by injection of antisense morpholino oligonucleotides resulted in the loss of early neural crest markers.

In addition to their epidermal expression, members of the Wnt family are expressed throughout the embryo in domains that are compatible with a role in neural crest induction, including the dorsal neural tube[35] and the paraxial mesoderm.[41] In fact, the ability of paraxial mesoderm to induce neural crest markers in neuralized animal caps is lost in the presence of a dominant negative form of Wnt8, suggesting that Wnt signaling mediates the inducing ability of paraxial mesoderm.[7,8,41]

FGFs

A recent study proposed that a member of the fibroblast growth factor (FGF) family, FGF-8, mediates the inductive effects of paraxial mesoderm on frog animal cap essays and that it is sufficient to induce the transient expression of several neural crest markers.[7] A requirement for FGF signaling in neural crest induction had been observed previously in an experiment where injection of a dominant negative FGF receptor prevented expression of neural crest markers.[30] In a subsequent study it was shown that FGFs ability to induce neural crest in frog embryos was dependent on Wnt signaling.[8]

The involvement of Wnts and FGFs in neural crest induction is consistent with previous observations that this process requires posteriorizing signals, at least in amphibians.[43] Interestingly, recombinants of Hensen's node and neuralized animal caps can induce expression of early border markers even in the absence of FGF, Wnt or retinoic acid signaling, suggesting that the node is also a source of a yet unidentified signal that has the capacity to induce neural crest.[41]

Notch

Experiments in chick, frog and zebrafish suggest a role for Notch signaling in neural crest induction.[44-48] In chick embryos, Notch ligands are expressed in non neural ectoderm adjacent to the Notch expression domain in the neural plate. Over-expression of an activated form of Notch results in down-regulation of the neural crest marker *Slug* and a reduction in the number of HNK-1+ migratory neural crest cells in the head region.[46] Surprisingly, inhibition of Notch signaling through electroporation of a dominant negative form of the Notch ligand Delta (Delta^{stu}) had the same effect. Gain and loss of function experiments negatively modulated the levels of BMP-4 in the neural plate border region. Over-expression of a BMP-4 construct was able to rescue the loss of Slug after the Delta^{stu} electroporation but not after Notch activation. The authors proposed that Notch acts upstream of BMP-4 in the specification of neural crest, but that loss of neural crest by Notch activation is independent of BMP-4. In addition, the effect of Notch on BMP-4 transcript levels was mediated by Deltex, (another component of the Notch pathway), in a non canonical manner independent of suppressor of hairless. These experiments suggest that Notch signaling could be acting on neural crest formation through parallel pathways at different times.[45] Because both activation and inhibition of Notch signaling result in the loss of neural crest markers, the authors conclude that a threshold level of Notch activation is required in order to achieve BMP-4 expression and subsequently proper neural crest specification. Similar experiments in Xenopus also revealed somewhat different results. In frogs, activation of the Notch pathway leads to a decrease in the levels of BMP-4 and subsequently to an expansion of neural crest markers.[44] Inhibiting the pathway results in an expansion of BMP-4 expression and a reduction in neural crest markers. Over-expression of hairy2, a downstream target of Notch, has the same effect as activating the Notch pathway, suggesting that the effects of Notch on BMP-4 levels and neural crest induction are mediated by this bHLH transcription factor (Glavic et al 2004). The apparent discrepancies between chick and frog could be explained by different requirements of BMP-4 at the border between neural plate and non neural ectoderm for neural crest induction, the timing at which the manipulations were performed, or both. In zebrafish, the evidence for a role of Notch in neural crest formation comes from the analysis of mutants. Fish carrying a mutation in the deltaA gene (*dlAdx2*) generate an excess of Rohon-Beard sensory neurons in the trunk at the expense of neural crest cells. These findings suggest a role for Notch in fate decisions amongst common precursors of these two cell types.[48] Interestingly, depletion of neurogenin-1 in deltaA mutants restores expression of neural crest markers in the trunk, suggesting that the main function of Notch is to repress the sensory neuron fate rather than specifying neural crest fate.[47] It is worth noting that in contrast to chick and frog, Notch signaling in zebrafish has little effect on cranial neural crest and the major effects were observed in the trunk region. This observation could have evolutionary implications regarding the origins of the neural crest at different rostrocaudal levels, and the conservation of the inducing signals.

Neural Crest Induction Is a Multistep Process

Neural crest induction in *Xenopus* requires inhibition of BMP signaling to set the epidermal, neural and border fates within the ectoderm. The ectoderm at the border between epidermis and neural plate is then competent to respond to a second signal that enhances and maintains neural crest induction. Both Wnt and FGF signals have been proposed to play a role in this process.[8,16,30] Induction of neural crest occurs during or shortly after neural induction and the formation of the neural plate. In chick embryos, there is also evidence pointing to the existence of several steps in the induction of neural crest. In vivo and in vitro experiments have shown that neural crest formation has temporally distinct periods of sensitivity to the BMP antagonist Noggin. Addition of Noggin prevents specification of neural crest when added to neural folds of the closing neural tube, but not when added to neural folds at the level of the open neural plate of stage 10 HH chick embryos.[23] This result suggests that BMP signals are required for the maintenance of specified neural crest. In addition, isolated caudal neural folds of stage 10HH embryos begin to express Slug after 18 hours in culture in the absence of any further signals, suggesting that neural crest cells are specified long before the expression of specific markers. However, this expression is transient.[14] Taken together, these data suggest that neural crest induction requires at least an initial specification event and subsequently, the sustained action of further signals for its maintenance.

Conclusions

From the data presented above, it is clear that interactions between the epidermal ectoderm and/or mesoderm with the neural plate can generate neural crest. Members of the wingless/INT (Wnt), bone morphogenetic proteins (BMPs), Notch and fibroblast growth factors (FGF) families have been shown to participate in the process of neural crest induction. The data gathered from different organisms is still not enough to propose a unified model for neural crest induction. Even though all vertebrates seem to use the same set of signals for neural crest formation, the precise hierarchy and timing in which these signals are received by the responding tissues seems to differ slightly in the different model organisms. These discrepancies however, may just reflect the differences in the experimental approaches used to study each organism, rather than an intrinsic difference in the process of neural crest induction. From experiments in chick, we know that the induction of neural crest is a continuous process that can be disrupted at several points in time by manipulating some of these signaling pathways. In frogs, analyses of neural crest induction are largely based on the expression of early neural crest markers, an event that is a consequence of the induction itself.

We can distinguish at least two steps in the process of neural crest induction. First, a region of the ectoderm has to receive instructive signals to become specified as neural crest precursors. Second, these neural crest precursors need to receive further signals that will allow them to maintain their identity in the developing embryo. The concerted action of multiple signaling pathways at the border between neural and nonneural ectoderm defines the domain from which the neural crest will form. Ultimately, the identity of the neural crest will be established by the combination of the downstream targets of the converging signals in this territory.

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CHAPTER 3

Transcriptional Regulation at the Neural Plate Border

Thomas D. Sargent*

Introduction

The neural crest (NC) is usually defined as a cell type arising at the border of the neural plate and the epidermis in vertebrate embryos. While accurate, this definition implies that the border exists as a distinct boundary, which is not really the case. Like other domains in the early embryo, the NC is not a sharply delimited territory, but rather is an overlapping zone of specification that has characteristics of both epidermis and neural plate, with additional characteristics of its own. This can be seen in the spatial pattern of regulatory factors that have been implicated in NC induction, many of which are shared.[1] For example Msx1 and AP2 are also expressed in the epidermis, at lower levels, and c-myc is likewise transcribed in neural plate in addition to NC. Nor is this limited to regulatory factors. The epidermal keratins, which constitute the intermediate filament cytoskeleton of epidermal cells are also expressed in NC, at a lower level than the epidermis. This can be seen by in situ hybridization (Fig. 1A) as a region of relatively weak but significant signal in the cranial neural crest region. Another indication of the fuzzy nature of NC comes from lineage mapping experiments in the chick embryo, which show that cells fated to differentiate as NC, neural plate, epidermis and placodal derivatives are all intermingled in the neural-epidermal boundary region.[2] Nor is there always a clear gap visible between neural plate and epidermal gene expression domains, for example in a double in situ with epidermal keratin and the pan-neural marker NCAM the two domains are contiguous (Fig. 1B). Expression of keratin genes is also evident in animal caps that have been dissected from embryos injected with BMP antagonists, such as chordin, along with a canonical Wnt signal molecule, such as Wnt3a. This strongly induces NC gene expression, but at the same time induces keratin gene expression, again to a level lower than that of epidermis, but much higher than the background seen in animal caps from embryos injected with BMP antagonist alone (Fig. 1C). The point of this discussion is that the premigratory NC is a somewhat ambiguously defined cell type, comprising overlapping and intermixed domains of gene expression, morphology and developmental fate. This should be kept in mind when thinking about the genes that control the NC program, i.e., regulatory mechanism giving rise to complex, overlapping and transient cellular identities will likely reflect this complexity. Indeed, many regulatory factors have been associated with NC,[1] a list that continues to grow. Even organizing the undoubtedly incomplete roster into a regulatory flow diagram results in a complex picture.[3] This review will focus primarily on two sets of transcription factors implicated in the earliest stages of NC induction, the Msx/Dlx and TFAP2 families.

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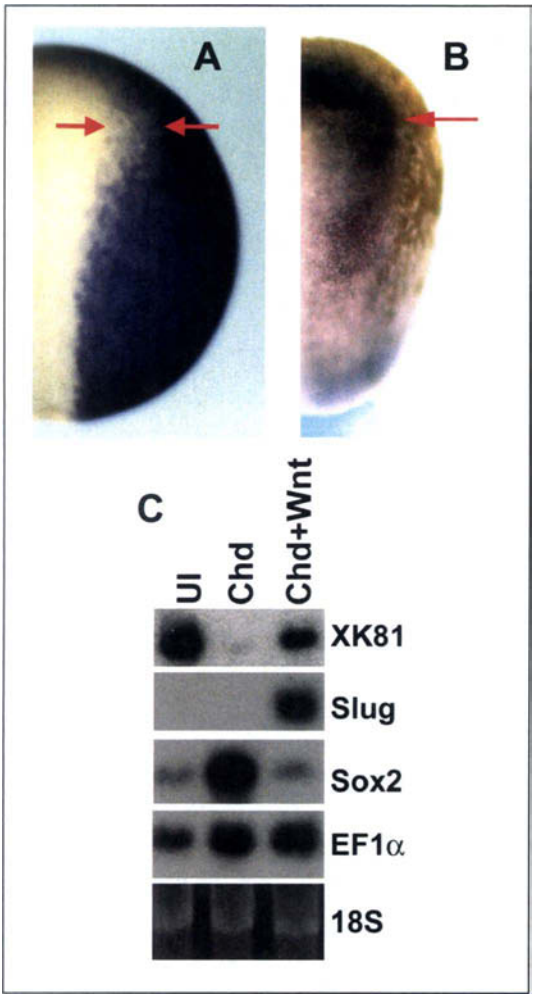

Figure 1. Overlap of epidermis and neural crest. A) Whole mount in situ hybridization to stage 14 (open neural plate) Xenopus embryo with an epidermal keratin probe (XK81). The arrows indicate the cranial NC region, where XK81 hybridization signal is clearly visible, although at a lower level than in the epidermis. B) Double in situ hybridization of similar staged embryo to neural plate (NCAM; purple color) and epidermal (XK81; brownish color) probes. The arrow indicates the cranial NC region, where no gap can be discerned between the epidermal and neural plate domains. Dorsal views, anterior towards top of figure. Panel B reproduced with permission from reference 61 and The Company of Biologists, Ltd. C) Northern blot analysis of an animal cap induction experiment. Embryos were injected with 250 pg of RNA encoding chordin (Chd) or a mixture of 250 pg each chordin and Wnt3a RNA (Chd+Wnt). Animal caps cultured to stage 14 show neuralization by Chd (Sox2 probe) and complete repression of epidermis (XK81 probe). NC is strongly activated by the chordin + Wnt3a treatment (Slug probe), which represses neural plate but also significantly reactivates the epidermal marker. EF1alpha and 18S RNA signals are shown as loading controls.

Dlx and Msx Genes

Dlx factors are homeodomain proteins, related to the Drosophila Distal-less (Dll) gene.[4] They behave as transcriptional activators, at least in coexpression assays.[5,6] In mouse and

human there are six Dlx genes, present in three pairs of convergently transcribed genes, each linked to a separate Hox cluster.[7] Only four Dlx genes have been described to date in Xenopus, of these three are known to be transcribed at the mid/late gastrula stage when NC is induced, these are Dlx3, Dlx5 and Dlx6.[8,9] Two other Dlx genes, Dlx1 and Dlx2, are also expressed in Xenopus NC, but later in development.[10] There are eight Dlx genes in zebrafish, with Dlx3b and Dlx4b representing the fish orthologs of the linked pair, Dlx3 and Dlx4, respectively.[11] There is no evidence for the existence of a Dlx4 ortholog in Xenopus either in EST databases or in the Xenopus tropicalis genome, which has been largely sequenced (http://genome.jgi-psf.org/Xentr3/Xentr3.home.html), so perhaps Dlx4 is absent in amphibian genomes. In mouse there are three Msx genes. These are also homeodomain factors, related to the Drosophila muscle segment homeodomain (msh) gene.[5] The Dlx and Msx homeodomains are quite similar, and bind to essentially identical DNA sequences in vitro.[6] Msx factors behave as transcriptional repressors. Msx1 can repress target gene transcription by binding to the TATA binding factor TBP,[12] and also by interacting with a linker histone H1b.[13] Importantly, Dlx and Msx factors can form heterodimers, and this interaction can antagonize the mutual effects on target gene activity.[14,15] In Xenopus, Msx1 and Msx2 are expressed beginning at or shortly following the midblastula transition, as are the Dlx genes. Both gene families are expressed in ventral and lateral tissues, Msx in mesoderm and ectoderm, and Dlx in ectoderm only.[5,9] This expression is regulated by BMP signaling. Msx1 is a direct target of such signaling, via a regulatory element in the 5' flanking DNA that associates with Smads factors.[16,17] Msx2 is likewise controlled by BMP signaling, and in the case of the mouse gene, the regulator element includes binding sites for both a Smads factor and an additional unknown protein that probably confers additional target specificity.[18] In contrast to Msx, the Dlx factors are not direct targets of the BMP pathway, requiring protein synthesis following BMP exposure.[6] In the ventral ectoderm, there is a graded dependence of Msx1, Dlx3 and Dlx5/6 on BMP signaling, which can be visualized by treating with increasing dosages of a BMP antagonist such as chordin. The lateral boundaries of Dlx3 and Dlx5/6 also differ, and this is consistent with the notion that a BMP signaling is locally weaker near the neural plate boundary compared to more laterally.[8]

There are several lines of evidence suggesting that Dlx and Msx play important roles in patterning the neural-epidermal border, including the NC. Before discussing these experiments, it is worth reviewing the tools that have been used in them. Gain of function has been accomplished by injecting synthetic mRNA into early cleaving embryos, which results in ectopic protein synthesis. For loss of function, two methods have been successfully employed; antisense and dominant negative overexpression. Most antisense work has been carried out with oligonucleotides based on morpholino backbone chemistry, which yields stable molecules that can base pair with target RNAs and either inhibit translation initiation or intron splicing. Dominant negative interference has been based on the concept that as transcriptional activators, Dlx factors can be antagonized by overexpressing derivatives in which the DNA binding domain has been fused to a transcriptional repressor, such as the repression domain from the Drosophila engrailed protein.[19] These experimental approaches can be quite informative, but they also have disadvantages and concomitant interpretational caveats. In the case of factor overexpression, the most obvious potential problem is that the level of the expressed protein is usually not known, and could be considerably higher than endogenous protein concentrations. This might lead to spurious interactions with genes or other factors, leading to regulatory artifacts. This is especially relevant to Dlx and Msx genes due to the similar DNA binding properties and opposite effects on target gene transcription. An illustrative example of this problem can be seen in the conclusion that Dlx5 functions in setting up the dorsoventral axis in Xenopus,[20] based on ectopic overexpression. Since Dlx5 is not expressed in mesoderm,[9] which is where the axial specification takes place, the ventralizing effect on this tissue is presumably an artifact.

Xenopus Dlx3 is excluded from the presumptive cranial neural crest domain, and when Dlx3 mRNA is delivered to this area by microinjection, repression of slug and other NC

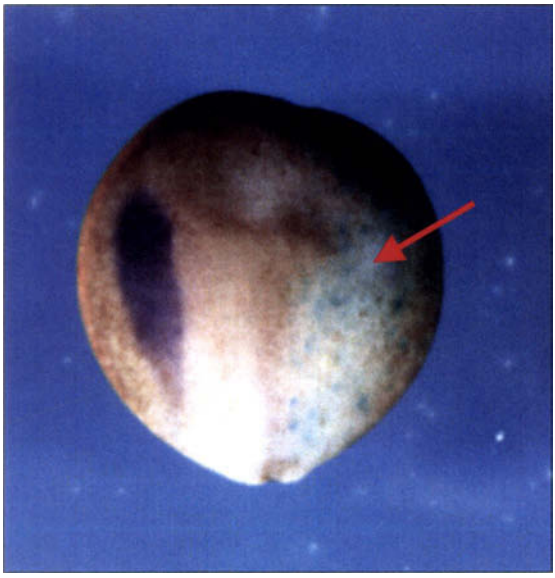

Figure 2. Repression of NC by truncated Dlx3. Stage 14 Xenopus embryo injected into one dorsal ectodemal cell at the eight-cell stage with 100 pg RNA encoding Xenopus Dlx3 that has been modified to delete amino acids 1-43 at the N-terminus and 200-277 at the C-terminus, leaving the homeodomain and about 60 residues N-terminal to it, and removing most of the transcriptional activation function of the Dlx3 protein.[6] LacZ RNA was also injected as a linear tracer (blue color). Hybridization with NC marker Slug shows complete inhibition on the injected side (arrow). Dorsal view, anterior towards top of figure. D. Khadka and T. Sargent, unpublished data.

markers is observed.[9] Dlx3 overexpression also inhibits expression of neural plate marker genes.[21] Dlx3 overexpression also antagonizes Wnt-beta catenin signaling, and this could be the basis for the negative effect on NC.[9,22] Similar results have been obtained using a zebrafish Dlx3 homeodomain fused to a VP16 activation domain, suggesting that this effect depends on target gene activation.[23] However, the inhibitory effect on NC is also observed with a truncated Dlx3 lacking the entire C-terminal and most of the N-terminal domains, a deletion construct that retains very little capacity to upregulate an artificial target gene[6] (Fig. 2). This makes it less likely that Dlx3 inhibits NC induction via a conventional transcriptional activation mechanism. Could Dlx3 be acting as an antagonist for Msx1 by competing for DNA binding, or interfering with Msx factors by protein-protein interactions? This model would predict a similar inhibitory effect for Dlx5, which is not what has been observed.[9] Furthermore, morpholino knockdown of Dlx3 alone does not seem to have much effect on the neural crest boundary, although there is a transient loss of some otic placode gene expression in Xenopus.[24] A similar effect on otic placode has also been observed in the zebrafish using morpholinos targeting Dlx3 and the linked Dlx7 genes,[25] with no obvious effect on neural crest boundaries. These results suggest that Dlx3 plays a role in ear development, but if this factor also functions in controlling the lateral limit of NC, there must be an alternate or alternates that are fully redundant. Dlx5 is not a good candidate in Xenopus, as its expression domain differs from that of Dlx3 in the NC. Another variation on the reduncancy theme is a model in which absence of Dlx3 from the NC domain is a permissive requirement for NC induction, while the limits of the induced NC domain could be determined by instructive signals.

Dlx5 is expressed more medially and overlaps with NC in the frog embryo, although this might be a bit misleading as the Dlx5-positive cells in the NC region are mostly limited to the

outer cell layer, whereas NC regulators such as Slug are mainly expressed in the inner, sensorial layer (Fig. 3). Overexpression of Dlx5 in Xenopus has not been tested extensively, although it has been shown to have little negative effect on NC induction compared to Dlx3 in animal cap assays.[9] In the chick embryo, ectopic Dlx5 expression, achieved by electroporation, alters the neural/nonneural boundary.[26] Artinger and colleagues used a dominant negative strategy to test Dlx function in frog embryos; the homeodomains from either zebrafish Dlx3 or Xenopus Dlx5 were fused to the engrailed repressor, intended to antagonize the normal activation function of Dlx factors. These constructs also included the ligand binding domain of glucocorticoid receptor to confer inducibility by dexamethasone.[27] This is important because mis-expression of Dlx factors in mesoderm results in severe axis disruption, as noted above. As already discussed, such a strategy also is essentially a combined loss of any and all Dlx function in the target embryos and tissues. In these experiments, both Engrailed fusions yielded loss of both neural plate and neural crest markers, or lateral shift of both.[23] This question has also been recently addressed in zebrafish by this group. In these experiments, loss of function was accomplished by the use of morpholinos designed to block translation or splicing of Dlx3a and Dlx4b, which are a linked pair in zebrafish and mammalian genomes. Other Dlx genes are not expressed at the relevant stages in the fish embryo.[28] Since these two genes have been shown to have largely redundant function in ear development,[25] they were treated in tandem in this study. Loss of Dlx3a and Dlx4b resulted in elimination of Rohon-Beard cells and trigeminal ganglia, and a rather slight effect on the neural crest, significant but considerably less extensive than what was observed using the dominant negative approach in Xenopus. Similar results were obtained with an Engrailed fusion in the zebrafish embryo, however, so this difference may reflect species individuality rather than pointing to a problem with the dominant negative approach per se. It would be interesting to see the effects of gene-specific knock-downs for Dlx genes in Xenopus, but this might require targeting three factors, Dlx3, Dlx5 and Dlx6, which might exceed the limits of morpholino oligonucleotides that the frog embryo can tolerate. However in the absence of such data, the interpretation of dominant negative overexpression in Xenopus should be considered somewhat tentative. In conclusion, Dlx genes are likely to be involved in the early specification of placodes, particularly the otic vesicle, but probably play a relatively minor role in NC induction, and are more important in fine-tuning the response to signals that determine the spatial limits of NC. Other regulators operate at a more upstream level in the initial formation of NC.

Two such factors may be the Msx1 and Msx2 homeoproteins. At the end of gastrulation, Msx1 and Msx2 RNAs, both of which are transcribed in epidermis, begin to accumulate at higher levels in the neural/epidermal border region. This pattern differs from that of most NC genes in that it is as strong or even stronger in the trunk region as in the cranial domain[29] (Fig. 4). This spatial difference suggests that the NC expression of Msx genes is controlled by somewhat different signaling compared to genes like Slug that are expressed more broadly in the anterior NC but in a narrow band in the trunk region. One possible difference is that Msx genes are more dependent upon canonical Wnt signaling, which would be expected to be locally stronger in the posterior, distant from Wnt antagonists elaborated by anterior tissues.[30] Using a microarray screening procedure, Willert et al[31] determined that in human embryonic carcinoma cells Msx1 and Msx2 were direct targets of this signaling pathway, and it is conceivable that this is also the case in the Xenopus embryonic NC. At any rate, the abundance (or conspicuous absence) of any regulatory factor in an embryonic domain is good preliminary evidence for a developmental role. This has been tested using the dominant negative approach by Mayor and colleagues.[29] These experiments were carried out in a similar manner to the Dlx experiments just described, except that to generate the negative interfering form of Msx1 the N-terminal third of the protein was deleted. Fusions to activation or repressor domains were not employed. As with the previous work, fusion to the glucocorticoid receptor ligand binding domain was used to enable dexamethasone induction, to avoid the axis disruption resulting from Msx1 overexpression during gastrulation. Overexpression of

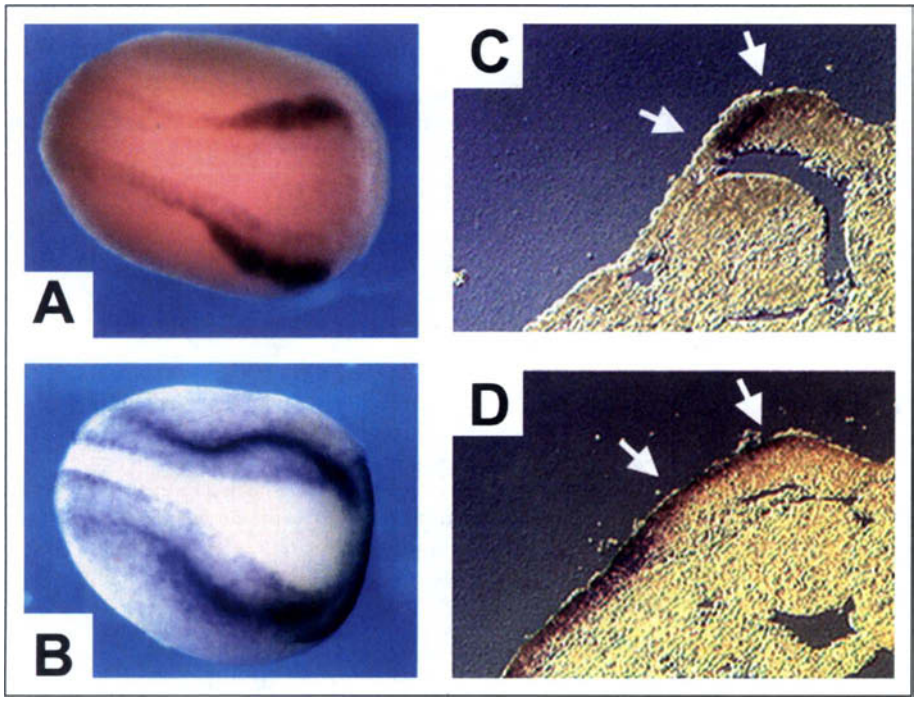

Figure 3. Deep versus superficial expression of Dlx5 and Slug genes. Panels A and B show whole mount in situ hybridizations of neurula stage Xenopus embryos to Slug and Dlx5 probes, respectively. Dorsal views, anterior to right. Transverse sections (dorsal side up) show that Slug is primarily transcribed in the deep ectodermal cells (C; arrows), whereas Dlx5 expression is restricted to the superficial cells in the NC domain (D; arrows). T. Luo and T. Sargent, unpublished data.

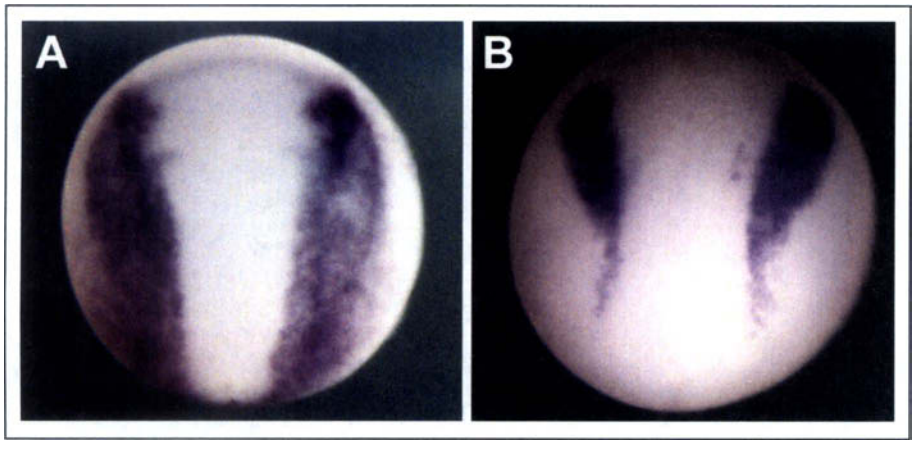

Figure 4. Comparison of Msx1 and Slug expression. Neurula stage Xenopus embryos hybridized in situ to Msx1 (A) and Slug (B) probes, showing that the Msx1 expression is much broader in the posterior region (bottom). D. Khadka and T. Sargent, unpublished data.

Msx1 in one side of Xenopus embryos after gastrulation resulted in an expansion of NC, visualized with FoxD3, slug and snail markers. This was accompanied by reduction in both epidermal (keratin XK81) and neural plate (Sox2) domains, although this effect is somewhat more difficult to appreciate due to the relatively large size of these domains compared to NC. The negative Msx1 had the opposite effect, reducing NC and expanding neural plate and epidermis. Interestingly, this loss of function phenotype could be significantly rescued by either slug or snail overexpression, while Msx1 was not able to rescue the effects of dominant negative forms of either factor. This suggests that Msx1 functions upstream from both slug and snail, possible acting as one of the first transcriptional effectors of NC induction. In subsequent work by Mayor and colleagues suggests that the transcriptional relationship between Slug and Msx ends by stage 15, after which time these two factors may act in an antagonistic manner to regulate apoptosis in NC cells.[32]

Thus we are left with the intriguing possibility that NC specification at the level of transcription is initiated by a repressor molecule. This of course raises the question of what is repressed, and how this in turn leads to the up-regulation of Snail and slug, which are also repressors. Another issue is that the use of dominant negative overexpression, as with the Dlx work, is open to the criticism regarding inappropriate targets and possible interference with other regulatory proteins. At this writing, we are completing a series of experiments on Msx1 and Msx2 loss of function using splice-inhibitory morpholino antisense oligonucleotides. These data confirm the importance of Msx genes in NC induction, although some aspects of NC seem to be independent of Msx function, as is epidermal development and dorsoventral axis specification (Khadka and Sargent, unpublished).

TFAP2

The transcription factor AP2 (TFAP2) is a family encoded by a total of five genes scattered at diverse sites in mouse and human genomes, designated alpha through epsilon, or TFAP2a, b, c, d and e. TFAP2 proteins have a basic helix-span-helix DNA binding and protein dimerization domain near the carboxy terminus. All five bind similar DNA elements with a consensus sequence of GCCNNNGGC[33] TFAP2 factors function as transcriptional activators via a proline/aromatic-rich activation domain located near the amino terminus. TFAP2 function also requires dimerization, and dominant interfering variants of TFAP2 have been generated by deletion of the activation domain, and also occur naturally, such as the variants associated with Char syndrome.[34] Members of the TFAP2 family have been implicated in many biological processes, including development and disease.[35] A number of directly regulated TFAP2 target genes have been identified, ranging in function from structural proteins such as keratin[36] to regulatory factors such as Hox genes.[37,38]

TFAP2a, the archetypical member of this family, has long been recognized as a characteristic factor expressed in neural crest.[39] Gene targeting in the mouse for TFAP2a was initially reported in 1996.[40,41] The null phenotype is a highly dysmorphic perinatal lethal. Among the most severely disrupted tissues were the facial bones, particularly the mandible, maxilla and frontonasal prominence. In addition, cranial ganglia and the heart outflow tract were abnormal. All of these derive largely from NC, supporting a role for TFAP2a in this lineage. TFAP2b and TFAP2c are also expressed in NC[35] but disruption of these genes does not have an apparent effect on NC induction or development in the mouse.[42,43]

An important aspect of the TFAP2a null phenotype is that the induction of NC and at least the early migratory processes do not seem to be affected much. Expression of Pax3 and Twist, two early NC markers, is essentially normal, and multiple aspects of NC migration can be observed.[41,44] Consequently, TFAP2a function in mouse appears to be more important in the terminal stages of NC cell differentiation.[40,41,44] Since TFAP2a is strongly expressed in NC, it might be expected that the loss of this factor would result in a largely cell-autonomous phenotype. However, this does not seem to be the entirely the case in the mouse, based on the results of a conditional TFAP2a knock out phenotype.[45] These embryos were generated by crossing a

line containing an TFAP2a allele including lox recognition sites with mice expressing Cre recombinase driven by a Wnt1 promoter,[46] resulting in ablation of TFAP2a expression in NC lineages (floxdel). Floxdel mice exhibited deficiencies in NC derivatives, reminiscent of the TFAP2a null phenotype, but with lower penetrance and with generally less severe craniofacial defects. For example, palatal shelves formed and elevated, but did not fuse, whereas in TFAP2a null pups these structures are absent.[40,41] Also, cranial ganglia were not affected by NC-specific TFAP2a loss, in contrast to severe reduction in the TFAP2a null phenotype. The less drastic phenotypic of floxdel TFAPa pups enabled further analysis of NC derivatives. For example, surviving floxdel pups exhibited defects in melanocytes that could not be evaluated in the original TFAP2a nulls due to the early lethality.[45] Similarly, the stapes bone, absent in the TFAP2a null, was particularly reduced compared to other middle ear ossicles. This is interesting in light of the TFAP2a null phenotype in zebrafish, discussed below. The most important lesson from these experiments is perhaps that TFAP2a functions in NC not only in a cell autonomous fashion, accounting for the similarities between the original null and the NC floxdel null phenotypes, but also in a noncell autonomous manner, accounting for the differences. Thus TFAP2a must regulate the expression of genes in NC-adjacent tissues encoding signaling factors that influence the terminal differentiation of NC cells. The identification of these TFAP2a-dependent genes and signals will be an exciting area of research in the coming years.

In zebrafish embryos, TFAP2a is first expressed in nonneural ectoderm, then is strongly upregulated in the NC. Interestingly the NC expression domain overlaps only partially with broadly expressed NC markers like Foxd3 and Sna2, which contrasts with the pattern in Xenopus (see below) and could account for some of the interspecies differences in loss of function phenotypes. TFAP2a is also expressed in intermediate and lateral plate mesoderm, which is not the case in Xenopus. Mutagenesis screens for zebrafish embryonic craniofacial anomalies led to the identification of two allelic recessive lethal mutations, montblanc (*mob*)[47] and lockjaw (*low*),[48] both of which are due to loss of function point mutations in the zebrafish TFAP2a gene.[49,50] The TFAP2a null phenotype in zebrafish is similar to the floxed TFAP2a mouse. The zebrafish TFAP2a null craniofacial skeleton is dysmorphic, but this is less devastating than in the TFAP2a null mouse and is mainly restricted to hypoplasia of derivatives of pharyngeal arches 2 (hyoid) through the branchial (gill) arches. Particularly affected is the hyosymplectic cartilage, which develops into a supporting bone in teleosts, but is the precursor to the stapes ossicle in mammals, and also greatly reduced in the floxed mouse. Also, like the floxed TFAP2 mouse, the zebrafish mutants exhibit reductions in pigments cells. This supports a phylogenetically conserved function for this factor in NC development, but implies some differences in the level of functional redundancy or the status of TFAP2a within the regulatory framework.

Both *low* and *mob* embryos have been analyzed using in situ hybridization with several molecular markers of NC, and the results are in basic agreement. Some early markers such as foxd3 and sna2 are not much affected, while others such as crestin and Sox9 show fairly conspicuous reductions in certain subdomains. At later stages migratory markers such as Dlx2 and EphA4 are reduced in the affected hyoid and more posterior arch NC.[38,49,50] This suggests that TFAP2 plays a less essential role during the early phases of NC development compared to later differentiation stages, and differs in function in different subsets of the NC. In the absence of TFAP2, affected NC cells undergo apoptosis and the target structures are absent or hypoplastic, similar to the mouse knockout data.

The question of cell autonomy for TFAP2 has been addressed in zebrafish by cell transplantation.[50] Wild type NC cells transplanted into the region of NC that gives rise to second pharangeal arch migrated and differentiated normally, and yielded some rescue of tissue missing in mutants. Mutant cells transplanted into wt recipients for the most part did not migrate normally into the branchial arches or contribute to cartilage. These results are consistent with the conclusion that TFAP2 functions in a subset of NC, but in a cell-autonomous manner. This is somewhat in contrast to the mouse, but an exact comparison is difficult due to the differences in experimental approaches.

In Xenopus the TFAP2 family is not as well characterized. All of the existing data have been obtained with the ortholog of TFAP2a. Other TFAP2 family members exist, however, represented as ESTs in Xenopus laevis and X. tropicalis databases. As of this writing our laboratory has characterized TFAP2b and TFAP2c, both of which are expressed at approximately the same time and place as TFAP2a (Y. Zhang, T. Sargent, unpublished). TFAP2a is first expressed throughout the ectoderm in Xenopus, then becomes cleared from the prospective neural plate during gastrulation. By the end of gastrulation TFAP2a is up-regulated in NC, but still remains expressed at a significant level in the epidermis, which continues throughout development.[36] Loss of function experiments have been done using both antisense and dominant negative approaches. Morpholino antisense oligonucleotides injected into one blastomere at the two-cell stage resulted in substantial, inhibition of Slug and Sox9 expression[51] but had little if any effect on Pax3, another early NC marker. Similar results have been obtained with a dominant negative TFAP2a lacking the activation domain. These results suggest that TFAP2a may be essential for early induction events in Xenopus, contrasting with zebrafish and mouse, where loss of TFAP2a leaves induction and migration largely unaffected. Another apparent difference between Xenopus and the other two species is the effect of global loss of TFAP2a, accomplished by radial injection of antisense olignonucleotide. These experiments were done using diethylethylene diamine (DEED) antisense oligonucleotides, which unlike the morpholino antisense strategy results in RNaseH-dependent cleavage of target RNAs.[52,53] Such embryos failed to gastrulate. The ectoderm was unable to engulf the mesoderm and collapsed into a convoluted mass on the animal hemisphere. This phenotype could be largely rescued by coinjection of a TFAP2a derivative with silent mutations rendering it resistant to the DEED oligonucleotide. A similar phenotype was also obtained using dominant negative TFAP2a. The gastrulation failure was accompanied by strong inhibition of epidermal marker genes (keratin). The dominant-negative TFAP2a, in which the activation domain had been replaced with a repressor domain from the Drosophila engrailed protein, also resulted in activation of neural genes concomitant with repression of epidermis. This was not observed with the DEED oligonucleotide, presumably indicating incomplete destruction of TFAP2a RNA. Thus in Xenopus it appears that TFAP2a is required for induction of NC, and also required earlier for epidermal function and gastrulation. One possibility is that the early loss of some NC markers in TFAP2a-inhibited embryos is a consequence of blocking the production of one or more inductive signals from epidermis that are needed for NC induction. This could be tested by targeting either a dominant negative TFAP2 or antisense specifically to the NC, avoiding expression in adjacent epidermis. This is impractical in Xenopus—even at the 32-cell stage, single blastomeres will give rise to both of these tissue types. In any case it is clear enough from the experiments outlined above that in the mouse there are TFAP2-dependent signals necessary for NC development that emanate from outside the NC, while this is apparently not the case in zebrafish based on the cell transplantation data. Thus it seems likely that TFAP2a, and perhaps other TFAP2's may function somewhat differently in various vertebrates.

In Xenopus it is relatively easy to carry out gain of function experiments by injecting synthetic mRNA into fertilized eggs, or into blastomeres fated to become particular tissues. TFAP2a RNA injected into one dorsal ectodermal blastomere at the 8 or 16 cell stage resulted in the expansion of Slug and Sox9 expression into the neural plate, accompanied by downregulation of neural markers indicated a neural to NC transfating had taken place as the result of ectopic TFAP2a activity.[51] This was also accomplished in animal cap explants. In these experiments, NC induction was achieved first by injecting synthetic mRNA for Wnt3a and for chordin.[54,55] When the chordin concentration was increased to very high levels, sufficient to extinguish BMP signaling entirely, as indicated by the loss of Dlx5 expression, the explanted ectoderm reverted to a posterior neural identity, as indicated by loss of all NC markers and activation of Sox2 expression. If the same treatment was performed with TFAP2a RNA added to the cocktail, the response was largely reversed. Most NC marker

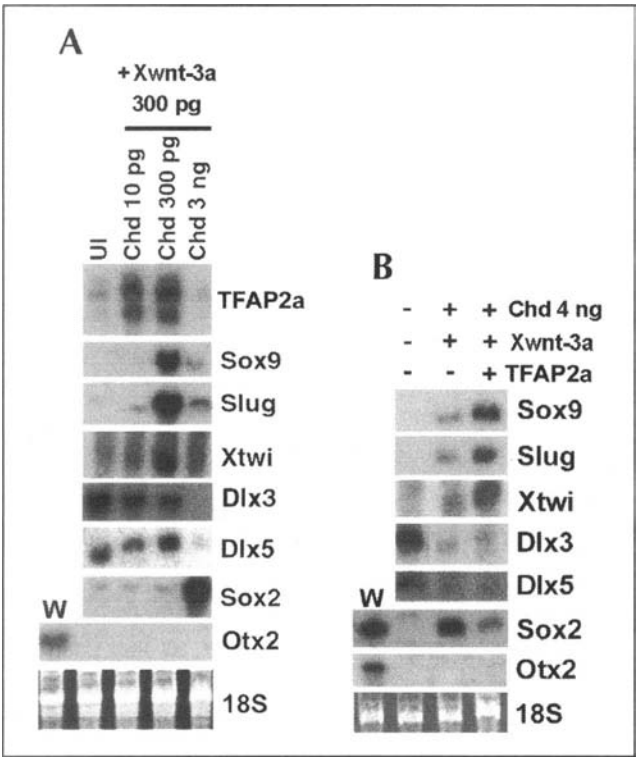

Figure 5. Dependence of NC induction on BMP signal strength and AP2 in Xenopus animal cap experiments. A) Increasing doses of RNA encoding chordin (Chd) from 10 pg to 3 ng, injected along with RNA encoding Wnt-3a induces NC gene expression (TFAP2a, Sox9, Slug, Xtwi) and represses Dlx genes and neural gene expression (Sox2, Otx2), except at the highest dose of Chd, which results in induction of the pan-neural Sox2, but not the anterior neural Otx2 genes, and silences the NC genes. B) Addition of TFAP2a reverses the NC to neural plate transition shown in (A). Modified from Luo et al, 2003.[51]

genes became active, neural genes were silenced (Fig. 5). This supports a model in which TFAP2a mediates the attenuated BMP signal which, in conjunction with a canonical Wnt signal (or FGF,[56] induces NC).

NC Regulatory Pathways Problems and Prospects

Does TFAP2 fit into a hierarchy of NC regulators? A good place to start is the epistatic pathway proposed for Xenopus by Mayor and colleagues in which Msx1 is upstream from Snail, followed by Slug.[29] In animal caps primed with Wnt/beta catenin signaling, and in embryonic neurectoderm, TFAP2a can induce Slug. There are no data regarding Msx1 or Snail, so for the sake of discussion we can place TFAP2a between Snail and Slug. TFAP2a is not a direct target of BMP signaling since it is not induced by BMP in the presence of cycloheximide,[36] and therefore there is presumably at least one activator factor upstream from TFAP2a that is synthesized in response to BMP. Both Msx1 and Snail are repressors, so this hypothetical TFAP2a activator (HTA) must be inserted in the pathway, i.e., downstream from Snail. Furthermore, as a repressor, the simplest way for Snail to activate HTA would be to repress a repressor of HTA (RHTA). Likewise, for Msx to positively regulate Snail, a repressor of Snail (RS) could be inhibited by Msx. This gives the following linear

pathway: Msx1 -| RS -|Snail -| RHTA -|HTA -> TFAP2 -> Slug. Additional steps downstream from Slug would presumably entail another repressed repressor event. Nor is it clear that another step is not required for TFAP2 to activate Slug. This pathway is complicated further by the apparent direct regulation of Slug by Wnt beta catenin signaling,[57] and the possibility that Msx is also a beta catenin target (see above). This is a complex event chain, all of which must take place within a fairly brief time interval, between late gastrula and early neurula. Indeed, it is difficult to discern any temporal differences in the expression of any of these known genes in the NC domain. Some steps, such as repression, could occur by protein interactions that might be essentially instantaneous, but it would seem that the linear model is probably too slow, cumbersome and too simple to account for all the available information.

Another argument against linear control of NC is the differences that exist between species. For example, Slug and Snail differ in expression pattern and presumably in function in various vertebrates.[58] Likewise, Msx1 and Msx2 null phenotypes in mouse are quite different,[59,60] and neither results in massive loss of NC. This could reflect redundancy, but could just as easily be due to differences in function in mouse compared to frog. There are other examples, and as data become available for orthologous genes in multiple species, this number will probably increase. A strict linear pathway would be inherently less tolerant of evolutionary change, compared to a network with plenty of redundancy and positive and negative feedback. Altering the status of one or a few regulatory factors in such a network could lead to alterations in the end product, as opposed to its elimination. This might help explain the tremendous variation in facial morphology among the vertebrates, a factor that has contributed very significantly to the adaptive success of this phylum.

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CHAPTER 4

Neural Crest Delamination and Migration: Integrating Regulations of Cell Interactions, Locomotion, Survival and Fate

Jean-Loup Duband*

Abstract

During the entire process of neural crest development from specification till final differentiation, delamination and migration are critical steps where nascent crest cells face multiple challenges: within a relatively short period of time that does not exceed several hours, they have to change drastically their cell- and substrate-adhesion properties, lose cell polarity and activate the locomotory machinery, while keeping proliferating, surviving and maintaining a pool of precursors in the neural epithelium. Then, as soon as they are released from the neural tube, neural crest cells have to adapt to a new, rapidly-changing environment and become able to interpret multiple cues which guide them to appropriate target sites and prevent them from distributing in aberrant locations. It appears from recent studies that, behind an apparent linearity and unity, neural crest development is subdivided into several independent steps, each being governed by a multiplicity of rules and referees. Here resides probably one of the main reasons of the success of neural crest cells to accomplish their task.

Introduction

The early development of the neural crest in vertebrate embryos can be likened to the history of a number of European peoples during the last centuries. Briefly, it starts with a long and obscure time period when minorities are dominated by their potent neighbors, their original territories occupied and often divided into separate entities, and their traditions and identities severely repressed. As time goes by, the minorities express signs of identity, first timidly and cryptically, then progressively more markedly. This period terminates in a sudden and paroxystic step, typical of revolutions, with the revolts of minorities, their rejection of all external influences, and their declarations of independence. This is inevitably followed by an unfortunate cohort of conflicts, wars and population displacements and emigrations. However, as populations are completely separated, they can express freely their individual characteristics and conflicts become less acute. Quite often, this situation is favorable for establishment of new, more stable and balanced contacts between the previously-fighting populations, and it is accompanied by the mutual recognition of the identities as well as the revival of a common past. This state is supposed to persist unless a new, emerging empire strikes again, but obviously this has not occured yet in Europe and is pure science fiction.

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Thus, the initial step of neural crest development would correspond to the domination period. The original territory of crest cells lies at the boundary between the ectoderm and the neural tube and is not well delimited. Prospective crest cells are most likely recruited from cells located on either side of the boundary but cannot be identified with certainty during early neurulation. Nascent neural crest cells become progressively specified and express a number of specific markers. However, they remain integrated at the dorsal aspect of the neural tube and are morphologically indistinguishable from the other neural epithelial cells. Then, the delamination step occurs. Undubiously, this event is the best sign of crest cells individualization from the rest of the neural tube and constitutes their declaration of independence. It is associated with profound changes in crest morphological and molecular features, with loss of previous properties and acquisition of new characters. At this step, neural crest cells are clearly distinct from neural tube cells and are easily recognizable both molecularly and morphologically. In most species, once cells are segregated from the neural tube, they immediately venture away from the tube in multiple directions. During migration, neural crest cells may face hostile environments that may repulse them or cause their death, but they may also occupy more accessible areas where they survive, grow and sometimes settle to undergo differentiation. Interestingly, as observed among European countries, neural crest cells that once have made secession with the neural tube often reestablish intimate contacts with it, such as at the sensory and motor nerve entry and exit points, and express a number of common molecular markers with the central nervous system. Thus, the analogy between neural crest cells and the origins, migrations and fates of European peoples appears to be very large, particularly during delamination and migration, the two critical steps when neural crest traits become manifest: how to become different while sharing a common history and how to survive, move and develop in a sometimes hostile environment.

Neural crest ontogeny has been extensively covered in the past by numerous reviews.[1-6] Here, I will focus on the recent advances and trends in some specific aspects that have considerably modified our view of the delamination and migration stages and discuss the current questions that are being examined and those that remain unexplained.

Neural Crest Delamination: Their Declaration of Independence

Delamination (also refered as to emigration, individualization or segregation from the neural tube) encompasses the series of events that allow the physical separation of neural crest cells from the rest of the neural tube. Although the term delamination is now widely employed, it may not be entirely appropriate and may be misleading to some extent, because neural crest cells do not appear by a process involving splitting of apposed laminae. Delamination can be viewed as both the final step of the whole process of neural crest cell specification, allowing cells to become irreversibly segregated from the neural tube, and the transition toward migration. It must be, however, clearly distinguished from the specification and migration steps since, as discussed below, these events appear now to be driven by independent and discernable cellular and molecular mechanisms. Yet, analyzing delamination has long been a difficult and elusive task: indeed, this step lags during a rather limited time period (so far it has not been possible either to predict or even to catch the moment when an individual neural crest cell dissociates from the neural fold); until recently, there was virtually no good molecular markers for this step in contrast to specification and migration (the best sign for delamination is the presence of cells at the periphery of the neural epithelium, in between the basal surfaces of the neural tube and the ectoderm); and there are no clear separations or transitions between the specification, delamination and migration steps which instead overlap at the level of the population, as the whole process evolves continuously in most species (thus, when the pioneer crest cells are undergoing migration, others are still being specified or delaminating). However, because it constitutes the first tangible sign of neural crest formation as an individual cell population, delamination is a key step during neural crest ontogeny that has long attracted great interest. In addition, it provides a paradigm to analyze cell dissemination, a process often

encountered under normal and pathological situations both during embryogenesis and adulthood, e.g., during tumor metastasis and tissue repair.

Because of the lack of adapted experimental designs, neural crest delamination has been at first exclusively the matter of descriptive studies. However, in the early 1990s, following the pioneer studies of Newgreen,[7] a breakthrough has been made possible essentially using an ex vivo culture approach, based on the comparison of the molecular and cellular properties of cells migrating out of neural tube explants at different stages of neurulation.[3,8] But it is only quite recently that the process of delamination has been amenable to in situ experimentation, in particular owing to the rapid development of transgenic approaches in mouse, zebrafish, frog, and chick. Finally, because it is highly suitable for both in vitro/ex vivo cultures and in ovo electroporation allowing real time analyses, the chick embryo has become the most popular model for studying neural crest delamination, but interesting information have been obtained with the Xenopus and mouse embryos. Curiously, although the zebrafish embryo allows a direct visualization of neural crest delamination and proved to be a valuable model for studying determination and migration, so far it has not been the matter of intense studies to address specifically the question of delamination.

Morphological and Cellular Events Define Neural Crest Cell Delamination Primarily as an Epithelium-to-Mesenchyme Transition

Morphological and immunohistological studies in various species ranging from fish to mammals have permitted a detailed description of the main cellular events that accompany neural crest cell delamination.[3] Although the topographies of the embryos in Vertebrates are notably different and influence timing of neural crest emigration relative to neural tube folding and closure (for example, cranial neural crest emigration in the mouse occurs when the neural tube is wide open while in the chick it appears coincident with neural fold fusion), a number of basic events can be recognized in the processes leading to the separation of the neural crest population from the neural tube (Fig. 1). These events are in many aspects similar to those occuring in any epithelium-to-mesenchyme transition (EMT). However, although neural crest cell delamination is often regarded as a model of EMT, it presents a number of specific features that make it somewhat different and atypical compared with other EMT such as somite disruption, tumor dissemination, wound healing, or dispersion of epithelial cell lines in vitro. In particular, in the trunk region, at the time nascent crest cells depart from the neural tube, the neural epithelium does not manifest signs of complete disorganization and disruption of tissular cohesion. Rather, it remains morphologically intact and crest cells are only gradually expelled from the epithelium in the extracellular matrix underneath, suggesting that the process of delamination is tightly controled both spatially and temporally and that there are mechanisms ensuring continuously the replacement of cells that have emigrated.

As they segregate from the neural tube, neural crest cells change shape progressively: from regular, elongated, and radially-oriented, they become at first more rounded and irregular in shape with increasing numbers of filopodia protruding out of the neural tube.[9-11] Once cells are entirely separated from the neural tube, they flatten over the surface of the neural tube and extend tangentially to it. These morphological changes correlate at least in vitro with reorganizations of the actin cytoskeleton, from a dense fibrillar network at the cell periphery in association with junctions to a more diffuse and labile organization.[12]

Important alterations in cellular cohesion have also been reported. Interestingly, these appear considerably more complex than originally appreciated. Indeed, at onset of neurulation in the chick, all cells in the neuroectoderm exhibit junctional complexes typical of polarized epithelial cells with tight junctions and adherens junctions containing E-cadherin. However, not all epithelial features exist in neuroepithelial cells: there are no desmosomes and the intermediate filaments are not composed of cytokeratins.[13] As the neural folds elevate to form the neural tube, i.e., long before any sign of crest cell delamination, tight junctions are lost gradually from the prospective neural epithelium along a ventro-dorsal gradient, but remain intact in the

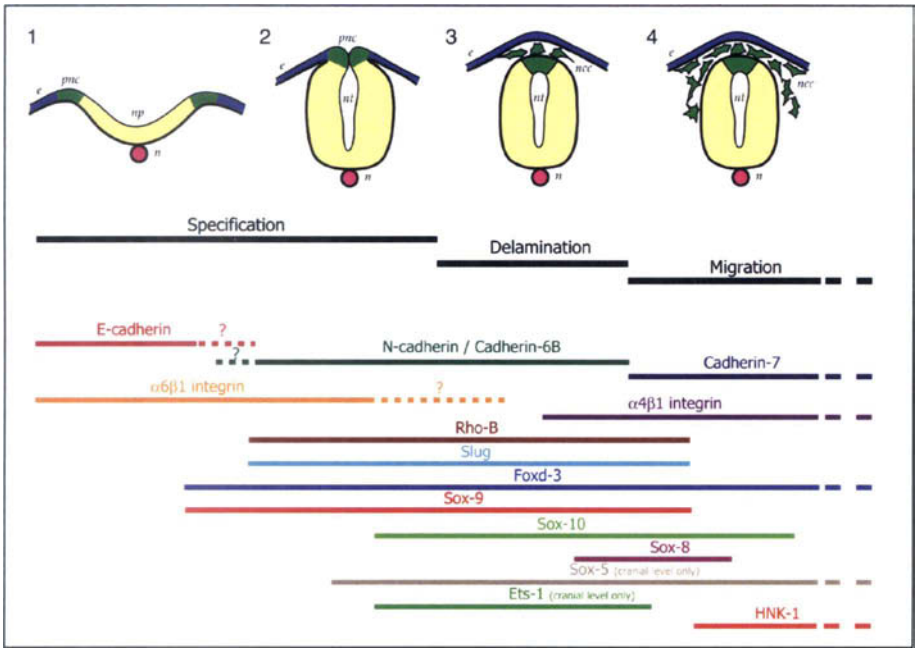

Figure 1. Temporal expression patterns of the molecular determinants of delamination and early migration in nascent neural crest cells as observed in the trunk of the chick embryo. The top panel indicates the timing and duration of the specification, delamination and migration steps with regard to neural tube morphogenesis : 1, opened neural plate; 2, closing neural tube; 3, delaminating neural crest; 4, migrating neural crest. The neural epithelium (neural plate, np; neural tube, nt) is indicated in yellow, the notochord (n) in red, the ectoderm (e) in blue, and neural crest cells either prospective (pnc) or migratory (ncc) in green. The bottom panel shows the temporal expression patterns of adhesion molecules (cadherins, integrins), GTPases (Rho-B), transcription factors of the Snail, Fox, Sox and Ets families, as well as the HNK-1 marker. The continuous lines represent the time windows at which genes are expressed. The long dotted lines indicate that expression persists during the later phases of migration while the short dotted lines with question marks represent incertainties about the patterns of the molecules considered. For example, it is not well established whether E- and N-cadherin patterns overlap in prospective crest cells at the time of neural tube closure.

superficial ectoderm.[14] In addition, although adherens junctions are retained, E-cadherin is replaced by N-cadherin and cadherin-6B until onset of migration.[15-17] Whether N- and E-cadherins can coexist transiently in individual cells is not known precisely. Slightly before emigration, coincident with change in cell shape, adherens junctions become disrupted though expression of N-cadherin on the cells' surface is not repressed.[17] N-cadherin and cadherin-6B are lost from neural crest cells, but only after their complete exclusion from the neural epithelium.[17-19] Finally, neural crest cells undergoing migration start expressing cadherin-7 and/or cadherin-11,[19,20] two types of cadherin expressed by fibroblastic cells. In the mouse, this cadherin sequence is different as neural crest cells start expressing cadherin-6, a close relative of chick cadherin-6B, prior to delamination but retain it on their surface during early migration, instead of shifting to cadherin-7.[21] The overall significance of this complex series of changes in the repertoire of cadherins in the neural epithelium during neural crest cell emigration is unknown at present, but it is likely that it is a prerequisite for the step-by-step occurrence of defined cellular events leading to the correct segregation of cells from the neural tube. Indeed, qualitative and quantitatives differences in cadherin expression in neighboring cells have been found to intruct cell segregation and influence cell fate,[22] and the fact that cells have never been

seen delaminating from the superficial ectoderm may be related with the absence of cadherin shift in this tissue. In addition, perturbation experiments aimed at altering this sequence all result in severe deficiencies in neural crest delamination. Thus, mouse embryos mutated for SIP-1, a repressor of E-cadherin expression, show persistent E-cadherin labeling thoughout epidermis and neural tube, and this is associated with a complete lack of neural crest delamination in cranial regions.[23] Likewise, forced expression of N-cadherin in prospective neural crest cells causes a deficit in their emigration and their accumulation into the lumen of the neural tube.[19] Finally, precocious overexpression of cadherin-7 in neural crest cells also prevents migration instead of producing anticipated emigration.[19]

Beside changes in cell-cell adhesion, prospective neural crest cells undergo a number of modifications in their interactions with the extracellular matrix that are believed to favor their release from the neural tube. First, there is no basement membrane covering the dorsal aspect of neural tube where cells delaminate, but this absence does not correlate strictly with the onset of delamination, indicating that although it is necessary, it is not a key triggering event.[24] Nevertheless, in vitro studies clearly indicate that neural crest cells respond differently to extracellular matrix material prior to and after delamination,[8] but it is not clear yet whether these changes result chiefly from modifications in the repertoire of integrins as observed in the chick embryo[25,26] or from activation of distinct downstream signaling pathways. In addition, in vivo and in vitro perturbation experiments suggest that neural crest cell delamination is fostered by matrix metalloprotease-2 (MMP-2), a type IV collagenase, even though it is only produced in the late phase of delamination, once cells are released in the extracellular environment.[27] Thus, neural crest interactions with the extracellular matrix are clearly altered during delamination, but the means and kinetics of these modifications remain ill-defined.

An important issue concerns the establishement of the spatial, temporal, and functional hierarchies in the various events affecting cell shape, cell-cell adhesion and cell-matrix interactions. In other words, what is the exact sequence of cellular events that accompany crest delamination; which of them play a critical role; how are they coordinated; are some of them dispensable and which is the last event necessary to trigger complete delamination ? At the present time, there are no clear answers to these questions. However, several clues suggest that delamination is not a linear cascade of events in which each step relies directly on the occurrence of the previous ones. For example, the fact that N-cadherin down-regulation is a late event taking place just prior to crest cell dissociation from the neural tube would suggest that this is a prerequisite for triggering delamination, and the N-cadherin overexpression experiments mentionned above tend to support this view.[19] Yet, recent in vivo studies reveal that complete delamination of neural epithelial cells followed by active migration can occur without down-regulation of N-cadherin from the cells' surface.[28,29] Likewise, using an in vitro approach, Newgreen and colleagues showed that affecting N-cadherin-mediated cell adhesion, blocking atypical PKC or challenging integrin-dependent matrix adhesion result in the same outcome, clearly demonstrating that delamination can be achieved by various routes, thereby excluding any obvious hierarchies among the different cellular events occuring during EMT.[12,30]

Candidates for orchestrating cellular events during neural crest EMT involve Rho GTPases -Cdc42, Rac and Rho- known to control cell adhesion and motility through dynamic regulation of the actin cytoskeleton. So far, two members of the family, Rho-A and Rho-B, have been identified in the neural epithelium at the time of neural crest cell migration.[31] Nonsurprisingly, Rho-A, the most common and best characterized member of the family implicated in actin bundling and focal contact formation, is ubiquitous in the neural tube. In contrast, Rho-B, a more divergent member whose function and cellular targets remain largely unknown, exhibits a very dynamic expression pattern in prospective crest cells prior to and during early migration, thereby suggesting that it plays a specific role during delamination. Indeed, blockade of Rho activity using the C3 exotoxin inhibits neural crest delamination in vitro,[31] but it should be stressed that as the C3 exotoxin shows no selectivity for any particular Rho, this experiment did not allow to ascribe a delamination-promoting activity exclusively to Rho-B. Nevertheless,

forced expression of a dominant-active form of Rho-B in the neural tube has been found to cause massive cell delamination, resulting in a severe distortion of the neural tube morphology.[29] The cellular events regulated by Rho-B in neural crest cells remain to be identified. Studies in cultured cell lines have shown that it is poorly involved in cytoskeletal organization, and its close association with endocytic vesicles argues instead for a role in intracellular transport of cell-surface receptors.[32] Interestingly, cells delaminating from neural tubes transfected with an activated form of Rho-B show a marked exclusion of N-cadherin staining from adherens junctions.[29] It is therefore plausible that Rho-B might promote delamination of neural crest cells by affecting N-cadherin trafficking in the cells. Alternatively, it cannot be excluded that it may also function in the dynamics of crest cell locomotion as primary mouse embryo fibroblasts derived from Rho-B-/- strains display a marked defect in cell motility.[33] Although Rho-B presents a number of additional features pertinent to a prominent role in the control of neural crest delamination (unlike most GTPases which are relatively stable, it is turned over quickly and its synthesis is tightly regulated by growth factors), it is likely that it operates in concert with other Rho GTPases, notably Rac and Cdc42. Indeed, in other examples of EMT, changes in cell cohesion has been found to correlate with subtle modifications in the balance between the different Rho GTPases. In particular, during condensation of the lateral plate mesoderm into the somite, Cdc42 activity levels appear critical for the binary decision that defines the epithelial and mesenchymal somitic compartments whereas proper levels of Rac-1 are necessary for somitic epithelialization.[34]

A Combinatorial Transcriptional Code for Controling EMT during Neural Crest Delamination

The search for early molecular determinants of neural crest cell specification led to the identification of a large variety of transcription factors that appeared to be expressed in prospective crest cells at least until early migration (Fig. 1). This suggested that specification of dorsal neural epithelial cells into neural crest progenitors is necessary and sufficient to initiate a linear signaling cascade characterized by a precise sequence of expression of transcription factors and ultimately leading to their delamination and migration, provided cells are confronted with the appropriate environment. However, in the absence of studies at the single cell level, there is no direct proof for a strict correlation between expression of early neural crest markers and the cellular capacity to undergo delamination. Rather, several observations made essentially in chick and Xenopus suggest that neural crest specification, delamination, and migration are causally independent events.

The Snail Family of Zn-Finger Transcription Factors

Slug and Snail were the first transcription factors to be identified in the neural crest, about a decade ago.[35] They belong to the Snail family of Zn-finger transcription factors and are most commonly used as neural crest markers.[36] In the chick, neural crest cells express Slug but not Snail,[37] whereas in the mouse and zebrafish,[37,38] it is the reverse situation, crest cells express Snail instead of Slug, and, lastly, in Xenopus, both factors coexist in crest cells but are induced separately.[39] Slug and Snail have been shown to play similar roles and be interchangeable in some experimental systems,[40] although they may also perform distinct functions in cells.[39] There are numerous indications that Snail transcription factors are involved in EMT.[36] Beside prospective neural crest cells, they are expressed in multiple embryonic regions known to undergo EMT such as in the primitive streak and mesoderm during gastrulation, during sclerotome dispersal, and during formation of the heart cushions. Snail mutant mice die at gastrulation, most likely due to defective ingression of the mesoderm in the primitive streak.[41] In tumors, Snail triggers EMT through direct repression of E-cadherin, and its expression correlates with the invasive phenotype in cell lines as well as in vivo, in chemically-induced skin tumors.[42,43] Likewise, overexpression of Slug in cultured epithelial cells causes desmosome dissociation followed by cell dispersion, upregulation of vimentin, and fibronectin redistribution.[44] With regard to the neural crest,

loss-of-function experiments in chick and Xenopus based on antisense oligonucleotides to Slug or Snail result in a strong deficit in migrating neural crest cells.[35,39,45] For all these reasons, Slug has been presented as a major player necessary for neural crest delamination. However, other studies tend to contradict this view and suggest that Slug may be neither sufficient nor necessary for delamination at least at some axial levels. First, with the possible exception of the cranial levels in the mouse,[42] Slug expression is often delayed with respect to the exclusion of E-cadherin from the neural tube particularly at truncal levels. It can be argued that Slug might also repress N-cadherin expression, but this has never been described so far. More significantly, in Xenopus, although Slug or Snail overexpression in whole embryos leads to the expansion of prospective neural crest territory and a greater number of melanocytes, their effect is limited to areas contiguous with endogenous neural-crest-forming regions.[39,45] In addition, Slug is unable to induce by itself crest formation in ectodermal explants, suggesting that its sole expression is insufficient to direct a program of neural crest ontogeny.[45] This is further supported by cell-tracing experiments which revealed that not all Slug/Snail-expressing cells are fated to become migrating neural crest cells.[46] In chick, overexpression of Slug in the neural tube using in ovo electroporation increased the number of neural crest cells migrating out of the dorsal side of the neural tube associated with an increase in Rho-B expression, but this occured only at cranial levels.[40] In addition, as observed in Xenopus, only cells situated in the most dorsal side of the embryo, i.e., in the neural crest prospective region, were able to emigrate, whereas cells situated immediately more ventrally exhibited no signs of delamination and expressed no neural crest markers.[29,40] Slug overexpression in the trunk region caused only a slight expansion of the prospective crest cell region, but this was not accompanied by greater numbers of migrating cells. Finally, neural crest delamination can be severely affected in trunk of chick embryos, without detectable repression of Slug expression.[47] Thus, paradoxically, despite convincing data on Slug/Snail function in numerous examples of EMT, their precise role in neural crest delamination remains elusive: they may promote delamination in a specific cellular context in the dorsal part of the neural tube but may be insufficient to drive by themselves EMT of neural tube cells situated more ventrally.

The Winged Helix-Forkhead Transcription Factor Foxd-3

Foxd-3 is a transcription factor of the winged helix-forkhead family whose temporal expression also closely matches neural crest induction, delamination, and migration. In Xenopus, chick, and mouse, its expression starts early during neural crest induction approximately coincident with or slightly before that of Slug, but in contrast to the latter, it remains expressed in most neural crest cells throughout migration, except for melanocyte precursors.[48-51] As for Slug/Snail, targeted inactivation of Foxd-3 in the mouse is embryonic lethal at very early stages of development, before implantation[52] and, thus, informations about its possible contribution to neural crest specification and delamination come essentially from gain- and loss-of-function analyses in frog and chick. In the chick, forced expression of Foxd-3 in the trunk neural tube was found to suppress interneuron differentiation and induce precocious and robust expression of HNK-1, a marker for migrating neural crest cells, in the whole neural tube by 24 hours post-transfection. Foxd-3 is also able to provoke ectopic delamination of cells but not until 24 hours post-transfection. Delamination is evident only after 36-48 hours and is accompanied by down-regulation of N-cadherin, up-regulation of integrins and cadherin-7, and disruption of the basement membrane lining the neural tube.[28,29,49] In Xenopus embryos, when ectopically overexpressed, Foxd-3 is a potent inducer of neural crest markers, including itself and Slug, but it also promotes expression of neural markers.[50] Interestingly, in contrast to Slug, Foxd-3 can induce expression of neural crest markers in distant locations from the neural crest region. Conversely, attenuation of Foxd-3 activity by overexpression of a dominant-negative form of the molecule inhibits neural crest differentiation.[50] Thus, although these studies did not directly address Foxd-3 function in delamination, they clearly highlight its critical role in neural crest formation and support observations made in the chick. In conclusion, unlike Slug, Foxd-3 seems to be a potent inducer of crest cell specification and delamination, but the delay for its

effect indicates that it does so only indirectly, possibly by activating intermediate genes, and in an uncontroled manner as it induces markers of migrating neural crest cells prior to delamination and at the expense of other cell populations of the dorsal neural tube.

The Sox-E Subgroup of HMG-Box Transcription Factors

Recently, the Sox-E subgroup of HMG box-containing transcription factors attracted much attention, because in all species and at all axial levels examined, they are specifically expressed in a temporal order in neural crest cells from early determination to late migration.[28,29,53] Sox-9 is the first to appear in prospective crest cells and it is closely followed by Sox-10 and, later, by Sox-8, just before neural crest cells exit the neural tube. At the onset of migration, while Sox-10 is retained by migrating cells, Sox-8 and Sox-9 are rapidly down-regulated. Later during differentiation, Sox genes are reexpressed in distinct neural crest subpopulations and have been shown alternatively to contribute the maintenance of neural crest multipotency or to participate to specific differentiation programs. Thus, Sox-E genes, and particularly Sox-10, compose at present the most universal neural crest cell markers. As observed for Foxd-3, production of neural crest cells is strongly agcted in Xenopus embryos upon knockdown or overexpression of Sox-9 and Sox-10. However, while both factors are critical for early crest determination and melanocyte differentiation, they are apparently relatively dispensable for delamination and subsequent migration.[54-56] Likewise, in mouse embryos lacking Sox-9, neural crest cells are specified and able to start migration but they rapidly undergo programmed cell death shortly after.[29] Very recently, implication of Sox-E transcription factors in delamination has been further investigated in detail in chick by two different laboratories, but their results differ in some significant aspects.[28,29,53] Both studies found that forced expression in the trunk neural tube of either Sox-8, Sox-9 or Sox-10, but not of Sox-2, a Sox gene from a different subgroup, convert within less than 24 hours neural tube cells into neural crest-like cells expressing the HNK-1 marker. In addition, both studies showed that Sox-E genes induces cadherin-7 expression but no downregulation of N-cadherin and that they rapidly turn off Rho-B expression. However, while Cheung and coworkers did not document any marked ectopic delamination of neural tube in the electroporated side of the neural tube and concluded that Sox-E genes by themselves are not capable of triggering cell delamination, McKeown and colleagues observed at variance extensive migration. This was seen at all levels along the dorsoventral axis, including in the floor plate, about 36 hours after electroporation, i.e., rather late after induction of a neural crest phenotype and, 48 hours after electroporation, the transfected side of the neural tube was almost entirely disrupted and almost all cells were released into the neighboring sclerotome.[28] At present, there are no obvious explanations for these discrepancies, but whatever the exact role of Sox-E genes in neural crest development, it appears that, like Foxd-3, they can elicit cell delamination only secondarily, after induction of HNK-1 and cadherin-7, two markers normally expressed after delamination. In addition, delamination induced by Sox-E genes as well as by Foxd-3 is massive, leaving the neural tube as an empty bag or a flat tire from which the whole content has been poured out, a situation which is never observed normally, therefore suggesting that these transcription factors cause delamination by an aberrant and uncontroled sequence of events at the expense of the other neural cell types.

Recently, beside Sox-E genes, another Sox transcription factor of the Sox-D subgroup, Sox-5, has been characterized at cranial levels in the chick.[57] It is expressed in premigratory crest cells, slightly later than Slug and is maintained in most neural crest cells during migration as well as in glial cells of cranial ganglia. Misexpression of Sox-5 in the cephalic neural tube leads to an exquisite phenotype contrasting with the massive effects obtained with Sox-E genes. In the dorsal neural tube, it augments both spatially and temporally the production of crest cells, associated with up-regulation of Foxd-3, Slug, Pax-7, Sox-10 and Rho-B, whereas in more ventral regions of the neural tube, it induces Rho-B expression, but not Foxd-3 or Sox-10 and its capacity to induce delamination is only marginal. Thus, like Slug, Sox-5 effect might be dependent on the cellular context within the neural tube.

Other Families of Transcription Factors

Neural crest cells have been found to express several additional transcription factors at the time of delamination, among which Pax-3, AP-2, Myc and members of the Zic family are the most remarkable.[58] The role of these factors in neural crest delamination has not been addressed directly and their possible implication in this process cannot then be formally excluded. However, it is clear that because they are not restricted to prospective neural crest cells, they cannot pretend to play a major role by themselves. Pax-3, for example, has been shown to be genetically upstream of Foxd-3[49] and mouse Splotch embryos in which its gene is mutated exhibit strong defects in neural crest cell generation and migration, possibly as a result from decreased cell-cell adhesion due to oversialylation of N-CAM molecules.[59,60] Yet, the precise role of Pax-3 in the control of cell adhesion remains unclear, as it has been also observed in vitro that its forced expression in mesenchymal cells may induce their aggregation of into multi-layered condensed cell clusters with epithelial characteristics.[61] The protooncogene Myc has been implicated in Xenopus in crest cell determination independently of its proliferation role[62] and, in the chick, it has been shown to stimulate massive crest cell migration followed by their differentiation into neurons.[63] Finally, mice deficient in the AP-2 gene show severe defects causing embryonic lethality and affecting primarily development of the neural crest: failure of neural tube closure, craniofacial anomalies and absence of cranial ganglia.[64]

It is striking that, among the different transcription factors characterized in neural crest cells at the time of their segregation from the neural tube, none of them exhibit expression patterns matching precisely with delamination, suggesting that this step is essentially dependent on transcriptional events occuring during the previous specification step. However, recent studies allowed to pin down factors that mark precisely crest cell delamination more reliably than Slug or Rho-B for example. Ets-1, a member of the Ets family of winged helix-turn-helix transcription factors has been found to be dynamically expressed in delaminating crest cells at cranial levels (ref. 65 and E. Théveneau, M. Altabef, and J.-L. Duband, unpublished results). At the midbrain level, for example, its expression starts in prospective crest cells just after apposition of neural folds, at the 5-6 somite stage, i.e., about 4-6 hours before onset of migration, and it persists in the dorsal neural tube until cell delamination ceases, i.e., at the 11-somite stage. In addition, migrating neural crest cells almost immediately turn Ets-1 expression off as soon as they become fully segregated from the neural tube and leave its vicinity. Ets-1 has been previously implicated in various EMTs and migratory events during embryonic development and, in contrast to most other transcription factors expressed by crest cells, a detailed list of its potential target genes has been established: these include key molecules for cell locomotion such as integrins, cadherins and MMPs.[66-69] Ectopic expression of Ets-1 in the chick neural tube by in ovo electroporation results in delamination of neural tube cells, at both cranial and truncal levels, although Ets-1 is not prominent in trunk crest cells. Interestingly, Ets-1-induced cell delamination presents unique characteristics that are not observed with forced expression of Foxd-3 or Sox-E genes (E. Théveneau, M. Altabef, and J.-L. Duband, unpublished results). It is rapid, cells being seen delaminating within 12 hours posttransfection; it occurs primarily at the basal side of the neural tube, but also less frequently at its apical (luminal) side; unlike Foxd-3, Sox-9 or Sox-10, it is not massive but rather progressive, leaving the neural tube intact in a very similar manner to the normal delamination of neural crest cells; cells exiting from the neural tube upon Ets-1 overexpression do not express neural crest cell markers such as HNK-1 or Slug, but show local disruption of the basement membrane, indicating that Ets-1 most likely triggers delamination by activating expression of MMPs; finally, delamination is not followed by migration, cells remaining for a while at the close vicinity of the neural tube, before undergoing apoptosis. Thus, at least at cranial levels, Ets-1 might regulate late cellular events accompanying neural crest cell delamination independently of a neural crest phenotype, thereby illustrating that specification, delamination, and migration are separable events.

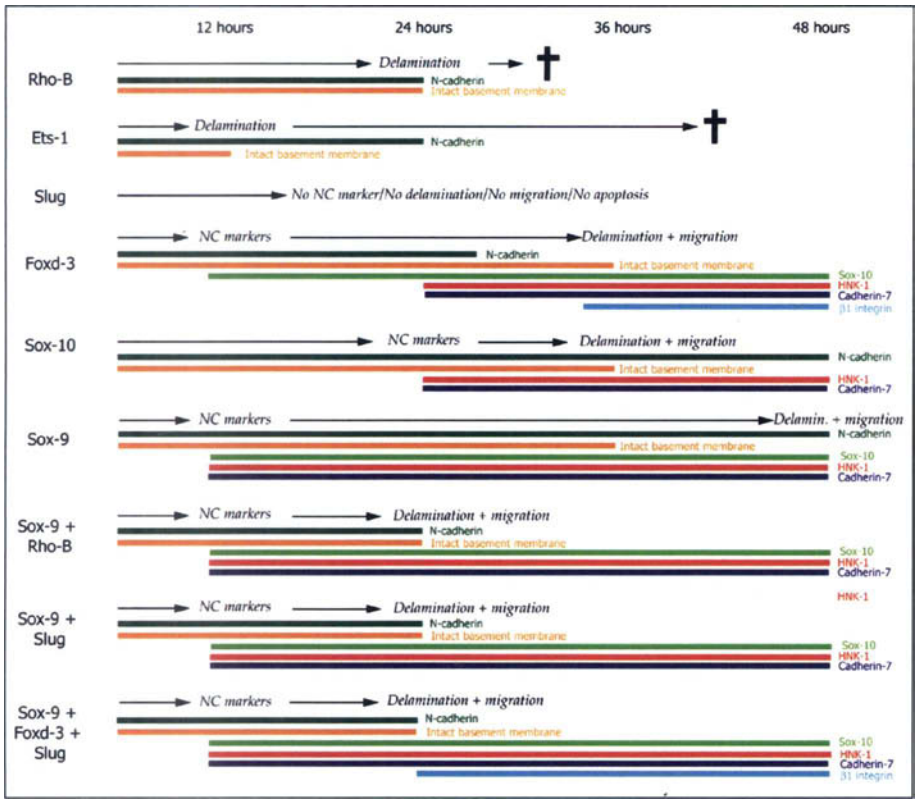

Figure 2. Roles of the Rho-B GTPase and transcription factors of the Snail, Fox, Sox and Ets families in neural crest EMT as deduced from gain-of-function experiments in the trunk neural tube of the chick embryo. Rho-B and various transcription factors (indicated on the left) were electroporated either alone or in combination in the lateral and ventral sides of neural tube at stages prior to neural crest migration and the consequences of their forced expression on the ectopic expression of neural crest markers, cell delamination, survival and migration, on celllular events, such as disruption of the basement membrane, and on expression patterns of molecules involved in EMT are indicated at the indicated time periods following electroporation. Data were collected from references 28, 29, 49 and from E. Théveneau, M. Altabef and J.-L. Duband, unpublished.

Cooperative Activity of Transcription Factors during Neural Crest Cell EMT

The above studies reveal that delamination elicited by transcription factors ectopically expressed in the intermediate and ventral neural tube is either partial or disordered, and that none of them is able to induce a complete neural crest phenotype (Fig. 2). Thus, they do not allow to draw a coherent sketch of the transcriptional network controling neural crest delamination. A possible clue is to identify the epistatic relationships between these transcription factors in prospective neural crest cells.[58] In Xenopus, gain- and loss-of-functions approaches revealed highly complex crossregulation of Snail, Slug, Sox-9, Sox-10 and Foxd-3 genes which can influence each other via direct transcriptional activation of repression or through secondary factors, thereby excluding any obvious linear hierarchy among these factors.[39,50,55] In chick, the situation contrasts with that observed in Xenopus in that Slug, Foxd-3, and Sox-9 signals are apparently independent and display distinct sets of targets.[28,29,40,49] In addition, Foxd-3 and Sox-9 lie upstream the Sox-10 and Sox-8 genes, consistent with their precocious expression in prospective crest cells. In the mouse, lastly, while the Foxd-3 and Snail mutants were

not informative because of premature lethality, the Sox9 mutant provided interesting informations about the functional interactions between Foxd-3, Snail, Sox-10 and Sox-9. It appears that Snail, but not Foxd-3 and Sox-10, is dramatically downregulated in premigratory crest cells in mutant embryos compared with their wildtype littermates, indicating that in this species, Snail is downstream of Sox-9.[29] Recently, using the chick system, the Briscoe's laboratory further investigated possible cooperative activities between Slug, Foxd-3 and Sox-9 in the control of truncal crest delamination by comparing the effects of these factors individually or in various combinations.[29] While forced expression of Slug showed no obvious effect 24 hours after electroporation, Sox-9 induced HNK-1 expression within 12 hours in the intermediate and ventral neural tube but, after 24 hours, it was not capable of triggering significant cell delamination with no disruption of the basement membrane and of N-cadherin junctions and no integrin upregulation. In contrast, Sox-9 and Slug in conjunction induced robust HNK-1 expression, disorganization of the neural epithelium with degradation of the basement membrane, delocalization of N-cadherin out of adherens junctions, but no increase in integrins. Thus, confirming previous observations, Slug is effective in inducing cell delamination only if cells are specified as neural crest cells. Foxd-3 by itself was sufficient to induce first Sox-10 after 12 hours followed by HNK-1 expression after 24 hours associated with a decrease in N-cadherin expression. Ultimately, after 36-48 hours, it provoked an increase in integrins, a breakdown of the basement membrane allowing delamination and migration. Finally, combination of all three factors in neural tube cells caused cells to express neural crest markers, to delaminate entirely from the neural tube and to move actively in the surrounding tissues: this was associated with the complete breakdown of the basement membrane, the disappearance of N-cadherin from the cell surface, and the up-regulation of integrins. Thus, Sox-9, Foxd-3 and Slug ectopically expressed in the neural tube can recapitulate most of the events observed during neural crest delamination except that, unlike for endogenous neural crest cells, delamination is massive and uncontroled and leaves the neural tube totally disorganized (Fig. 2).

From these studies, some of the basic traits of the interplay between transcription factors during the transition from neural crest determination to early migration are now taking shape. First, delamination as well as specification and migration require the cooperating activities of at least three members of distinct families of transcription factors, Snail/Slug, Foxd-3 and Sox-9. Second, there is no simple linear hierarchies among these factors, rather a complex network of mutual interactions. Third, deployment of delamination is complete and efficient only if its basic cellular events are properly ordered in connection with crest cell specification and migration. For example, although neural crest specification is not sufficient to induce complete delamination (as suggested by experiments of Sox-9 overexpression) and that, conversely, delamination can be induced independently of specification (as suggested by overexpression of Rho-B or Ets-1), delamination is followed by active migration only if cells are specified into neural crest cells.

Regulation of Neural Crest EMT by a Balance between BMP-4, Noggin and Sonic Hedgehog

Long before transcription factors were identified in prospective neural crest cells as a response to inducing signals, it has been established that neural crest delamination is under the control of extrinsic factors released in the environment by the neighboring tissues, i.e., ectoderm, neural tube and paraxial mesoderm.[3] Because of their implication in the regulation of cell-substrate adhesion, members of the transforming growth factor-β (TGF-β) family have been suspected to play a critical role in neural crest delamination. Thus, our laboratory has been able to show that TGF-β1 and TGF-β2 induces a precocious emigration of neural crest cells from avian neural tube explants possibly by increasing adhesion of cells to their substrate.[8] The kinetics of TGF-β effect suggests that it functions primarily through integrin activation. More recently, the Kalcheim's laboratory confirmed and further extended this observation also in the avian system.[47] In particular, it was found that BMP-4 is expressed in the dorsal neural tube, and that addition of BMP-4 alone to neural tube explants stimulates production of neural crest

cells. Consistent with this, delaminating and early migrating neural crest cells express the BMP receptor IA.[70] However, BMP-4 expression is not restricted to the time window when neural crest cells are released, and instead it is expressed uniformly throughout a long portion of the neural axis, in a pattern consistent with a role not only in delamination but also in specification and early migration. Interestingly, Noggin, a BMP-4-specific antagonist, shows a dynamic expression in the dorsal neural tube along a caudorostral gradient that coincides precisely with onset of neural crest emigration, thereby suggesting that BMP-4 activity may be regulated spatially and temporally by Noggin in relation with delamination. In agreement with this assumption, addition of Noggin to neural tube explants or grafting Noggin-producing cells in embryos in the vicinity of the neural tube at the time of neural crest migration prevent neural crest cell migration. Later studies by the same laboratory demonstrated that Noggin expression is under the control of the paraxial mesoderm.[71] More specifically, the dorsomedial region of the dissociating somite was found to be the source of an inhibitory factor of an as-yet unknown nature that downregulates Noggin expression in the dorsal neural tube. Hence, neural crest delamination would be triggered by a signaling cascade elicited by BMP-4 interacting with its receptor BMPR-IA. The timing of crest emigration would be dictated by factors extrinsic to the neural epithelium, such as the dorsomedial portion of somite that controls Noggin expression in the dorsal neural tube. Additional cross-talks between the somite and neural tube cannot be excluded in order to further coordinate neural crest delamination and neural tube patterning with the maturation and subdivision of the paraxial mesoderm. However, this appealing model may not apply to all truncal levels but only for a very limited portion as it has been known for long that neural crest departure is not strictly synchronized with somitogenesis.[2] Additional regulatory mechanisms may then be required for controling timing of emigration. Furthermore, it remains to be determined whether this model can be transposed to cranial levels where the paraxial mesoderm is not partitioned into somites like in the trunk. Nonetheless, cranial neural crest formation and migration in the mouse has also been found to be under the influence of BMPs, as BMP-2 mutants show marked defect in neural crest development.[72]

The BMP-4-signaling cascade controls neural crest cell delamination primarily through regulation of adhesion events associated with EMT. As mentionned above, when added to chick neural tube explants, both TGF-β and BMP-4 promote substrate-adhesion of neural crest cells through activation of β1-integrins (ref. 8 and A. Jarov, C. Fournier-Thibault and J.-L. Duband, unpublished). In addition, BMP-4 can induce in a temporal sequence expression of, first, Slug and cadherin-6B, then Rho-B and finally cadherin-7.[31] Conversely, inhibition of BMP-4 by Noggin in chick embryo causes a severe repression of Rho-B, cadherin-6B but, surprisingly, not of Slug.[47] A likely explanation is that Slug is regulated by several independent processes that might compensate for the lack of BMP signals. In support of this, functional Lef-binding sequences have been isolated in the Xenopus Slug-gene promoter, suggesting that it might also be controled by Wnt signals.[73] However, the timing of appearance of these factors in prospective neural crest is not compatible with the expression pattern of BMP-4 and Noggin. Cadherin-6B and Slug, for example, are expressed in the dorsal neural tube long before the downregulation of Noggin and therefore prior to the time when BMP-4 signals are activated. This suggests that BMP-4 is not the endogenous inducer of these genes but that it is merely involved either in their maintenance or in their upregulation. Alternatively, these genes may be induced at thresholds of BMP-4 doses much lower than those necessary to cause delamination. As to the other key players in neural crest EMT are concerned, i.e., Foxd-3 and Sox-E genes, the possible influence of BMPs on their expression has surprisingly not been explored and documented yet. Of interest, members of the forkhead family to which Foxd-3 belongs have been found to be part of TGF-β-signaling pathways[74] whereas, in other systems such as limb cartilage differentiation, Sox9 has been proposed to function independently of, but in concert, with BMP.[75]

Although neural crest cells are the only cell type within the neural tube that is endowed with delaminating and migratory properties, it has become clear over the last decade that other neural epithelial cells also possess at least transiently some migratory capacity (Fig. 3a-e). When challenged with BMPs, ventral portions of neural tubes explanted in vitro can

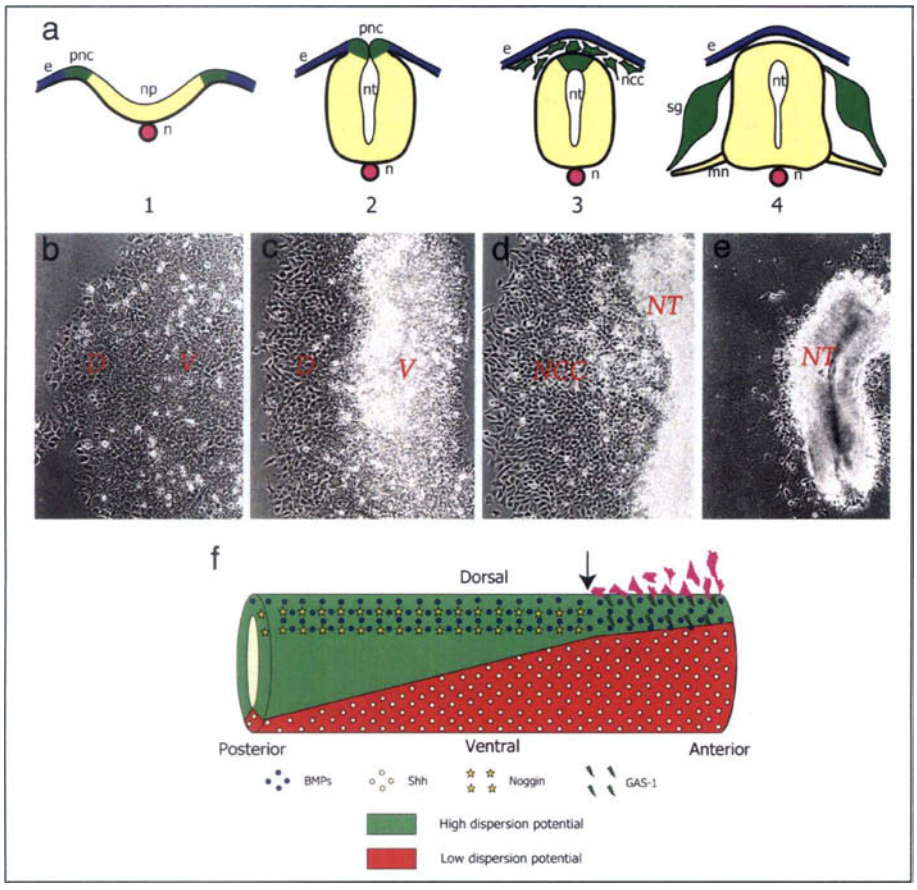

Figure 3. Morphogenetic control of cellular dispersion in the developing neural tube. a) Schematic representations of the main steps of neural tube formation at the truncal level in the chick embryo. 1, open neural pate; 2, closing neural tube; 3, closed neural tube at the onset of neural crest delamination and migration; 4, early spinal cord after cessation of neural crest migration and at the time of neuronal differentiation. e, ectoderm; mn, motor nerve; n, notochord; ncc, neural crest cells; np, neural plate; nt, neural tube; pnc, prospective neural crest; sg, spinal ganglion. b-e) Temporal changes in the ability of neural epithelial cells to disperse in vitro. Neural plate or neural tube explants at the brachial level were collected at the embryonic stages corresponding to the 4 above-mentionned steps of neural tube morphogenesis and cultured for 24 hours in fibronectin-coated dishes. In early neural plate explants, all cells are able to spread on the dish and disperse as an epithelium-like sheet (b). At the time of neural tube closure, in contrast, only cells originating from the dorsal half show the propensity to disperse, the remainder of the neural tube retaining its compact, original structure (c). At the time of neural crest cell migration, only crest cells are able to move actively on the dish (d). Finally, when neural tubes are explanted after the last neural crest has emigrated from it, no cells are able to adhere to the dish and move. Only neurites extending from motor nerves are able to grow out of the explant. D, Cells originating from the dorsal half of the neural tube; V, cells originating from the ventral half; NT, neural tube, NCC, neural crest cells. f) Putative model depicting the spatio-temporal control of neural epithelial cell dispersion by the antagonistic activities of the BMP-4 and Shh morphogens. See text for details.

generate cells that display some of the migratory characteristics of neural crest cells.[76,77] Likewise, as extensively described above, ectopic expression of Rho-B, Foxd-3, Sox-9 or Ets-1 in the intermediate or ventral neural tube can induce cell delamination sometimes followed

by migration. Furthermore, our laboratory has observed that all neural epithelial cells can naturally disperse in vitro on fibronectin or laminin substrates even in the complete absence of exogenous factors, provided they were derived from early, immature neural plates in the most caudal region of the embryo.[78] Interestingly, the migratory potential of neural epithelial cells is only transient and declines gradually along a ventrodorsal gradient with the progressive maturation of the neural tube. Thus, in contrast to cells from early neural plates which are all able to disperse, only cells from the dorsal half retain this ability in more mature neural tube adjacent to the anterior, unsegmented mesoderm. At axial levels corresponding to the epithelial somites, only neural crest cells situated at the apex of the neural tube are able to migrate. Later, after the last neural crest has emigrated, the neural tube remains compact when explanted in vitro, and virtually no cell is seen delaminating from it. This progressive restriction in the migration potential of the neuroepithelium along a ventrodorsal gradient is suggestive of an inhibitory action of diffusible factors originating from the ventral neural tube. This inhibitory activity is most likely attributable to Sonic hedgehog (Shh), a morphogen produced by the notochord and the floor plate that plays a citical role in the patterning of the neural tube. Indeed, in addition to its well-characterized function in driving differentiation of ventral neural tube cells and promoting their survival and proliferation, Shh has been found to control the substrate-adhesive properties of both dorsal and ventral neuroepithelial cells.[78,79] When Shh is presented under an immobilized form onto their substrate or produced by neuroepithelial cells themselves after transfection, neural tube explants or neural crest cells fail to disperse and instead form compact structures. Shh effect on cell adhesion is immediate, reversible and can be accounted for by inactivation of surface β1-integrins combined with an increase in N-cadherin mediated cell cohesion. In agreement with these in vitro data, forced expression of Shh in the dorsal neural epithelium after in ovo electroporation results in the cellular detachment of neural tube cells from the basement membrane followed by their collapse into the lumen, most likely due to inhibition of integrin function (C. Fournier-Thibault and J.-L. Duband, unpublished).

All together, these observations suggest that the adhesive properties of neural epithelial cells, both cell-cell and cell-substratum, are essentially regulated by the antagonistic activities of BMPs and Shh although they do not exclude the possible implication of other signaling molecules such as FGF, Wnt or retinoic acid. Dorsally, BMP-4 is produced by the ectoderm and the roof plate and its migration-promoting activity is restricted, spatially to the margin of the neural tube, because of its limited diffusion properties[80] and, temporally due to the antagonistic activity of Noggin. Conversely, Shh produced ventrally in the floor plate gradually diffuses toward the dorsal aspect of the neural tube and progressively restricts the capacity of neuroepithelial cells to disperse by lowering the activity of integrins and reinforcing cadherin contacts. The activity of Shh in the dorsal neural tube would be limited at least transiently by the production of GAS-1, a specific Shh antagonist.[81,82] The outcome of this exquisite regulation of cell adhesion by the interplay between morphogens and their respective antagonists would be that neural crest EMT is restricted spatially and temporally to the dorsal side of the neural tube (Fig. 3f).

Several Possible Scenarios for Neural Crest Delamination

Segregation of neural crest cells has so far been essentially regarded as an EMT. However, most the experiments aimed at manipulating EMT in the neural tube ended with the same striking outcome: the complete disorganization of the neural tube and the absence of replacement of the emigrating cells. Therefore, additional mechanisms are to be required to operate in parallel to or in combination with EMT to account for all the aspects of crest cell delamination, including for the correct spatio-temporal coordination and regulation of the molecular cascades and cellular events elicited by BMPs, Slug, Foxd-3 and Sox-9. Insight into these mechanisms can be gained if specification and delamination of neural crest cells are viewed as a question of generation of cellular diversity among neuroepithelial cells. Generating cellular

diversity is usually achieved through different means, such as EMT coupled or not with cell migration, proliferation of precursor or stem cells, asymmetric cell division and lateral inhibition, and all contribute to the establishment of frontiers between neighboring cell populations or to the segregation of individuals or groups of cells that subsequently follow distinct fates and acquire specific functions (Fig. 4).

Proliferation of Precursor Cells

There are now convincing, both direct and indirect evidence that formation of the neural crest involves proliferation of precursor cells situated in the dorsal neural tube. On the basis of genetic analyses in the mouse, Foxd-3 has been proposed to play a critical role in the establishement or maintenance of proliferating and self-renewing progenitor cell populations.[52] Although it has not been formally demonstrated in the case of neural crest cells, this role most likely also applies to them since Foxd-3 is restricted to few embryonic cell types, all exhibiting properties of multipotent progenitor cells, i.e., the blastocyst, epiblast, neural crest, neuroblasts and ES cells. Moreover, recent elegant experiments in the chick and Xenopus embryos have shown that segregation of neural crest cells from the neural tube is intimately coupled with cell division. In Xenopus, Kee and Bronner-Fraser found that depletion of Id3, a member of the Id family of helix-loop-helix transcription regulators expressed in nascent and migrating neural crest cells, results in the absence of neural crest progenitors.[83] This appears to be mediated by cell cycle inhibition followed by the death of the pool of neural crest precursors, rather than a cell fate switch. Conversely, overexpression of Id3 increases cell proliferation and results in a greater number of migrating neural crest cells. These observations therefore highlight a critical role for cell proliferation in the generation of neural crest cells and ascribe to Id3 a unique regulatory role in mediating the decision of neural crest precursors to proliferate or to die, independent of cell fate determination. In the chick, Burstyn-Cohen and Kalcheim established that truncal neural crest cells synchronously emigrate from the neural tube in the S phase of the cell cycle.[84] Inhibition of the G1/S transition in vivo or in explants specifically blocks delamination, without affecting expression of Slug, cadherin-6B, Rho-B or Pax-3. In contrast, arrest at the S or G2 phases has no immediate effect on delamination. It has been known for long that in neuroepithelial cells, the nucleus shuttles from the luminal side at mitosis to the basal side in the S phase. Neural crest cells would then delaminate at the favor of the most proximal position of their nuclei to their site of release. This appealing model accounts for the very progressive release of neural crest cells at truncal levels where delamination has been estimated to last during at least 24-30 hours, and it is compatible with the constant replacement of cells that have exited. Interestingly, at cranial levels where delamination is more sudden and massive (it lasts during less than 12 hours), fewer cells are in the S phase once they are released out of the neural tube, suggesting that in this region, delamination may be driven chiefly by EMT (E. Théveneau, M. Altabef and J.-L. Duband).

However, it remains to determine whether cell division in the dorsal neural tube is sufficient to account for the total number of cells that delaminate from the neural tube. This will require a precise estimate of the number of divisions that occur in the neural tube as well as the number of neural crest cells that are produced at each axial level. Moreover, it will be necessary to determine why cells are released out of the neural tube only in its dorsal aspect since this nuclear migration event occurs in all neuroepithelial cells. The lack of an organized, continuous basal lamina along the dorsal neural tube may be one of the cues to allow the release of cells once they become located basally. Alternatively, cell cycle would be coupled with other cellular events involved in the detachment of the cells from the neural tube. In this regard, further studies in the avian embryo reported recently by the Kalcheim's laboratory propose that G1/S transition in neural crest cells accompanying cell delamination is linked to BMP/Noggin signaling through the canonical Wnt signaling.[85] They found that Noggin overexpression inhibits G1/S transition, while blocking G1/S transition abrogates BMP-induced EMT. Moreover, Wnt-1 expression is stimulated by BMP and interfering

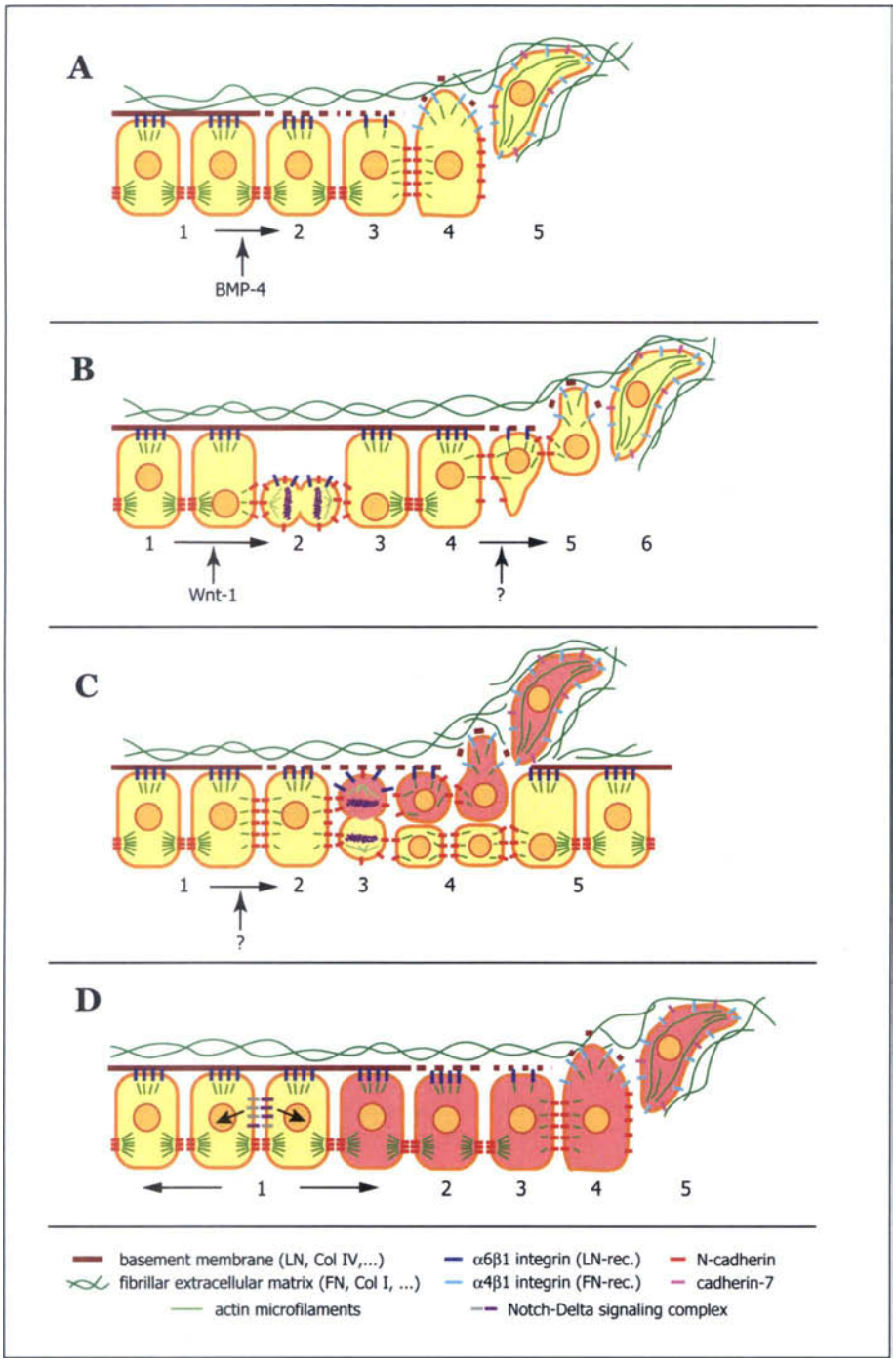

Figure 4. Four possible scenarios of neural crest cell delamination. Figure legend continued on next page.

Figure 4, continued. Four possible scenarios of neural crest cell delamination : A) epithelium-to-mesenchyme transition; B) proliferation of precursor cells; C) asymmetric cell division; D) lateral inhibition. It should be stressed that these scenarios should not be considered as mutually exclusive but may rather occur in combination or sequentially to account fully for the entire process of delamination and for the diversity of the neural crest cell population. A) Epithelium-to-mesenchyme transition. Prior to onset of delamination (1), neural epithelial cells are assembled into a epithelial sheet of a single cell layer lined by a basement membrane. Note that, as opposed to the conventional representations of epithelial cells, the basal side of the cells have been represented at the top and their apical side at the bottom to account for the normal orientation of cells in the neural epithelium. Cells present well-organized adherens junctions toward their apical sides. Upon the effect of BMP-4, cells secrete matrix proteases in their basal side, allowing them to digest the basement membrane (2). Then, they progressively downregulate integrin receptors for constituents of the basement membrane, such as laminin (3) and, instead, express integrin receptors for fibrillar matrix elements, e.g., fibronectin (4). Adherens junctions are disrupted resulting in redistribution of N-cadherin molecules along the lateral surfaces of the cells and in actin cytoskeleton reorganization, permitting cells to change shape and emit pseudopodia protruding into the overlying extracellular matrix (4). Finally, N-cadherin molecules are completely downregulated and replaced by cadherin-7, allowing cells to dissociate completely from the rest of the epithelial sheet and to interact with the fibrillar extracellular matrix for locomotion (5). B) Proliferation of precursor cells under the control of Wnt-1 signals. In neural epithelial cells, the nucleus shuttles from one pole of the cell to the other during the cell cycle. At the G2 and M phases, it is situated toward the apical side of the cell. During mitosis, the entire cell body is shifted apically and the cell loses contact with the basement membrane (2). Due to the presence of stable adherens junctions, the mitotic spindle is oriented perpendicular to the apicobasal axis of the cell, and division occurs symmetrically, leading to two identical daughter cells which reform contacts with the basement membrane immediately after cleavage. The nucleus progressively migrates toward the basal side during the G1 and S phases (3,4). At the S phase, when the nucleus is closest to the basal side, cells may lose contact with the neighbors at their apical side and dissociate from the rest of the epithelium provided an additional, as yet unidentified signal triggers degradation of the basement membrane and disorganization of adherens junctions (5,6). C) Asymmetric cell division. If neural epithelial cells receive signals that causes adherens junctions to deteriorate (2), the orientation of the mitotic spinde during cell division is shifted from orthogonal to parallel to the apical-basal side and cells divide asymmetrically (3). This may result to the generation of two distinct daughter cells that differ in their content of cell fate determinants (4). While the apically-located cell retains contact with the rest of the epithelium and contributes to the maintenance of its integrity, the basal cell may be expelled at the favor of local disruption of the basement membrane (5). D) Lateral inhibition. Two originally-identical neural epithelial cells exchanging information through the Notch-Delta signaling complex may adopt distinct cell fates and follow different genetic programs. One may retain its epithelial features and remain integrated in the neural epithelium whereas the other one may undergo epithelium-to-mesenchyme transition, e.g., under the control of Slug (2-5).

with Wnt signaling by blocking β-catenin and Lef-Tcf inhibits G1/S transition, cell delamination and transcription of several BMP-dependent genes. However, several previous observations are at variance with this study, and the precise function of Wnt signals in neural crest emigration remains obscure. First, although Wnt-1 presents a localized expression in the dorsal neural tube at the time of crest delamination, onset of its expression matches closer with somitogenesis than with neural crest delamination and, unlike BMP4, it persists in the dorsal neural tube long after cessation of emigration. Such an expression pattern is therefore not compatible with a direct role in the triggering of emigration. Second, targeted inactivation of Wnt-1 and Wnt-3a as well as genetic analyses in the mouse aimed at altering the canonical Wnt signaling pathway in neural crest cells mostly implicated Wnt signals in lineage specification rather than in delamination.[86-88] Third, neural crest cells produce Wnt antagonists at the time of their emigration, thereby raising the intriguing possibility that they may not be responsive to Wnt signals at this step.[89-91] Finally, overstimulation of Wnt-β-catenin signals in neural crest cells in vitro has been found to provoke a severe inhibition of delamination and migration, as a result of a decrease in substrate adhesion and reduction of proliferation.[92]

Asymmetric Cell Division

During asymmetric cell division, cells become polarized in response to extrinsic or intrisic signals before mitosis. As a result, cell-fate determinants present in the cytoplasm become localized to one pole of the cell in alignment with the mitotic spindle and, upon cytokinesis, they are unequally partitioned to the two daughter cells.[93] At present, there are no definitive proof for the contribution of asymmetric cell division to neural crest cell delamination. In particular, although both horizontal (i.e., symmetrical) and vertical (i.e., asymmetrical) cleavage plans can be detected in the dorsal neural epithelium at the time of neural crest delamination, the cell-fate determinant Numb does not show a polarized distribution in mitotic neural epithelial cells prior to neurogenesis.[94] Several indirect observations would in contrast argue in favor of the implication of asymmetrical division in neural crest cell delamination. Cell tracing experiments using lipophilic dyes such as DiI revealed the existence of a common precursor for neural crest and dorsal neural tube cells.[95,96] In Drosophila, neuroepithelial cells are polarized along the apical-basal axis and divide symmetrically; upon deterioration of adherens junctions, cell divisions are converted from symmetric to asymmetric,[97] suggesting that orientation of the mitotic spindle is under the control of adherens junctions. Although this mechanism has not been specifically addressed in neural crest cells, disruptions of adherens junctions have also been detected among dorsal neuroepithelial cells, coincidently with delamination.[17] In Drosophila again, Snail proteins play an essential role in the generation of neuroblasts by controling expression of cell fate determinants during asymmetric cell division.[98]

Lateral Inhibition

Lateral inhibition is the process by which a cell both adopts a distinct fate from its neighbors, through signal echanges mediated by Notch and its receptor Delta, and prevents them to follow the same differentiation program. It allows to explain how a regularly-spaced array of structure can develop from a uniform field such as the development of the nervous system in Drosophila.[99] It is not clear yet whether Notch signals directly affect neural crest cell delamination or whether they are merely involved in the establishement of frontiers between the ectoderm, neural crest and neural tube domains, but it is worth-mentionning that Notch has been found to promote EMT via Snail during cardiac cushion tissue formation in Zebrafish,[100] a process presenting numerous similarities with crest cell delamination. In addition, it has been shown in the avian embryo that Notch has a dual function during neural crest formation, first, in maintaining expression of BMP-4 in the ectoderm and, second, in inhibiting Slug expression also in the ectoderm, possibly to prevent aberrant delamination of cells from this tissue.[101] Conversely, in Drosophila, during differentiation of the mesoderm, Snail shows a dual activity on the Notch signaling pathway: it stimulates Notch signaling in some cells while repressing Notch target genes in others, thereby contributing to create precise boundaries among the tissue.[102] Finally, at the time of neural crest delamination, lunatic fringe, a positive modulator of Notch signaling, is expressed throughout the neural tube with the notable exception of the prospective neural crest area, thereby delineating a border between the neural crest and neural tube domains. Overexpression of lunatic fringe in the cranial neural tube by retrovirally-mediated gene transfer causes a significant increase in the number of migrating neural crest cells as a result of activated cell proliferation.[103]

Other Possible Means

Other morphogenetic events may influence emigration of neural crest cells from the neural tube. One possibility is the elevation of the neural folds and the closure of the neural tube. In most species, delamination of neural crest cells is coupled to neural tube closure, but untill recently there was no easy and appropriate mean to manipulate the mechanical events accompanying neurulation to investigate their possible impact of neural crest delamination. Recent studies identifying new genes involved in the dynamics of neurulation may however provide new insights into this process.[104] The other mechanism that can be put forward is repulsion

from the neural tube. Although this possibility has not been seriously explored yet, it cannot be excluded since expression of molecules with repulsive activities has been reported in the dorsal neural tube at the time of neural crest delamination. For example, both Slit-1 and Slit-2, two proteins known to be potent chemorepellents for a variety of axons in Drosophila and in Vertebrates show a conspicuous expression in the roof plate. The available informations about the temporal expression of slits in relation with neural crest emigration are not sufficient to speculate on their implication in this process, but it is worthmentionning that trunk neural crest cells show a dual response to Slit-2 in vitro: they avoid cells expressing slit-2 and they migrate farther when exposed to soluble slit.[105] BMP-7 is also a candidate molecule for repulsing neural crest cells out of the neural tube by analogy with commisural neurons. These neurons, located near the dorsal midline, send axons ventrally and across the floor plate but not dorsally through the roof plate. The latter has been found to express a diffusible factor that repels commisural axons and orients their growth within the dorsal spinal cord, and this chemorepellent activity is mediated by BMP-7 produced by roof plate cells.[106]

If it is confirmed that these mechanisms actually participate to the control of neural crest cell delamination, it will be of importance to determine at the single cell level whether they are purely independent or whether they may reflect a genuine requirement for distinct signaling processes. Moreover, such a multiplicity of cellular events is likely to represent a mechanism to establish precocious heterogeneity within the neural crest, even prior to delamination.[107] It is then conceivable that different subsets of neural crest progenitors are specified independently and sequentially by distinct signaling cascades and are released at the periphery of the neural tube by different cellular processes (Fig. 4).

Cessation of Delamination

Important efforts have been put on the signaling cascades that induce neural crest cell delamination and migration and we are now relatively close to a fair appreciation of the basic mechanisms that govern these events. In comparison, very few is known about the mechanisms controling cessation of delamination, although it is likely to be precisely regulated in connection with the further development of the spinal cord. Several mechanisms may account for cessation of emigration. If neural crest cells are generated by a limited pool of precursors, cessation of emigration naturally occurs with the last precursor cell to segregate from the neural tube. This hypothesis supposes that the neural crest precursor, yet to be identified, lacks the capacity to give rise to cells other than neural crest cells and, reciprocally, that roof plate cells cannot differentiate into neural crest-like cells after cessation of emigration. However, although the existence of a unique neural crest precursor cannot be ruled out, cell lineages studies have clearly shown that neural crest or neural tube cells can be generated at the expense of one another, therefore arguing in favor of a great flexibility in the differentiation potential of neural epithelial cells at the time of neural crest emigration. Moreover, even if neural crest stem cells have been identified, they are very rare and their proportion cannot account for the production of all emigrating neural crest cells. Consistent with this, as mentionned above, strong heterogeneity has been observed among neural crest cells even before delamination. A more likely alternative then is that neural crest cells are generated from multipotent cells in the dorsal neural tube and that cessation of migration results as an impoverishment of this potential due to an intrinsic developmental program defined by a sort of internal clock and/or in response to external influences. If it is so, it would be then conceivable to induce experimentally a prolonged production of neural crest cells without major alteration in the morphology of the neural tube. So far, the only documented example concerns the secreted glycoprotein Noelin-1. In chick, Noelin-1 messages are expressed in a graded pattern in the closing neural tube and later they are restricted to the dorsal neural folds and migrating crest. Overexpression of Noelin-1 causes an excess of neural crest emigration and extends the time that the neural tube is competent to generate neural crest cells.[108] How Noelin-1 mediates its effect at the molecular level has not been investigated yet partly because it has been found to exhibit divergent expression patterns in frogs, mouse and chick and thus may not perform the same functions.[109]

Neural Crest Migration: How to Survive, Move and Develop in a Hostile Environment

Much data has accumulated over the years on the process of neural crest cell migration, notably the road maps and the driving code. Interestingly, new concepts are progressively emerging from recent studies using different model systems and new insights are to be expected with the development of powerful real-time imaging techniques.

Transition between Delamination and Migration

With the exception of the Axolotl in which neural crest cells stand for a while on top of the neural tube prior to undergoing migration (hence the term neural crest), migration immediately follows the delamination step; yet, as discussed above, there are experimental evidence that both events are driven by independent and discernible mechanisms. However, the nature of the signals triggering onset of migration is not known at the present time. A candidate molecule for triggering neural crest motility is the hepatocyte growth factor (HGF) also known as scatter factor, but so far its expression has not been reported in neural crest cells, although they can respond to exogenous HGF in in vitro cultures (M. Delannet and J.-L. Duband, unpublished). Efficient neural crest migration may be achieved in vitro in the complete absence of external growth factors added to the culture medium or originating from the neural tube, indicating that induction of migration may be cell autonomous and would depend directly on cellular events occuring during delamination. Disruption of cadherin-mediated cell contacts and activation of integrins may be one of such events. We have shown previously that, in migrating neural crest cells, surface distribution and activity of N-cadherin are precisely regulated by intracellular signals elicited by integrins, thereby revealing that direct coupling between adhesion receptors provides the necessary interplay between cell-cell and cell-substrate adhesion during migration.[110] Indeed, in vitro, neural crest cells express intact N-cadherin molecules on their surface but, contrasting with nonmotile cells, the bulk of these molecules is maintained excluded from the regions of cell-cell contacts, thus causing their instability. Stable contacts can be restored upon addition to the cells of specific inhibitors of integrin function and signaling activity. A possible target of this signaling pathway is β-catenin, known to play a critical role in both intercellular adhesion and cell signaling.[111-113] Interestingly, it has been found in other cellular systems that cadherin binding can cause a massive recruitment of β-catenin to the cell membrane, thereby sequestering it and preventing its nuclear localization.[114] This ability of cadherins to regulate the pool of β-catenin available for signaling therefore raises the intriguing possibility that β-catenin function in neural crest cells would be possibly driven by modulations in cellular cohesion during migration. Thus, in migrating neural crest cells, N-cadherin activity would be repressed by signals emanating from integrins, thereby resulting in an increase in the cytoplasmic pool of β-catenin that would be in turn allocated to the nucleus. Conversely, upon cessation of cell migration, e.g., after inhibition of integrin function, N-cadherin-mediated cell-cell contacts would be restored and β-catenin would be mostly recruited to them and would no longer be available for signaling. Consistent with this model, we reported recently that β-catenin is essentially associated with N-cadherin at the cell surface of actively migrating neural crest cells and that it is detected in their nuclei in association with Lef-1 only at the time of their segregation from the neural tube. However, manipulating N-cadherin-mediated cell contacts in migrating neural crest cells had no obvious impact on the nuclear localization of β-catenin, indicating that the membrane and nuclear pools of β-catenin are not directly connected at least during migration.[92] Thus, the putative role of β-catenin as an inducer of neural crest cell migration awaits further developments and the identity of the molecular switch between delamination and migration remains elusive.

Maintenance of Survival during Neural Crest Migration

The problem of neural crest cell survival after delamination and during migration has long been underestimated. It was generally believed that, once released at the periphery of

the neural tube, neural crest cells are naturally endowed with the ability to move, proliferate, and invade tissues and are marginally concerned with survival problems except in regions that they are not supposed to occupy. However, during the 1990s, it became obvious that in all animal organisms, only with rare exceptions, each cell type is programed to survive in a very peculiar tissular environment. Consequently, when a cell escapes from its tissue, it is immediately confronted with a new environment that may present very different features and does not support their survival: such cells unavoidably undergo apoptosis, thus eliminating any risk of aberrant cellular interactions. This "environmentalist" view has for example revolutionized our conception of cancer, which can be defined as the series of intracellular alterations allowing a cell to proliferate in an uncontroled manner, to be freed from its environmental constraints and to become able to occupy new territories. The same view also applies to migrating neural crest cells.

The importance of maintaining cell survival during neural crest ontogeny emerged in recent studies investigating the function of molecular players originally thought to play a role in cell specification and delamination. Neural epithelial cells forced to undergo EMT by expressing Rho-B or Ets-1 fail to migrate and to survive after their release at the periphery of the neural tube, in contrast to cells forced to express Foxd-3 or Sox-9 that do not show any sign of apoptosis. Phenotypic characterization of these cells reveal that Rho-B- or Ets-1-transfected cells show a striking difference with the Foxd-3- or Sox-9-transfected cells in that they express none of neural crest cell markers, such as HNK-1, Slug and cadherin-7 (refs. 28, 29, 53 and E. Théveneau, M. Altabef, and J.-L. Duband, unpublished data), suggesting that acquisition of a neural crest phenotype may protect cells from apoptosis once they are segregated from the neural epithelium. Accordingly, neural crest cell death can be rescued in Rho-B-transfected neural tubes by cotransfecting Sox-9.[29] Among the numerous genes expressed in premigratory neural crest cells, Slug (or Snail in mouse) is likely to play a major role in maintaining neural crest survival. In Sox-9 mutant embryos, neural crest cells are specified and are able to undergo EMT, yet they die soon after the onset of migration. Interestingly, almost all the essential neural crest genes, including Foxd-3, are expressed in neural crest cells from these embryos, with the notable exception of Snail.[29] In C. elegans, several lines of evidence point to a direct role for Snail superfamily members in the control of cell death.[36] Finally, further experiments using cultured cell lines and mouse and chick embryos convincingly demonstrated that Slug and Snail confer resistance to cell death induced by the withdrawal of survival factors or by pro-apoptotic signals.[115] Thus, Snail proteins may not play an essential role in triggering EMT among prospective neural crest cells, but they may directly connect cell survival with EMT and possibly with other cellular events such as cell division and Notch signaling, thereby providing to crest cells a selective advantage to migrate and populate distant territories. *It is not much their ability to migrate actively that make neural crest cells a peculiar cell population among the neural epithelium, but it is mostly their capacity to survive once they become irreversibly separated from the neural tube.*

Though it may be important for cell survival, Slug may not be the sole factor involved in protection from cell death. In particular, ectopic expression of Foxd-3 in the neural tube induces cell delamination but no apoptosis despite the absence of Slug induction.[29,49] It cannot be excluded that Foxd-3 itself may drive a survival program in neural crest cells directly or indirectly by alternative means. Indeed, ectopic expression of Foxd-3 has been found to upregulate adhesion molecules of the cadherin and integrin families, which have been found to prevent apoptosis of epithelial cells, a process called anoikis.[116] Moreover, Slug is only transiently expressed by migrating neural crest cells, particularly at truncal levels, therefore raising the problem of how this system is relayed to ensure maintenance of cell survival. A possibility is that neural crest cells produce their own survival factors or become responsive to growth factors secreted by tissues neighboring their migration routes. In this respect, it has been demonstrated that the neural tube produces factors that selectively support survival and proliferation of neural crest sub-populations and that this activity can be mimicked by FGF-2 or BDNF.[117,118]

Integrin Function Is Not Limited to the Control of Cell Adhesion and Locomotion

The organization and molecular composition of the migration routes of neural crest cells have been amply documented. Neural crest cells follow defined, restricted pathways that contain a fibrillar network of extracellular matrix material and are lined by the basal laminae of epithelia,[2,119] although it is plausible that under certain conditions, they may also use the surface of cells encountered during migration as a support for locomotion. Numerous functional studies have provided convincing evidence that the matrix encountered by crest cells serves as a scaffold onto which they migrate and that integrin receptors for matrix molecules play a prominent role in this process. In vitro, neural crest cells are able to spread and migrate in an integrin-dependent manner onto a variety of matrix components, including fibronectin, laminin-1, vitronectin and collagens[120-123] and, in vivo, antibodies to matrix molecules or integrins, competitor peptides, or antisense oligonucleotides to integrins or matrix constituents all perturb crest cell migration.[124-127] Thus, integrin play a critical role in the mechanics of neural crest cell migration.

However, it is now increasingly clear that integrin functions during neural crest development are not limited stricly to substrate anchorage and cell motion. Integrins are heterodimers of α and β subunits that are present and conserved in all metazoan animals and are primarily involved in physical aspects of cell adhesion to the substrate, in cell traction and matrix assembly, by providing a bridge between the matrix and the cytoskeleton.[128,129] Central to their function is their unique ability to promote actin assembly to generate tension locally via the recuitment of a wide array of molecules that directly activate the actin polymerization machinery or physically link it to adhesion sites.[130,131] In addition to their structural and mechanosensory roles, integrins are able to activate upon engagement with their ligands a large variety of tyrosine kinases and GTPases to induce multiple downstream signaling pathways.[132,133] Furthermore, integrin signals have been found to cooperate with and modulate signaling events initiated by growth factor receptors.[134] Thus, because of this dual physical and chemical signaling activity, integrins influence numerous aspects of cell behavior, including migration, proliferation, survival, and differentiation.

It has been found that crest cells migrating in vitro express a multiplicity of integrins (at least three vitronectin receptors, three laminin-1 receptors and up to seven fibronectin receptors), and that not all of them are implicated in adhesion and migration.[120,121,135] Such a diversity of matrix receptors certainly reflects the very changing nature of the environment to which crest cells are confronted during migration, but is also presumably related with additional roles for integrins not directly related to matrix adhesion. For example, as discussed earlier, integrins have been found to control cell-cell interactions during migration by repressing the surface distribution and activity of N-cadherin, thus ensuring rapid and flexible coordination between adhesion systems.[110] Integrins are also involved in the maintenance of cell survival as revealed by functional studies in avian embryos[136] and by genetic analyses in mouse and zebrafish.[137-139] In fact, the primary defect observed in neural crest cells of embryos depleted in individual integrin α subunits is anoikis during migration, thereby revealing that anchorage-dependent survival signals elicited by integrins are of paramount importance for cells confronted with a continuously-changing environment in which supplies in growth factors are limited. Finally, both changes in the integrin repertoire at the time of neuronal differentiation in peripheral ganglia and the numerous alterations observed in conditional β1-integrin gene deletion argue for a possible implication of integrins in late neural crest development, such as lineage segregation, cell differentiation, and final maturation of the peripheral nervous system.[25,140,141] All together, these observations suggest that integrins may control multiple cellular events during neural crest development. Yet, the signaling cascades that are responsible for their coordination over time and space remain to be established.

Novel Guidance Mechanisms for Neural Crest Cells

One of the main goals of migration is to segregate neural crest lineages from a uniform population and to drive them to specific locations at the right time so that they can undergo differentiation and establish appropriate contacts with their neighboring tissues. Hence a complex driving code which guides cells to their targets and prevents them from invade wrong pathways and occupying aberrant tissues. In most cases, this code is essentially a repressing code, with a complex combination of repulsive molecules (Fig. 5). This is particularly well-documented in the case of the somite which imposes a partition of the truncal neural crest population into several streams, each at the origin of the sensory, sympathetic, Schwann cell and melanocyte lineages. At least 10 different molecules have been implicated in the restriction of neural crest cell migration into the rostral half of the somite.[142] These molecules are primarily recruited among extracellular matrix components or surface molecules released into the matrix, e.g., tenascin, F-spondin and semaphorins, as well as among surface receptors such as the ephrins and slits. The same mechanism is likely to apply also to the colonization of the branchial arches by hindbrain neural crest cells.[143,144] Recently, two studies provided interesting information about the mechanisms by which the various neural crest subpopulations can interpret this code and distribute differently along separate pathways. In the first one, De Bellard and coworkers found that slit-1, slit-2, and slit-3 are expressed in the mesenchyme adjacent to the ventral aorta and the gut, which is selectively invaded by vagal, but not truncal, neural crest cells, suggesting that they may prevent ventral migration of trunk crest cells.[105] Accordingly, truncal, but not vagal, neural crest cells express Robo-1 and Robo-2, two receptors for slits, and avoid Slit-expressing cells in vivo and in vitro, clearly indicating that Slit may contribute to the differential ability of neural crest population to populate and innervate the gut.[105] In the second study, Santiago and Erickson investigated how neural crest cells fated to sensory and sympathetic lineages only migrate ventrally and are prevented from migrating laterally into the skin, whereas melanoblast precursors are directed only toward the lateral pathway.[145] They found that ephrin-B ligands are expressed in the lateral pathway at the stage when neural crest cells migrate ventrally, consistent with a putative repulsive activity. Non surprisingly, inhibition of ephrin receptor function by addition of soluble ephrin-B ligand relieves the blockade of migration, thus allowing precocious invasion of the lateral pathway by neural crest cells normally migrating only ventrally. However, ephrin-B ligands unexpectedly continue to be expressed at later stages during melanoblast migration. Furthermore, when signaling of the Eph receptors for ephrins is disrupted in vivo, melanoblasts fail to migrate laterally, suggesting that ephrin-B ligands not only favor but are required for melanoblast migration. Thus, ephrins act as bifunctional guidance cues: they first repel sensory and sympathetic neural crest precursors from the lateral pathways, and later stimulate migration of melanoblast precursors into this pathway. The mechanisms by which ephrins regulate repulsion or attraction in crest cells are unknown, but most likely reside in the downstream signaling cascades elicited by the Eph receptor.

Chemotactism has long been proposed to account for the precision by which neural crest cells reach their target sites, particularly for those subpopulations that colonize sites situated at long distances from the source. But numerous arguments have been opposed against chemotactism in the case of neural crest cells. First, neural crest migration is essentially centrifugal, from a single source, the neural tube, to multiple target sites and this is not considered as compatible with chemotaxis which instead is highly efficient for centripetal migrations towards a unique final destination, as e.g., germ cells invading the gonads. Second, neural crest cells undergo migration sometimes well before their target tissue are elaborated in the embryo and this is difficult to reconcile with chemotactism. Finally, manipulations of the neural tube in ovo such as rotation along the dorsoventral axis revealed that neural crest cells can move backward along their migratory paths. However, although the existence of a unique chemotactic mechanism is unlikely to account for all neural crest directions, it is plausible that this process may regulate locally migration of distinct neural crest subpopulations such as melanoblast precursors and enteric neural crest cells. Thus, the dermis has been proposed to attract

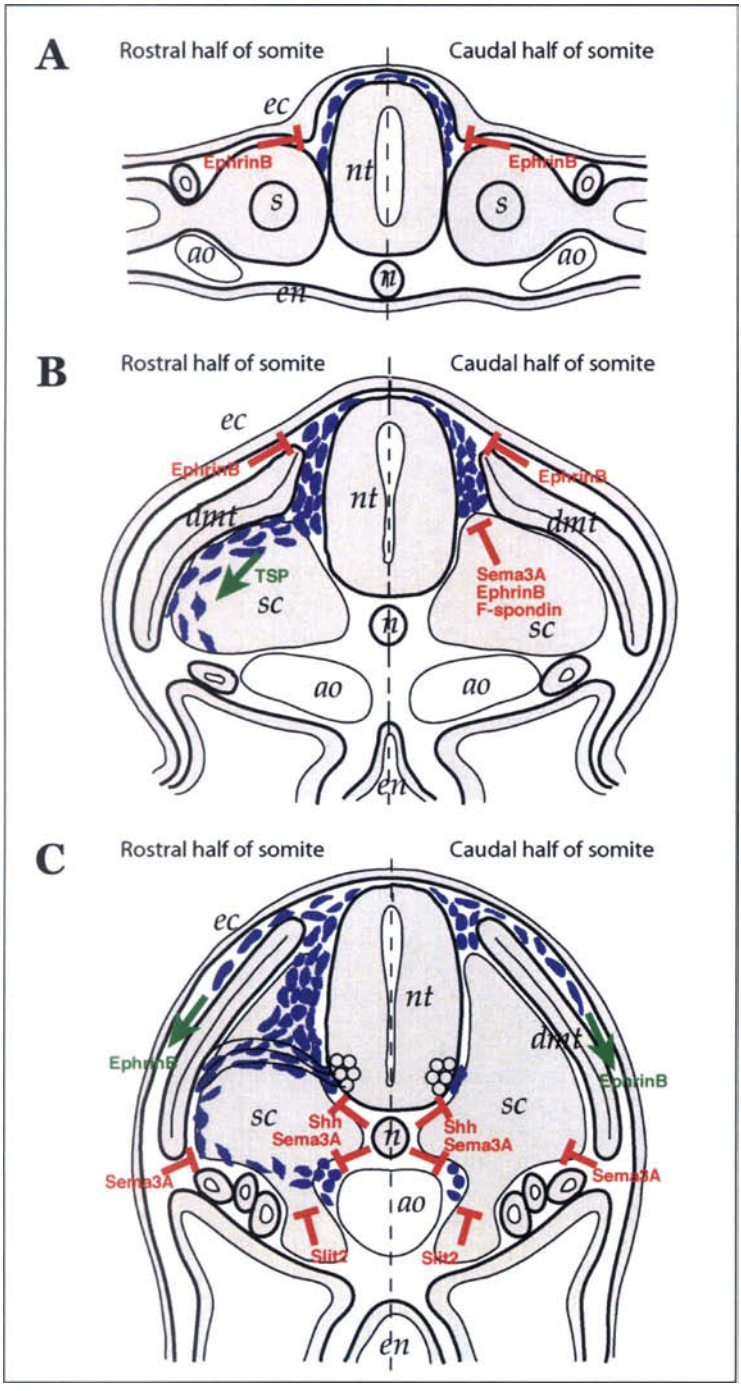

Figure 5. Environmental cues involved in the guidance of truncal neural crest cells. Figure legend continued on next page.

Figure 5, continued. Schematic representations of the trunk region of chick embryos viewed transversally at three typical stages of neural crest migration : early migration (A), ventral migration through the somite (B), late migration phase (C). For each step, the position of neural crest cells and the guidance cues are indicated at the levels of rostral and caudal halves of the somite, shown on the left and right sides of the picture, respectively. Neural crest cells are depicted in dark blue and the other solid tissues in grey : ao, aorta; dmt, dermomyotome; ec, ectoderm; en, endoderm; n, notochord; nt, neural tube; s, somite; sc, sclerotome. A) At the onset of migration, neural crest cells migrate along the neural tube and rapidly reach the dorsomedial edge of the somite. Their lateral migration under the ectoderm is inhibited by ephrin-B ligands. B) As neural crest cells enter the somitic area, they are prevented to migrate farther through the sclerotome in the caudal half of the somite by a variety of repulsive molecules : sema-3A, ephrin-B and F-spondin. Such cues are not produced in the sclerotome in the rostral side of the somite, instead, positive cues stimulating migration, such as thrombospondin, are released, allowing crest cells to migrate along the developing myotome to reach the ventral side of the embryo. At this stage, neural crest cells still cannot invade the lateral pathway and stand in the so-called staging area, between the ectoderm, the neural tube and the dorsomedial aspect of the dermomyotome. C) During the last period of migration, crest cells migrating ventrally are guided toward the aorta and are forced to coalesce into the primordium of the sympathetic ganglia due to the combination of repulsive cues that prevent them migrating laterally to the limb (sema-3A), ventrally to the endoderm (slit-2) and medially to the aorta (sema-3A and Shh). Cells that accumulate along the neural tube cannot migrate farther ventrally toward the notochord due to Shh effect and, consequently they regroup into the primordium of the spinal ganglia. Conversely, in the lateral pathway, ephrin-B repulsive cues turn into stimulating factors, thereby favoring migration of the melanocyte precursors through the dermis. Repulsive cues are depicted by red arrows while cues favoring migration are indicated by green arrows.

melanoblast precursors to the lateral path by a chemotactic mechanism, based on the observations that melanoblasts cannot enter the lateral path until emergence of the dermis and that grafts of dermis explants distally in the lateral pathway elicits precocious migration of neural crest cell into this pathway.[146] Likewise, GDNF, a growth factor critical for the survival of enteric neural crest cells, has been found to promote oriented migration of neural crest cells throughout the gastrointestinal tract and to prevent them from dispersing out of the gut.[147,148]

Cell Communications during Migration

During the course of their migration, neural crest cells continuously establish contact with the neighboring tissues as well as with the other neural crest cells. Analyses of static images of embryonic sections stained with neural crest markers, notably HNK-1, and videomicroscopic studies of cells cultured in vitro clearly established that neural crest cells migrate as a cohort of cells remaining in close contact and that only few pioneer cells migrated as individuals (e.g., see ref. 149). Partly based on these observations, Thomas and Yamada proposed in 1992 the new, intriguing concept that migration could be stimulated by cell-cell contacts instead of being inhibited as originally described for isolated fibroblasts by Abercrombie.[150,151] Unfortunately, the molecular cascade involved has not been deciphered and this hypothesis has been poorly visited since then. It is only recently that the importance of cell communication during migration has been reconsidered seriously owing to numerous technical developments, including embryo or explants cultures, high resolution confocal microscopy and in ovo imaging methods combined with the great improvement of cell labeling and transient transfection techniques. Young and coworkers used mice in which the expression of GFP is under the control of the ret promoter to visualize enteric neural crest cell migration in the embryonic gut in organ culture.[152] In a similar approach, Teddy and Kulesa and Kasemeler-Kulesa and coworkers used chick embryos in which the neural tube is electroporated with GFP to explore hindbrain and truncal neural crest migration in whole embryo explants maintained in vitro.[153,154] These studies revealed that neural crest cell migrate in chain-like formations that displayed complicated patterns of migration with sudden and frequent changes in migratory speed and trajection very much like in in vitro cultures. Pioneer cells formed a scaffold onto which following cells migrated. Morover, cells maintained nearly constant contacts with other migrating neural crest cells both at short and long distances up to 100 μm. Interestingly,

cell-cell contacts often stimulated a cell to change direction, thereby revealing intense communication between cells and their possible implication in directional guidance.

The identity of the mediators of cell communication between neural crest cells is currently unknown but plausible candidates include cadherins and connexins. For example, cadherin-7 which is expressed in neural crest cells as soon as they segregate from the neural tube may be involved in cell recognition among navigating neural crest cells although this possibility has not yet been addressed directly. This putative function is compatible with the fact that cadherin-7 does not mediate strong cell-cell interactions in comparison with N-cadherin or E-cadherin and that its expression does not interfere with cell locomotion.[155] Additional studies on mouse neural crest cells revealed that, in N-cadherin-deficient embryos, neural crest cell migration is affected. Videomicroscopic analyses further indicated that neural crest cells lacking N-cadherin exhibit an elevated speed of locomotion but that cell directionality was reduced, therefore pointing to the implication of N-cadherin in cell-cell communication during migration.[156] Likewise, transgenic mice exhibiting inhibition of connexin-43 in cardiac neural crest cells showed a deficit in neural crest cells in the outflow tract due to a reduced cell migration. Conversely, an elevation of connexin-43 expression caused outflow tract obstruction and conotruncal heart malformation as a result of an enhanced rate of neural crest migration and an increase in the abundance of neural crest-derived cells in the outflow tract.[157]

Cessation of Migration

As for delamination, cessation of migration has been poorly explored and numerous questions remain open. Supposedly, it involves the converse sequence of events of delamination and onset of migration, i.e., inactivation of integrins consecutive to the loss of substrate adhesion, increase in cell cohesion, reorganisation of the cytoskeleton and more in-depth modifications of cells, such as engagement into a differentiation program, thereby rendering the process of cessation of migration irreversible. Curiously, although neural crest cells navigate through a large variety of territories and interact with numerous cell types, once they stop migration, they regroup into homogenous clusters composed essentially of neural crest cells and they do not mingle with other cells. This is particularly true for the major neural crest derivatives such as peripheral ganglia (spinal, sympathetic, ciliary, cranial and enteric) and for the skull. There are however a few notable exceptions to this rule, such as the melanocytes, some cranial ganglia and connective tissues of the face and neck, where neural crest cells mingle with the other cell populations in the invaded tissues.

Several processes have been put forward to explain in causal terms the cessation of migration of neural crest cells, but they mostly apply to the first category of neural crest derivatives. Spatial restriction of migration involving coincidently the lack of available space, the absence of a suitable extracellular matrix and/or the presence of physical barriers (such as dense connective tissues or epithelia) certainly contributes at least to a transient blockade of migration, but so far it has not been possible to design appropriate experimental devices to test this hypothesis directly. In contrast, factors implicated in the directional guidance of neural crest cells have been clearly demonstrated to induce arrest of migration. For example, mouse embryos deficient in the semaphorin Sema-3A or its neuropilin receptor exhibit marked alterations in the formation of the sympathetic nervous system. In these animals, sympathetic precursors are not accumulated at their target sites around the dorsal aorta but dispersed widely. Consistent with this, when confronted with Sema-3A-secreting cells in vitro, sympathetic neurons lose their locomotory activity, coalesce into compact cell masses and emit thick bundles of neurites. These data therefore indicate that Sema-3A functions both as a stop signal to prohibit migration of the neural crest cells of sympathetic neuron lineage into inappropriate regions of embryos and as a signal to promote aggregation of sympathetic neurons into tightly packed cell masses at defined target sites to produce the stereotyped sympathetic nerve pattern (ref. 158 and Fig. 5).

Recently, morphogens have also been shown to control arrest of migration. In particular, while BMP have been implicated in the initiation of migration by activating integrins and

downregulating cadherins, Shh appeared to play an opposite function during cessation of migration. Shh can directly modulate substrate adhesion of neural crest cells in vitro by shifting integrins from an active to an inactive state.[79] Moreover, migrating neural crest cells cannot penetrate embryonic regions where Shh is produced, notably the perinotochordal area,[159] and tend to accumulate and aggregate at the periphery of these regions: for example the spinal ganglia form in a region along the ventral neural tube which harbors no obvious clues for spatial restriction of migration or repulsive cues, but which is situated at the vicinity of the notochord. Thus, like Sema-3A, Shh activity may have on neural crest cells a broader impact that simple repulsion: it may inhibit migration, induce compaction and promote differentiation, as already observed for neuroepithelial cells.[78] Accordingly, grafts of notochord or of Shh-producing cells along the dorsal mesencephalon in the chick induce formation of ectopic, trigeminal-like sensory ganglia while mice deficient in Shh show poorly-condensed trigeminal and spinal ganglia distributed in aberrant sites.[160] Similarly, Zebrafish mutations in the Shh signaling pathway result in the absence of spinal ganglia and in the loss of expression of neurogenin-1, a gene required for determination of DRG precursors, albeit early neural crest migration occurs almost normally (ref. 161 and Fig. 5).

Certain neural crest populations, such as melanoblasts and enteric neuron precursors, display the striking ability to leave cells behind them during migration so that they finally distribute evenly along their migratory pathway while others, e.g., sensory and sympathetic ganglia, migrate en masse and accumulate in a unique site. This raises the puzzling question of the molecular and cellular mechanisms that selectively promote migration of cells at the front of the population or induce their arrest at the rear. Although this complex problem is far from being elucidated, time-lapse imaging studies and analyses of mouse mutants revealed an intricate interplay between morphogens and growth factors in the coordination of enteric neural crest migration, proliferation and differentiation that might contribute to the typical pattern of enteric ganglia along the entire gut.[152,162] Thus, GDNF promotes proliferation, differentiation and oriented migration of enteric neural crest cells; Shh in contrast inhibits differentiation, but it promotes proliferation, and restricts GDNF-induced migration; finally, endothelin-3 inhibits cell differentiation and shows the same effect on migration that Shh.

Conclusion

An overview of the molecular mechanisms underlying the neural crest development reveals apparent paradoxes that have greatly influenced appreciation of this process and the ways problems were tackled: a great diversity of independent events combined into an apparent linear and unique process. Intriguingly, the same paradox applies to Europe: a great diversity of peoples with distinct cultures and languages sharing a long History and many values. Indeed, albeit ontogeny of the neural crest evolves as a continuous process apparently obeying to a preestablished genetic program, each step is independent and can be at least experimentally separated from the others. This is in accord with the situation observed in a number of pathological situations, where neural crest derivatives can be generated in aberrant positions despite the fact that cells failed to delaminate or migrate properly. Rather than a linear cascade, neural crest cell delamination and migration must then be considered as the result of a conjunction of a great variety of cellular events that ultimately control cell and matrix interactions, cell proliferation, cell fate and cell survival. On the other hand, neural crest cells are most likely generated by multiple processes rather than a single one and they follow numerous migration paths, each governed by specific rules rather than a unique, common one. Such a multiplicity of processes and rules is likely to contribute to establish precocious diversity among the cell population.

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CHAPTER 5

Neural Crest Cell Plasticity:
Size Matters

Lisa L. Sandell and Paul A. Trainor*

Abstract

Patterning and morphogenesis of neural crest-derived tissues within a developing vertebrate embryo rely on a complex balance between signals acquired by neural crest cells in the neuroepithelium during their formation and signals from the tissues that the neural crest cells contact during their migration. Axial identity of hindbrain neural crest is controlled by a combinatorial pattern of *Hox* gene expression. Cellular interactions that pattern neural crest involve signals from the same key molecular families that regulate other aspects of patterning and morphogenesis within a developing embryo, namely the BMP, SHH and FGF pathways. The developmental program that regulates neural crest cell fate is both plastic and fixed. As a cohort of interacting cells, neural crest cells carry information that directs the axial pattern and species-specific morphology of the head and face. As individual cells, neural crest cells are responsive to signals from each other as well as from non-neural crest tissues in the environment. General rules and fundamental mechanisms have been important for the conservation of basic patterning of neural crest, but exceptions are notable and relevant. The key to furthering our understanding of important processes such as craniofacial development will require a better characterization of the molecular determinants of the endoderm, ectoderm and mesoderm and the effects that these molecules have on neural crest cell development.

Introduction

The neural crest is a population of migratory cells that arise transiently along the dorso-lateral margins of the closing neural folds during the neurulation stage of embryogenesis in vertebrates. Neural crest cells give rise to a remarkable array of cell types and contribute to a striking number of tissues and organs of the vertebrate body during embryonic development. In the trunk, neural crest cells produce neurons, glial cells, secretory cells and pigment cells—contributing to the peripheral nervous system, enteric nervous system, endocrine system and skin. Cranial neural crest cells exhibit an even more surprising diversity of derivatives, giving rise to pigment cells, nerve ganglia, smooth muscle, and connective tissue, as well as most of the bone and cartilage of the head and face (Fig. 1).[1,2]

Neural crest cells clearly contribute to many critical tissues and systems but it is the major role that neural crest cells play in building the vertebrate head and face that is particularly significant. The acquisition of neural crest cells, which drove evolutionary development of the head, is considered a key factor in the origin and evolution of vertebrates.[3] The important evolutionary contribution of the neural crest is clearly evident in the remarkable diversity of

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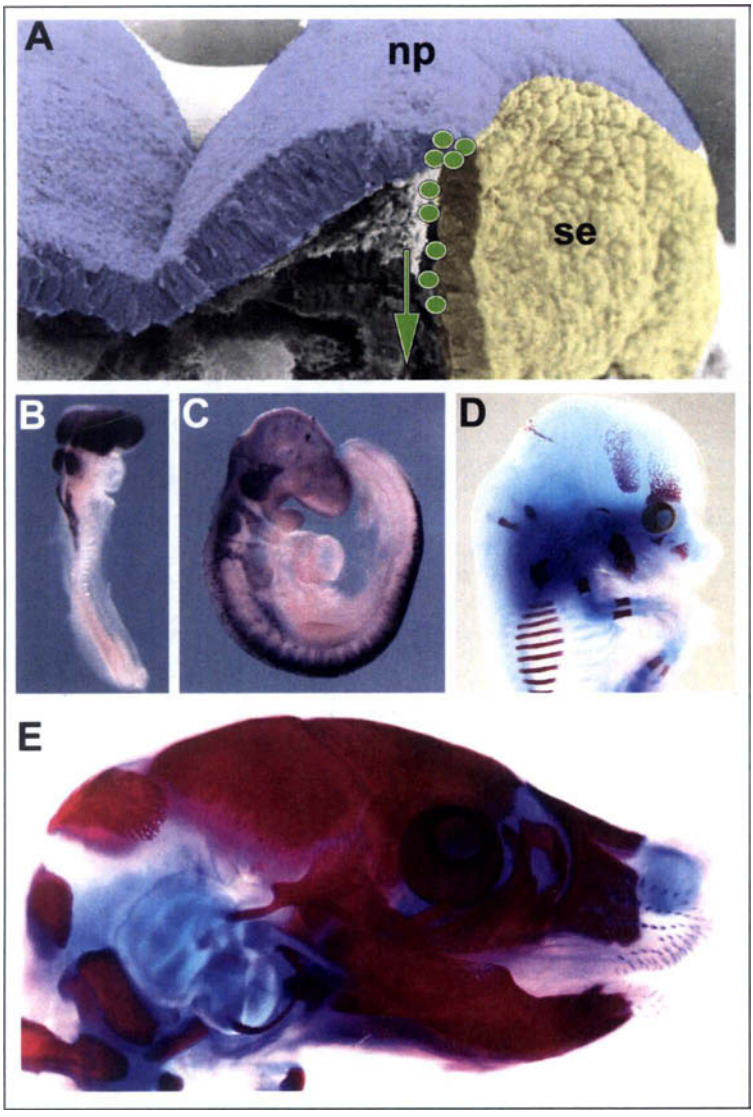

Figure 1. Migratory neural crest cells give rise to bone and cartilage of the head and face. A) Coloured scanning electron micrograph of head region of a mouse embryo at 8.0 days of development. Neural crest cells (represented by green dots) are induced to form at the border between the neural plate (blue—np) and surface ectoderm (yellow—se). Neural crest cells migrate away from the neural plate following a path beneath the surface ectoderm and over the surface of the mesoderm. B) Patterns of neural crest cell migration in mouse embryo at 8.5 days of development as detected by Sox10 RNA in situ hybridization. At this stage neural crest cells have emigrated from the forebrain, midbrain and hindbrain. Neural crest cells completely cover the frontonasal region and the maxillary component of the first branchial arch. C) At embryonic day 9.5, Sox10 positive neural crest cells are associated with neurogenic neural crest cells that contribute to the craniofacial placodes and ganglia. D) A skeletal preparation of a mouse embryo at 14.5 days of development reveals early condensations of bone (red) and cartilage (blue), which, in the head and face, are primarily derived from neural crest. E) The precise morphology of craniofacial bone and cartilage elements are evident at 17.5 days of development.

avian beaks. Different species of birds exhibit striking differences in the shape, size and function of their beaks. Yet despite this amazing morphological diversity, the beak in each avian species is developmentally derived from comparable tissues, with the cartilage and connective tissue being formed primarily by neural crest cells.

Proper head and facial development requires the intricate integration of multiple distinct tissue types such as ectoderm, endoderm, mesoderm and neural crest. One of the defining features of craniofacial development is that it is an extraordinarily complex process that is characterized by massive active cell relocation (during neural crest cell migration) as well as passive displacement (head flexure). Given the multipotency of neural crest cells, their migratory character, and the importance and diversity of structures to which they contribute, the nature of the patterning program that directs neural crest differentiation is a key question in vertebrate developmental biology. Proper development of an embryo requires the patterning of progenitor cells to produce structures, such as the bones of the inner ear for example, of appropriate morphology in defined axial positions. Therefore, of particular interest is the question of whether neural crest cells play an instructive role and direct the patterning of craniofacial development or whether they exhibit plasticity and differentiate according to cues they receive from their environment. This question has been a subject of intensive research and debate over many years, with convincing arguments that neural crest cells govern species specific morphogenesis of the head and face and that the specification of neural crest cells is regulated by tissue specific environments.

The focus of this review is to firstly, examine the evidence concerning the instructive or responsive nature of neural crest patterning with the goal of elucidating factors and contexts that influence the plasticity and determination of this remarkable cell type. Secondly, we highlight the extraordinary complexity of the 3-dimensional tissue interactions that regulate normal craniofacial morphogenesis.

Hindbrain, *Hox* Genes and Axial Identity

The cranial neuroepithelium can be divided into forebrain, midbrain and hindbrain regions, each of which is a source of migrating neural crest cells. Neural crest cells do not appear to migrate randomly, rather they follow precise, species and region specific pathways moving sub-ectodermally over the surface of the mesoderm.[4] In the head, neural crest cells typically migrate in discrete segregated streams, the pattern of which is highly conserved in vertebrate species as disparate as amphibians, teleosts, avians, marsupials and mammals (for review see ref. 5). Briefly, forebrain and rostral midbrain neural crest cells colonize the frontonasal and periocular regions. Caudal midbrain derived neural crest cells populate the maxillary component of the first branchial arch. The hindbrain is divided into seven distinct segments known as rhombomeres and neural crest cells emigrate from each rhombomere, but predominantly from rhombomeres 2, 4 and 6 in discrete segregated streams that populate the first, second and third branchial arches respectively.[6]

Exquisite fate mapping analyses, particularly in avians, have revealed that neural crest cells derived from each region of the cranial neural plate and in particular from each individual rhombomere generate specific parts of the craniofacial complex including unique components of the viscero- and neurocraniums as well as the peripheral nervous system.[2,7,8] For example, neural crest cells that populate the maxillary and mandibular prominences of the first branchial arch form the upper and lower jaws respectively. In contrast neural crest cells that migrate into the second branchial arch give rise to elements such as the retroarticular process, columella and facial/vestibular cochlear nerve. The segmental organization of the hindbrain therefore, is important for the proper organization of the cranial ganglia, branchiomotor nerves and pathways of neural crest migration and as such is a conserved strategy used by vertebrates to establish the foundations or the blueprint of craniofacial development.[9-18] Hence the hindbrain is one key source of patterning information for the migrating neural crest cells during early craniofacial development.

There are several families of genes whose patterns of expression are restricted to different segments of the brain and to specific rhombomeres within the hindbrain.[19] Of particular interest

are the *Hox* genes, a family of evolutionarily conserved transcription factors that play important roles in axial patterning.[20] Interestingly, within the hindbrain, rhombomere segments are distinguished by specific patterns of overlapping *Hox* gene expression.[16,17,21] Individual members of the *Hox* gene family are expressed in either single (e.g., *Hoxb1* in rhombomere 4) or multiple overlapping segment domains (*Hoxa2*, *Hoxb2*, *Hoxa3*, *Hoxb3*), with distinct anterior limits of expression such that the combinatorial *Hox* code expressed by each rhombomere regulates its segmental identity.[17,20,22-29]

In addition to their expression in the neural tube, the segment-defining *Hox* genes are also expressed in neural crest derivatives within the ganglia and branchial arches in a manner that reflects the specific *Hox* pattern of the rhombomeres from which they originated.[16,21,30-34] For example, *Hoxa2* and *Hoxb2* are expressed in the hindbrain up to an anterior limit that corresponds with rhombomere 3. These same two *Hox* genes are expressed in the vii/viii ganglion complex and the mesenchyme of the second and third branchial arches, which are derived from neural crest that emigrate from rhombomere 3 and more posteriorly. Similarly, within the hindbrain, *Hoxa3* and *Hoxb3* are expressed up to an anterior limit that coincides with rhombomere 5 and they are also expressed in third and fourth branchial arch neural crest cells and the ix/x ganglion complex.

Proper segmental restriction of *Hox* gene products is critical for normal craniofacial development. Ectopic expression of *Hox* genes outside their segmentally restricted domains causes defects in development of the craniofacial skeleton.[35-37] Similarly, null mutations in mice have revealed the particular importance of *Hoxa2* and *Hoxa3* for patterning the branchial arch skeleton during craniofacial development.[38-42] Neural crest cells that emigrate from the forebrain and midbrain, which give rise to distinct skeletal components of the upper face and jaw, do not express any *Hox* genes. This *Hox*-negative neural crest is necessary and sufficient for development of the facial skeleton[43] and ectopic expression of *Hox* genes within the normally *Hox*-negative forebrain and midbrain neural crest disrupts cranioskeletal development.[37] These results imply that a *Hox* code and axial patterning are important components of the regulatory mechanisms that govern normal neural crest cell development and craniofacial morphogenesis.

Environment Influences Neural Crest/Neural Crest Influences Environment

One mechanism that has been hypothesized to describe neural crest cell development and craniofacial patterning is the neural crest cell preprogramming model (for review see ref. 44). This model essentially consists of two important components. Firstly, neural crest cells are programmed prior to their emigration from the neural tube and secondly that neural crest cells control the patterns of cell differentiation around them. This model was based largely on two pieces of experimental evidence. The first is that the same combinatorial and rhombomere specific domains of *Hox* gene expression observed in the hindbrain were emulated by hindbrain-derived migrating neural crest cells. For example, rhombomere 4 expresses *Hoxb1*, *Hoxb2* and *Hoxa2* as do neural crest cells derived from rhombomere 4 that subsequently populate the second branchial arch. That the pattern of *Hox* expression in neural crest derivatives mirrors the pattern of expression in the neural tube from which the neural crest were derived suggested that segmental identity of neural crest could be preprogrammed within the neural folds and maintained passively as the cells migrate into the branchial arches.

The second piece of evidence came from a landmark study in which presumptive first branchial arch neural crest cells were grafted in place of more posterior hindbrain segments. These grafts resulted in duplications of the proximal first arch facial skeletal elements such as the squamosal, pterygoid, angular and quadrate bones in avian embryos.[2] Interestingly, the muscle alignments and attachments associated with these ectopic jaw structures were typical of the first arch and not of the second arch tissues from which they were derived. This indicated that myogenic populations and other cell types receive spatial cues from the invading neural crest-derived connective tissue.[2]

Collectively, these observations prompted the model that neural crest cells acquire their genetic identity and regional fate specification before they emigrate from the neural tube. The patterning information acquired in the neural tube is then passively transferred to the periphery of the head and body by the neural crest cells where it directs the development not only of tissues derived from the neural crest cells themselves but surrounding myogenic and connective tissues as well.

Contradictory to this model however is substantial evidence in numerous species that *Hox* gene regulation in the neural tube and neural crest are independently controlled and can be dissociated from one another. The evidence is clearest in relation to the regulation of *Hoxa2* which is critical for second branchial arch neural crest cell patterning.[38,39] *Hoxa2* is expressed in the hindbrain of vertebrate embryos up to an anterior limit that correlates with rhombomere 2. However neural crest cells derived from rhombomere 2 never express *Hoxa2*, implying that neural crest cells are not preprogrammed or specified prior to their emigration from this particular axial level of the neural tube.

However dissociation of *Hox* regulation between hindbrain and neural crest is also observed at other axial levels within the neural tube. Transgenic analyses of *Hoxa2* in mice have revealed distinct *cis*-regulatory elements that mediate expression specifically within the caudal hindbrain or the neural crest.[45-47] The transcription factor *AP-2α* is one of the key players regulating *Hoxa2* expression in second branchial arch neural crest cells. An *AP-2α* specific binding element has been uncovered in the 5' regulatory region of *Hoxa2* and mutations in this binding element eliminate *Hoxa2* expression from second branchial arch neural crest cells.[47] In contrast, the expression of *Hoxa2* in rhombomere 4, from which the majority of second branchial arch neural crest cells are derived, was unperturbed and maintained at a high levels.

Furthermore, the differences in *Hox* regulation between neural crest and hindbrain neural tube are particularly evident in the analyses of embryos that lack *AP-2α* function. *AP-2α* is required in mice and zebrafish for survival and differentiation of certain subsets of neural crest emigrating from the hindbrain and spinal cord.[48-52] In mice, mutation of the *AP-2α* gene compromises expression of *Hoxa2* in the neural crest but not in the hindbrain.[47] Similarly, in zebrafish mutants such as *montblanc* and *lockjaw*, which disrupt the *AP-2α* gene, *Hox* expression is dramatically reduced in second branchial arch populations of migratory neural crest but is not affected in the hindbrain regions from which the cells emigrated.[51,52]

Neural crest cell and hindbrain patterning are also independent from one another with respect to sensitivity to certain perturbations. Appropriate patterning of the neural crest-derivatives in the second branchial arch can be compromised by a reduction in *Hoxa2* expression of only ~50% whereas patterning of the anterior hindbrain remains relatively unaffected even when *Hoxa2* expression is reduced to as little as 20% of normal levels.[42] Conversely, retinoic acid treatment can induce phenotypic changes in the segmental identity of the hindbrain without affecting the derivatives of the neural crest.[53] Thus, neural crest cells and the hindbrain from which they are derived differ both with respect to the regulation of *Hox* expression and also to the extent to which *Hox* gene products are required for their specification. Taken together these data compellingly argue that migratory neural crest cells do not simply carry an axial specification pattern programmed within the neural tube.

Neural Crest Plasticity Is Influenced by Graft Size and Developmental Age

Further convincing evidence that neural crest cells are not necessarily autonomously preprogrammed was revealed by neural crest cell transplant experiments in mouse and zebrafish that demonstrated the influence of environment on engrafted neural crest cells.[54,55] Importantly, the fish and mouse experiments revealed a possible explanation for the seemingly contradictory instructive and responsive nature of neural crest cells. The key factor of the mouse and zebrafish studies is that they involved transplantation of relatively small numbers or even single neural crest cells. In contrast, early transplant experiments between chick and

quail embryos typically involved transposition of relatively large numbers of cells, which led to conflicting ideas about the autonomy or plasticity of neural crest cells. Avian grafts of whole rhombomeres or larger neural tube segments involve relocation of hundreds or thousands of cells.

In the mouse system, a combination of lineage tracing and genetic markers were utilized to show that the *Hox* gene axial identity of neural crest cells translocated from the second branchial arch to first branchial arch environments could be dictated by new environment rather than the origin of the transplanted tissue.[54] Interestingly, grafted cells that remained in coherent groups within the neural tube after transplantation, autonomously maintained their original *Hox* gene axial patterning information. Surprisingly, individual cells that dispersed from the cohort and remained within the confines of the neural tube adapted their *Hox* gene axial patterning information to fit with their new location. Specifically, groups of cells that maintained contact with their original community of cells retained *Hox* expression pattern of their original presumed fate and cells that became isolated from their community of grafted cells altered their *Hox* expression to a pattern appropriate for their new environment. Dispersed neural crest cell populations lack the strong cell community effects characteristic of individual rhombomeres that help to maintain segmental hindbrain identity. The autonomy observed in neural tube cells versus the plasticity uncovered in neural crest cells reflects the fact that neural tube cells typically exist in tight association with one another whereas neural crest cells migrate as a dispersed population. These results suggested that craniofacial development is not patterned by autonomous preprogrammed neural crest cells but instead is regulated by cellular interactions including neural crest cell interactions with each other as well as interactions with non-neural crest tissues in the branchial arch environments.[56]

The effect of graft size on neural crest plasticity was further emphasized by experiments in zebrafish in which neuroepithelial cells were transplanted as single cells or small groups of cells.[55] As in the mouse experiments, the translocated zebrafish neuroepithelial cells were regulated, in some examples by their original position and in other instances by the environment into which the cells had been grafted. Again, the degree of plasticity of the transplanted neural crest cells correlated with the size of the tissue transplanted. When small grafts of 10 or fewer cells were transposed, plasticity of *Hox* gene expression was observed in all cases. When larger groups of up to 30 cells were translocated, cells at the center of an undispersed group maintained their original *Hox* expression while cells at the periphery of the graft exhibited plasticity. The neural crest cells that were derived from transplanted neuroepithelium always exhibited plasticity of axial identity to the extent that they incorporated into elements of the craniofacial skeleton that were typical of their new location.

That individual grafted cells are influenced by their new environment whereas large clusters of cells that remain in a coherent group are resistant to reprogramming strongly implicates interactions of neural crest cells with each as well as the tissues they contact during their migration in the regulation of their patterning and morphogenesis. In support of this idea, timelapse imaging of avian embryos has revealed that individual neural crest cells maintain contact with each other and the adjacent mesoderm, ectoderm and endoderm environments via extensive and dynamic filopodial and lamellipodial connections.[57]

As might be expected, the plasticity of neural crest cells becomes restricted with developmental age. In the zebrafish transplantation experiments described above, plasticity of *Hox* expression diminished as developmental age increased. When neural crest cells were transplanted at the 5 somite stage, *Hox* expression was regulated by the new environment in more than 80% of transplanted cells. In contrast, when neural crest cells were transplanted at the 15 somite stage, *Hox* expression was altered by transplantation in only 40% of the cells.[55] Corresponding temporal restrictions in cell fate are also observed when mature avian neural crest cells are transplanted back into the immature environment of much younger avian embryos.[58]

The ability of transplanted neural crest cells to adopt fates appropriate to their new location provides clear evidence that prior to their migration from the neural tube, the program

specifying their axial identity remains flexible and cells remain responsive to their environment. However, these results seem contradictory to those obtained when rostral hindbrain neuroepithelium was transplanted more caudally in avian embryos resulting in jaw duplications.[2] How can the convincing evidence that neural crest fate is influenced by the environment into which the cells migrate be reconciled with the evidence from classic quail to chick chimera transplantation experiments that promoted the idea that neural crest cells are autonomously programmed while still in the neural tube?

An often ignored aspect of Drew Noden's landmark study is that in addition to forming duplicated first branchial arch jaw skeletal structures, the transplanted neural crest cells also contributed to the formation of relatively normal second arch skeletal elements, an indication that the patterning of some of the transplanted progenitor cells was directed by their new environment. Furthermore, within the same study transplantation of presumptive frontonasal neural crest in place of hindbrain neural crest and transplantation of first branchial arch neural crest cells each produced similar duplicated first arch structures. This is despite the fact that frontonasal neural crest cells do not normally give rise to bones such as the squamosal, pterygoid and quadrate. Thus, the same inappropriate duplicated jaw skeletal elements could be formed by two distinct populations of neural crest cells translocated from two completely different axial levels. Drew Noden was astutely cautious in the interpretations of his results because of these observations of neural crest cell plasticity and concluded that "these results indicate there are no irreversibly specified frontonasal, maxillary and trabecular segments of the premigratory neural crest".[2]

One possible explanation for how these two different axial populations of avian neural crest cells give rise to similar jaw structures in ectopic transplants could reside in their proximity adjacent to the isthmus.[59] The isthmus is a clear, well-characterized neuromeric landmark which lies at the junction between the midbrain and hindbrain. Unbeknown in the 1980s, the isthmus is an important organizing centre that regulates patterning within the hindbrain and hindbrain tissues, specifically restricting *Hox* gene expression in the anterior hindbrain.[60-62] As described above, *Hox* genes are critical determinants of neural crest and branchial arch patterning. Given that the isthmus is contiguous with both the presumptive frontonasal and anterior hindbrain neural crest cell populations, it seems likely that isthmus organizer patterning was highly influential in the jaw duplications that were observed by Noden. In support of this idea, posterior transplantations of just the isthmus in place of presumptive second branchial arch neural crest cells can also lead to the formation of duplicated jaw structures such as the quadrate.[59]

At the level of gene expression, the posterior transplantations of the isthmus led to the suppression of *Hoxa2* in the second branchial arch. This is significant because *Hoxa2* is a key regulator of second branchial arch patterning. In *Hoxa2* null mutant mice, the identity of the second branchial arch is transformed into that of a first branchial arch such that the mice exhibit duplications of jaw skeletal elements.[38,39] The similarity between the jaw duplications observed in avian and mouse studies provocatively suggested that the transplantations of avian neuroepithelium from distinct axial levels, each of which possibly included some isthmus tissue, effectively created a conditional knockout of *Hoxa2* in the second branchial arch thereby transforming its identity into that of a first branchial arch. These results demonstrate the plasticity and adaptability of neural crest cells and highlight the effects of local signaling centers on anterior-posterior patterning.[63]

The idea that neural crest cells exhibit an inherent plasticity or adaptability that can be regulated by their microenvironments is supported by other classic transplantation experiments that utilize regional differences in trunk neural crest cell derivatives. For example, parasympathetic neurons are formed by the vagal neural crest (at the level of somites 1-7). These neurons, known as cholinergic neurons, line the gut and produce the neurotransmitter acetylcholine. Sympathetic neurons form in the thoracic region from trunk neural crest cells. These cells produce norepinephrine and are called adrenergic neurons. When chick vagal and thoracic neural crest cells are reciprocally transplanted or exchanged, the formerly thoracic neural crest

gives rise to cholinergic neurons of the parasympathetic ganglia and the formerly vagal neural crest gives rise to adrenergic neurons in the sympathetic ganglia.[64] In both situations the exchange of trunk-level neural tube segments from one axial position to another resulted in neural crest that migrated, differentiated and contributed to tissues appropriate for their new location rather than their origin. It is interesting to note however that both vagal and thoracic presumptive neural crest cells express the enzymes for synthesizing both acetylcholine and norepinephrine and yet during normal embryonic development this dual potential is never realized by either the vagal or thoracic neural crest. Therefore this implies that the differentiation of a neural crest cell depends largely on its local environment and that neural crest cells may typically have a broader differentiation capacity than is usually realized.[65]

Neural crest cell ablation experiments provided further evidence that properties of neural crest cell identity are not imprinted before migration. Small regions of midbrain, occipital and cervical neural crest cells can be surgically ablated without significantly compromising development of the neck and face.[66-68] The absence of major craniofacial abnormalities in these instances was due to the regeneration of neural crest cells which in-filled and produced the tissues that would have otherwise been formed by the ablated cells. This again demonstrates the regulative nature of embryonic development and the plasticity and adaptability of the neural crest cell population as a whole.

While neural crest cells from each axial level can form neurons, glia and melanocytes, a significant difference exists between cranial and trunk neural crest cells with respect to their endogenous ability to generate skeletal derivatives such as bone and cartilage. These hard tissue derivatives are typical of cranial neural crest cells while, in contrast, trunk neural crest cells are thought to generally lack skeletogenic potential. However this idea was recently overturned via both in vitro and in vivo analyses.[69] By exploiting culture media typically used for growing bone and cartilage cells, trunk neural crest cells were demonstrated to have the ability to generate these hard cell and tissue types in vitro. Furthermore, this study also revealed that if the same trunk neural crest cells are placed directly into developing facial structures, these cells will contribute to cranial skeletal elements in vivo.

The ability of trunk neural crest to form skeletal elements is consistent with earlier demonstrations that rostral trunk neural crest cells could contribute to tooth formation when challenged with mandibular epithelium.[70,71] Interestingly, despite the skeletogenic potential of trunk neural crest cells not being realized during normal amniote development, fossil fish display extensive caudo-cranial exoskeletal coverings of dermal bone and dentine, tissues that are typical neural crest cell derivatives.[69] This suggests that although neural crest cells from each axial level may have a similar intrinsic potential, the ability to generate the wide repertoire of possible derivatives is controlled through extrinsic environmental factors in the embryo and as such plasticity may be an inherent property of neural crest cells.

Plasticity in Neural Crest Cell Migration

Numerous experiments have also shown that premigratory neural crest cells are not irreversibly programmed with respect to their routes of migration. Neural crest cells that are anteriorly or posteriorly transplanted to different positions along the neural tube, do not, for the most part, seek out their original path or final targets but rather migrate along pathways that are typically appropriate of their new location.[55,64,72] Recent investigations emphasize the importance of the microenvironment adjacent to the neural tube in regulating the pathways of neural crest cell migration.[54,63,73,74]

The influence of the microenvironment is clearly evident from lineage tracing and time-lapse imaging of neural crest cell migration in avian embryos. Rhombomeres 3 and 5 of the hindbrain generate small numbers of neural crest cells, but rather than migrating laterally like neural crest cells derived from the rest of the hindbrain, odd rhombomere derived neural crest cells migrate anteriorly and posteriorly to join the segregated neural crest streams. On occasions when a rhombomere 3 derived neural crest cell does delaminate and migrate laterally, its filopodia

collapse as it contacts the mesenchymal environment adjacent to rhombomere 3.[75] These results underscore the importance of the environment adjacent to the neural tube in regulating the pathways of neural crest cell migration. Furthermore they demonstrate that the presence of neural crest free zones and the segregation of neural crest cells into discrete streams is not an intrinsic property of the vertebrate hindbrain or the neural crest cells.

Importantly, the mechanism of regulation of neural crest migration pathways by the adjacent environment may be conserved between mouse and chick. In *ErbB4* null mutant mice neural crest cells from rhombomere 4 acquire the ability to migrate through the dorsal mesenchyme adjacent to rhombomere 3, which is normally free of neural crest cells.[74] The aberrant migration arises as a consequence of changes in the paraxial mesenchyme environment and is not autonomous to the neural crest cells. Since *ErbB4* is normally expressed in rhombomeres 3 and 5 this phenotype reflects defects in signalling between the hindbrain and the adjacent environment branchial arches in mutant embryos. To date few molecules that influence the path finding of cranial neural crest cells have been identified, however evidence obtained primarily from analyses in frog embryos suggests that bi-directional *Eph/ephrin* cell signalling plays an important role in keeping the neural crest cell streams segregated ventrally.[76]

Although overall the migratory patterns of broad regions of the neural crest appear to be extrinsically regulated and generally conserved between species, specific patterns within these populations emerge which may be exceptions to the general rule. In the trunk, neural crest cells traverse two distinct migration pathways.[77,78] Initially trunk neural crest cells migrate ventrally between the somites and the neural tube. Some of these cells continue migrating ventrally until they reach the dorsal aorta whereas a subpopulation migrates laterally along the basal surface of the myotome, penetrating only the anterior half of each somite. This patterned migration through the somites is critical for establishing the segmental layout of the adult peripheral nervous system. Approximately one day after the ventrally migrating neural crest cells emigrate, trunk neural crest cells also begin to migrate dorsolaterally between the ectoderm and somites. The sub-ectodermally migrating neural crest cells are destined to become pigment cells. This melanoblast neural crest cell lineage is the only neural crest cell population capable of following the dorsolateral route at the trunk level. When early emigrating nonmelanogenic neural crest cells are transplanted into the neural tube of avian embryos at a time and place where both migratory pathways are accessible, the transplanted cells migrate only along the ventral pathway.[79] This suggests that in the trunk, to migrate along the dorsolateral pathway, a neural crest cell must have already committed to the melanocyte lineage. While this first, and to date only, evidence that cell autonomous characteristics dictates neural crest migratory properties, it is unlikely to remain the only example.

These studies highlight the complexity in regulating neural crest cell patterning and the balance between signals acquired in the neuroepithelium during formation versus signals received from the extrinsic tissue environments during migration. This intricate regulation affects not only the migratory properties of neural crest cells but also dramatically influences their differentiation.

Neural Crest Cells Direct Species-Specific Craniofacial Morphology

The ability of neural crest cells to influence species-specific facial structure was first illustrated by classical experiments in which neural tube segments were transplanted between frog and salamanders.[80-83] More recently, interspecies transplants in avians have been used to further elucidate the role of neural crest in patterning face morphology.[84,85] For example, the beaks of ducks and quails are distinct in shape. The duck has a long relatively flat bill, whereas quails possess a shorter and more pointed beak. Despite similar coordinated differentiation of multiple tissue types, beak morphogenesis can be distinguished in these two species by differences in timing of expression of key molecular markers during development. These

differences have been utilized to demonstrate the ability of neural crest cells to direct the species-specific pattern facial morphogenesis.[84] Analysis of reciprocal transplants of neural crest cells between duck and quail embryos revealed that transplanted donor neural crest cells direct the species-specific morphology of host beak formation. Transplantation of duck neural crest into quail embryos produced embryos with a duck-like beak and, conversely, transplantation of quail neural crest into duck embryo resulted in embryos that developed with a quail-like beak. More importantly however, analyses of molecular markers whose expression was temporally different between the two species demonstrated that the transplanted neural crest cells maintained their own species-specific temporal expression program and, moreover, imposed their species-specific temporal expression program on the adjacent host ectodermal tissue. This in turn led to alterations in the position and timing of differentiation of structures such as the egg tooth which is entirely derived from host tissue. The ability of transplanted neural crest to carry species-specific patterning information is also manifest in the morphology of the branchial cartilages, from which the mature beak and facial skeleton develop.[85]

These results demonstrate that at least one component of the original neural crest programming model holds true in that neural crest cells can directly influence the spatial patterns of cell differentiation around them. Given that the neural crest cells form most of the cartilage and bone that constitute the foundations or scaffolding in the head, it is not surprising that the progenitors of the framework directly impact formation of tissues and structures such as muscle fibers and the egg tooth which must be functionally integrated into the scaffold.

In theory one could interpret the results of interspecies transplants as evidence for autonomy or preprogramming of neural crest cell patterning in addition to the presentation of species specific characteristics. However, two things should be borne in mind when considering these studies. Firstly, large populations of neural crest cells were typically transplanted in these interspecies grafts. Secondly, the spatiotemporal patterns of morphogenesis signaling molecules and epithelial determinants that regulate branchial arch and craniofacial patterning are highly conserved between species. That the signals mediating interactions of craniofacial development are highly conserved is clearly evident in the dramatic example of ectopic tooth formation in the beaks of chickens. Teeth have been missing in birds for 60 million years or more.[86] In toothed vertebrates, teeth develop from a combination of oral ectoderm and neural crest-derived mesenchyme. In mouse, the source of instructive signals that initiate tooth development is the oral epithelium, which induces tooth formation in neural crest-derived mesenchyme.[71,87] In vitro coculture assays between chick oral epithelium and mouse neural crest-derived tooth mesenchyme suggest that chick epithelium retains its capacity to induce tooth formation, whereas the avian neural crest has lost the ability to respond to the inductive signals.[88-90] Tellingly, when tooth-forming mouse cranial neural crest is transplanted into chick, evidence of tooth-like structures were observed.[91] Thus, the chick epithelium retains the ability to instruct neural crest to form teeth and the cranial neural crest of mouse is capable of interpreting those signals from the chick epithelium. The donor cells still undergo their typical differentiation program due to the highly conserved nature of branchial arch patterning between these species. These results should not be taken as evidence of neural crest cell preprogramming or autonomy because recombination of the same population of first branchial arch neural crest cells with second branchial arch epithelium from mice does not result in tooth differentiation.[70] The spatiotemporal cascade of signaling molecules and determinants is more highly conserved for the first branchial arch between species than it is for the first and second branchial arches within a species. Ectopic tooth formation in chick branchial arches thus demonstrates the influence of neural crest cells on craniofacial patterning but also the complexity of the tissue interactions that regulate head and facial morphogenesis.

Patterning of the Head and Face by Non-Neural Crest Tissues

While the importance of neural crest cells to craniofacial development is clear, it is also true that neural crest is not the only tissue that plays a role in patterning early morphogenesis of the head. Some aspects of facial patterning require no contribution from neural crest. Ablation of neural crest destined for the second and third branchial arches in chick embryos does not compromise early development or regional specification of those arches nor the formation of epibranchial placodes.[92] Formation of the epibranchial neurogenic placodes is induced by signals from the pharyngeal endoderm and does not require a contribution from the neural crest.[93] Similarly, *Hoxa1/Hoxb1* double null mutant mice that lack specifically second branchial arch neural crest develop second arches in which early patterning is relatively normal.[94] Although the skeletal derivatives fail to form since they are derived from neural crest cells which are missing in these instances, these studies demonstrate that early branchial arch formation and patterning can be initiated appropriately, independent of any contribution from migratory neural crest cells.

In contrast, many aspects of craniofacial patterning require cross-talk between neural crest and non-neural crest tissues. For example, epithelial-mesenchymal interactions between the epithelia of the frontonasal and mesenchymal neural crest regulate outgrowth of the facial primordial.[95] Tooth formation also involves epithelial-mesenchymal interactions between the oral ectoderm and mesenchymal neural crest (for review see ref. 96). The endoderm of the foregut has the ability to instruct neural crest cells with respect to orientation, shape, size and position of the facial skeletal elements.[43] Other endodermal tissues also influence neural crest patterning. In zebrafish, endoderm is required for survival and differentiation of the chondrogenic neural crest cells that give rise to the ventral head skeleton.[97] Mesendodermal cells of the pharyngeal arches also play a role in patterning neural crest derived structures in zebrafish. Mutation of the *tbx1* gene, which acts cell autonomously in that tissue, disrupts development of the neural crest-derived pharyngeal arch cartilages and associated structures.[98,99]

Ectodermal tissues can also direct the patterning of neural crest within the developing head and face. Signals from neural epithelium and surface ectoderm direct the path finding of neural crest that stream from the hindbrain into the mesenchyme adjacent to rhombomeres 2, 4 and 6.[100] Removal of the neuroepithelium and surface ectoderm overlying rhombomere 3 disrupts the normal segregation of the streams of neural crest and results in aberrant migration into the mesenchyme adjacent to rhombomere 3. An ectodermal region within the frontonasal process of a developing chick embryo functions as a signaling center directing outgrowth and patterning of the mandibular facial skeleton.[101] When transplanted ectopically this frontonasal ectodermal zone causes duplications of neural crest-derived distal beak structures, the orientation of which is controlled by the position and orientation of the grafted tissue fragment.

Cranial mesoderm is also capable of influencing patterning of neural crest cells. When transplanted in isolation, mouse neural crest cells translocated from the second branchial arch level to the first branchial arch level exhibit plasticity, as shown by the down-regulation of a *Hox* reporter transgene. However, if mesodermal cells from the second branchial arch are included with the transplanted neural crest, expression of the *Hox* transgene is maintained.[54] Thus, signals from the cranial mesoderm are important for regulating the identity of the second branchial arch neural crest cells.

Molecular Pathways Involved in Neural Crest Signaling

In molecular terms, interactions between neural crest cells and other tissues is likely to involve many of the same signaling molecules that are known to regulate the patterning of neural tube and limb buds. These include retinoic acid and members of the *Sonic hedgehog* (*Shh*), Bone morphogenic factor (BMP), and fibroblast growth factor (FGF) pathways.[102-105]

The *Shh* pathway is implicated in patterning head and face development by virtue of the dramatic human and murine craniofacial abnormalities that result from mutation of the *Shh*

gene.[106,107] In chick also, reduction or overexpression of SHH results in malformations of the frontonasal and maxillary processes.[105,108] SHH also acts synergistically with FGF8 to promote outgrowth of neural crest-derived cartilage in the developing chick head.[109]

Several lines of evidence implicate the BMP pathway in regulating craniofacial patterning. Ectopic addition of retinoic acid and the BMP antagonist Noggin into developing chick maxilla causes repatterning of the frontonasal mass and results in duplication of neural crest-derived skeletal elements.[110] Ectopic addition of BMP2 and BMP4 or reduction of BMP4 causes alterations in the development of the facial skeleton of chick.[102,111] Recently, analysis of gene expression patterns in developing embryos of Darwin's finches has established a clear role for Bmp4 in craniofacial patterning.[112] In this study, variation in *Bmp4* expression correlated with naturally occurring differences in beak morphology between species of finch. Species with beaks shaped more broad and deep than long express *Bmp4* earlier and at higher levels than species whose beak shape is relatively narrow and shallow. In addition, overexpression of *Bmp4* in chick embryos altered beak morphogenesis and did so in a tissue-specific manner. Overexpression of *Bmp4* in the facial ectoderm produced beaks that were smaller and narrower than normal and overexpression of *Bmp4* in mesenchyme of the frontonasal process, much of which is neural crest-derived, resulted in beaks that were broader and deeper than normal. These results illustrate that beak morphogenesis is directed by BMP4 signaling via interactions of different tissue types.

FGFs are another family of signaling molecules that play an important role in patterning craniofacial skeletal development. Formation of the ventral head skeleton has been shown to require FGF3 signaling from the endoderm.[97] In the developing embryo *Fgf8* is expressed in the isthmus at the midbrain-hindbrain junction and also in the telencephalon and in the branchial arch ectoderm. Rostral-caudal polarity of the first branchial arch is determined by an FGF8 signal from the rostral epithelium.[113] Reduction of *Fgf8* expression in the forebrain disrupts structures such as the upper beak and deletion or overexpression of *Fgf8* in the branchial arches disrupts the patterning of the arches.[105,114-116]

Reciprocal Interactions

Many of the organs and tissues to which the neural crest contributes are composed of multiple tissues types whose intimate interconnection is essential for function. For example, a bird beak requires contributions from multiple germ layers. The cartilage and connective tissues are derived from neural crest, the outer cornified layer comes from facial ectoderm, blood vessels and muscles are produced by mesoderm and the oral cavity is lined by endodermal cells.[1,7,117] In addition to their juxtaposition with other cell types at their site of differentiation, neural crest cells are also exposed to a variety of different tissue environments as they migrate along their route from the neural tube to their final destination (Fig. 2). Clearly these associations of neural crest with other cells types offer abundant opportunities for interactions and cross-signaling between neural crest and other tissues.

Regulation of craniofacial patterning by reciprocal inductions between neural crest and other tissues is clearly demonstrated by a recent study of FGF8 signaling in frontonasal process and branchial arch development. In this example the *Hox*-negative anterior neural crest were found to influence the expression of *Fgf8* in other cell types and in turn, the neural crest were influenced by FGF8 signaling emanating from the non-neural crest tissue.[118] FGF8 activity in branchial arch ectoderm and the telencephalon was shown to require the presence of *Hox*-negative neural crest as ablation of the *Hox*-negative neural crest resulted in loss of *Fgf8* expression in the arch and neural tissue. Exogenous addition of FGF8 to the surface ectoderm of presumptive first branchial arch largely rescued formation of the facial skeleton in embryos from which the *Hox*-negative neural crest had been ablated. These results demonstrate that anterior neural crest induces FGF8 activity in the ectodermal and epithelial tissues of the branchial arch ectoderm and telencephalon, and subsequently that FGF8 signaling from these tissues signals the maintenance and survival of the neural crest cells.

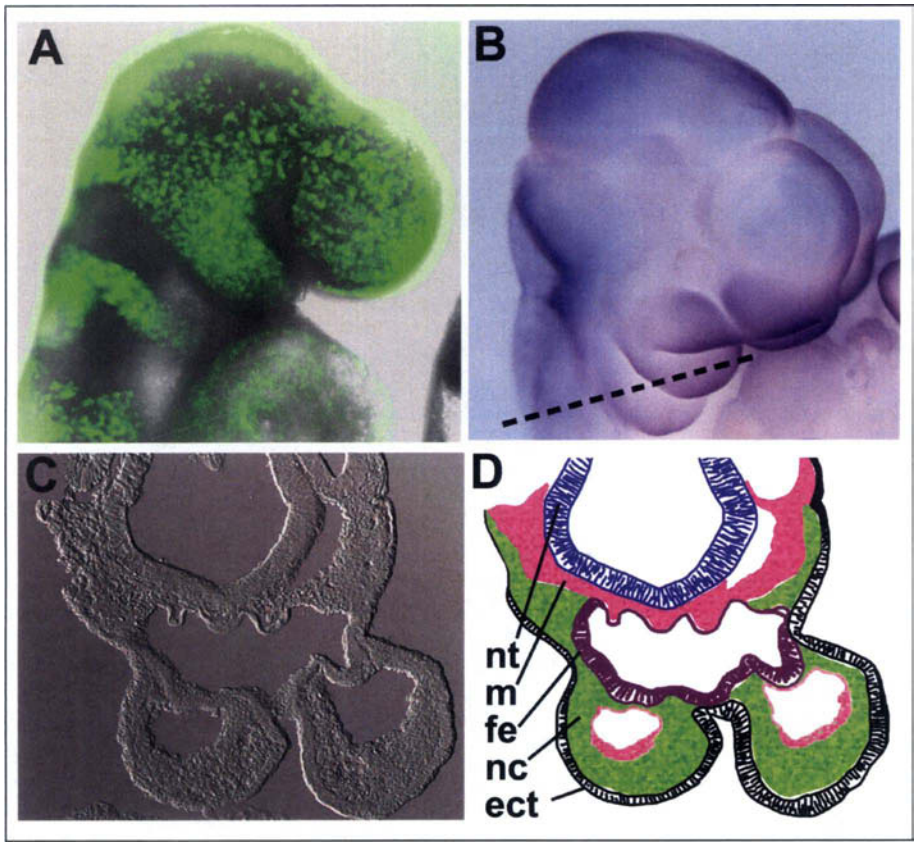

Figure 2. Migratory neural crest cells contact multiple tissue types during craniofacial morphogenesis. A) Neural crest cells emigrating from the closing cranial neural folds of a mouse embryo at 9.0 days of development are revealed by GFP expressed from the Pax3 locus. Cells stream into the frontonasal region and the first and second branchial arches. B) Pax3 in situ hybridization reveals the morphogenesis of the frontonasal region and first and second branchial arches at embryonic day 10.5. Dotted line represents approximate location of section shown in (C). C) Transverse section through the neural tube and first branchial arch of an e10.5 mouse embryo. D) Schematic representation of tissue types present in section of (C). Neural crest derived cells within the branchial arches contact multiple tissues including mesoderm (m), foregut endoderm (fe), and surface ectoderm. (nt) neural tube.

Conclusions

Patterning and morphogenesis of neural crest-derived tissues within a developing vertebrate embryo are regulated by reciprocal interactions between neural crest and other tissue types. These cellular interactions involve signals and organizing centers mediated by the same key molecular families that regulate other aspects of patterning and morphogenesis within a developing embryo, namely the BMP, SHH and FGF pathways. Axial identity of neural crest is controlled by a combinatorial pattern of *Hox* gene expression, which is regulated independently within the hindbrain and migratory neural crest. The developmental program that regulates neural crest cell fate is plastic in the sense that the neural crest cells are influenced by signals from each other as well as tissues in their migratory environment. At the same time, neural crest cells are capable of directing patterning and morphogenesis within a developing embryo, controlling

the formation of structures composed of multiple tissue types. The distinction between plasticity and maintenance of identity is a matter of scale. As a cohort of interacting cells, neural crest cells carry information that directs the axial pattern and species-specific morphology of the head and face. As individual cells, neural crest cells are responsive to signals from each other as well as from non-neural crest tissues in the environment.

Neural crest cell patterning therefore relies on a complex balance between signals acquired in the neuroepithelium during their formation together with the signals from the tissues that the neural crest cells contact and interact with during their migration. The studies described in this review clearly highlight the difficulty in trying to condense the complexity of neural crest cell patterning into simple general models. There will always be species specific, region specific or context dependent exceptions to the general rules and models hypothesized. As much as these general fundamental mechanisms have been important for the conservation of basic patterning it is important to note that the exceptions to the rules are intricately connected to evolutionary diversity and should also be embraced.

Craniofacial development is a complex 3-dimensional morphogenetic process and most craniofacial abnormalities arise through defects in neural crest cell formation, migration and differentiation. However, the analyses described in this review clearly demonstrate that the cause of craniofacial anomalies are not limited to defects intrinsic to the neural crest cells but may sometimes result from a primary defect in the ectoderm, endoderm or mesoderm tissues with which the neural crest cells interact. The key to furthering our understanding of congenital craniofacial abnormalities is a better characterization of the molecular determinants of the endoderm, ectoderm and mesoderm and the effects that these molecules have on neural crest cell development. Elucidating these interactions will also enhance our appreciation of the basis of neural crest cell and craniofacial evolution and diversity.

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CHAPTER 6

The Contribution of the Neural Crest to the Vertebrate Body

Elisabeth Dupin, Sophie Creuzet and Nicole M. Le Douarin*

Abstract

As a transitory structure providing adult tissues of the vertebrates with very diverse cell types, the neural crest (NC) has attracted for long the interest of developmental biologists and is still the subject of ongoing research in a variety of animal models. Here we review a number of data from in vivo cell tracing and in vitro single cell culture experiments, which gained new insights on the mechanisms of cell migration, proliferation and differentiation during NC ontogeny. We put emphasis on the role of *Hox* genes, morphogens and interactions with neighbouring tissues in specifying and patterning the skeletogenic NC cells in the head. We also include advances made towards characterizing multipotent stem cells in the early NC as well as in various NC derivatives in embryos and even in adult.

Introduction

The neural crest (NC) is a transient structure of the vertebrate embryo which was discovered in 1868 by the Swiss embryologist Wilhelm His.[1] He described it in the chick embryo as a band of cells lying between the neural tube and the future epidermal ectoderm. Since the neural crest cells (NCCs) leaving the dorsal aspect of the closing neural tube, can be seen aggregating laterally into the spinal ganglia, it used often to be called *'ganglion crest'*.

Following its first description, the NC was found in selachians, teleosts, amphibians and all forms of vertebrates (for a review see ref. 2).

Because its component cells are endowed with migratory properties and become widely distributed over the whole embryo, the NC has readily attracted much interest. The work carried out on the NC during the first half of the 20th century and which concerned essentially lower vertebrates, was reviewed in a well-acknowledged monograph by Sven Hörstadius.[3]

A prerequisite for studying the behaviour and fate of NCCs is the availability of markers providing experimental long-term lineage labelling, which allow this cell type to be specifically identified throughout ontogeny.

The quail-chick marker system based on the construction of embryonic chimeras between two species of birds whose cells can be identified, either by the structure of their nucleus or by species-specific monoclonal antibodies, was instrumental in deciphering the contribution of the NC to the various anatomical and histological structures in birds and, by extrapolation, in mammals[4,5] (for a review see refs. 2,6).

Recently, molecular methods have been devised to label the NCCs in mammals by constructing transgenic mice in which expression of either β-galactosidase or of the green

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fluorescent protein (GFP) is driven by promoters of genes that are expressed in the NCCs. The genes that have so far been used for this purpose are essentially *Wnt1*,[7-9] *Sox10*[10] and *tissue plasminogen activator (tPA)*.[11] In the mouse and in other vertebrates, *Wnt1* is transiently expressed during embryogenesis in the dorsal neural primordium,[12-17] except in the first rhombomere (r1) (see ref. 10 online supplement) and in the early migrating NCCs (see ref. 18 and references therein). Early migratory cephalic and trunk NCCs and their derivatives can also be identified by genetic labelling using the gene encoding tPA,[11] a proteolytic enzyme synthesized by NCCs during the epithelio-mesenchymal transition.[19] *Sox10* transcription factor gene is expressed transiently in the early NC and later remains activated only in neural and melanocytic NC lineages while it is never detected in mesenchymal cells, whether derived from mesectoderm or from mesoderm (see ref. 10 and references therein).

Sox10 has been used to permanently label post-otic NCCs and trace their fate in the neck and shoulder in transgenic mice.[10] Permanent single cell labelling was obtained in double transgenic mice in which the production of the Cre-recombinase is conditioned upon expression of either *Wnt1* or *Sox10* genes. The *Wnt1-Cre* and *Sox10-Cre* founder mice were crossed to Cre-conditional *R26R-LacZ* and *-GFP* reporter lines.[20,21] Neither LacZ nor GFP carry a nuclear localization sequence, thus blue staining or fluorescence is mainly in the cytoplasm. After Cre-mediated excision of the floxed cassettes, F1 offspring expresses *LacZ* (or *GFP*) permanently in cells that have expressed *Wnt1* or *Sox10*, even if the endogenous *Wnt1* and *Sox10* genes ceased to be activated during development.[10] This elegant technique is thus a way to get single cell resolution and long-term labelling for NCC fate mapping in mammals. Recombinase-based fate mapping using *Gdf7* gene encoding a Bone morphogenetic protein (BMP) family member, has recently led to characterize a subset of late-emigrating NCCs present in the roof plate, which yield sensory-restricted progenitors.[22]

The Derivatives of the NC

The NCCs are remarkably invasive. There is virtually no tissue in the embryonic and adult body that does not possess cells originating from this highly pluripotent structure.

Investigations carried out in the avian embryo, using the quail-chick chimera system, have allowed the fate map of the NC to be established (Fig. 1).

The NC is at the origin of all the neurones and glial cells of the peripheral nervous system (PNS) except for the neurones of certain cranial nerve sensory ganglia.[6]

The PNS includes the sensory ganglia, the sympathetic chains and plexuses, the parasympathetic ganglia and the enteric nervous system (ENS) often called the *'second brain'*[23] because it includes sensory, intermediate and motor neurones and can function independently from the central nervous system (CNS) which modulates its activity through sympathetic and parasympathetic innervations.

The NC is also at the origin of sensory receptor cells (e.g., Merkel cells and Grandry corpuscules) associated with sensory nerve endings in the skin.[18,24,25]

The NC provides all the peripheral nerves with Schwann cells and the peripheral ganglia with their glial component. The pigment cells of the body (except those of the pigmented retina) derive from the NC, which is also at the origin of endocrine cells: the adrenal medulla and the calcitonin-producing cells (C cells). The latter develop in the ultimobranchial bodies (UB) in all vertebrates except in mammals, where the UB join the thyroid gland in which the C cells form the population of *'clear cells'* (also called *'parafollicular cells'*) located between the thyroid follicles.[6]

Another derivative of the NC is the *mesectoderm* i.e., mesenchymal cells which are derived from the ectodermal germ layer, in contrast to the other mesenchymal cells of the body, which are of mesodermal origin.

Mesectodermal cells exit from the cephalic and trunk NC in lower vertebrates (e.g., fish in which they yield the dorsal fin mesenchyme; see ref. 6 and references therein). In amniotes they are mainly restricted to the cephalic level of the neural axis (Fig. 1). However it was recently shown in the mouse that the trunk NC is able to give rise to endoneurial fibroblasts.[26]

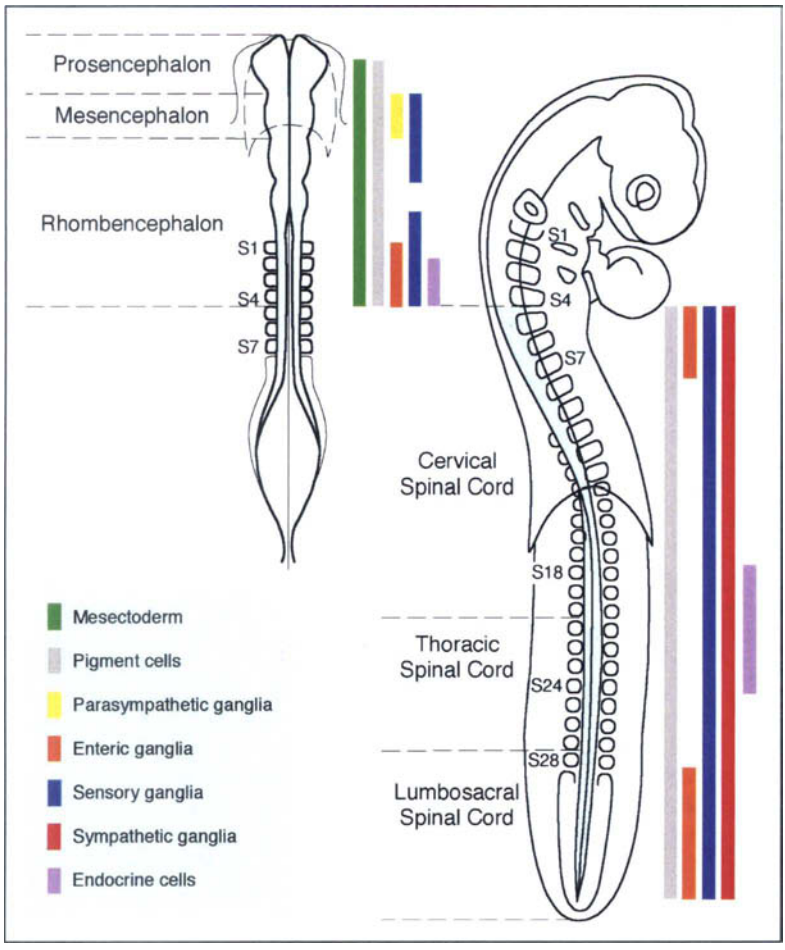

Figure 1. Fate map of NC derivatives. As established in the avian embryo,[38] the different phenotypes (see box) yielded by the cephalic (left panel) and the trunk (right panel) NC along the NCC-producing neural fold (shaded in light blue) are represented in 7- and 28 somite-stages embryos. S, somite.

Pathways and Molecular Control of NCC Migration

The early specification of the NC from the neural plate depends on the function of both soluble growth factors and regulatory genes, the relative influences of which have been reviewed elsewhere.[27-30] Soon after NC formation, NCCs undergo an epithelium to mesenchyme transition and acquire the ability to navigate along complex paths.[6] A number of molecules are known to participate in NCC delamination and migration such as cadherins, Rho GTPases, Noggin and molecules of the extra-cellular matrix (ECM).[31-34]

The routes through which NCCs navigate, have been mostly characterized in the avian embryo.[6] In the trunk region, NCCs follow two main patterns of migration, which differ spatio-temporally. First, NCCs of the dorso-ventral migratory wave travel between the neural tube and the somites, through the anterior half of each somite as well as between consecutive somites. These cells will give rise to most of the PNS by coalescing to form the dorsal root ganglia (DRG) and, more ventrally, the sympathetic ganglion chains and the adrenal medulla.

Second, a late-emigrating population of NCCs stays in the so-called *'migration staging area'*[35] circumscribed by the neural tube, the superficial ectoderm and the dorso-medial somite. When the dermatome becomes individualized, these NCCs take a medio-lateral route, penetrating the dermomyotome beneath the superficial ectoderm. These cells, which become committed to the melanocyte lineage, are fated to give rise to the pigment cells of the skin and feathers.[6]

In the cephalic region, the migration pathways and target sites of NCCs have been mapped along the antero-posterior axis[36,37] (see refs. 6,38 for additional references). Although the formation and emigration of NCCs is basically unsegmented along the dorsal hindbrain as it is in the trunk, NCCs adopt a stereotypic migratory pattern along three main streams.[39-42]

Therefore the NCCs that migrate from the various levels of the neural axis, are rapidly patterned by their environment. At the trunk level, the migratory pattern of NCCs is closely correlated with somitic development. The migration of NCCs can take place only in the rostral half of each somite, whereas its caudal half is inhibitory to the penetration of both NCCs and growth cones from the motoneurones. The segmental origin of NC progenitors of the DRG was described in an elegant series of experiments where small fragments of the quail neural primordium were grafted orthotopically in chick embryos.[43] These experiments showed that the NCCs facing two consecutive somites participate in the formation of each DRG (Fig. 2A). Moreover, the NCC migration and DRG formation are perturbed subsequently to replacement of contiguous somites by either multiple rostral or multiple caudal somitic halves. If a succession of rostral somitic halves is created, a continuous and giant ganglion forms. In contrast, following grafting of multiple caudal somitic halves, the NCCs give rise to very small, dorsally located ganglia with irregular segmentation.[44] Therefore, the normal morphogenesis and segmentation of the DRG depend upon the integrity of the rostro-caudal succession of somitic regions (Fig. 2B).

The mechanisms that direct NCCs to the appropriate migration pathways and how NCCs interprete external signals into different locomotion behaviours start to be understood. Time-lapse confocal imaging in intact chicken embryos has allowed the cell-cell interactions between individual migratory NCCs and their motile behaviour at trunk and cephalic levels to be visualized.[45-48]

NCCs whose individualization results from proteolytic activity,[19] are guided by both inhibitory and permissive cues that they encounter en route. These cues modify the expression and activity of integrins and cadherins responsible for intercellular detachment, cytoskeletal changes and cell motility.[49-51]

Signals permissive for NCC migration are provided by basal lamina and several ECM components, such as fibronectin,[52] laminin, collagens, thrombospondin and hyaluronic acid (reviewed by Perris and Perissinotto).[32] Inhibitory molecules expressed in particular regions of the embryo impair invasion by NCCs, such as chondroitin sulfate proteoglycans in the peri-notocordal mesenchyme. In the caudal somite, F-spondin[53] and lectin-binding glycoproteins[54] can function as a barrier for NCCs and thus participate in segmentation of the NC migratory streams in the trunk.

Recent studies have uncovered the role in directing NCC migration of secreted proteins originally characterized for their function in axonal guidance such as Ephrin, class III Semaphorin, Slit and Netrin families of ligands, which transduce repulsive or attractive signals through interactions with the cognate receptors expressed by NCCs.

Ephrins-B ligands expressed in the caudal somite act as repulsive signals to restrict trunk NCC migration to the anterior somite and the dorso-ventral pathway.[55-57] However, at a later stage, NCCs switch their response to Ephrins-B, which then act as positive cues required for the lateral migration of presumptive melanocytes.[57] Other inhibitory elements supplied by the epithelial dermatome include secreted Slit proteins, which interact with Robo receptors expressed by early NCCs to confine them to the dorso-ventral pathway.[58] Slit 2 produced by the splanchnic mesenchyme repels trunk but not vagal migratory NCCs, inhibiting the former cell population to invade the gut.[59]

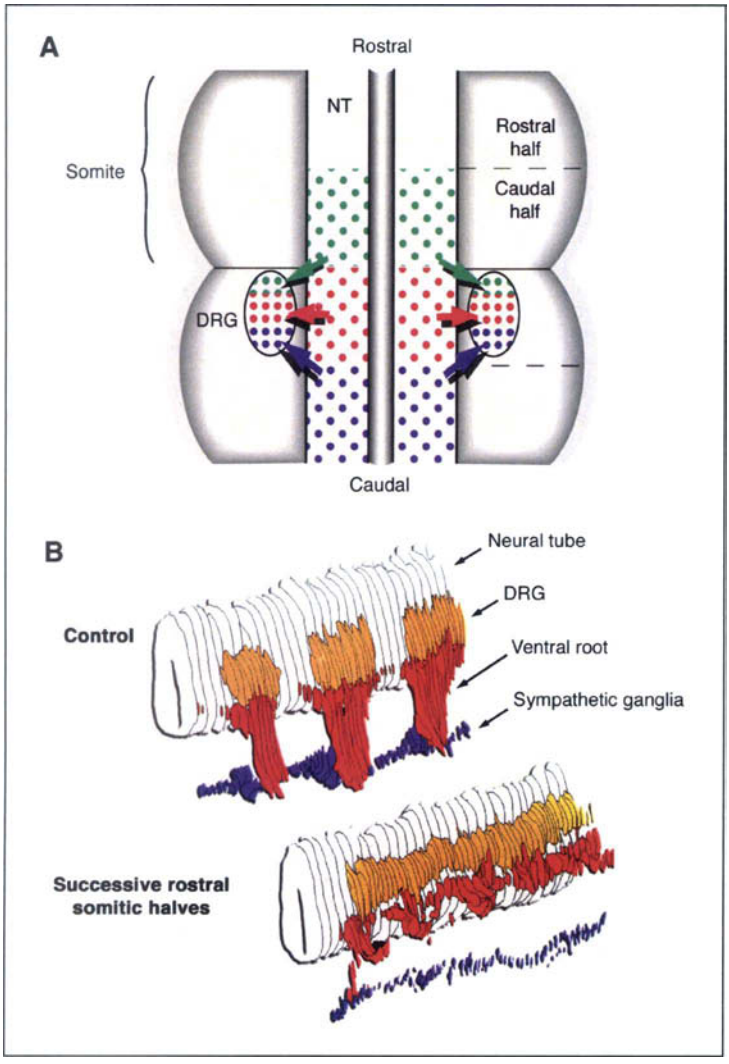

Figure 2. Segmental pattern of peripheral ganglia: influence of the somite. A) Each dorsal root ganglion (DRG) forms opposite the rostral part of the somite and is colonized by NCCs migrating from the part of the neural tube (NT) facing the same somite (80% of DRG cells). NCCs corresponding to the caudal half of the preceding somite populate the rostral 20% of the DRG (adapted from ref. 43). B) Three-dimensional reconstruction of the neural tube and peripheral nervous structures in E4.5 embryos.[6] In controls, the DRG, ventral roots and primary sympathetic ganglia are segmentally patterned. In embryos implanted with successive rostral somitic halves, NCCs generate a continuous dorsal polyganglion and yield unsegmented nerves and sympathetic ganglia.

Recent grafting experiments to perturb the spatio-temporal relationships between the NCC medio-lateral migration and dermis formation have suggested that the emerging dermatome supplies a long-range diffusible attractant to stimulate NCC entry into the dorsal path.[60] In other regions as well, NCCs may display chemotactic responses to growth factors such as Fibroblast growth factor 2 (FGF2) in the mesencephalon.[61] Glial cell line-derived neurotrophic

factor (GDNF) peptide is chemoattractant to NC enteric precursors that express Ret tyrosine kinase receptor during colonization of the gut.[62,63] At later stages of ENS development, Netrins have been reported to attract the enteric nerve cells expressing the Netrin receptor DCC (for '*deleted in colorectal cancer*'), thus promoting the secondary formation of sub-mucosal and pancreatic ganglia.[64]

Semaphorin 3A, a secreted chemorepellent of class III Semaphorins, which signals via Neuropilin 1 receptor, plays a role not only in the patterning of the sympathetic innervation but is also required for the accumulation of neuronal precursors that form the sympathetic ganglia.[65] In addition, Semaphorin 3A reduces the migration area of trunk NCCs in vitro.[66] Semaphorin 3/Neuropilin 2 signalling has been suggested recently to play a crucial role in the segregation of hindbrain NCCs into migration streams in the chick[67] and zebrafish.[68] Semaphorins 3 however function in concert with other inhibitory signals to create crest-free zones in the mesenchyme adjacent to r3 and r5. Abnormal NCC invasion of these zones occurs in mice mutated for genes encoding Twist transcription factor[69] and ErbB4 receptor of Neuregulins.[70,71] This phenotype has been suggested to result from alterations of Ephrin-B/Eph receptors signalling. In particular, conditional mutation of *Ephrin-B1* in mouse NC has recently shown that Ephrin-B1 regulates NCC directional migration to craniofacial target tissues.[72]

The Mesectoderm Is at the Origin of Most Head Structures

The crest-derived mesenchyme, which arises from the posterior diencephalic level, is destined to populate the upper facial primordia corresponding to the naso-frontal and naso-lateral buds (Fig. 3A). Mesencephalic and anterior rhombencephalic (r1-r2) NCCs fill up the branchial arch 1 (BA1). In the more caudal BAs, migration streams are delimited by several factors (see above) including by apoptotic foci of the cells in r3 and r5, which restrict the NCC scattering to a segmented pattern:[36,37,39-41] while r4-derived cells colonize BA2, NCCs that originate from r6 to r8 migrate into caudalmost BAs (BA3, BA4-6). Moreover, the final fate of these mesectodermal cells could be revealed thanks to the stability of the labelling provided by the chimeric quail-chick combination. The BA1 NCCs form the maxillo-mandibular skeleton while mesectodermal cells from the most posterior arches differentiate into the ventral part of the neck where they give rise to the hyoid bone[6] (Fig. 3B). In mammals, the NC is at the origin of mesenchymal cells in the dentine and the pulp of the teeth.

As a whole, the investigations carried out so far in the avian embryo have allowed the delimitation of the mesectodermal and mesodermal components of the skull (Fig. 4A). Ventrally, this limit corresponds to the rostral end of the notocord between the basipost- and the basipre-sphenoid, both forming the *Sella turcica* on which the pituitary gland sits (Fig. 4B). Dorsally, the posterior mesectodermal limit was placed at the transition between the parietal and the occipital bones, the latter being mesoderm-derived.[73] The parietal itself differentiates late during embryogenesis and was primitively thought not to belong to the NC-derived skeleton.[73,74] An approach based on the expression of *Wnt1* in the neural fold of the mouse, has excluded the parietal bone from the NC-derived territory.[9] These results could however be due to the fact that the r1 NC, the main source of mesectoderm for building the parietal bone, does not express *Wnt1* (see ref. 10, online complementary information and our own unpublished observations). More investigations are required to ascertain the origin of the parietal bone in mammals.

Long-term fate maps established in the avian embryo have revealed differences in the ability of the NCCs to form the various types of skeletal tissues: yielding cartilage and endochondral bone appears as a ground property of the entire skeletogenic NC domain. In contrast, the ability to form membrane bones is restricted to its most rostral part, corresponding to NC derivatives of the naso-frontal and maxillo-mandibular processes. Striking is the fact that, as discussed below, this partition matches different molecular traits involving *Hox* gene expression.

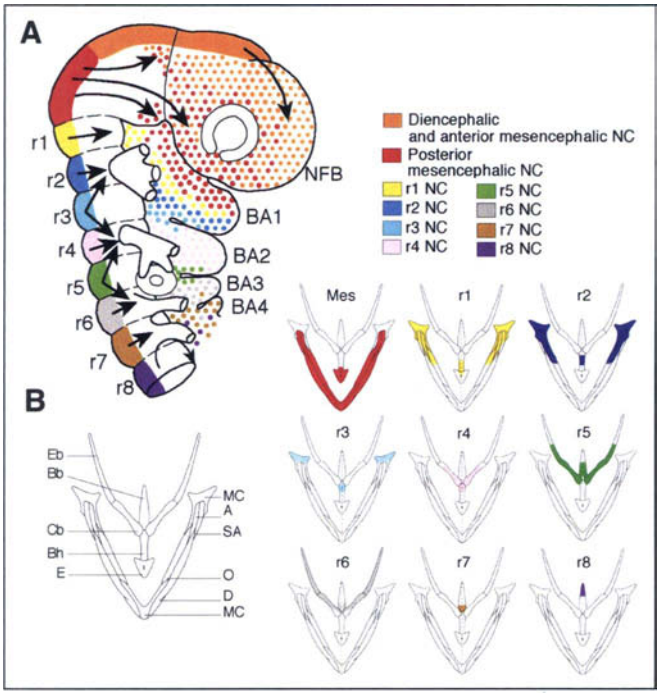

Figure 3. Cephalic NCC migration and contribution to visceroskeleton. A) Migration streams of cephalic NCCs to the naso-frontal bud (NFB) and branchial arches (BA), according to their level of origin. B) Composite origin of the maxillo-mandibular and hypo-branchial skeleton (the level of NCC origin is colour-coded as in (A). A: angular; Bb: basibranchial; Bh: basihyal; Cb: ceratobranchial; D: dentary; E: entoglossum; Eb: epibranchial; MC: Meckel's cartilage; Mes: mesencephalic level; O: opercular; r: rhombomere; SA: supra-angular.

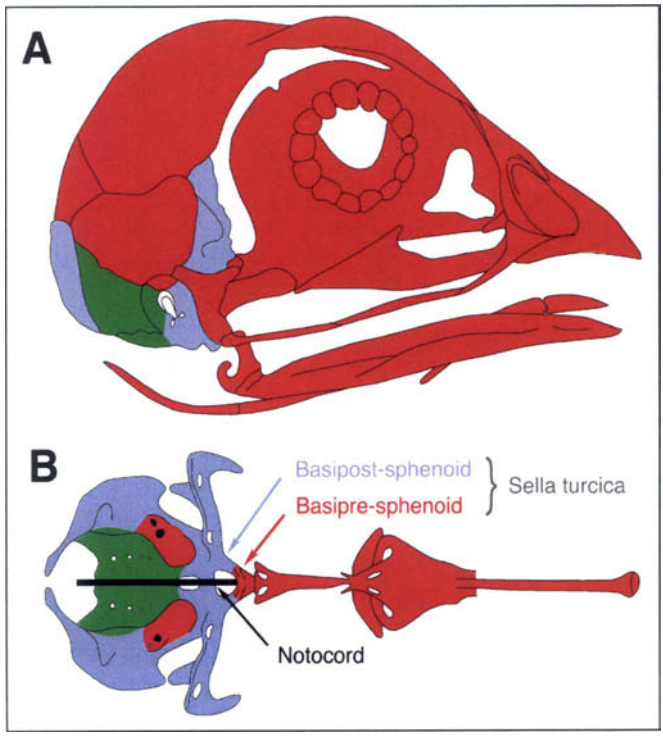

Figure 4. Triple origin of the head skleleton. A) Contribution of the cephalic NC, cephalic paraxial mesoderm, and somitic mesoderm to the craniofacial skeleton at E14. B) Ventral view of E10 chondrocranium showing the transition between the NC- and the mesoderm-derived skeleton.

The Posterior Limit of the NC-Derived Skeleton in Mammals

Labelling of the mammalian post-otic NC by taking advantage of the transgenic mice approach mentioned above, has provided novel information on the participation of the NCCs to the neck and shoulder skeleton.[10]

The neck region has been subjected to major changes during evolution from fish to terrestrial vertebrates, and the respective boundaries of NC and mesoderm contribution in the neck transition zone had not been previously investigated. According to the classical view,[75,76] the dermal bones of the forelimb girdles are of NC origin while their endochondral components are considered mesodermal. This so-called *"ossification model"* to account for either a NC or a mesoderm origin of the skeleton had however not been experimentally tested in any vertebrate.

As noticed by G. Koentges' group, there are variations in the mode of ossification (either endochondral or dermal) of the shoulder bones in various organisms, whereas the pattern of neck muscle attachments on these bones is very conserved throughout evolution.[10] In fact, the origin of the muscle connective component and the attachment points of the muscles on the skeleton were shown to be similar in both cranial and trunk regions throughout the vertebrate phylum.[37,77] On this ground, Matsuoka et al[10] have proposed a competing model according to which morphogenesis of the bones in various forms of vertebrates has obeyed constraints imposed by a strict muscle attachment scaffold. This idea is based upon the homologies that can be drawn concerning the relationships that exist between the muscles and bones in the head and neck regions of different vertebrates. It was found that the cellular distributions of NC and mesoderm correspond to the muscle attachment scaffold rather than to the type of ossification of the bones: if the muscle connective tissue is of NC origin, its attachment site on a given bone will be made up of NC-derived cells, irrespective of whether the bone is of dermal or endochondral type.[10]

Thus in mouse, the post-otic NC extends to the anterior lining of the shoulder girdle at the sites where the trapezius muscle is fixed, whose connective component is of NC origin. The post-otic NCCs undergo dermal ossification of the anterior clavicle and form endochondral bone at the insertion of branchial muscles (i.e., sternocleidomastoid and the fascia of omohyoid).[10] Generally speaking, the branchial muscles that have a NC connective component extend their attachment site to NC-derived bone in all skeletal structures of the neck and shoulder regions. However, in the cervical vertebra whose body and neural arches are somitic in origin,[73] the NC contribution is cryptic since it is limited to spots in the spinal process, which correspond to muscle attachment sites.

Similar results had been obtained for the attachment of the cleidohyoid muscle on the medial part of the clavicle of birds by McGonnell and coworkers[78] through the use of several NCC labelling procedures. It is noticeable that the scapula was found to be entirely mesoderm-derived in birds.[79]

Contribution of the Mesectoderm to the Cardiovascular and Periocular Structures

As evoked above, the cephalic NC scaffolds the fascicular organization of the myogenic primordia derived from cephalic paraxial and somitic mesoderm; regarding the splanchnopleural mesoderm, NCCs play a similar role. The first insights of NCC contribution to conotroncal structures in vertebrates were gained from experiments using quail-chick chimeras. They demonstrated that mesectodermal cells nested in BAs 3 to 6 were lining the endothelial cells of the large aortic arteries (from brachiocephalic down to pulmonary arteries).[80] These results prompted Margaret Kirby and colleagues to further investigate the migration and fate of NCCs into the heart anlage and to question their functional role. It turned out that NCCs form the aorticopulmonary septum and the sigmoid valves.[80-83] This NCC distribution was later on generalized to mammals by transgenic approaches.[81-85] The large-scale defects in cardiac outflow tract septation recorded in the absence of the posterior rhombencephalic mesectoderm corresponding to the r6-r8 area of the neural fold, led Kirby and coworkers to put forward the notion of *'cardiac neural crest'*. This territory was shown to play an important role not only in its

contribution to the heart anlage but also in regulating various aspects of the proliferation, differentiation and physiology of the cardiomyocytes.[86,87]

Long-term cell tracing investigations revealed for the first time the composite origin of the cephalic blood vessels.[80] Two notions came out from these observations. First, the endothelium was found to be of mesoderm origin all over the body. Later on, it was shown that the angiogenic lineage shares a common *'hemangioblastic'* precursor with hematopoietic cells.[88] These precursor cells can be early evidenced by expression of VEGFR2 (for '*vascular endothelial growth factor receptor 2*').[88-90] Second, cephalic blood vessels from the large arteries arising from the heart up to the very thin ramifications of cerebral capillaries of the vascular tree in the face and the anterior brain, are surrounded by cells of mesectodermal origin (Fig. 5A-C). NC provides the craniofacial and

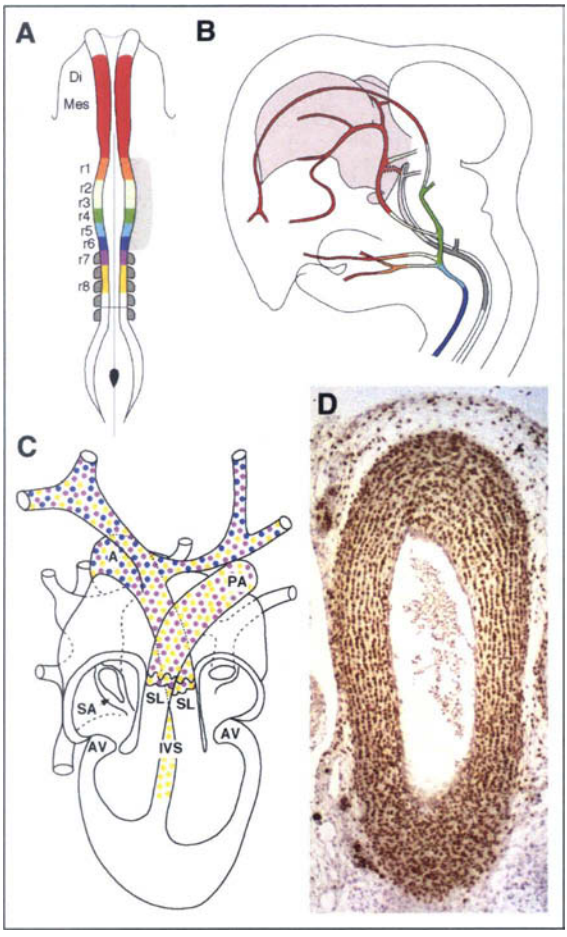

Figure 5. Cephalic NCC contribution to the cardiovascular system. A) Refined colour-coded cephalic neural fold of a 5 somite-stage chick embryo and NCC contribution to the musculo-connective wall of artery tree in the head. B) Prosencephalic meninges (in pink) derive from the diencephalic and mesencephalic NCCs; from mesencephalon down to more caudal cephalic structures, meninges (in grey) are of mesoderm origin. C) Contribution of the posteriormost rhombencephalic NCCs to the conotruncal structures of the heart. D) NCCs form the musculo-connective wall of chimeric cephalic artery (QCPN immunoreactivity of quail rhombencephalic NCCs grafted into chick host embryo). A: aorta; AV: atrioventricular valve; IVS: intraventricular septum; PA: pulmonary artery; SA: sinoatrial valve; SL: semilunar valve.

Table 1. Origin of the avian ocular and periocular structures

Ectoderm	Neural Crest	Mesoderm
Corneal epithelium	Corneal stroma and endothelium	Blood vessel endothelium
Lens	Iridial stroma and muscles	
Iridial epithelium	Ciliary muscles	Myofibers of extra-ocular
and lamella	Mesenchyme of ciliary corpus and process	muscles
Pigmented and	Wall of Schlemm's canal	
neural retina	Sclero-corneal limbus	
Eyelid epithelia	Sclerotic ossicles	
	Choroid membrane	
	Sclerotic cartilage	
	Muscles and mesenchyme of eyelids and nictitating membrane	
	Mesenchyme of lacrimal gland	
	Connective tissue of extra-ocular muscles	
	Pericytes of ocular blood vessels	
	Frontal, lacrimal, ethmoïd, maxilla, zygomatic bones	

prosencephalic blood vessels with their media, meaning that these cells are locally induced to acquire pericyte and smooth muscle cell phenotypes[80,91] (Fig. 5D). In this respect, the ocular and periocular region offers a unique site where the NC gives rise not only to the smooth muscles of the ciliary bodies but also to the striated muscle components of the iris.[92] Altogether, the variety of NC derivatives identified in the developing eye concurs in maintaining homeostasis of the anterior optic chamber and in endowing the vertebrate eye with refracting media (Table 1).

Hox Gene Expression in the Cephalic NCCs and Their Derivatives

As first established in the mouse[93] and later confirmed in the chick,[94,36] the caudal domain of the cephalic NC (from r4 down to r8) expresses *Hox* genes of the first four paralogous groups, whereas in the rostral domain (from diencephalon down to r2), these *Hox* genes are not expressed. At the edge of both, NCCs from r3 exhibit a versatile *Hox*-status according to the environment into which they migrate. If r3 NCCs invade *Hox*-negative BA1, they lose *Hoxa2* expression while, if they migrate into *Hox*-positive BA2, they maintain it.

The evidence that *Hox* gene expression exerts a crucial influence on BA identity has been provided by seminal experiments based on gene targeting in mice: the null-mutation of *Hoxa2* generates the duplication of BA1-type skeleton (mandible) at the expense of BA2 hyoid bone structures.[95,96] By contrast, if *Hoxa2* expression is targeted to all BA1 tissues, a partial homeotic transformation of BA1 into BA2 is observed in the chick[97] and in *Xenopus*.[98]

The phenotype resulting from surgical extirpation of the anterior *Hox*-negative NC is the absence of the facial skeleton together with severe defects of forebrain and midbrain development[99] (Fig. 6A,B). If the *Hox*-expressing neural fold from r4 to r8 is heterotopically transplanted into the *Hox*-negative forehead environment, no regeneration of the skull and facial skeleton takes place. Despite their capacity to migrate and invade facial processes, *Hox*-expressing NCCs fail to form either cartilage or bone, and therefore are unable to substitute for *Hox*-negative NCCs in facial skeletogenesis[100,101] (Fig. 6C,D). A strikingly different result is obtained when the *Hox*-negative anterior domain of the NC (from mid-diencephalon down to r3 excluded) is replaced by small fragment of NC from either the posterior diencephalon, the mesencephalon or r1-r2 levels coming from a stage-matched quail. As shown in Figure 6E,F, the morphogenesis of the head and brain is rescued and all the NC-derived structures of the face are made up of quail cells.

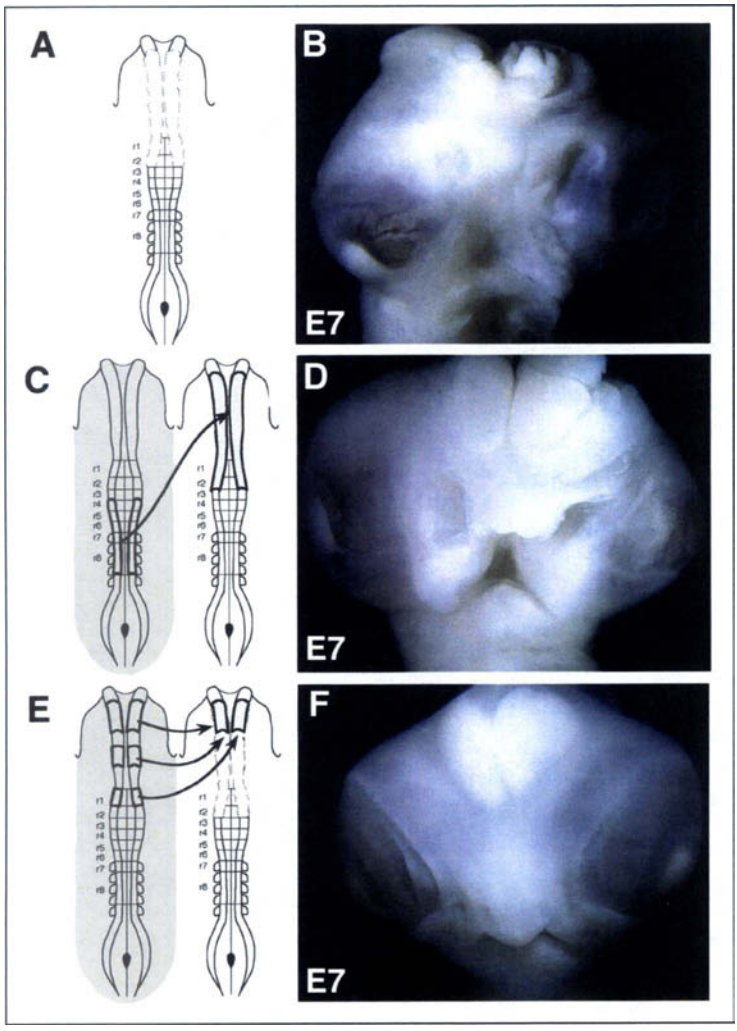

Figure 6. Facial development relies on the *Hox*-negative NC. A) Removal of the *Hox*-negative NC in early chick neurula (dotted lines) results in absence of facial structures and exencephaly in E7 operated embryos (B). C) Replacement of the anterior *Hox*-negative NC by an exogenous fragment of *Hox*-positive NC severely hampers head morphogenesis at E7 (D). E) Following removal of the anterior *Hox*-negative NC (as in A), the implantation of only a fragment from either di-, mes- or anterior rhombencephalic level (i. e., *Hox*-negative NC) restores normal development of the face and forebrain at E7 (F).

Therefore, the *Hox*-positive NC cannot replace the *Hox*-negative domain to construct a facial skeleton. In addition, *Hox*-positive NCCs transplanted rostrally yield the PNS derivatives corresponding to the level of their implantation but do not form any facial skeletal tissues. We have shown that the anterior *Hox*-negative domain is more plastic: when posteriorly transplanted, anterior *Hox*-negative NCCs are able to participate in the formation of the hyoid bone, thus adopting a fate corresponding to their new location.[100,101] Moreover, these NCCs are necessary for brain morphogenesis.

These experiments have demonstrated that the NC territory extending from the mid-diencephalon down to r2 (included) shows equivalent developmental potential whatever the

level considered and thus its component cells behave as an *'equivalence group'*. This implies that the anterior domain of the NC relies on environmental cues for patterning the facial skeleton.

Shifting Rostrally the Limit of *Hox* Gene Expression

As previously mentioned, the NC ability to form skeletal tissues is not uniformly distributed along the cephalic neural axis since the *Hox*-expressing rhombencephalic NC is unable to yield facial skeleton.[100,101] That this trait could essentially rely on *Hox* gene expression was further investigated by forcing *Hox* gene activation in the anterior *Hox*-negative neural fold fated to yield facial skeleton.[102] If gain-of-function for *Hoxa2* is restricted to mesectodermal cells, *Hoxa2*-transfected embryos are utterly deprived of facial skeleton. On the other hand, forced expression of *Hoxa3* and *Hoxb4* promotes different phenotypes. While neither *Hoxa3* nor *Hoxb4* transfection affects NCC migratory properties, it strongly reduces their skeletogenic potential. In these embryos that have virtually no face, some rudimentary skeletal elements still develop in either the nasal bud (*Hoxa3*) or the mandibular bud (*Hoxb4*). According to the same paradigm, cotransfection of *Hoxa3* and *Hoxb4* completely abolishes the development of the facial skeleton.[102] This experiment therefore led to phenocopy of the facial defects resulting from the replacement of the endogenous *Hox*-negative NC by more posterior *Hox*-positive neural fold or from the ectopic activation of *Hoxa2* in the anterior neural fold.

Aside from skeletal defects, forced expression of *Hoxa3* was also detrimental to the differentiation of transfected mesectodermal cells into pericytes, causing lethal haemorrhages at the head level.[102] Together with the effects of *Hoxa3* null mutation in mice, these data show that *Hoxa3* plays an essential role in patterning the cardiovascular system.[103-105]

Altogether, these experiments show that *Hox* gene gain-of-function by the rostral NCCs prevents the formation of the skeletal (and, for *Hoxa3*, the vascular) apparatus. It thus appears that the *Hox*-free rostral domain of the neural fold is the site of striking diversity of NC-derived structures.

Patterning the Facial Skeleton by the Pharyngeal Endoderm

In lower vertebrates, tissue associations in organotypic culture have indicated that intimate contact between the NCCs and the pharyngeal endoderm is required for cartilage differentiation.[106,107] More recently, it has been put forward that skeletal morphogenesis relies on the pharyngeal segmentation. The absence of NC did not significantly perturb the early patterning of the pharyngeal endoderm.[108] Conversely, mutations affecting the endoderm resulted in aberrant organization of the BAs in zebrafish.[109] Recent studies have enlightened the synergistic effect of *Fgf3* and *Fgf8* genes in this process, by directing lateral progression of endodermal cells in order to sculpt the pharyngeal pouches.[110] Hence the segregation of NCC migratory flows into the BAs tightly depends on the early segmentation of the pharyngeal endoderm.[109]

Experiments carried out in the avian embryo have established the capacity of defined regions of the pharyngeal endoderm to convey instructive patterning cues to the NCCs during facial skeleton morphogenesis.[101,111] Extirpation of endodermal stripes at neurula stage results in the disruption of pieces of the facial skeleton, which vary according to the level of the ablation (Fig. 7A-C). Reciprocally, grafting similar endodermal stripes into otherwise intact host embryos along the migration pathway of cephalic NCCs, induced predicted duplications of facial skeletal components (Fig. 7D-F). Moreover, changing the orientation of the grafted stripes accounted for modifying the polarity of the induced supernumerary skeletal pieces.[101] Therefore, the *Hox*-negative NC behaves as a naïve entity to which the ventral foregut endoderm confers patterning cues in order to specify the shape and spatial orientation of the maxillo-mandibular skeleton.

Later on, these results were extended to the formation of the hyoid bone that arises from both *Hox*-negative and *Hox*-positive NCCs.[111] Thus, the patterning ability of the more caudal levels of the pharyngeal endoderm (facing the mid- and posterior rhombencephalic structures in the neurula) to design the pharyngeal skeleton was demonstrated.

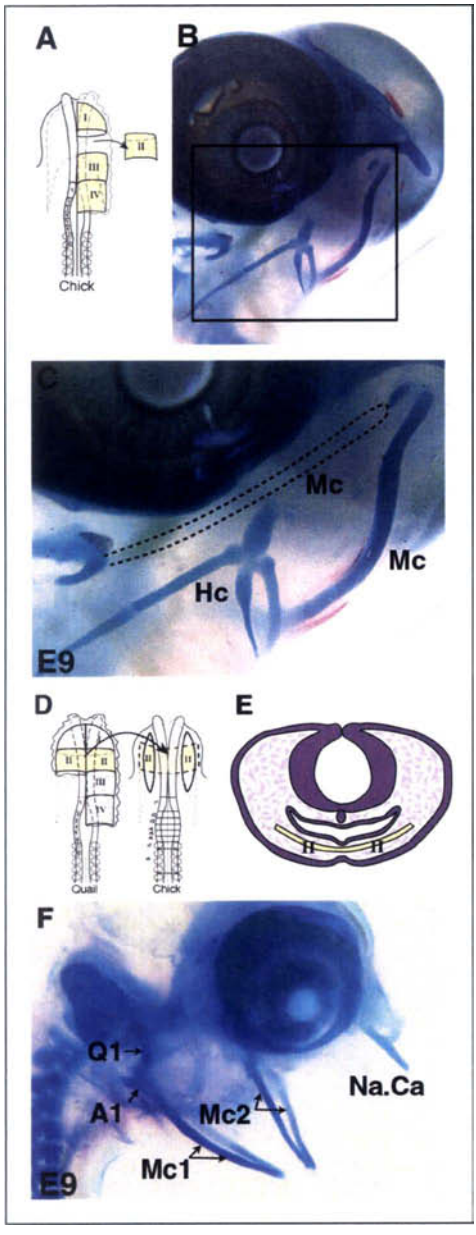

Figure 7. The ventral foregut endoderm patterns the NC-derived skeleton. A) Unilateral surgical extirpation of the zone II of the ventral foregut endoderm (shaded) facing the anterior mesencephalon in chick embryo. B) In E9 operated embryo, this extirpation leads to ipsilateral loss of Meckel's cartilage as evidenced on higher magnification (C; dotted line). Bilateral transplantation of endodermal zone II taken from a quail embryo (D) implanted ventrally to the endogenous ventral foregut of the host (E). F) In E9 recipient embryo, the additional endodermal stripe has induced a supernumerary mandible (Mc2) interposed between the endogenous upper and lower jaws. A1: endogenous articular; Hc: Hyoid cartilage; Mc1: endogenous Meckel's cartilage; Na.Ca: nasal capsule; Q1: endogenous quadrate.

Role of the Superficial Facial Ectoderm in Shaping the Face

In the mouse, *Fgf8* gene inactivation in the BA ectodermal component impairs lower jaw development.[112] Moreover, isthmus-derived FGF8 has been shown to be involved in the patterning of the visceral skeleton.[113]

One of the striking and early consequences of the surgical extirpation of the anterior cephalic NC which is responsible for building up the entire facial skeleton (i.e., NCCs from the di-mesencephalon and r1-r2), is the strong down-regulation of *Fgf8* expression in the anterior neural ridge (ANR, i.e., the prosencephalic superficial and neural ectoderm) (Fig. 8A,B).

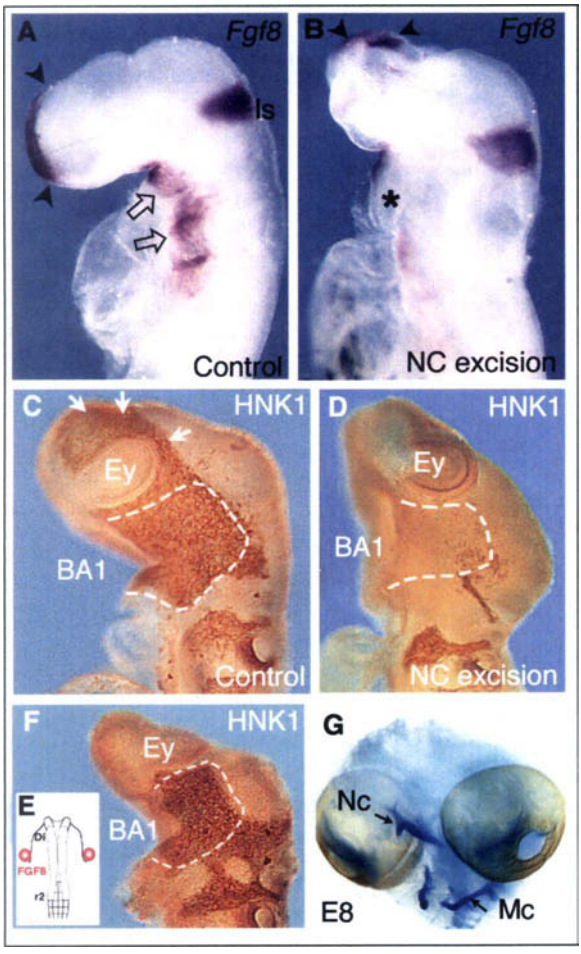

Figure 8. Reciprocal interplay between NCCs and *Fgf8* expression by the superficial and neural ectoderm. A) In E3-control embryos, *Fgf8* is expressed in the anterior neural ridge (ANR; i.e. the prosencephalic superficial and neural epithelium; arrowheads), in the ectoderm lining BA1 (open arrows), and in the isthmus (Is). B) Ablation of the *Hox*-negative NC entails a dramatic decrease of *Fgf8* expression in the ANR and BA1 ectoderm (*) 24 hours after surgery. C) In control embryos, the cephalic NCCs (HNK1 immunoreactivity) massively populate the forming BA1 (dotted line) and colonize the nasofrontal bud (arrows). D) After *Hox*-negative NC excision, only rare HNK1-labelled NCCs are present in BA1. E) Bilateral treatment of NC-excised embryos with FGF8 rescues the colonization of BA1 by r3-derived cells at E3 (F). G) At E8, these embryos have developed a maxillo-mandibular skeleton.

Moreover, this operation totally suppresses *Fgf8* expression in BA ectoderm. As mentioned above, the phenotype resulting from surgical extirpation of the cephalic NC is the absence of facial skeleton together with severe defects of forebrain development (Fig. 6A,B). If, in this experimental system the endogenous protein is substituted for by exogenous FGF8, the NCCs from r3 (which normally provides only a few cells to BA1) entirely colonize BA1, induce *Fgf8* expression in BA1 ectoderm and generate a normal jaw skeleton (Fig. 8C-G). In addition, the NCCs, even if they belong to the *Hox*-positive posterior domain of the neural axis, induce *Fgf8* expression in the prosencephalic superficial and neural ectoderm. Restoration of *Fgf8* expression in the prosencephalon is followed by normal forebrain development.[99] Therefore, (i) the cephalic NCCs are necessary for *Fgf8* expression by the epithelial ectodermal cells (ii) in turn FGF8 exerts a strong proliferative effect on cephalic NCCs.

These experiments pointed out to the strong regeneration capacities of r3-derived NCCs and to the role of FGF8 in regulating NCC proliferation and migration. In addition, it has been proposed that, in mice, *Fgf8* expression by the superficial ectoderm specifies the mesectoderm to form either the mandible or the tooth germs and refines the position of the developing jaw joint.[114,115]

The ectoderm of the fronto-nasal process was also shown to play an important role in positioning and refining the shape of the upper beak. This effect is mediated by the two morphogens Sonic hedgehog (SHH) and FGF8, the expression domains of which abut in the frontonasal ectodermal zone, which plays a role in the growth of the underlying mesenchyme of NC origin.[116,117]

Signals emanating from the NCCs are also required for shaping the face. This has been demonstrated by recent experiments in which NCCs were orthotopically exchanged between embryos of quail and duck. These two species were chosen because they show different bill morphologies and develop according to different timings of incubation (17 days for the quail and 28 days for the duck). These experiments revealed that NCCs convey the timing for *Shh* and *Pax6* gene expression to the host ectoderm. NCCs thus impose a donor- rather than host-type molecular pattern for bill morphology.[118]

In similar experiments, Tucker and Lumsden[119] have further analysed the morphology of the quail cartilages that develop within the duck environment (and vice-versa). They found that the shape of the facial cartilages (e.g., the entoglossum and the retroarticular process which strikingly differ between the duck and quail) is always of NC donor-type.

Therefore, once induced by the endoderm to develop into a particular cartilage, NCCs follow a species-specific genetic programme involving a particular growth and morphogenetic pattern. Another important conclusion drawn from these studies is that the dermal ossification of mandibular bones follows a species-specific timing of differentiation.

Thus, during facial morphogenesis, a temporally regulated and multi-step cross-talk occurs between the epithelia (endoderm and ectoderm) and the NCCs.

Heterogeneity of Progenitors and Stem Cells in the Early NC

The final phenotype adopted by NCCs depends mainly on local cues encountered by the cells during and at the end of their journey (see ref. 120 for a review). Single cell studies, essentially carried out in vitro, have revealed that migratory NCCs include highly pluripotent as well as oligopotent progenitors and fate-restricted precursors.[121-127] Some of the pluripotent and bipotent progenitors in the early NC have been shown to be stem cells endowed with self-renewal capacity.[125,127]

In the trunk NC, the pluripotent cells give rise in vitro to pigment cells, glial cells and PNS neurones as well as to myofibroblasts/smooth muscle cells expressing α-smooth muscle actin (αSMA), the latter cell type arising from the NC only at the cephalic level in normal development.[125,127,128]

Quail mesencephalic-rhombencephalic NCCs in culture generate mesectodermal derivatives such as chondroblasts and myofibroblasts.[121-123,127] Subsets of these NCCs exhibit the potential to develop into these mesenchymal as well as glial, neuronal and/or melanocytic cells, with diverse combinations of phenotypes. These cells however constitute a relatively small

proportion (7%) of the clonogenic cephalic NCCs in our culture conditions.[38] Such progenitors, which give rise both to neural-melanocytic (trunk-like) and to mesenchymal (cephalic-like) NC derivatives, could be identified late in NC ontogeny, including the migratory stages, thus arguing against the contention that mesectoderm might be segregated from other NC lineages already in the dorsal neural primordium.[129] The existence of common progenitors for neurones, pigment cells, myofibroblasts and chondrocytes has been also shown in the posterior rhombencephalic NC of the quail[130] and the mouse.[131]

Altogether, these results support the emergence of diversified cell types from highly pluripotent NC stem cells, through the generation of various intermediate progenitors endowed with stem cell properties (Fig. 9).

Resident NC Stem Cells Are Present in Differentiated Tissues up to Adulthood

At post-migratory stages, pluripotent NC stem cells were identified in the PNS nerves, DRG and in the gut of fetal and postnatal rat.[132-135] These findings further document the results of previous in vivo back-transplantations of quail NC derivatives, wherein grafted PNS ganglia gave rise to migratory cells able to colonize new targets and to differentiate into alternative phenotypes in the chick host tissues.[6]

The '*boundary cap cells*' of the dorsal root entry zone and motor exit points in the embryonic spinal cord were identified recently as a source of NC stem cells during neurogenesis.[136,137] These cells, which are derived from late-emigrating trunk NCCs, produce Schwann cells but also differentiate into subsets of sensory neurones and satellite glial cells in the mouse DRG, as shown by Cre-recombinase fate mapping using the *Krox20* locus.[136] Moreover, mouse '*boundary cap cells*' in clonal cultures comprise self-renewing progenitors for glia, sensory neurones and myofibroblasts.[137]

In the mammalian skin, progenitors isolated either from the dermal papillae[138,139] or the epidermal bulge area[140,141] of hair follicles, behave in vitro as pluripotent stem cells endowed with both neural and mesenchymal lineage potentials. Genetic tracing using *Wnt1-Cre* transgenic mice revealed that these progenitors are of NC origin. In the head region, the NCCs invade the presumptive skin early in mouse embryogenesis and comprise pluripotent stem cells, which thus persist up to adulthood in the vibrissae whisker follicles and may serve to regenerate multiple cell types during skin repair.

Therefore, a certain degree of differentiation plasticity and regeneration capacities characterize subsets of NC-derived cells long after PNS and skin organogenesis is completed. In mammals, the opportunity to prospectively isolate NC stem cell populations using cell surface markers combined with gene targeting mutations, allows further understanding the mechanisms of self-renewal[142] and maintenance of pluripotency.[143,144]

Plasticity of NC-Derived Phenotypes

How the NC-derived phenotypes are specified and thereafter maintained in NC derivatives is still poorly understood. We have shown recently that pigment cells and Schwann cells can change their phenotype and convert into each other in vitro upon mitogenic stimulation by endothelin-3 (ET3) peptide.

Schwann cells isolated from quail embryonic nerves are capable of changing their lineage programme and generate pigment cells in long-term cultures supplemented with ET3.[145] In addition to melanocytes, these cells give rise to myofibroblasts, which are obtained in culture earlier than melanocytes and independently of the cytokine ET3.[146] Moreover, after transplantation into the BA1 of younger chick embryos, the Schwann cells spread into the host mesenchyme and generate myofibroblasts that are recruited to participate in the formation of the peri-vascular smooth muscle layer.[146]

The melanocyte phenotype also displays a high plasticity in vitro. Single pigment cells purified from the quail epidermis until hatching stage, are able to yield glial and myofibroblastic cells in addition to parental-like melanocytes, following exposure to ET3 (see ref. 147 and Real et al submitted) (Fig. 10). This phenotypic reprogramming involves the dedifferentiation of

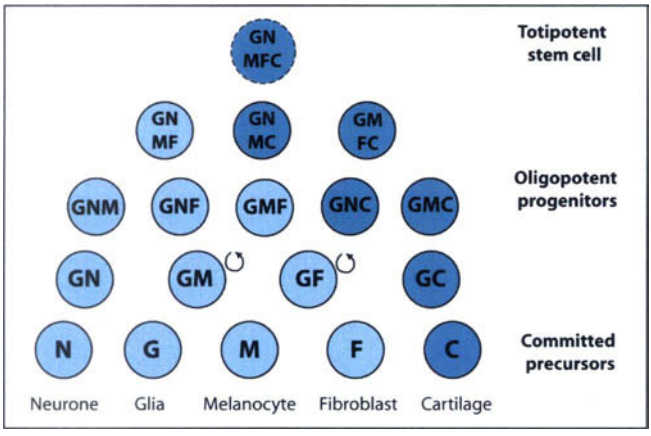

Figure 9. Model for the diversification of NC-derived lineages. The progenitors identified in the quail NC by in vitro clonal cultures are classified according to their number of potentialities. Data[122,127] support a hierarchical model for lineage segregation, according to which progressive restrictions of a putative pluripotent stem cell (broken circle) give rise to oligopotent progenitors and finally to precursors committed to each of the main NC-derived phenotypes, namely, glia (G), neurones (N), melanocytes (M), myofibroblasts (F) and cartilage (C). All the progenitors able to yield cartilage (dashed circles) are present in the cephalic but not in the trunk NC. At least some of the oligopotent progenitors exhibit the ability to self-renew in vitro (curved arrows).

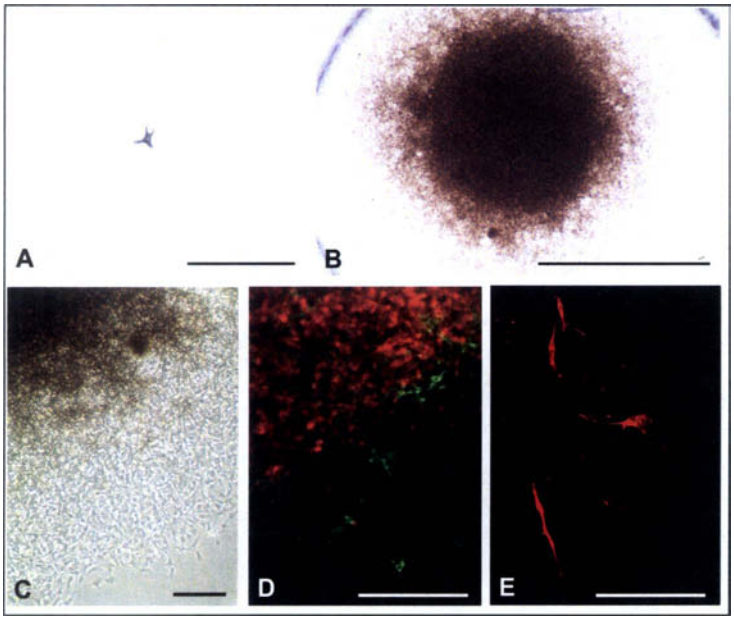

Figure 10. Pigment cells generate multiphenotypic clonal progeny in vitro. A) Culture of a single melanocyte from quail embryonic epidermis 4 hours after clonal seeding. B) After 13 days in the presence of endothelin-3 (ET3), the melanocyte has generated a large colony with a highly pigmented core. C) Detailed view of (B)—the periphery of the colony comprises both unpigmented and pigmented cells. D,E) Phenotypic analysis show that colony contains (D) melanoblasts and glial cells (immunoreative to MelEM and SMP markers)[147] as well as (E) myofibroblasts (expressing α-smooth muscle actin αSMA). Bars: 100 μm in (A) and (E), 200 μm in (C) and (D), 1 mm in (B).

dividing pigment cells into cells that reexpress NC early transcription factors such as *Sox10*, *FoxD3*, *Pax3* and *Slug*. Single melanocytes were shown to generate multipotent progenitors able to self-renew along serial subcloning, thus exhibiting characteristic properties of stem cells. Some of these stem cells harbour the HNK1 cell surface marker that is expressed by early NCCs and which had been extinguished in the parental differentiated pigment cells (Real et al submitted).

Therefore, when removed from their *'niche'* and subjected to new environmental in vitro conditions, embryonic pigment cells and Schwann cells can reverse their differentiation programme and recapitulate early properties of their NC ancestors. These findings suggest that differentiated cell types derived from the NC are phenotypically unstable and capable of broad differentiation plasticity. In the skin and nerves, the dedifferentiated NCCs thus might be mobilized for tissue repair, alternatively or complementary to resident undifferentiated NC stem cells.

Concluding Remarks

During the last ten years, the NC has become a popular subject in cell and developmental biology. This is justifiable by the widely diversified interests offered by this system. First, in the group of chordates characterized by a common body plan, the NC is present only in vertebrates. It is considered to have been essential in the transition from cephalochordates to vertebrates since specific vertebrate traits within the chordate phylum such as skeletal tissues, PNS and spectacular head and brain development, are linked to the NC and its derivatives.

The first emphasis on the role of the NC in the evolution of chordates was brought about in a well-acknowledged article by Carl Gans and Glenn Northcutt in 1983.[148] These authors proposed that the NC, which gives rise to most of the skull, the meninges and the vascularization of forebrain and face (as revealed by embryological studies, see ref. 2 for references), was responsible for the formation of a *'New Head'* characterized by the spectacular development of the forebrain and associated sense organs. According to Gans and Northcutt, these attributes were related to a radical change in the life style of the vertebrates as compared to the cephalochordates: vertebrates became able to look for their food and even became predators, whereas the cephalochordates, like their extant form the *Amphioxus* (considered as closely related to the vertebrate ancestors), were filter-feeders. It is noticeable that the first organ of predation that appeared in vertebrates is the jaw, which is entirely NC-derived (see above).

In addition to its interest in vertebrate evolution, the NC is a system on which a large register of developmental problems can be studied in a privileged manner. As mentioned above, the NCCs are endowed with migratory properties and, in this respect they can be a model for studying the molecular mechanisms that regulate cell migrations which commonly occur in normal and pathological conditions.

The NC is a highly pluripotent structure. Several laboratories have demonstrated the presence of NC stem cells both in the embryo and in the adult derivatives of this structure. NCCs of birds and mammals can be cultivated in vitro and their plasticity was shown to be remarkable. A lot remains to be discovered, in terms of cytokines active on the various NC-derived lineages and of their mode of action in these well-defined experimental models.

Finally, by its ubiquitous ramifications all over the organism, the PNS is critical in coordinating the functions of various cellular components of the vertebrate body.

Many key problems remain to be investigated about the NC, among which, the genetic control of its formation in the embryo is particularly interesting owing to its possible evolutionary implications.

Acknowledgements

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CHAPTER 7

Cranial Neural Crest and Development of the Head Skeleton

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Abstract

The skeletal derivatives of the cranial neural crest (CNC) are patterned through a combination of intrinsic differences between crest cells and extrinsic signals from adjacent tissues, including endoderm and ectoderm. In this chapter, we focus on how CNC cells positionally interpret these cues to generate such highly specialized structures as the jaw and ear ossicles. We highlight recent genetic studies of craniofacial development in zebrafish that have revealed new tissue interactions and show that the process of CNC development is highly conserved across the vertebrates.

Introduction

The skull and jaws were key innovations in vertebrate evolution, vital for a predatory lifestyle.[1] Much of the skull and all of the pharyngeal skeleton, including jaws, hyoid and gill structures, also have a unique embryonic origin from CNC, unlike the more posterior axial and appendicular skeletons which are derived from mesoderm. These CNC-derived cartilages and bones are modified in different vertebrate lineages, such that in mammals the branchial elements form laryngeal bones in the throat, in contrast to the primitive function in fish where they support the gill arches.[2] Similarly, the mammalian homologue of the hyoid bone that primitively supports the jaw in fish, instead forms the stapes of the mammalian middle ear. These remarkable changes in function and shape of vertebrate cranial skeletal elements reveal how subtle differences in patterning of CNC in the embryo can result in significant morphological differences between species.

Such changes are best understood if the skeletal elements are considered as units within larger head segments that are established in the embryo.[3] The pharyngeal skeleton forms within a reiterated series of arches that surround the anterior foregut. Several factors influence the fate of CNC within the arches. These include the site of origin of the CNC in the neural tube, position within the arch following migration and proximity to local signaling centres including the surface ectoderm and the pharyngeal endoderm. Interpretation of these signals provides the spatial information required for the development of the final skeletal pattern.

Specification and Migration of the Skeletogenic CNC

A CNC origin for the skull was first suggested near the end of the 19th century by ablation experiments in amphibians.[4-6] These results were treated with skepticism at the time, since

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they contradicted the germ layer theory in which skeletal tissues were thought to be exclusively of mesodermal origin. Lately, new debates on the origin and significance of this "ectomesenchymal" population have emerged, suggesting that cranial skeletal elements arise from a migratory cell population distinct from the CNC.[7] Fate mapping studies in chick first showed that CNC contributes directly to the "neurocranium", which surrounds the brain, as well as to all of the pharyngeal arches of the "viscerocranium",[8-10] and these results have been confirmed in other species.[11-13] CNC cells delaminate from the ectoderm overlying the dorsal neural tube and migrate as separate streams into the pharyngeal arches (Fig. 1). Neurocranial precursors emerge from midbrain levels and migrate between the eyes to form the palatal shelves, while viscerocranial precursors emigrate in three distinct streams from the hindbrain into the mandibular (stream 1), hyoid (stream 2), and five branchial (stream 3) arches. Recent data also reveal a contribution of the most anterior (mandibular) stream to the ventral neurocranium[14,15] suggesting that the mandibular stream has a more significant contribution to the skull than has been appreciated. As discussed below, this has implications relating to signals received early in development during CNC migration.

In all vertebrates, segmental differences between streams of CNC are conferred through the nested expression of homeobox (Hox and Otx) genes (Fig. 1). This is thought to coordinate A-P patterning of the hindbrain with the arches that it innervates (reviewed in ref. 16). For example, CNC cells migrating in stream 2 (hyoid) are the most anterior neural crest cells to express Hox genes. They express only Hox group 2 genes, and arise from the Hox-2 expressing region of the hindbrain. In contrast, CNC cells in stream 1 (mandibular) that will give rise to the jaws do not express Hox genes and arise from Hox-negative regions of the anterior hindbrain and midbrain. Loss of Hox group 2 gene function results in a homeotic transformation of hyoid skeletal elements derived from CNC of stream 2, to a Hox-negative, mandibular fate.[17,18] Grafts of CNC along the neural tube from Hox-negative regions to more posterior Hox-positive levels in the chick results in ectopic mandibular structures at inappropriate A-P positions.[19] In contrast, overexpression of Hox genes in CNC of stream 1 results in ectopic hyoid skeletal elements in place of the jaw.[20,21] Taken together, these results suggest that the Hox expression status of CNC confers a subsequent positional identity, which is dictated by the site of origin of the CNC in the hindbrain.

Recent evidence, however, suggests that this identity is not completely fixed prior to migration into the periphery. The maintenance of Hox expression in migrating CNC depends on the Hox expression of immediately neighbouring cells and other signaling centers within the arches.[22,23] This implies that the positional origin of CNC in the neural tube is important for subsequent CNC fates in the pharyngeal arches, but that this is not sufficient to confer identity along the A-P axis. Indeed, the molecular regulation of Hox group 2 gene expression in migrating CNC is independent to that in the hindbrain, indicating that CNC do not simply inherit their A-P identities and patterns of Hox expression based on their hindbrain origins.[26]

Recent genetic studies in zebrafish have provided several insights into the mechanisms of Hox gene regulation in CNC. For example, mutations in the histone acetyltransferase *moz* (monocytic leukemia zinc finger) disrupt both *hoxa2* and *hoxb2* expression in the hyoid CNC, but not in the hindbrain.[24] This results in partial transformations of hyoid arch cartilages to a mandibular fate, resembling a loss of *hox2* function (Fig. 2A-C).[21] The first signs of these tranformations are changes in early gene expression in CNC condensations within each arch, prior to overt skeletal differentiation (Fig. 3). Two examples of this are *goosecoid (gsc),* expressed in medial first arch and lateral second arch CNC condensations of cartilage precursors and *bapx1,* an orthologue of *Drosophila bagpipe*, expressed only in first arch joint precursors (asterisks in Fig. 3). *moz* mutants develop mirror-image patterns of *gsc* expression in first and second arch medial cartilage precursors, and *bapx1* expression in both the first and second arch joints. Such mirror-image transformations are also seen in the skeleton in *Hoxa2*$^{-/-}$ mice and *hox2*-deficient zebrafish, pointing to the existence of a polarizing influence from cells at the junction between first and second arches (Fig. 2C).

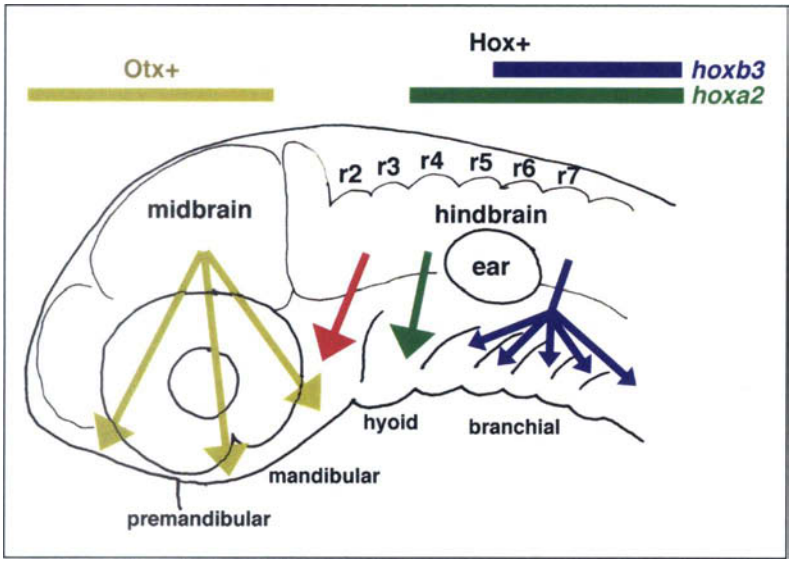

Figure 1. Pathways of migration and patterns of homeodomain gene expression in the skeletogenic CNC. Camera lucida drawing of the head of a zebrafish embryo at 24 hours postfertilization, lateral view. Hindbrain rhombomeres (r2-r7) give rise to streams of CNC in each pharyngeal arch: stream 1 (red) forms the mandibular arch; stream 2 (green) forms the hyoid arch; and stream 3 (blue) forms the five branchial arches. Only stream 2 (*hoxa2+*) and stream 3 (*hoxb3+*) express Hox genes. Premandibular CNC cells migrate in more anterior streams (yellow), between the eyes, and express Otx genes.

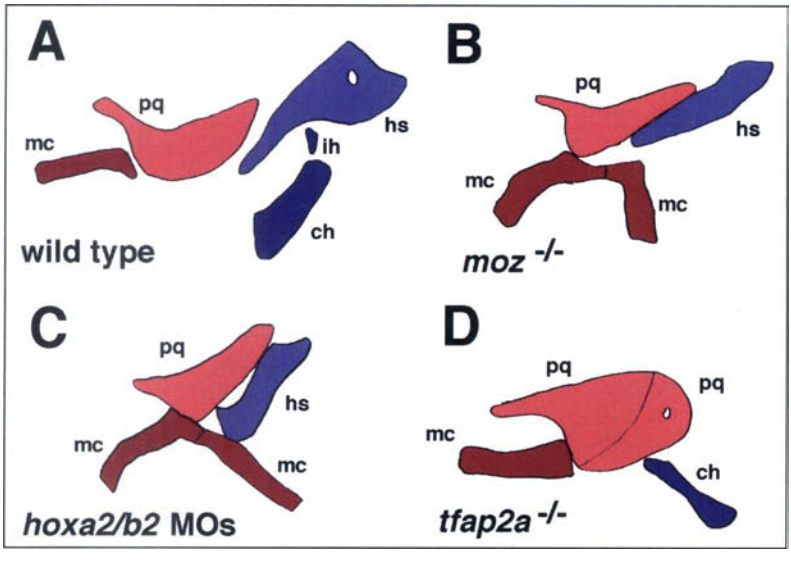

Figure 2. Mandibular and hyoid cartilages in wild type zebrafish and mutants or morphants with partial homeotic skeletal transformations. Camera lucida drawings of flat-mounted cartilages dissected from the left side of the head in wild type (A), *moz* mutants (B), morphants coinjected with *hoxa2* and *hoxb2* morpholinos (C), and *low/tfap2a* mutants (D). Abbreviations: ch, ceratohyal; hs, hyosymplectic; ih, interhyal; mc, meckel's cartilage; pq, palatoquadrate.

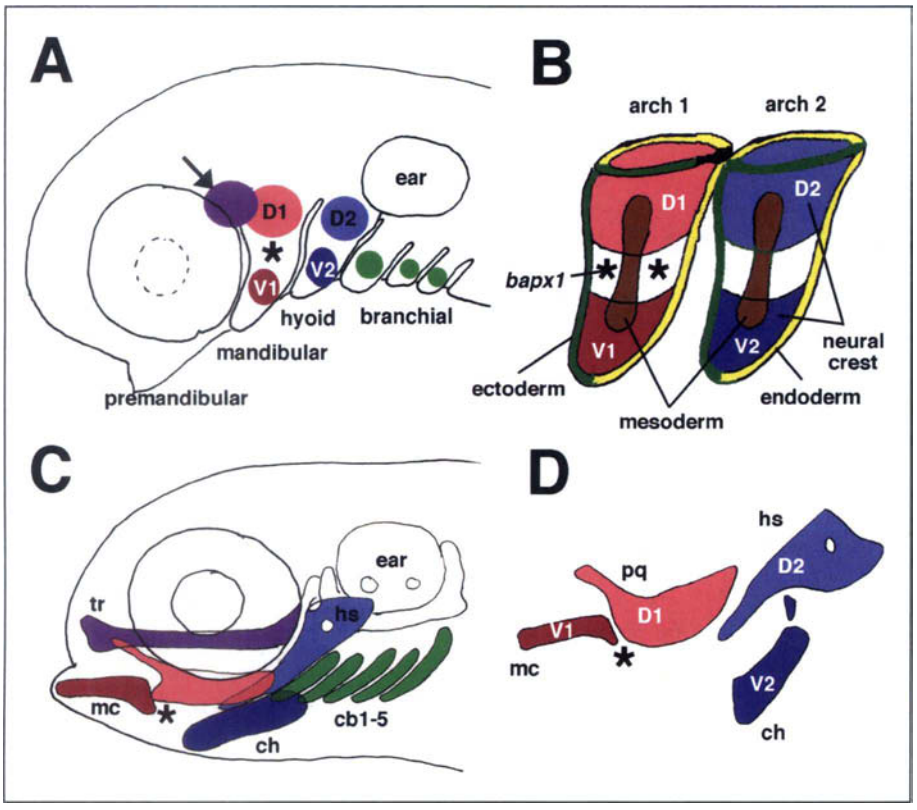

Figure 3. Pharyngeal arch primordia and CNC condensation patterns that form craniofacial cartilages in zebrafish. Lateral views at 28 hours (A,B) and 72 hours (C,D) postfertilization. A) Schematic illustrating dorsal (D1,D2) and ventral (V1,V2) groups of skeletal precursors. Green circles, ventral branchial arches. Purple oval (arrow), precursors of the neurocranium. Asterisks indicate the mandibular joint. B) Schematic illustrating the cylindrical organization of arch primordia. Mesoderm (brown) is surrounded by CNC (red, white and blue) which form dorsal and ventral condensations. Joint precursors (white) express *bapx1* in the mandibular arch. These groups of arch mesenchyme are surrounded by endodermal (yellow) and ectodermal (green) epithelia. C) Camera lucida drawing of larval cartilages of the mandibular (red), hyoid (blue) and branchial (green) arches, as well as the neurocranium (purple). D) Camera lucida drawing of flat-mounted, dissected cartilages of the mandibular and hyoid. Abbreviations: ch, ceratohyal; hs, hyosymplectic; ih, interhyal; mc, meckel's cartilage; pq, palatoquadrate; tr, trabeculae.

The zebrafish *lockjaw (low)* mutant disrupts the transcription factor AP-2 alpha (*tfap2a*), which also regulates Hox gene expression. Like *moz*, homozygous *low* mutants exhibit segment-specific changes in CNC fate in the arches, but not in the hindbrain.[25] *low* mutants develop partial hyoid-to-mandibular transformations, particularly in the dorsal region of the arch, which closely correlate with reductions in *hoxa2* expression (Fig. 2D).[27] *hoxa2* gene expression is lost in CNC of the hyoid arch in mutants, but expression in the hindbrain remains unaffected. This CNC-specific requirement for *tfap2a* in *hoxa2* regulation is conserved with mammals; the mouse *Hoxa2* promoter contains an AP-2 binding site essential for expression in the CNC but not in the hindbrain.[26] Disruption of specification during CNC development correlates with a lack of neural crest-derived pigment cells, enteric neurons, and craniofacial cartilage in *low (tfap2a)* mutants. These correlate with early defects in the specification of the premigratory neural crest (expression of *foxd3* and *sox9a* is reduced in

tfap2a mutants) and cell survival in *low* mutants. These studies in *low* and *moz* support the paradigm of independent regulation of *hox2* genes in CNC and the hindbrain and reveal that the final fate of the CNC is not dictated simply by its hindbrain level of origin, but requires signals from adjacent tissues.

Patterning Influences of Skeletogenic CNC on Surrounding Tissues

The most anterior CNC cells that form the upper and lower jaws as well as the anterior neurocranium do not express Hox genes (Fig. 1). However this CNC, perhaps more than any other, possesses an identity independent of adjacent tissues once it has migrated into the head periphery. This was dramatically demonstrated by Noden[19] in a classical series of transplantation experiments in avian embryos, in which midbrain CNC (Hox negative) was grafted to more posterior hindbrain (Hox positive) regions. Surprisingly, grafted cells retained the identity appropriate for their original positions in the donor embryo and formed an ectopic mandibular skeleton. Notably, these grafts reorganized the surrounding mesoderm to form an ectopic set of mandibular muscles, indicating that CNC is instructive for muscle development.[19,28] Similar results were obtained more recently with interspecific transplants of CNC contributing to the frontonasal process, between duck and quails, which showed that at least some aspects of beak shape are dictated by the CNC from the donor.[29,30] Likewise, in this case the transplanted CNC reorganized surrounding soft tissues including feather placodes derived from ectoderm and muscles derived from the mesoderm, indicating an instructional influence of the CNC on these tissues.

How do CNC cells that form the skeleton interact with the muscles to which they attach? In the head, this coordination appears to be regulated by specialized attachment cells (putative tendon or ligament precursors) that are also derived from CNC (Fig. 3D). These resemble attachment cells in the limb, which derive from lateral plate mesoderm and are able to assemble a correct spatial pattern in the complete absence of myocytes.[31] Within each arch primordium the CNC cells surround a central core of mesoderm that will form the muscles (Fig. 3B). Here the initial "pioneer" muscle cells form attachments at precise locations along the skeleton through CNC-derived tendons. Fate mapping studies of CNC in chick-quail chimeras revealed a striking correlation in the embryonic origins of these two tissues. Tendon precursors attaching a muscle to a particular bone originated from the same A-P location in the CNC as the precursors of that bone.[32] This suggests a mechanism whereby muscle attachment and bone shape are coordinated through a common process that determines the A-P identities of CNC cells prior to their migration, possibly through their Hox expression (or lack thereof). Through this process, CNC-derived skeletal and tendon precursors may coordinate the formation of the entire functional skeletomuscular system.

Mutant studies in zebrafish also support the model that CNC cells play instructive roles in patterning cranial muscles. Histological analysis of the *chinless (chn)* mutant revealed that the pharyngeal skeleton and cranial muscles fail to form entirely.[33] Surgical replacement of the mandibular CNC with wild-type cells rescued both skeletal and muscle development. Rescued myocytes were confined to regions immediately adjacent to CNC derived from the donor, demonstrating the local nature of the interaction. Likewise, in *moz* and *low (tfap2a$^{-/-}$)* mutants, reductions/transformations of the second arch (hyoid) skeleton correlate with disruptions of cranial muscles.[24,25]

Patterning within Pharyngeal Arch Primordia

After CNC migration, each pharyngeal arch is organized cylindrically, with a core of mesoderm surrounded by CNC (Fig. 3B). These in turn are surrounded by endodermal and ectodermal epithelia. Separate dorsal and ventral condensations (proximal and distal in mice) form in the CNC and these are serially reiterated in every arch (Fig. 3A; reviewed in ref. 35). For example, the ventral/distal element (V1) of the first arch (mandibular) forms Meckel's cartilage (mc), the template for the lower jaw, while the dorsal/proximal element (D1) forms the

palatoquadrate (pq), or upper jaw. Until recently, less was known about the trabeculae (tr) of the so-called "premandibular" skeleton (Fig. 3C). Fate maps both in amphibian and avian embryos now suggest that adjacent condensations in the mandibular arch contribute not only to the jaws, but also to the neurocranium (arrow in Fig. 3A). Dorsal condensations of the mandibular CNC give rise to cartilage of the neurocranium and fronto-nasal process, while both upper and lower jaws arise from more ventral condensations.[14,15] At face value, this challenges established notions of first arch development in which the only derivatives are thought to be mandible and maxilla. It also appears to differ from previous fate maps, including maps of mandibular CNC in zebrafish.[36] The precise locations of condensations, however, with respect to arch boundaries are difficult to compare between such widely divergent species and need to be defined relative to patterns of gene expression (as discussed below).

Comparative studies of vertebrate embryos suggest that many of the key vertebrate innovations evolved through changes in patterning along the D-V axis. It is interesting to speculate that such apparent differences in CNC condensations within the arches underlie: (1) the origins of jaws, (2) divergent patterns of jaw articulation between teleosts and amniotes, and (3) the eventual evolution of ear ossicles from dorsal skeletal components of the arches.[2,3,37] Teleosts exhibit a "hyostylic" jaw suspension, in which the mandibular arch is not attached directly to the neurocranium, but instead its dorsal element (D1; pq) forms a joint with the dorsal hyoid arch (D2; hs), and this in turn articulates with the skull (Fig. 3C). In contrast, in amniotes the suspension is "autostylic", with the lower jaw (V1) hinging directly upon the skull. Currently, it is thought that modification of a hypothetical primitive autostylic suspension in an ancestral vertebrate, led to a "amphistylic" condition seen in some primitive sharks, in which there is a direct articulation of the jaw (V1) with the braincase plus support from the dorsal hyoid arch (D2; hs). Subsequent modifications of this amphistylic articulation led toward hyostyly in teleost fish and autostyly in tetrapods with the consequence of the eventual freeing of mammalian homologues of the palatoquadrate (incus) and hyosymplectic (stapes) to function exclusively as sound transducers. Thus, modifications in the spatial and temporal sites of these D-V skeletal condensations have had major consequences for modes of feeding and hearing during vertebrate evolution.

One signaling molecule that plays a critical role in D-V skeletal patterning in the arches is Endothelin 1 (Edn1), and recent studies in fish and in mice have revealed conserved functions in formation of the lower jaw (Fig. 4; reviewed in ref. 35). Edn1 acts through a type-A, G-protein coupled endothelin receptor (EdnrA), which is expressed in CNC and required for lower jaw development.[38-41] Kimmel et al[42] have argued based on phenotypic changes in bony elements of the zebrafish *edn1* mutant *sucker*, that Edn1 acts as a "morphogen" to specify fates along the D-V axis of an arch. This is based on the observation that while some *sucker* mutants simply lack the lower jaw, others show what appear to be partial duplications of dorsal skeletal elements in the ventral arch, possibly the result of a ventral to dorsal transformation caused by a loss of Edn1. Likewise, EdnrA receptor mutant mice show proximal-distal (D-V) transformations that result in small ectopic dorsal (maxillary) structures in place of the mandible and ectopic whisker barrels ventrally on the chin.[43] This suggests that Edn1 acts to pattern D-V identities of CNC in the arches, as well as surrounding facial ectoderm, and that cells exposed to the highest concentrations of Edn1 form ventral fates such as Meckel's cartilage.

Consistent with this model, expression of a number of transcription factors in ventral arch CNC is lost in Edn1 and EdnrA mutants. Of particular note are *Dlx5* and *Dlx6* (Fig. 4). In mice, these genes are coexpressed broadly throughout the arches, though not to such a dorsal (proximal) extent as *Dlx1/2*. Mice lacking Dlx1/2 function lack proximal (maxillary) bones within the arches. Conversely, the combined loss of Dlx5/6 function results in a loss of distal (mandibular) structures and duplicated maxillae. Similar to *EdnrA*$^{-/-}$ mutants, not only bones but also dorsal ectodermal tissues (marked by the whisker barrels) expand into the ventral arch.[44-46] The similarity between the transformations of cartilage and bone in *Dlx5/6* and *EdnrA* mutant mice, points to a role for *Dlx* genes in potentiating the Edn1 signal. This is achieved

through a nested pattern of *Dlx* gene expression along the D-V axis of the arch (Fig. 4), which subsequently confers positional identity in combination with other ventrally expressed genes, such as *HAND2*, another target of Edn1 signalling.[47] D-V fates of CNC within the arch in this model are induced by a graded Edn1 signal from the distal part of the arch that activates expression of different combinations of *Dlx* genes. This distal Edn1 signal arises from either the pharyngeal ectoderm or endoderm, suggesting that the CNC does not possess an intrinsic D-V identity (ref. 41, unpublished results). These results amid others, have led to a reappraisal of the importance of other tissues in subsequent patterning of the CNC. Recent studies in chick and zebrafish now suggest that much of the positional information within an arch is initiated independent of the CNC.

Endoderm Patterns CNC and Is Required for Skeletal Development

All vertebrates form a series of pharyngeal pouches or pharyngeal slits that subdivide the arches in the embryo, and these are derived from endoderm (Fig. 5). The pouches form as slender, bilateral outgrowths from the lining of the foregut during early segmentation of the embryo. Classic extirpation studies in amphibians and avians showed a requirement for this endoderm in pharyngeal cartilage formation.[48,49] Genetic studies in zebrafish have recently confirmed a role for pharyngeal endoderm in cartilage development and patterning of the CNC-derived head skeleton. Mutants that lack endoderm, such as the *sox32* mutant, *casanova (cas)*, fail to form pharyngeal cartilage entirely, while more dorsal neurocranial cartilages remain unaffected. Furthermore, these skeletal defects can be restored by transplantation of wild-type endoderm into *cas* mutants.[50] This suggests that CNC cells contributing to pharyngeal cartilages depend on the presence of pharyngeal endoderm whereas those forming the neurocranium do not. Cartilage patterning is also disrupted in *tbx1* (*vgo*) mutants; pharyngeal pouches are variably lost and cartilages are reduced or fused.[51] Similar to *cas*, the cartilage defects in *vgo* can be rescued by transplantation of wild-type endoderm, revealing the importance of the endoderm in both skeletal differentiation and patterning.[52] Segmental development and patterns of gene expression within the endodermal pharyngeal pouches, on the other hand, occur independently of the presence of CNC.[53]

To identify signals from the pharynx required for cartilage development, David et al[50] chose to examine *fibroblast growth factor 3 (fgf3)*, which is expressed specifically in the endoderm of the pouches during cartilage condensation. By creating mosaic animals in which Fgf3 function was removed specifically from the endoderm, they showed that *fgf3* is required for the formation of posterior, branchial cartilages (Fig. 5D). Similar defects are seen in the *fgf3* mutant *lia*.[54] Subsequent work suggests that Fgf3 acts together with Fgf8 in pharyngeal patterning.[55,56] Loss of both Fgf3 and Fgf8 function, following gene knockdown using antisense morpholino oligonucleotides, results in a loss of all head cartilages, including most of the neurocranium. In mice, loss-of-function mutations in *Fgf8* targeted to the facial ectoderm of the first arch also disrupt skeletal patterning (ref. 57, see below). Thus, although these and other Fgfs are expressed in multiple cranial tissues throughout development, Fgf3 and Fgf8 in particular play critical roles in the epithelial-mesenchymal interactions that pattern the CNC-derived head skeleton.

When pharyngeal pouches are missing or malformed, streams of migrating CNC often do not separate from one another, pointing to a role for the pouches in guiding CNC migration. This does not affect the segmental identity of the CNC streams initially, as assayed by *Hox* expression during migration, but does result in subsequent loss of regional gene expression in the arches. The shape of the pouch also seems crucial in molding the shapes of certain skeletal elements, and for at least one pouch in the zebrafish this is an integrin-dependent process (Fig. 5B).[58] In this respect, endoderm may stabilize adjacent skeletal precursors in the CNC. In the integrin-α5 mutant *polypterus*, the first pharyngeal pouch is reduced as are the adjacent dorsal cartilages of the hyoid arch. Time-lapsed movies of CNC development, using a transgenic line in which GFP is expressed in the postmigratory neural crest (fli1-GFP), revealed that cartilage precursors align along the edge of the pouch. Twists and bends in the pouches in the absence of

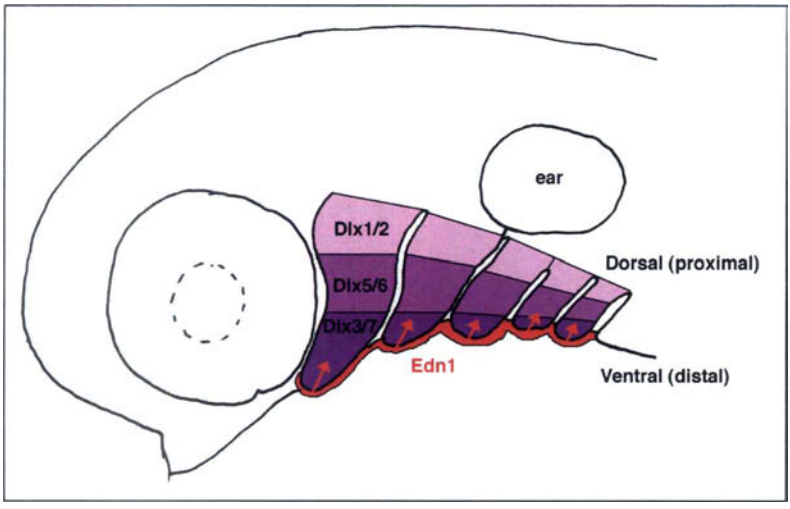

Figure 4. Dorsal-ventral patterning within the pharyngeal arch primordia. Schematic of the zebrafish head, lateral view, at 28 hours postfertilization. Three domains of Dlx gene expression are indicated along the D-V axis in each arch. A ventral source of Edn1 (red) promotes ventral fates (arrows) in the arch skeleton.

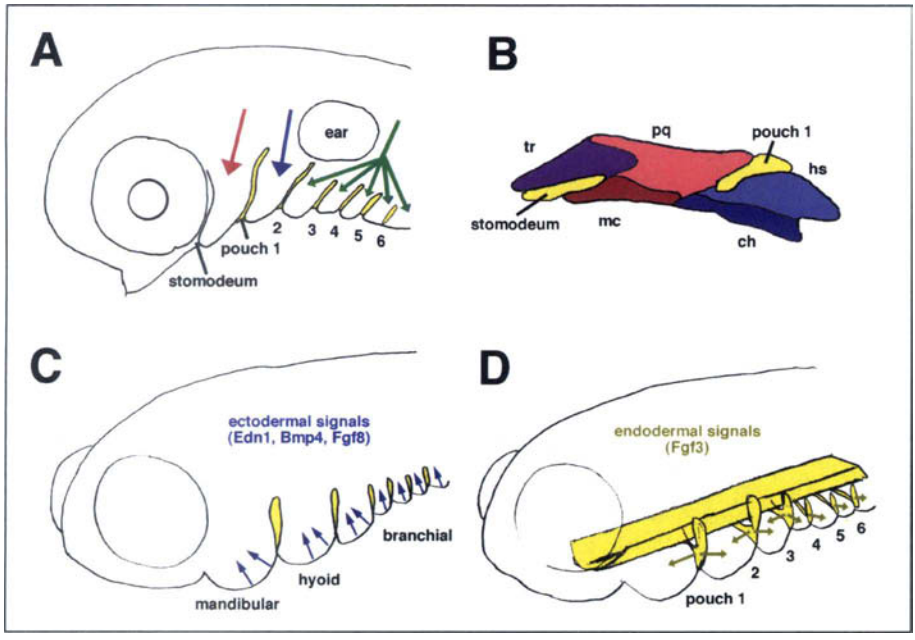

Figure 5. Pharyngeal endodermal pouches and epithelial signals that regulate cranial skeletal development. Lateral views. A) Schematic illustrating pouches (yellow) and their relationship to CNC streams. B) Camera lucida drawing of the early primordia of the mandibular (red) and hyoid (blue) arches in zebrafish at 28 hours postfertilization, showing the relationship of individual cartilage shapes to the pouches (yellow). C,D) Schematics of generalized vertebrate embryos showing pouch organization and putative signals from the ectodermal (C) and endodermal (D) epithelia. Abbreviations: ch, ceratohyal; hs, hyosymplectic; ih, interhyal; mc, meckel's cartilage; pq, palatoquadrate; tr, trabeculae.

integrin-α5 function lead to misalignment of GFP-positive CNC. These data suggest that the endoderm acts not only to pattern the CNC regionally and induce chondrogenesis through Fgf signaling, but may also impose the subsequent shape of each cartilage element.

Tissue-grafting studies in chick have also shown that endoderm plays an instructive role in maintaining the identities of CNC along the A-P axis and establishing the skeletal pattern.[59] Simply reversing the A-P orientation of the endoderm at the level of the midbrain can lead to ectopic mandibular structures. The anteriormost region of the endoderm is vital for formation and orientation of the nasal capsule, while more posterior endoderm promotes formation of the first pharyngeal arch. Importantly, it is only Hox-negative CNC cells that respond to the endoderm in this manner, suggesting that the Hox-positive CNC cells derived from hindbrain levels are patterned by different signals (see Fig. 1). Those CNC cells derived from the Hox-negative parts of the neural tube exhibit a larger range of plasticity than those expressing Hox genes, which may in part reflect their origins near the mid-hindbrain boundary.[28] This highlights the fundamental differences in how different populations of CNC respond to environmental signals, which depends in part on Hox gene function.

Pharyngeal Ectoderm Patterns CNC

A second set of epithelial-mesenchymal interactions in cranial skeletal development occur between CNC and the surface ectoderm, which forms epidermis. Ectoderm was first shown to promote chondrogenesis in CNC by culturing facial ectoderm together with chick mandibular and maxillary mesenchyme.[60] Removal of the surface ectoderm correspondingly results in a failure of skeletal development in adjacent CNC mesenchyme. Only CNC cells that migrate superficially within the arches contact overlying surface ectoderm, but ectodermal influences (like those from endoderm) extend throughout the skeletogenic population (Figs. 3B, 5C). A key ectodermal signal in the first arch is Fgf8. Mice with a targeted loss of Fgf8 function in the mandibular ectoderm lack all but the most distal (ventral) skeletal elements (Meckel's cartilage), implying that an Fgf signal is necessary for proximal (dorsal) mandibular development.[57] This requirement for Fgf8 by proximal skeletal elements in the mandibular arch correlates with the proximity of CNC to a domain of *Fgf8* expression in the oral ectoderm. More distal mandibular elements (Meckel's cartilage) do not develop adjacent to *Fgf8* expressing ectoderm in mice and may respond instead to other Fgfs in the ectoderm, as well as other sources of Fgfs.

In zebrafish, CNC cells migrating into the mandibular arch do not express the Fgf inducible gene *erm* until after migration, and expression is first detected adjacent to the first pharyngeal pouch.[51] How is this pattern regulated by Fgfs originating from the surface ectoderm versus Fgfs from the endoderm? Tissue-specific ablation of Fgf8 function in either ectoderm or endoderm during early mouse development indicates that the primary requirement for Fgf8 is in the ectoderm.[61] Removal of Fgf8 function in mandibular ectoderm causes reductions and fusions of arch elements. CNC cells die through programmed cell death in these conditional mutants, similar to hypomorphic alleles of *Fgf8*.[57,62] In the zebrafish, *fgf8* is also expressed in the mandibular ectoderm and the patterning of pharyngeal cartilages is disrupted in *acerebellar* (*ace*) mutants, which lack a functional Fgf8.[63]

Recent studies on the function of *tfap2a* and its close relative, *tfap2b*, also support a role for the ectoderm in craniofacial patterning in zebrafish. In both mouse and zebrafish *Tfap2a* is expressed in nonneural ectoderm as well as CNC. *Tfap2a* loss-of-function mutants exhibit craniofacial defects, including a lack of second arch (hyoid) skeletal elements and these, at least in part, are cell autonomous requirements in CNC (Fig. 2D).[25,64-68] However, transplants of wildtype CNC into zebrafish *tfap2a* mutants rescues hyoid arch outgrowth, but never completely rescues cartilage development, suggesting that there may be other functions for *tfap2a* in arch development, perhaps in the ectoderm. In zebrafish *tfap2b* is coexpressed with *tfap2a* in pharyngeal ectoderm but is not expressed in CNC.[68] Surprisingly, the combined disruption of *tfap2a* and *tfap2b* function using morpholino oligonucleotides results in the loss of both pharyngeal and neurocranial cartilages, a phenotype much more severe than

loss-of-function mutations in *tfap2a* alone. This suggests that these two closely related genes play redundant roles in chondrogenesis, and that both are required in the ectoderm. Consistent with this model, the facial ectoderm shows elevated levels of apoptosis in embryos lacking tfap2a and tfap2b function (Tfap2 deficient), and transplantation of wild-type ectoderm into these embryos rescues cartilage development. This demonstrates a nonautonomous role for *Tfap2* genes in CNC through their roles in promoting survival and patterning of the pharyngeal ectoderm.

An obvious target of Tfap2 genes in the ectoderm is the Fgf signaling pathway, given its role in chondrogenesis. Despite this, neither *fgf3* nor *fgf8* expression is perturbed in Tfap2-deficient animals. It is possible that *Tfap2* genes regulate expression of other *fgf* genes in the ectoderm. Of the more than 25 known *Fgf* genes in mammals, several are expressed in the facial ectoderm, and may have overlapping roles with Fgf3 and Fgf8.[69] In support of a role for Tfap2 proteins in regulating Fgf signaling, the expression of a known Fgf target gene, *sef*, is reduced in CNC of the arches of Tfap2-deficient zebrafish.[68] This expression can be rescued by wildtype ectoderm, further confirming the nonautonomous requirements for AP-2 proteins in CNC development.

Several other signaling molecules expressed in the facial ectoderm are known to influence the craniofacial skeletal pattern. Like Fgfs, these include factors also expressed in endodermal and mesodermal tissues, such as Edn1 (see above), Bone morphogenetic proteins (Bmps; reviewed in ref. 70) and Sonic hedgehog (Shh; reviewed in ref. 71) (Fig. 5C). A specialized region of facial ectoderm in the hyoid arch, known as the posterior ectodermal margin (PEM), expresses *Shh*. This pattern is conserved in the chick and mouse, and correlates with accelerated growth of the hyoid relative to the other arches. *shh* expression is lost in *low* mutants, which correlates with a failure of hyoid arch outgrowth.[27] Transplantation of wild-type CNC into *low* restores *shh* expression in the adjacent PEM, confirming the interaction and suggesting that it is direct. Ectodermal cells of the PEM may serve a role similar to the apical ectodermal ridge (AER) of the limb bud, maintaining growth and patterning of the underlying mesenchyme.[34] In turn, the reciprocal maintenance of the PEM by underlying CNC also resembles the AER. By comparison with the limb, we suspect that such epithelial-mesenchymal interactions coordinate development of various craniofacial tissues.

Shh and Fgf8 synergize in promoting cranial cartilage outgrowth in the chick.[72] Domains of *Bmp4* expression in the maxillary and mandibular ectoderm lie both anterior and posterior to *Fgf8* expression and this appears to restrict the response of the underlying mesenchyme to Fgf8.[70,71] Interestingly, recent evidence suggests that domains of early Fgf8 and Bmp4 expression in ectoderm are specified surprisingly early in the chick, prior to CNC migration, and are regulated by interactions with endoderm.[73] Subsequent Bmp and Fgf signaling from this ectoderm induces the expression of two homeodomain transcription factors, *Msx1* and *Barx1* respectively, in the underlying CNC, both in mice and chick.[74-77] These prefigure the sites of tooth buds in mice, such that Barx1 specifies molars and Msx1 specifies incisors.[78,79] However, this regional specification of fate in the underlying mesenchyme is not just restricted to tooth type, but as mentioned earlier, also plays a role in defining the identity of the underlying cartilages and bones. This interaction of Fgf and Bmp signaling in specifying identity in the mandibular mesenchyme appears to be a conserved feature in all vertebrates including the sister group of the jawed vertebrates, the jawless lampreys.

CNC Development and Evolution

Evolution of a biting jaw was a crucial step in the evolution of jawed vertebrates (gnathostomes) from their jawless ancestors (agnathans) over 400 million years ago. The classical model of jaw evolution arises from the segmental theory of Goodrich,[3] who proposed that a single cartilaginous element in the vertebrate ancestor became modified into a dorsal and a ventral element, the precursors of the upper and lower jaws in the mandibular arch. In accordance with this theory, the two extant agnathans, lampreys and hagfish (which form the sister

group to all gnathostomes), have a segmented pharynx and CNC, but their arches are not clearly subdivided into dorsal and ventral components (reviewed in refs. 80,81). One theory suggests that the jaw could have arisen by modification of the ventral cartilage element through changes in D-V patterning. Consistent with this idea, some genes that show ventrally restricted expression in the arches of gnathostomes, such as *Dlx5/6* and *Dlx3/7* (Fig. 4), are expressed throughout the D-V extent of the arches during lamprey embryogenesis.[82,83] This lack of nested Dlx gene expression in CNC of the arches is reminiscent of Edn1 signalling mutants in which ventral Dlx gene expression is lost, leading to a loss of a clear ventral skeletal identity. This suggests that progressive restrictions in gene expression evolved into discrete D-V domains within the arches, possibly through the acquisition of new ventral signals.

D-V identity of CNC, however, is imposed by Dlx genes in all of the arches and does not confer a specific mandibular identity. What processes led to segment-specific changes in the mandibular arch that led to an articulated jaw during vertebrate evolution? *Hox* genes are obvious candidates, due to their highly conserved roles in imposing A-P identity along the body axis in all bilaterian animals. There are no *Hox* genes expressed in the mandibular arch (see Fig. 1), and ectopic expression of *Hoxa2* in this arch results in a loss of the mandible.[84,85] This suggests that the jaw evolved, in part, as a consequence of a loss of *Hox* expression in the anterior CNC that contributes to the mandibular arch. Consistent with this model, the brook lamprey, *Lampetra fluviatilis*, appears to express one *Hox* gene (*HoxL6*) in the mandibular arch.[86] However, more recent studies of a larger number of lamprey *Hox* genes do not support the model. In the Japanese lamprey, *Lampetra japonicum*, *Hox* expression is restricted to the hyoid and branchial CNC streams and arches, similar to gnathostomes.[87]

This reveals that agnathans and gnathostomes show a similar conserved pattern of early Hox expression and CNC patterning. This implies that there are dramatic differences in later events that determine the eventual fates of CNC-derived skeletal structures between jawed and jawless vertebrates. These may include differences in signals from adjacent tissues, such as the endoderm or the ectoderm, as well as in CNC responses to these signals. Shigetani and colleagues[88] tested if Fgf and Bmp signaling from the pharyngeal ectoderm to the underlying CNC is conserved between gnathostomes and lampreys. They showed that the same target transcription factors, *Dlx* and *Msx* genes (see above), are similarly induced in the underlying CNC in both groups, but importantly, that this activation differs spatially in relation to CNC condensations. Thus, despite the conserved manner of CNC migration into the mandibular arch in agnathans, certain parts of the lamprey pharyngeal apparatus may not be directly comparable to the jaws of gnathostomes. Both share the same molecular cues for patterning CNC in the arches, but they differ in their spatial interpretation. Central to these features of jaw evolution may have been the interpretation of signals from adjacent epithelia to condense at specific locations and assume identities based on their spatial position within the arch. Future investigations into the nature of these signals will lead to a better understanding of both the development and evolution of the skeleton in different vertebrate lineages.

Summary and Conclusions

CNC cells enter the pharyngeal arches to become the cartilage and bones of the jaw and middle ear in mammals. They also form the bones of the frontonasal process, the dentine of the teeth and the peripheral neurons and glia of the cranial nerves. Specification and migration of the skeletogenic CNC depends on interactions with surrounding tissues. Paracrine factors from these regions induce the expression of transcription factors that control CNC migration and differentiation. This is particularly well known for patterning along the D-V axis within the pharyngeal arches, whereby Edn1 signaling and the nested expression of *Dlx* genes define distinct domains within each pharyngeal arch segment. Other signals received by CNC cells in the arches include Fgfs, Bmps and Shh which are secreted by the facial ectoderm and pharyngeal endoderm in a dynamic fashion. Changes in the sources of these signals and the responses by the CNC have been central to the evolution of craniofacial development.

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CHAPTER 8

Neural Crest Contribution to the Cardiovascular System

Christopher B. Brown* and H. Scott Baldwin

Abstract

Normal cardiovascular development requires complex remodeling of the outflow tract and pharyngeal arch arteries to create the separate pulmonic and systemic circulations. During remodeling, the outflow tract is septated to form the ascending aorta and the pulmonary trunk. The initially symmetrical pharyngeal arch arteries are remodeled to form the aortic arch, subclavian and carotid arteries. Remodeling is mediated by a population of neural crest cells arising between the mid-otic placode and somite four called the cardiac neural crest. Cardiac neural crest cells form smooth muscle and pericytes in the great arteries, and the neurons of cardiac innervation. In addition to the physical contribution of smooth muscle to the cardiovascular system, cardiac neural crest cells also provide signals required for the maintenance and differentiation of the other cell layers in the pharyngeal apparatus. Reciprocal signaling between the cardiac neural crest cells and cardiogenic mesoderm of the secondary heart field is required for elaboration of the conotruncus and disruption in this signaling results in primary myocardial dysfunction. Cardiovascular defects attributed to the cardiac neural crest cells may reflect either cell autonomous defects in the neural crest or defects in signaling between the neural crest and adjacent cell layers.

Introduction

The neural crest are a pluripotent population of cells responsible for the formation or remodeling of a large number of tissues and organ systems. Neural crest cells can form a wide variety of cell types including neurons, glia, Schwann cells, cartilage, bone, and smooth muscle.[1] The neural crest is divided into two major regions termed cranial (mid-diencephalon to somite 5) and trunk (cells arising caudal to somite 5) based upon the rostral-caudal position at which they arise and their subsequent developmental potential.[2] The cranial neural crest form ectoderm derived mesenchyme (ecto-mesenchyme or mesectoderm) that is characterized by the ability to differentiate into numerous cell types normally associated with mesoderm including muscle and bone.[3] Trunk neural crest however, are restricted in developmental potential to melanocytes, neurons and their support cells.[2] A sub-population of cranial neural crest provide material contribution of pericytes and smooth muscle to the cardiovascular system as well as the neurons and ganglia of symapthetic and parasympathetic cardiac innervation.[3,4] This population of neural crest cells has been termed the "cardiac neural crest" (Fig. 1A).[5]

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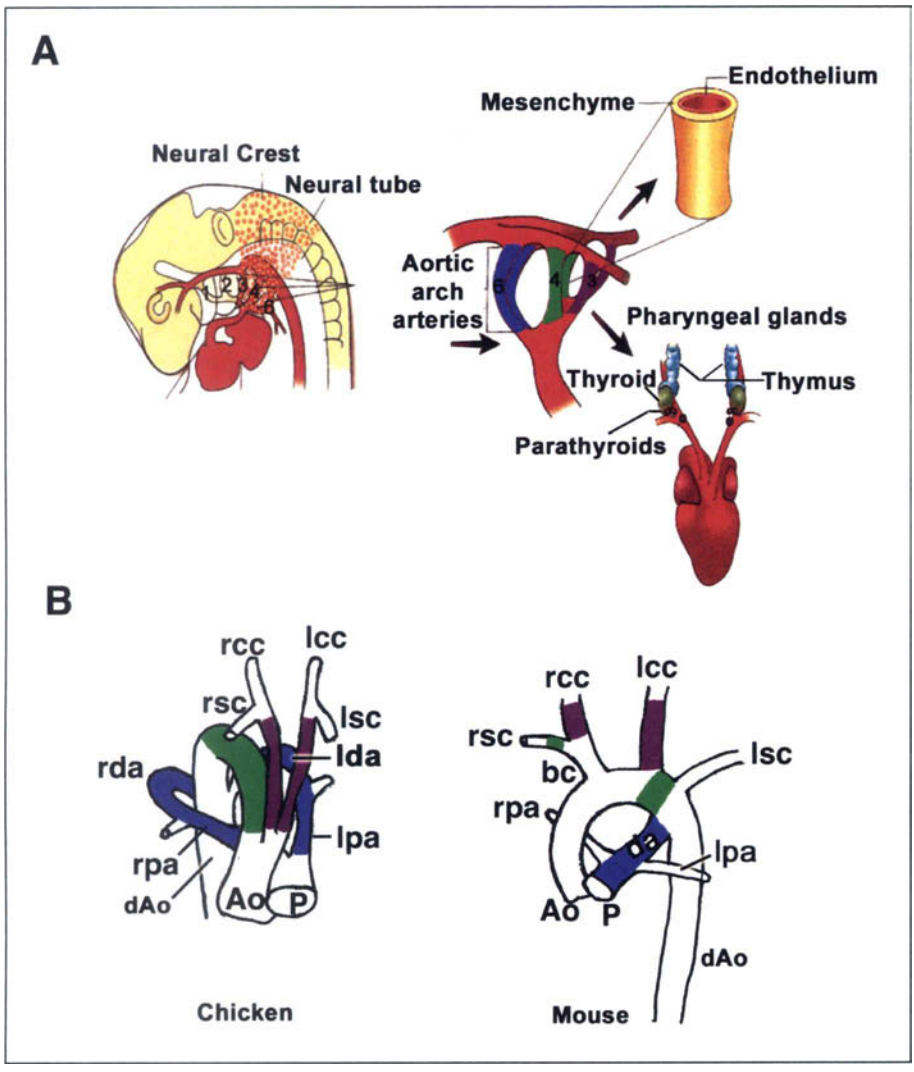

Figure 1. Diagram illustrating the contribution of cardiac neural crest cells to the pharyngeal arch arteries and pharyngeal glands. A) Neural crest form the smooth muscle of the great vessels and connective tissues of the thyroid, parathyroids, and thymus. B) Remodeling of the pharyngeal arches results in a right-sided aortic arch in chickens and a left-sided aortic arch in mouse and human. The third arches in chickens (purple) form the right and left brachiocephalic arteries and form portions of the common carotids in mice. In chickens, left and right ductus arteriosus are formed from the sixth arches (blue). A single ductus arteriosus forms in the mouse (blue). The right fourth arch (green) forms the aortic arch in the chicken, while the left fourth arch forms the transverse segment of the aortic arch in mice. Ao, aorta; P, pulmonary trunk; rpa, right pulmonary artery; lpa, left pulmonary artery; rda, right ductus arteriosus; lda, left ductus arteriosus; da, ductus arteriosus; rsc, right subclavian artery; rcc, right common carotid artery; lcc, left common carotid artery; lsc, left subclavian artery; bc, brachiocephalic artery; dAo, descending aorta. Adapted from: Kirby ML. Contribution of neural crest to heart and vessel morphology. In: Rosenthal RPHN, ed. Heart Development. New York: Academic Press, 1999:179-193;[156] ©1999 with permission from Elsevier.

Identification and Characterization of the Cardiac Neural Crest

Neural crest contribution to the cardiovascular system was first demonstrated in avian models of quail-chicken chimeras.[3,4] Le Douarin and colleagues transplanted the entire rhombencephalon primordium from quail into chicken and observed quail cell contribution to the walls of the brachiocephalic arteries, the carotid arteries, the pulmonary trunk and proximal aorta.[3] Margaret Kirby and colleagues utilized the quail nuclear marker QCPN coupled with small neural tube transplants in quail-chicken chimeras for more detailed analysis of neural crest contribution to the cardiovascular system.[4] These chimera experiments confirmed the results of Le Douarin and localized the cardiac neural crest to the region of the neural tube between the mid-otic placode and somite three (rhombomeres 6-8). Subsequent fate mapping analysis in chicken and mouse have suggested that the caudal boundary of the cardiac neural crest may extend to somite four.[6,7] These cells populate pharyngeal arches 3, 4 and 6 and the outflow tract (Fig. 1A).[8,9] Neural crest entering pharyngeal arches 3 and 4 interact with the endoderm to produce the pharyngeal glands (thymus, and parathyroid glands) (Fig. 1A).[10] This population of neural crest cells also forms the enteric ganglia of the midgut and hindgut (Fig. 2C).[11,12] Thus the term "cardiac neural crest" refers to the unique role of this cell population in cardiovascular patterning and does not imply that these cells are restricted to cardiovascular lineages.

One of the first direct comparative analyses of mouse and chicken neural crest contribution to cardiovascular patterning used a connexin 43 (Cx43) enhancer-*Lac Z* transgene.[13] These experiments demonstrated that mouse cardiac neural crest cells are targeted to the pharyngeal arches, outflow tract and cardiac ganglia consistent with results in the chicken (Fig. 1). Definitive fate mapping of cardiac neural crest was subsequently preformed using the CreLoxP system in transgenic mice. At least four transgenic mouse lines that drive expression of Cre in neural crest cell populations have been reported.[14-17] Three of these lines, *P0*-Cre, *Pax3*-Cre and *Wnt1*-Cre, are active in the dorsal neural tube and allow for lineage tracing of neural crest from the time of emergence into the final mature structures when mated to a *Lac Z* reporter line (R26R).[14-16,18] Human tissue plasminogen activator (*ht-PA*) Cre is not active in the neural tube and labels later migratory and post-migratory neural crest.[17] All four lines demonstrate cardiac neural crest contribution of smooth muscle to the aortic arch, the pulmonary trunk, brachiocephalic artery, the right subclavian artery, the right and left common carotid arteries and the remnant of the ductus arteriosus (ligamentum arteriosum)(Fig. 2A,C-E). The left subclavian artery, pulmonary arteries and descending aorta are not stained in neural crest lineage analysis, reflecting their mesodermal origin (Fig. 2A,E). Fate mapping using a Tbx1-Cre mouse line that labels pharyngeal mesoderm confirms the mesodermal origin of these vessels and demonstrates that the conal portions of the aorta and pulmonary trunk are mesodermally derived (Fig. 2B).[19] The cardiac ganglia and parasympathetic nerve fibers are also labeled as neural crest derivatives (Fig. 2C,D).[15-17] Cardiac neural crest are seen in the leaflets of the aortic and pulmonic valves and the interventricular septum although their role in these tissues is unknown.[15,17] A few fate-mapped cells of unknown function have also been observed in unexpected locations including in the inflow tract, the epicardium, the coronary arteries, the myocardium and in close apposition to the conduction system.[20-23] Although labeling of cells is highly consistent between the Cre lines, the *Wnt1*-Cre mouse line has become the predominant line used for fate mapping and conditional gene inactivation. Some controversy remains over the exact number of cells labeled with each mouse line, and over which mouse line most faithfully labels the true cardiac neural crest niche.

Cardiac Neural Crest Function in Cardiac Development and Remodeling

The first functional heart is a linear tube composed of an endocardial layer and a myocardial layer separated by specialized extracellular matrix. At the heart tube stage, blood enters through a common atria and ventricle, and exits through a common outflow tract (conotruncus). Blood flows through paired pharyngeal arch arteries and into paired dorsal

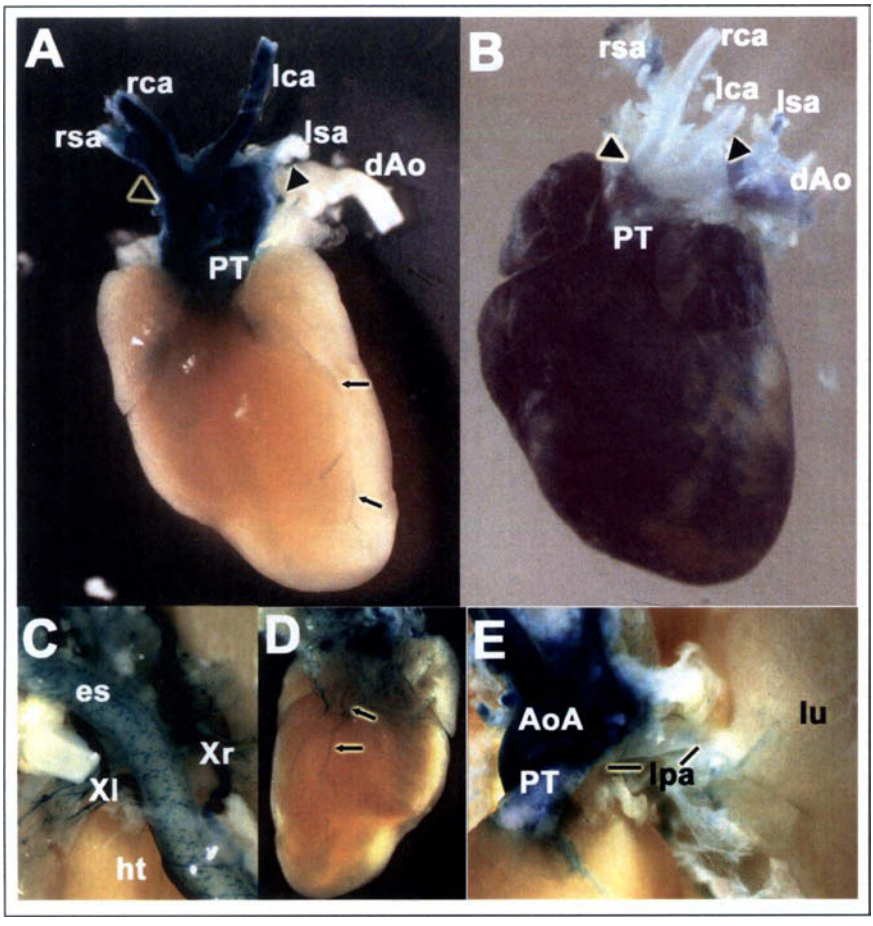

Figure 2. Fate mapping of cardiac neural crest cells with Wnt1-Cre and R26R. A) Ventral view of an X-gal stained neonatal Wnt1-Cre::R26R heart revealing cardiac neural crest derivatives. Note staining in the aortic arch to the level of the left subclavian artery (between black arrowheads). Small arrows denote neuronal staining associated with the coronary arteries. B) Ventral view of an X-gal stained Tbx1-Cre::R26R neonatal heart demonstrating mesodermal contribution to the aortic arch arteries. The proximal pulmonary trunk (PT) and aorta are labeled. The aortic arch is not labeled showing a reciprocal pattern to the neural crest staining (compare region between arrowheads in A, B). The right subclavian artery, left subclavian artery and dorsal aorta (dAo) are labeled using Tbx1-Cre and are not neural crest derived. C) Dorsal view of a Wnt1-Cre::R26R neonatal heart with esophagus and nervous tissue in place. Labeled neural crest contribute to the enteric ganglia in the esophagus, and the left (Xl) and right (Xr) vagal branches. D) Dorsal view of the heart in C with noncardiac tissue removed. Extensive labeling of the neural crest derived cardiac ganglia and nerve tracts is observed (arrows). E) Left lateral view of the Wnt1-Cre::R26R heart from C,D. Neural crest contributes extensively to the aortic arch (AoA). The pulmonary trunk shows lower levels of neural crest contribution with no expression in the conus. The left pulmonary artery (lpa) and lung (lu) show no neural crest contribution.

aorta (Fig. 1A) (for reviews see refs. 24,25). As development proceeds, the conotruncus is split into the pulmonary trunk and aorta while the symmetrical arch arteries are remodeled into the asymmetrical mature forms (for review see refs. 11,26). Ablation of the cardiac neural crest in chickens demonstrated that these cells are required for both septation of the

conotruncus, and for the remodeling of the pharyngeal arch arteries.[5,27] Final separation of the four heart chambers is accomplished by elaboration of atrial and ventricular septa and by the formation of the mitral and tricuspid valves. Chamber formation is not believed to be dependent on the activity of neural crest, although defects may be observed which reflect compensatory changes secondary to defective remodeling of the outflow tract and pharyngeal arch arteries.

Neural Crest Ablation

The functional requirement for cardiac neural crest in normal cardiovascular development was first demonstrated in the chicken embryo by mechanical or laser ablation (Fig. 3).[5,28] Following ablation, no new cells arise from the ablated regions, and these gaps are not repopulated by adjacent neural crest populations. The outflow tract fails to elongate resulting in altered cardiac looping.[29] Cardiovascular defects following ablation include double outlet right ventricle (DORV), Teratology of Fallot, persistent truncus arteriosus (PTA), and anomalous development of the pharyngeal arteries (Fig. 3).[28,30] The severity of

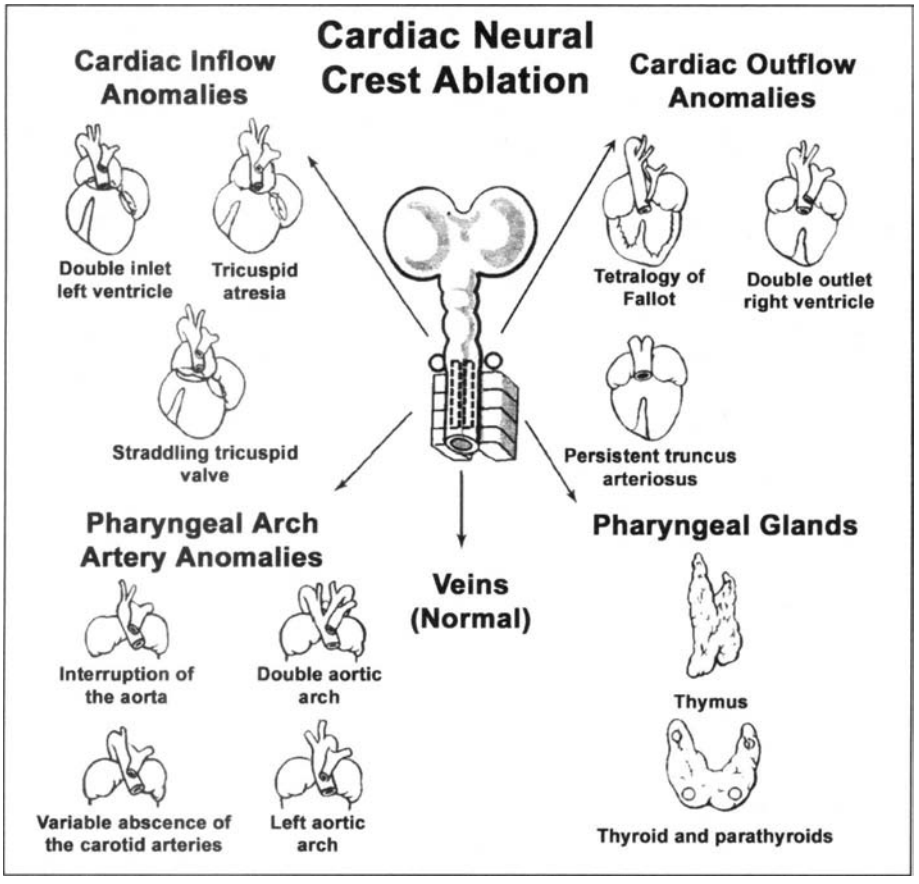

Figure 3. Phenotypic consequences of cardiac neural crest ablation in the chicken. Ablation of the cardiac neural crest causes defective inflow morphology, defective pharyngeal arch patterning, defective outflow septation and alignment defects. Hypoplasia or aplasia of the pharyngeal glands is also noted. Modified from: Kirby ML. Trends Cardiovasc Med 1993; 3(1):18-23;[157] ©1993 with permission from Elsevier.

the defects observed depends on the number of cells ablated.[30] In the absence of neural crest persistence of pharyngeal arch arteries is variable with unpredictable loss or maintenance of individual vessels. These experiments demonstrated that the cardiac neural crest cells are required for persistence rather than formation of the arch arteries.[31] Hypoplasia or aplasia of pharyngeal glands is also seen following ablation (Fig. 3).[10] Deletion of smaller numbers of neural crest cells causes misalignment of the pulmonary trunk and aorta without a septation defect. This suggests an indirect role for neural crest in alignment of the outflow vessels. In addition to defects in patterning, neural crest ablation also causes heart failure.[32-34]

Cardiac Neural Crest Function in Pharyngeal Arch Patterning

The pharyngeal arches are transient "bulges" of mesoderm that arise on the ventral surface of the head flanking the primitive foregut.[35] Each of the pharyngeal arches houses a single artery.[31,36] In chicken, mouse and human five pairs of pharyngeal arch arteries arise in symmetric pairs and connect the common outflow tract to the paired dorsal aortae (Fig. 1A). The arch arteries arise as endothelial tubes. The endothelial cells of the arteries are mesodermally derived and are one of the few cell types not formed by neural crest. Cardiac neural crest form pericytes and smooth muscle in the arteries as they mature.[37,38] During remodeling of the pharyngeal arch system, asymmetric contributions of neural crest cells is thought to be a major determinant of whether a particular vascular component persists or is reabsorbed.[36]

During remodeling, pharyngeal arch arteries 1 and 2 are remodeled into capillary beds.[39,40] Arch arteries three, four and six persist and are remodeled to become components of the mature vasculature (Figs. 1, 4). There are several important differences between birds and mammals in the remodeling of the pharyngeal arches (Fig. 1B).[11,39] In birds remodeling is predominantly right-sided while in the mouse the left side is dominant. In chicken, remodeling occurs between the initiation of circulation at Hamburger and Hamilton stage 12 (45-49 hours) and stage 34 (approximately day 8) (Fig. 5).[39] In birds, the third arch arteries are remodeled into two branchiocephalic arteries (left and right) (Figs. 1B, 4A,D). The left and right common carotid and subclavian arteries originate from the branchiocephalic arteries. The arch of the aorta forms from the right fourth arch while the left fourth arch regresses. Each sixth arch forms a ductus arteriosus (an embryonic vascular shunt that directs circulation away from the developing lungs).[39] It is important to note that while the pulmonary arteries connect to the sixth arch arteries during patterning, they are not "derived" by remodeling from the sixth arch vessels. Thus, pulmonary arteries with ectopic origin are formed in embryos lacking the sixth arch. In the mouse, remodeling occurs between embryonic day 10.5 and 13 (Fig. 5).[40] The right third and fourth arch arteries along with the proximal right dorsal aorta are remodeled to form a single brachiocephalic artery from which originates the right subclavian and right common carotid arteries (Figs. 1B, 4E). The left fourth arch forms the "bridge", or transverse segment, connecting the ascending aorta to the descending aorta while the right fourth arch regresses (Fig. 1B). The left common carotid and left subclavian arteries originate from the aortic arch (Fig. 4B,E). The left sixth arch forms a single ductus arteriosus while the right sixth arch regresses.[40] In both mammals and birds, the ductus arteriosus closes in the early neonatal period in response to a sudden increase in oxygen concentration.

Cardiac Neural Crest in Outflow Tract Septation and Alignment

In addition to mediating pharyngeal arch remodeling, cardiac neural crest cells are also required to separate the outflow tract into the pulmonary trunk and aorta (Figs. 5, 6).[4,5,13,27,41] While it is universally accepted that cardiac neural crest cells play a fundamental role in this process and provide material contribution of smooth muscle to the aorta and pulmonary trunk, the specifics of septation are a matter of controversy. In embryological terms, the conotruncus

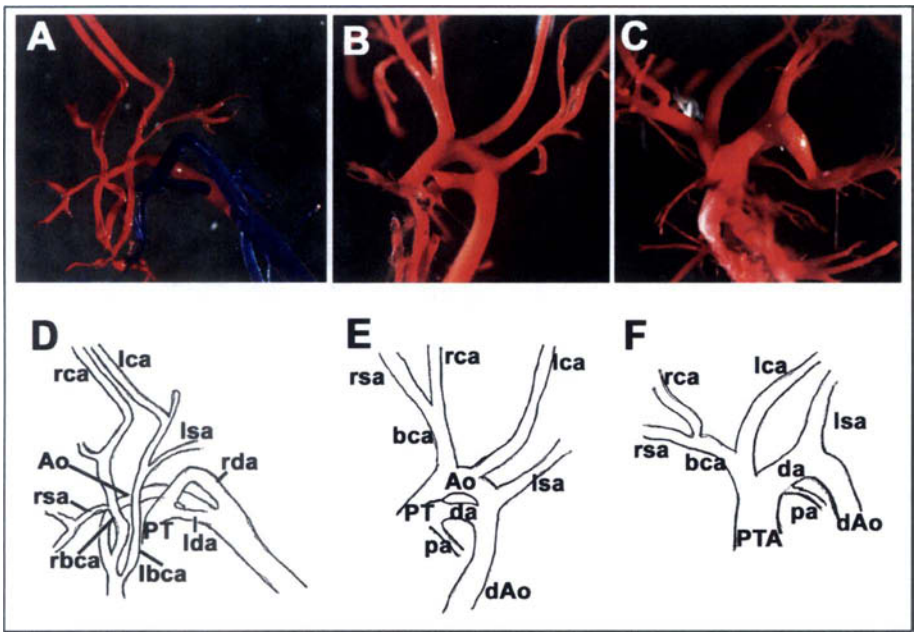

Figure 4. Acrylic resin cast analysis of chicken and mouse pharyngeal arch arteries. A,D) Acrylic resin cast of the pharyngeal arch arteries from a 10 day in ovo chicken embryo. Red acrylic resin was injected into the left ventricle and blue resin in the right ventricle. The pulmonary trunk (PT) has become dissociated from the ascending aorta during processing and is shifted up artificially in this image. D) Labeled sketch of the arteries shown in A. Note that the first branch from the ascending aorta(Ao) is the left brachiocephalic artery (lbca) followed by the right brachiocephalic artery (rbca). The carotid and subclavian arteries branch from the brachiocephalic arteries. There is both a left (lda) and right (rda) ductus arteriosus in chickens. B,E) Single color resin injection demonstrating normal pharyngeal arch structure in a neonatal mouse. E.) The pulmonary trunk (PT) arises anterior to the aorta (Ao) and here is backfilled through the single left sided ductus arteriosus (da). The first branch from the aortic arch is the single brachiocephalic artery (bca). The right subclavian (rsa) and right common carotid (rca) branch from the brachiocephalic. The left common carotid (lca) and left subclavian (lsa) arise directly from the aortic arch as independent branches. The pulmonary arteries (pa) originate from the pulmonary trunk. C,F) Abnormal arch patterning in a Semaphorin3C null embryo. F) This embryo has persistent truncus arteriosus (PTA) and an interrupted aortic arch type B between the left common carotid (lca) and left subclavian (lsa) arteries. The ductus arteriosus is greatly distended and serves as the vascular channel for systemic circulation in this embryo. Pulmonary arteries (pa) arise from the ductus arteriosus.

is generally synonymous with "outflow tract". The conus refers to the proximal portion below the level of the valves while the truncus refers to the region above the valves and below the aortic sac (Fig. 6A). The aortic sac is the nonmuscularized connection between the conotruncus and the arch arteries.[41] Septation involves a twisting or "spiraling" of the outflow tract, fusion of the endocardial cushions and significant investiture of the cushions with cardiac neural crest.[27,41-43] One popular model argues that the neural crest form an aorticopulmonary septum in the roof of the aortic sac that connects to prongs of neural crest that invade the truncal cushions (Fig. 6).[13,27] In this model the aorticopulmonary septum processes down the truncus separating the aorta and pulmonary artery. Other investigators argue that there is no aorticopulmonary septation complex and that separation of the vessels is achieved through cushion fusion and neural crest mediated formation of the facing walls of the aorta and pulmonary artery.[41] The conus region below the level of the valves is also extensively remodeled, and

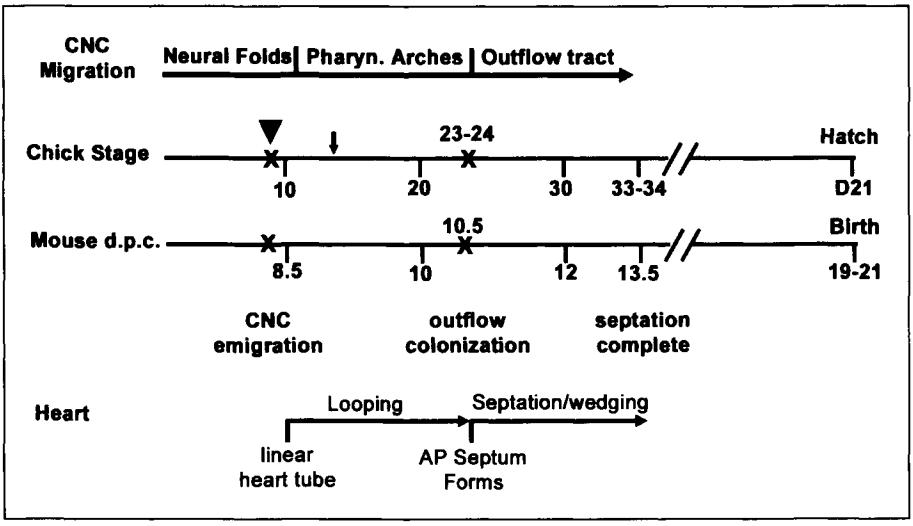

Figure 5. Timeline of cardiac neural crest migration in the chicken and mouse. Timelines for cardiac neural crest migration are presented indicating neural crest position and cardiovascular patterning events relative to developmental age. The black arrowhead on the chick timeline indicates the timing of neural crest ablation at Hamburger and Hamilton stage 9-10. The black arrow indicates the onset of detectable myocardial dysfunction at H&H stage 13-14. Cardiac neural crest do not contact primary myocardium until H&H stage 23-24. Cardiac neural crest cells colonize the mouse outflow tract around E10.5 and septation is completed by E13.5.

is likely to be involved in an additional contribution of myocardial cells (myocardialization) from the inner curvature of the heart.[44,45] Remodeling of the conus causes a change in position of the aorta and pulmonary trunk resulting in a shift from side by side alignment to the correct anterior-posterior positioning. The process of rearrangement and valve placement is called aortic wedging (Fig. 6B).[26,27] The aortic valve comes into fibrous continuity with the mitral and tricuspid valves, whereas the pulmonary valve is elevated by a band of muscle. Defective remodeling of the conus can produce malalignment in the presence of a fully septated truncus indicating that alignment and septation are independent processes.

Myocardial Dysfunction, Elongation, Alignment and the Secondary Heart Field

Myocardial dysfunction is observed in cardiac neural crest ablated chicken embryos. Following neural crest ablation, the heart has reduced ejection fraction and ventricular dilation accompanied by reduced calcium current and dysregulated excitation-contraction coupling. Similar myocardial dysfunction has been observed in the *Splotch mouse*.[46,47] Myocardial dysfunction is too early to be explained by direct neural crest interaction with myocardium in the heart tube (Fig. 5). Another unexplained outcome of neural crest ablation is the failure of outflow tract elongation that results in altered looping. Classic embryological experiments in the chicken embryo demonstrated that outflow tract elongation occurs through addition of cells from outside of the heart tube.[48] The issue of outflow tract elongation has recently been revisited using a variety of molecular markers. These experiments demonstrated that elongation is due to cell addition from a population of anterior pharyngeal splanchnic mesoderm (Fig. 7).[49-53] In the chicken this mesoderm population gives rise to the conotruncus and is called the secondary heart field.[51] In the mouse a similar region of pharyngeal mesoderm gives rise to the conotruncus and right ventricle and has been called the anterior heart-forming

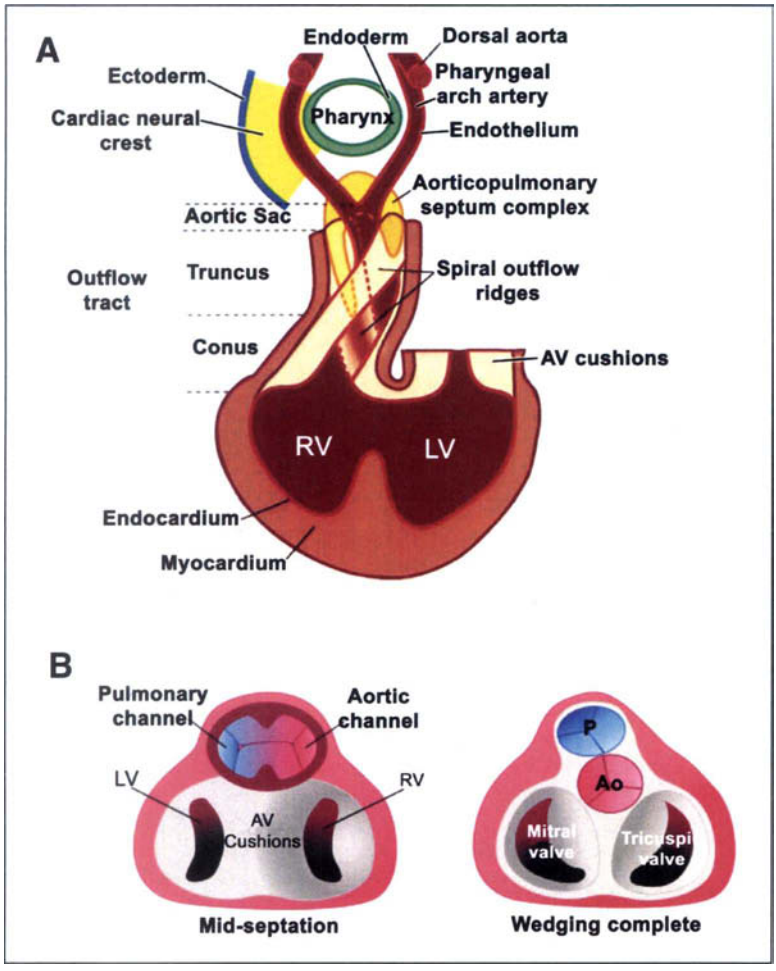

Figure 6. Diagram of neural crest mediated septation of the outflow tract and aortic valve wedging. A) Illustration of the popular model of outflow tract septation involving an aorticopulmonary septation complex and spiraling ridges of conotruncal cushions. B) Diagram of aortic valve wedging. The aortic valve is wedged between the mitral and tricuspid valves and the valves are in fibrous continuity with no intervening myocardium (pink). Panel A is from: Waldo KL et al. Dev Biol 1999; 208(2):307-323;[13] 1999 with permission from Elsevier. Panel B is from: Hutson MR, Kirby ML. Birth Defects Res C Embryo Today 2003; 69(1):2-13;[26] ©2003 with permission from Wiley-Liss, Inc., a subsidiar of John Wiley & Sons, Inc.

field[49,50] (reviewed in ref. 52). Loss of early reciprocal signaling between the cardiac neural crest and secondary heart field mesoderm in the pharynx presumably explains the myocardial dysfunction phenotype in neural crest deficient embryos, and this signaling in chickens appears to involve fibroblast growth factors.[33] Outflow tract elongation is deficient in ablated embryos due to lack of cell addition from the secondary heart field. It has also been proposed that continuous addition of cells to the truncus provides a mechanical force important for wedging.[29,54] Neural crest thus affects both elongation and septation of the outflow tract as well as alignment of the great vessels by influencing the elaboration or differentiation of cells from the secondary/anterior heart field.

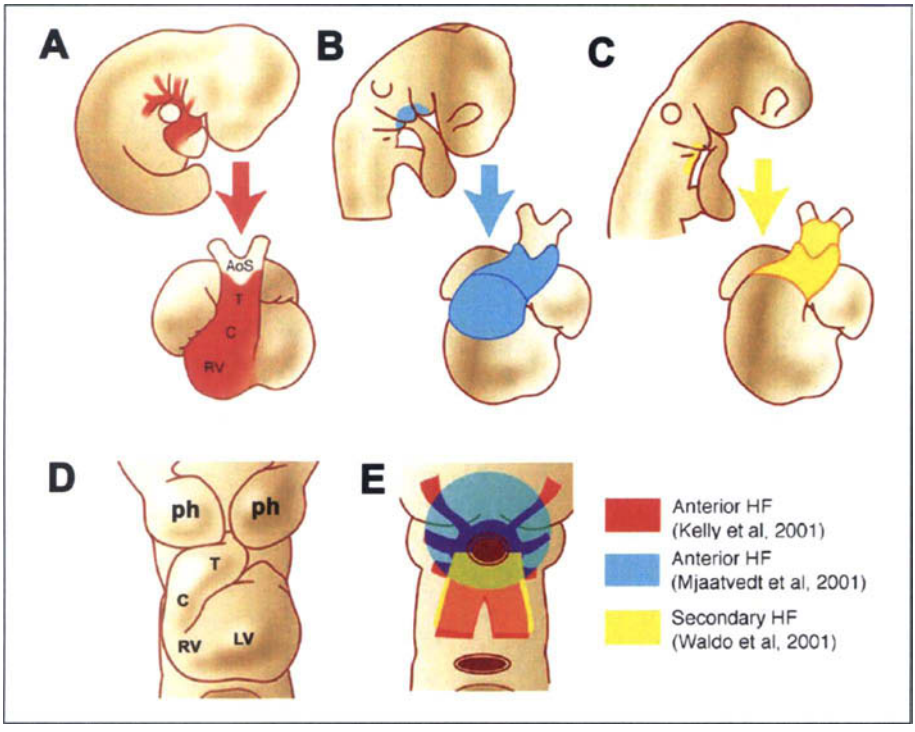

Figure 7. Comparison of the extent, location and contribution of cells from the anterior or secondary heart field to the outflow tract and right ventricle. A) Lateral view of an E9.5 embryo and ventral view of an E11.5 mouse heart demonstrating the origin and contribution of the "anterior heart field" (red) to the conotruncus and right ventricle as described by Kelly et al using an Fgf10-lacZ mouse line.[50] B) Lateral view of a chick stage 16 embryo and ventral view of a stage 22 heart demonstrating the origin and contribution of the "anterior heart field"(blue) mesoderm to the distal conus and truncus as defined by Mjaatvedt et al.[53] C) Lateral view of the "secondary heart field" (yellow) as described by Waldo et al. at stage 14 in the chick and limited contribution of secondary heart field mesoderm to the distal truncus myocardium of the heart at stage 22.[51] D) Ventral view of anE9.5 mouse (comparable to stage 12 chicken) demonstrating relationship of the heart and pharyngeal arches (ph). E) Ventral view of the embryo in D with the heart removed demonstrating the overlap in described secondary or anterior heart field pharyngeal mesoderm. Red ovals are sites of heart connection to the vasculature at the arterial (upper oval) and venous (lower flat oval) poles. Abbreviations: Aos,aortic sac; T,truncus; c, conus; RV,right ventricle; LV, left ventricle. Modified from: Abu-Issa R et al. Dev Biol 2004; 272(2):281-285;[52] ©2004 with permission from Elsevier.

Molecular Pathways

Numerous signaling pathways and individual genes have been implicated in cardiac neural crest induction, migration and differentiation following the observation of outflow tract or pharyngeal arch artery malformations in homozygous null mouse embryos. Analysis of the regulation of gene function and signaling pathways in the cardiac neural crest is complicated by the complex cellular interactions associated with normal cardiac morphogenesis. Defective development, or disruption of signaling pathways in any of these cell populations can result in cardiovascular abnormalities consistent with disrupted cardiac neural crest. The use of tissue specific-Cre mouse lines for conditional gene inactivation in the mouse is beginning to unravel the cell-specific gene requirements during neural crest mediated cardiovascular remodeling. Here we focus on a subset of genes that have known impact on cardiac neural crest mediated cardiovascular remodeling.

Mouse Model of Cardiac Neural Crest Deficiency

The *Splotch* mouse is the most extensively studied mouse model of cardiac neural crest dysfunction. The first *Splotch* allele was identified in 1954 and is named for the white belly spot apparent in heterozygous mice.[55,56] Homozygous *Splotch* mice die in utero by day 14 and resemble the chick neural crest ablation phenotype of persistent truncus arteriosus, pharyngeal arch patterning defects and hypoplasia or aplasia of the thymus and parathyroid glands.[56-58] The exact defect in cardiac neural crest in *Splotch* embryos is controversial. *Splotch* alleles represent mutations in the *Pax3* gene, a member of the paired box family of transcription factors.[59-61] *Pax3* is known to regulate cell migration through transcriptional regulation of the scatter factor receptor *c-met*.[62] Transgenic expression of *Pax3* in neural crest cells in *Splotch* null embryos rescues the cardiovascular defects arguing for a cell autonomous function.[63] Cardiac neural crest cells are formed and colonize the pharyngeal arches and conotruncal cushions in *Splotch* null embryos, albeit in reduced number and lacking proper positional identity arguing against a primary migration defect.[58] However, several investigators have suggested a delay in cardiac neural crest emigration from the neural tube that may be consistent with a role of *Pax3* in regulating factors required for migration.[6,57] It has also been proposed that cardiac neural crest cell numbers in *Splotch* may be decreased due to a failure of expansion of the neural crest stem cell population. Inappropriate or precocious differentiation would result in a decrease in the total number of neural crest cells.[64] This role in stem cell regulation would be consistent with the recent observation that *Pax3* in melanocyte stem cells plays a dual role in initiating lineage restriction and maintenance of the lineage restricted stem cell population.[65] A similar role is played by the related paired box gene *Pax7* in skeletal muscle stem cells.[66,67] The relative importance of *Pax3* in mediating lineage restriction and maintenance of the cardiac neural crest stem cell niche to the observed *Splotch* phenotypes remains to be determined.

Neurotrophins

The neurotrophins (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT4/5) are a family of growth factors that regulate neural survival and differentiation.[68] Neurotrophins signal through the Trk class of tyrosine kinase receptors.[68,69] Neurotrophin-3 (*NT-3*) and its receptor *trkC* are expressed in a subset of neural crest, in the neural crest-derived subendothelial mesenchyme of the aorta and pulmonary trunk, and in the neurons of the cardiac ganglia.[70-72] *NT-3* is also expressed by some endothelial cells.[73] Knockout of the *NT-3*[72] or it's receptor *trkC*[74] results in cardiovascular defects including atrial and ventricular septal defects, abnormal valves and conotruncal defects including persistent truncus arteriosus and Tetralogy of Fallot at low penetrance. Neurotrophins have been proposed to function in maintenance and lineage restriction of the neural crest stem cell niche. Youn and colleagues analyzed *trkC* null cardiac neural crest cells in explant culture.[73] Three types of neural crest stem cells were identified in explant culture. Cardiac neural crest stem cells (CNC-SC) were able to self renew and could assume any terminal fate including neurons, Schwann cells, pigment cells, chondrocytes and smooth muscle. Restricted cardiac neural crest cells (CNC-RC) form mostly smooth muscle cells, and do not form pigment cells or neurons. Smooth muscle stem cells (CNC-SmC) were committed to the smooth muscle lineage with little proliferative capacity.[73] Explants from *trkC* null embryos contained more lineage-restricted stem cells (CNC-RC) and reduced levels of uncommitted neural stem cells (CNC-SC). The decrease in uncommitted stem cell numbers in *trkC* null embryos suggests that neurotrophins function to retain pluripotency in the cardiac neural crest stem cell niche.

Forkhead Transcription Factors FoxC1/FoxC2

Foxc1/Mf1 and *Foxc2/Mfh1* are closely related forkhead/winged helix transcription factors.[75,76] Both genes are expressed in head mesoderm, pharyngeal arch mesenchyme and endothelial

cells and *Foxc1* is expressed in cardiac neural crest. Both *Foxc1* and *Foxc2* nulls exhibit cardiovascular defects including coarctation and interruption of the aortic arch and ventricular septal defects.[76,77] *Foxc1/Foxc2* compound heterozygote and homozygote embryos display more severe cardiovascular defects indicating that these genes compensate for one another and that function is dose dependant.[78] Migration of cardiac neural crest cells and expression of neural crest markers appears normal in *Foxc1* and *Foxc2* nulls suggesting that the cardiovascular defects observed are not due to a cell-autonomous neural crest defect.[76] *Foxc1* and *Foxc2* can regulate expression of the T-box transcription factor *Tbx1* in tissues where they are coexpressed.[79] *Tbx1* is a transcription factor implicated in the etiology of DiGeorge syndrome, the most common congenital heart syndrome in humans.[80,81] *Tbx1* has an important non cell-autonomous role in regulating cardiac neural crest maintenance and differentiation through a signaling cascade involving Fibroblast growth factor(Fgf) ligands.

Tbx1, Fibroblast Growth Factors and DiGeorge Syndrome

DiGeorge syndrome, velocardiofacial syndrome, and conotruncal anomaly face syndrome (DiGeorge spectrum disorders) are the most common human congenital cardiovascular disorders affecting as many as 1 in 4000 births. The majority of patients with these syndromes have chromosome 22q11 deletions.[82-86] These patients exhibit an incompletely penetrant phenotype including hypoplasia of the thymus and parathyroid, craniofacial and skeletal abnormalities, cardiac abnormalities, and speech and learning disabilities. Common heart defects include interrupted aortic arch type B, persistent truncus arteriosus, tetralogy of Fallot, tetralogy of Fallot with pulmonary atresia, and posterior malalignment ventricular septal defect.[87] These defects are consistent with neural crest dysfunction, and the DiGeorge spectrum disorders have long been considered "neurocristopathies". Mouse models of DiGeorge syndrome have been developed by deletions on mouse chromosome 16 in regions syntenic to human chromosome 22 (the DiGeorge critical region)[88,89] and these models helped to identify the transcription factor TBOX1 (TBX1) as a candidate gene.

Tbx1 is one of the genes contained within the DiGeorge critical region in humans and mice.[90] Targeted inactivation of *Tbx1* results in cardiac defects (similar to those seen in patients with 22q11 deletions) in mice heterozygous or homozygous for the mutations.[88,89,91] Recently, three independent cases of TBX1 mutations in human patients with conotruncal anomaly face syndrome were reported[92] confirming that TBX1 is a major genetic determinant in the DiGeorge spectrum disorders. Tbx1 is expressed in pharyngeal mesoderm and endoderm but not in neural crest cells indicating that the neural crest defects are non cell-autonomous.[93] Tbx1 is also expressed in precursors of the secondary heart field suggesting a cell autonomous role in conotruncal patterning.[94]

Fibroblast growth factor (*Fgf8* and *Fgf10*) signaling has been reported as a critical mediator of aortic arch development and conotruncal septation downstream of *Tbx1*.[95-97] *Fgf8* and *Fgf10* expression is down regulated in *Tbx1* nulls, and *Fgf8* hypomorphic embryos exhibit cardiovascular defects reminiscent of *Tbx1* nulls. Neural crest cells migrate normally in *Fgf8* hypomorphs, but there are increased levels of apoptosis of neural crest cells within the pharyngeal arches.[95,96] This suggests that *Fgf8* expression in the pharyngeal arches is required for neural crest cell survival. Tissue specific inactivation of *Fgf8* in the ectoderm results in defective pharyngeal arch artery patterning.[98] Deletion of *Fgf8* in the *Tbx1* expression domain results in defects of conotruncal septation and malpositioning of the proximal great vessels.[94] Thus, *Fgf8* is required both for neural crest survival/differentiation and in the secondary heart field. Hu et al recently identified an *Fgf8* enhancer that is dependent on *Tbx1* in vivo for regulating expression specifically in the cardiac outflow tract, but were unable to show direct transcriptional activation by *Tbx1*.[99] Direct transcriptional regulation of *Fgf10* by *Tbx1* has recently been demonstrated.[100] Determination of the relative roles of *Fgf8* and *Fgf10* in cardiovascular patterning is an area of active research.

Retinoic Acid

It has long been known that too much or too little Vitamin A, or the biologically active form retinoic acid, causes neural crest dependent cardiovascular defects.[101-103] Retinoic acid(RA) is synthesized from retinol by the action of retinol and retinal dehydrogenases. In mouse the enzyme retinaldehyde dehydrogenase 2 (RALDH-2) is apparently the primary rate-limiting enzyme for RA synthesis.[104] Deletion of RALDH-2 recapitulates the full spectrum of RA deficiencies.[105] RA signals through heterodimers of retinoic acid receptors (RAR α, β and γ) and retinoid X receptors (RXR α, β and γ).[106] RARs can be activated by RA or 9-cis RA[107] whereas only 9-cis RA efficiently activates RXRs.[108] Further complexity is generated through different receptor splice isoforms. RA also binds to cellular retinol binding proteins I and II and cellular retinoic acid binding proteins I and II and this binding may regulate RA signaling by decreasing free RA in the cell.[109]

Stereotypical retinoic acid deficient phenotypes are only observed in compound RAR (α1β2, αβ2, αγ) nulls indicating functional receptor redundancy.[109] The cardiovascular defects observed following retinoic acid exposure or compound receptor knockout include persistent truncus arteriosus, interrupted aortic arch and double outlet right ventricle. These defects are consistent with defects in the cardiac neural crest.[109] Neural crest fate mapping analysis in *RARα1/RARβ* compound null embryos demonstrated normal migration and differentiation of cardiac neural crest in animals exhibiting persistent truncus arteriosus.[110] Wnt1-Cre mediated tissue specific RAR deletion does not result in cardiovascular defects suggesting that RA effects on the neural crest are non cell-autonomous.[110] Cardiovascular defects may arise due to altered signaling between the cardiac neural crest and a retinoic acid responsive neighboring cell population. RA regulation of Fibroblast growth factor ligand expression may explain the observed cardiovascular defects. *Fgf8* is expressed in the pharyngeal ectoderm and endoderm and is required for maintenance or differentiation of both neural crest and secondary heart field cells. Retinoic acid has been shown to directly activate expression from an *Fgf8* genomic enhancer in vitro[111] and to induce *Fgf10* expression in vivo.[112] Both Fgfs are proposed to have a non cell-autonomous role in regulating neural crest function in the pharyngeal arches and conotruncus.

Cell-Cell Contact (Connexins)

Connexin 43, also known as alpha1 connexin, is a gap junction protein expressed in cardiac neural crest.[113] Gap junctions are membrane channels that allow passage of low molecular weight molecules and ions between cells. Dye coupling experiments have shown that cardiac neural crest cells maintain inter-cell continuity while migrating. Cx43 is involved in cardiac neural crest migration. Both increase and decrease of Cx43 levels in cardiac neural crest result in outflow patterning defects.[114-116] Deletion of Cx43 results in outflow tract obstruction and conotruncal defects.[114] Loss of Cx43 expression or expression of a dominant negative form of Cx43 in cardiac neural crest results in decreased migration and decreased cardiac neural crest in the outflow tract.[114,115] Overexpression of Cx43 causes the opposite effect with increased cell motility and more cells in the outflow tract.[116] These results suggest a primary role for Cx43 in regulating neural crest cell motility. In addition to neural crest, Cx43 is also expressed in the pro-epicardium and is required for normal formation of the coronary arteries. Cx43 deficient proepicardial cells display increased proliferation and decreased migration rates in culture.[117]

Platelet Derived Growth Factor (PDGF)

Platelet-derived growth factors are broadly expressed growth factors that have been implicated in regulation of cell migration, survival and proliferation.[118] Interest in PDGF stems from the observation that the PDGF α receptor (*PDGFRα*) is deleted in the *Patch* mouse *Patch* (Ph).[119] Heterozygous *Patch* mice exhibit defective melanocyte migration causing a white belly spot. *Patch* homozygotes exhibit PTA, interrupted aortic arch, decreased thymus and other defects associated with deficient cardiac neural crest.[120] Homozygous null *PDGFRα* mice phenocopy *Patch*.[121]

While the *Patch* deletion also encompasses some enhancers for the c-kit gene, similarity in phenotype suggests that loss of *PDGFRα* is the primary defect. Tissue specific inactivation of *PDGFRα* in neural crest causes PTA and abnormal patterning of the right subclavian artery.[122] However, the exact role of PDGF signaling in neural crest is unclear. There are no obvious defects in migration, proliferation or survival of cardiac neural crest in conditional null animals and patterning defects are seen in only slightly more than 50% of conditional null embryos.

Endothelin

Endothelins (ET) are a family of small signaling peptides (*ET-1, ET-2, ET-3*).[123] The active forms of Endothelin are generated from large precursor proteins through the activity of endothelin converting enzyme-1 and-2 (*ECE-1* and *ECE-2*).[124] Endothelins signal through two G-protein coupled receptors named *ET-A* and *ET-B. ET-A* binds *ET-1* and *ET-2* but not *ET-3*.[125,126] *ET-A* and its receptor *ET-1* are expressed in complimentary patterns. In the mouse, *ET-1* is expressed in the endothelium of pharyngeal arch vessels and *ET-A* is expressed on migratory neural crest and in neural crest derived mesenchyme of the pharyngeal arches.[127] Mice lacking the ligand *ET-1*, the receptor *ET-A*, or the converting enzyme *ECE-1* have defective pharyngeal arch and conotruncal patterning. The converting enzyme *ECE-1* is expressed in both endothelium and mesenchyme in the arches.[124,127] The most common malformations in *ECE-1* and *ET-A* null embryos are type B aortic arch interruption and absent right subclavian artery. Outflow tract defects include overriding aorta, double outlet right ventricle and rare cases of PTA. Most embryos also have a peri-membraneous ventricular septal defect. Cardiac neural crest appear to migrate normally in *ECE-1* and *ET-A* null embryos and cardiovascular defects appear to result from deficient paracrine signaling between pharyngeal arch endothelial cells and neural crest derived mesenchyme.[127]

TGFβ Superfamily Members

Bone morphogenetic proteins (BMP) and transforming growth factor beta (TGFβ) are members of the transforming growth factor beta superfamily of signaling molecules and are important mediators of embryogenesis.[128,129] TGFβ family ligands signal through heteromeric serine-threonine kinase receptor complexes of a Type II receptor (*TBR2*) and a Type I receptor (activin-like kinase 5 (*Alk5*), also known as *TBR1*).[130,131] BMPs similarly utilize a single BMP type 2 receptor (*Bmpr2*) but multiple type I receptors (*Alks*) to transduce signals from different ligands.[132] One type I receptor, *Alk2* appears to be utilized by both TGFβ and BMP in certain cell types.[133,134]

There are three Tgfβ ligands (Tgfβ1, Tgfβ2, and Tgfβ3). Tgfβ1 null embryos die in early gestation from a defect in yolk sac vasculogenesis.[135] Tgfβ3 knockouts die shortly after birth and display cleft palate.[136] Knockout of the ligand TGFβ2 results in cardiovascular defects including DORV, short or absent brachiocephalic artery, and retroesophageal right subclavian artery.[137] Both Type II TGFβ (*TBR2*) receptor and Type I TGFβ (TBR1) receptor null mice die before E10 from defective vascularization of the yolk sac and placenta.[138,139] Wnt-Cre mediated deletion of *TBR2* in neural crest cells results in PTA, ventricular septal defects, pharyngeal artery remodeling defects as well as defects in the thymus, parathyroids and craniofacial structures.[140,141] Neural crest migration and survival appear normal in conditional TBR2 nulls but neural crest fail to form smooth muscle. Traditional knockout of the bi-functional *Alk2* receptor results in embryonic lethality at gastrulation. Wnt1-Cre mediated deletion of *Alk2* in the neural crest results in PTA and abnormal pharyngeal arch maturation with right ventricular hypertrophy.[142] Cardiac neural crest migration is decreased and smooth muscle differentiation of neural crest is impaired in *Alk2* conditional nulls suggesting that *Alk2* may be the functional Type I Tgfβ receptor in cardiac neural crest.[142]

BMP signaling is known to play a role in induction and maintenance of the neural crest. Double knockout of *Bmp6* and *Bmp7* leads to cardiac outflow tract, valve and septal defects, and expression of a hypomorphic *Bmpr2* causes an interrupted aortic arch with an unusual

subvalvular PTA.[143] Neural crest specific deletion of the BMP receptor IA (*Bmpr1a* also called *Alk3*) causes shortened cardiac outflow tract, defective septation and acute heart failure with reduced proliferation of the myocardium.[22] These defects are consistent with the previously described indirect effect of reciprocal signaling from the cardiac neural crest to the secondary heart field and not a primary defect in neural crest differentiation. Nkx2.5Cre deletion of BMP4 (a *Bmpr1a* ligand) in the caudal pharyngeal arches, splanchnic mesoderm and truncus results in defective septation, aortic arch interruptions, abnormal arch artery remodeling with decreased smooth muscle recruitment, decreased myocardial differentiation in the truncus, and hypoplastic conotruncal cushions.[144] These defects imply a multi-tissue role for BMP4 in cardiac neural crest, secondary heart field and cushion tissues. BMP7 expression in the conditional BMP4 mutant embryos may prevent more severe conotruncal defects since BMP7 null embryos with reduced levels of BMP4 have a shortened outflow tract consistent with a BMP requirement in the secondary heart field.[144]

Semaphorins

Semaphorins were originally identified as neural pathfinding molecules providing predominantly repulsive axon guidance cues.[145] It has subsequently been appreciated that semaphorin signaling can be attractive or repulsive depending on the cell type and environmental context.[146] Class 3 semaphorins are secreted ligands known to signal through a heteromeric complex of class A plexins and either neuropilin-1 (*npn-1*) or neuropilin-2 (*npn-2*).[147] Semaphorin 3C (Sema3C) null embryos die at birth of interrupted aortic arch and PTA (Fig. 4C).[148] *Sema3C* is expressed in the conotruncal myocardium and pharyngeal arch mesenchyme at E10.5. The Semaphorin receptor *PlexinA2* is expressed in cardiac neural crest suggesting a role for semaphorin signaling in guidance during migration.[94] Decreased levels of cardiac neural crest are observed in the conotruncal cushions of *Sema3C* nulls, consistent with a defect in neural crest cell migration. However, normal levels of neural crest were observed in the pharyngeal arches in *Sema3C* nulls indicating that the *Sema3C* phenotype was not due to a global defect in neural crest homing or migration. Recent experiments suggest an additional role for class 3 semaphorin signaling through plexin D1 in endothelial cells of the pharyngeal arch arteries.[149,150]

Pitx2 and Laterality

One of the enduring mysteries of pharyngeal arch patterning is how stereotyped left sided (mouse) or right-sided (chicken) asymmetry is achieved.[151] The bicoid-related homeodomain transcription factor Pitx2 plays a critical role in directing asymmetric cardiovascular remodeling (for review see ref. 152). Three isoforms of Pitx2 are produced by alternate splicing and alternate promoter use but only the Pitx2c isoform is expressed asymmetrically in the developing heart.[153] In the cardiac crescent stage Pitx2c is expressed only in the left heart field. At the linear and looped heart stages Pitx2c retains left sided expression in the entire heart tube and extending into the body wall at both the arterial and venous poles. Between E9.5 and E10.5 Pitx2c is asymmetrically expressed in the left pharyngeal arch mesoderm, splanchnic mesoderm and outflow tract myocardium. This left-sided expression suggests a role for the Pitx2c isoform in asymmetric pharyngeal arch patterning.[153]

Pitx2 expression is regulated by a wnt signaling pathway involving disheveled 2 (*Dvl2*) and β-catenin.[154,155] The global knockout of all three Pitx2 isoforms (Pitx2abc) causes right atrial isomerism (RAI), PTA, DORV, and atrial and ventricular septal defects.[153] Knockout of Dvl2 or Wnt1-Cre mediated deletion of β-catenin results in loss of expression of all Pitx2 isoforms.[154] Global loss of Pitx2 expression results in decreased numbers of cardiac neural crest cells due to an arrest in proliferation.[154,155] Thus, Pitx2 has a cell autonomous effect in regulating cardiac neural crest proliferation.

Pitx2c null embryos exhibit most of the cardiovascular phenotype of the Pitx2abc null, but do not exhibit PTA. In addition, Pitx2c nulls display pharyngeal arch patterning anomalies seen only with this knockout.[153] Null Pitx2c mice display patterning defects including right-sided

aorta and double aortic arch, although apparently normal levels of cardiac neural crest are observed in the pharyngeal arches and outflow tract. These mutations are believed to result from a loss of reciprocal signaling between the cardiac neural crest expressing all three Pitx2 isoforms and pharyngeal mesenchyme expressing only Pitx2c.[153] The laterality pathway downstream of Pitx2 is unknown, however the asymmetric expression of Semaphorin 3C in the conotruncus is mediated by Pitx2c.[153]

Summary

Our understanding of neural crest contribution to cardiovascular development has increased greatly since the early observations of quail cells in the great vessels of chimeric chicken embryos. The primary challenges now facing the field involve deciphering the complex reciprocal signaling events between the cardiac neural crest and the myriad cell populations with which they interact, and in deciphering the pathway relationships between the ever expanding list of genes with cardiac neural crest associated phenotypes. There exists a surprisingly large number of knockout mice with "cardiac neural crest defects" in which there is no demonstrable defect in migration, survival or differentiation of the neural crest. The defects in these mice must lie in either poorly understood tissue layer interactions, or in as of yet undiscovered aspects of neural crest biology. The generation of new tissue specific Cre-recombinase mouse lines and conditional alleles will be critical for the careful molecular dissection of tissue specific gene function during cardiovascular patterning. The recent realization of the importance of reciprocal signaling between neural crest and the secondary/anterior heart field demonstrates that many important aspects of cardiac neural crest biology remain to be elucidated.

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CHAPTER 9

The Genetic Regulation of Pigment Cell Development

Debra L. Silver, Ling Hou and William J. Pavan*

Abstract

Pigment cells in developing vertebrates are derived from a transient and pluripotent population of cells called neural crest. The neural crest delaminates from the developing neural tube and overlying ectoderm early in development. The pigment cells are the only derivative to migrate along the dorso-lateral pathway. As they migrate, the precursor pigment cell population differentiates and expands through proliferation and pro-survival processes, ultimately contributing to the coloration of organisms. The types of pigment cells that develop, timing of these processes, and final destination can vary between organisms. Studies from mice, chick, *Xenopus*, zebrafish, and medaka have led to the identification of many genes that regulate pigment cell development. These include several classes of proteins: transcription factors, transmembrane receptors, and extracellular ligands. This chapter discusses an overview of pigment cell development and the genes that regulate this important process.

Introduction

The pigment cell is one of the most well characterized derivatives of neural crest. Early in development, the pluripotent neural crest population develops from the border between the neural tube and overlying ectoderm. The neural crest then migrates along two paths relative to the neural tube. The cells that migrate dorso-laterally become the pigment cells while those that migrate ventrally become various cell types including the entire peripheral and enteric nervous system. Variations in pigment cell development and function are easily identifiable and apparent as unique colorations and patterns amongst vertebrates. These and additional differences between pigment cells and other neural crest derivatives have made them an excellent model to dissect the molecular mechanisms underlying neural crest lineage specification, migration, differentiation, and related human disorders.

Vertebrate pigment cells can be divided into two types based upon their embryonic origins. One type, the retinal pigment epithelium, is found only in an outer layer of the eye and is derived from an outpocketing of the developing forebrain, the optic cup. The second type, the neural crest derived pigment cells, encompass the pigment cells of the integuement and the inner ear, the iris, and the internal organs. The primary function of these cells is to produce pigment for coloration of skin and appendages (hair, feather, scale). Depending on the organism, the type of pigment produced, and the developmental stage, neural crest-derived pigment cells are defined by different nomenclature such as melanoblasts, melanocytes and chromatophores (including melanophores, iridipohores, xanthophores, and erythophores).

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Since pigment cells are not essential for viability, mutations that affect their development are frequently not lethal yet cause obvious alterations in the coloring of adult vertebrates. This has led in the availability of many mutants to dissect developmental pathways. Studies using rodents, chick, quail, *Xenopus*, and zebrafish, have provided both complementary and overlapping information on pigment cell development. Each organism lends itself to unique experimental studies and the specific details of pigment cell development can vary amongst them. Thus pigment cell number, timing of migration and differentiation, and type of pigmentation produced varies across the vertebrates. Comparisons of studies with different organisms have revealed that the genetic regulation of pigment cell development is highly conserved.

Model Organisms

Work in amphibians such as *Xenopus* has been instrumental in understanding early aspects of pigment cell development, in particular pigment cell fate specification and the genetic hierarchies regulating it. While they are not genetically amenable, *Xenopus* are suitable for both loss and gain-of-function studies using anti-sense oligos and morpholinos, which block protein translation. In contrast to mammals, differentiated pigment cells are observed early during development which has facilitated developmental analyses.[1]

Zebrafish, and more recently a distant relative, medaka, have emerged as excellent model systems for studying pigment cell development primarily because this process can be visualized in live embryos. Zebrafish have three pigment cell types (melanophores, xanthophores, and iridophores) and medaka have a fourth type.[2,3] The distribution of the different types of chromatophores is the major determinant of the coloration pattern in adult fish. Similar to *Xenopus*, pigment is produced in the chromatophores before the completion of migration. The melanophores, which are similar to melanocytes and produce black pigment, appear around 24 hr laterally and then along the trunk. The xanthophores appear next followed by the iridophores, which produce yellow pigment and silvery pigment, respectively. Also unique to fish, their pigment cells migrate both dorso-laterally and ventrally, and do not cross the basement membrane into the epidermis.[3]

Zebrafish and medaka are amenable to both genetic and embryological experiments. Over 90 pigmentation mutants in zebrafish have been identified from large-scale ENU mutagenesis screens, and additional mutants have been created from insertional mutagenesis.[3,4] In medaka, over 40 spontaneous mutants exist.[2] Similar to *Xenopus*, zebrafish are also well suited to loss and gain of function analyses using genes and morpholinos, respectively.

Much of our understanding of the cell biology of pigment cell development has come from studies of the avian species. Although chick and quail are not well suited for genetic studies, neural tubes can be dissected from chick embryos, cultured, and loss and gain of function analyses can be carried out. Since quail cells can be distinguished from chick cells by the presence of a large amount of heterochromatin in their nuclei, cell transplantation experiments have been used to understand pigment cell fate and migration. These techniques provided the first evidence that pigment cells migrate dorso-laterally and that pigment cell fate, like other neural crest derivatives, is dependent upon the environment through which they migrate.[5-7] DiI analysis in chick also revealed that pigment cell precursors migrate a day after other neural crest-derived lineages have begun to emigrate from the neural tube.[8]

The rodent is another classical system that has been instrumental to the identification of genes required for pigment cell development. Mice and rats with black and white coat coloration patterns have been favorites of mouse fanciers for centuries. In mammals, the pigment cell precursor is called the melanoblast and the mature pigment cell is called the melanocyte. A major determinant of skin and hair color results from the regulation of the two types of pigment produced by melanocytes, eumelanin (brown or black) and pheomelanin (yellow/red). In many mammals including mice, the Agouti signaling pathway regulates this switching of melanin type within a cell. Genetic analyses of over 100 spontaneous coat color mutants in small rodents have led to the identification of at least ten loci essential for pigment cell development.[9,10]

Defects in pigment cell development in small rodents are often evident as white spots in an otherwise dark animal.

Melanoblast development in mice is well characterized by expression of molecular markers (Fig. 1A). The precursors to melanoblasts develop around embryonic day 8.5 (E8.5), concurrent with the migration of other neural crest, and in a cranial to caudal order.[11] These neural crest cells express Wingless/INT-related 1 (Wnt1), Paired homeodomain transcription factor 3 (Pax3), and SRY box containing transcription factor (Sox10).[12-15] The precise time that neural crest are specified to melanoblasts in not known, but at E10.0 - E10.5 a subset of crest begin to express the earliest known melanoblast precursor markers Kit and microphthalmia-associated transcription factor (Mitf).[16-20] A few hours later these cells express a melanogenic enzyme marker gene, *Dopachrome tautomerase* (*Dct*), and localize to the migration staging area, dorsal to the neural tube.[21] From this region they continue to divide while migrating through the dermis, and by E13.5 a large proportj3é of melanoblasts migrate into the epidermis. By E15.5 a subset of melanoblasts enter the developing hair follicle, where they express markers downstream of Mitf, including *Tyrosinase* (*Tyr*) and *Tyrosinase related protein 1* (*Tyrp1*). Between E15.5 and birth, once the melanoblasts begin to produce pigment, they become melanocytes.

The controlled differentiation of melanocytes from melanoblasts and their precursors does not end during development, as adult hair follicles require repopulation of melanocytes at each cycle (Fig. 1B). These pigment cells originate from a *Dct* positive melanocyte stem cell pool located in the bulge area of the hair follicle.[22] Precise regulation of cell division and differentiation is needed to maintain the stem cell population and instruct differentiation into melanocytes in repopulated follicles. Interestingly, defective maintenance of this population may be responsible for hair graying.[23] Mitf, Pax3, and Wnt signaling, which are all required for pigment cell development also regulate stem cell maintenance.[23-25]

Studies of neural crest in these model organisms have identified several genes that regulate pigment cell development (Figs. 1, 2). Some genes are required for early aspects of neural crest development, including their induction and epithelial to mesenchymal transition, yet also appear to be essential for later aspects of pigment cell development. Many genes are needed for development of multiple neural crest derivatives, including pigment cells. These include the *Wnt* signaling pathway, *Snail/Slug*, *Sox10*, *Pax3*, *AP-2*, and *Ednrb*. Other genes may be more specific for pigment cells, including *Kit* and *Mitf*. In this book chapter we will review the important genes that regulate the specification, cell survival, differentiation, and migration of pigment cells. Although we do not discuss uncloned mutants, it is important to note that many uncharacterized mutants generated in the above model organisms will continue to advance our understanding of pigment cell development.

Initial Induction and Expansion of the Pigment Cell Population

Components of the Wnt/β-catenin pathway are among the earliest proteins expressed during neural crest development, supporting their role in neural crest and pigment cell induction, specification and expansion. The canonical Wnt signaling pathway is activated when an extracellular Wnt binds to a coreceptor complex including Frizzled, resulting in the accumulation of cytoplasmic β-catenin and the subsequent shuttling of β-catenin into the nucleus.[26] Nuclear β-catenin then interacts with members of the Lef1/Tcf transcription factor family and regulates transcription of target genes. Wnts 1,3, and 3A are expressed in the dorsal portion of the neural tube in mice, chick, and zebrafish embryos prior to and during neural crest specification.[27-30] *β-catenin* is expressed in both premigratory and migratory neural crest in chick.[31]

Insight into the role of WNT signaling in pigment cell development has come from studies in multiple organisms. In *Xenopus* and zebrafish overexpression of canonical Wnt signaling causes an expansion of neural crest markers and inhibition of this pathway leads to a reduced neural crest population.[1,32-34] In mice, individual knockouts of either Wnt1 or Wnt3a do not display overt neural crest defects, however Wnt1 and Wnt3a double knockout embryos have reduced numbers of *Dct*-positive melanoblasts.[35] Since these mutant embryos do not exhibit

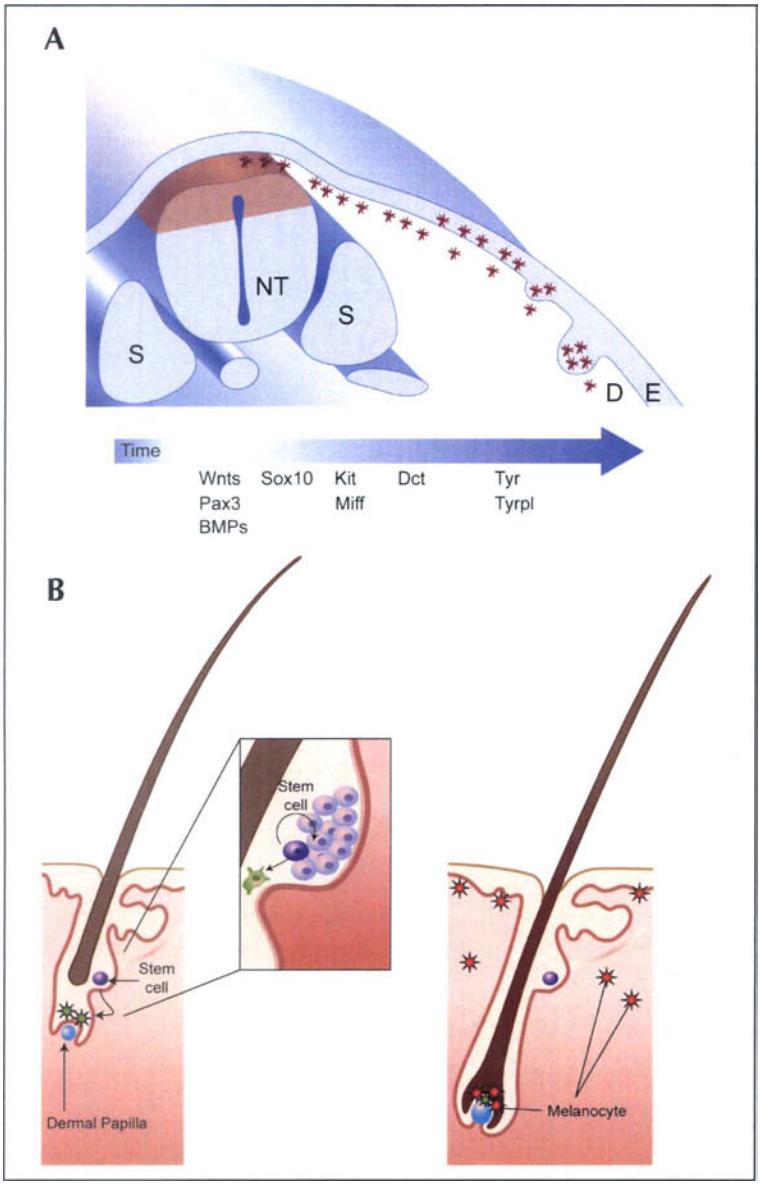

Figure 1. Cartoon depicting pigment cell specification and migration, and melanocyte stem cells. A,B) Pigment cell development and stem cell regeneration of pigment cells are based primarily on work done in mice. A) The neural crest population is induced from the dorsal edge of the neural tube (dark shading). The pigment cell precursor cells (asterisks) arise from a subset of the neural crest and migrate dorso-laterally beneath the ectoderm. As the cells migrate they divide and express more specialized markers of pigment cells, as indicated. The pigment cells migrate from the dermis (D) into the epidermis (E) and then a subset of these cells enters the hair follicle. B, left) Melanocyte stem cells are found in the bulge region of the mature hair follicle. B, middle) During the growth phase of the hair follicle, these cells divide generating a pluripotent stem cell and a population of melanocyte precursors. B, right) As the hair follicle cycle reaches resting phase the precursors become mature melanocytes and populate the hair follicle to produce pigment. Modified from Steingrimsson et al.[140]

increased cell death or reduced cell proliferation, this phenotype is likely to be due to defective melanoblast specification.

Neural crest-specific ablation of *β-catenin* in mouse blocks the formation of both melanoblasts and sensory neurons.[36] Surprisingly, over-expression of an activated form of *β-catenin* in descendents of Wnt1-cre neural crest cells induces sensory neurons but at the expense of melanoblasts.[37] These apparently contradictory results suggest that different neural crest derivatives may respond differently to Wnt signaling at various times in development. Deletion of *β-catenin* function specifically in melanoblasts will be necessary to assess its role in pigment cell development at later stages. In Zebrafish, over-expression of *β-catenin* in migratory neural crest cells promotes pigment cell formation at the expense of neurons and glia.[27] Moreover over-expression of a dominant-negative form of *tcf/lef* significantly reduces expression of *mitf*, without globally affecting neural crest populations.[32] Wnt signaling is proposed to specify neural crest to a pigment cell fate by direct regulation of the melanocyte transcription factor, Mitf. In support of this, the *Mitf* promoter contains Lef binding sites that are highly conserved between zebrafish and mice.[38,39]

In vitro studies using neural crest cultures also support a role for Wnt1 and 3a in promoting pigment cell differentiation and expansion at the expense of other neural crest derivatives. Addition of Wnt3a into neural crest cultures from mouse and chick induces pigment cell differentiation and an expansion in the number of pigment cells, probably at the expense of other neural crest derivatives.[28,40] Although the Wnt1 and 3a knockout mice suggest that these Wnts act redundantly, this might be temporally regulated as studies in mouse neural crest cultures indicate that Wnt3a but not Wnt1 can signal melanoblast differentiation.[41]

Members of the Bone Morphogenetic Protein (BMP) signaling family are also involved in neural crest and pigment cell formation. In mice, BMP2 expression has been reported in the surface ectoderm of E8.5 embryos.[42] Two independent studies have shown that treatment of chick neural crest cultures with BMP4, or with FGF2 and BMP2 together, reduces the overall number of pigment cells while expanding the population of glia and neurons.[28,43] These results suggest that the BMPs may inhibit pigment cell specification and/or expansion. At later stages of pigment cell development, BMPs may have a different role as BMP2 treatment of chick neural crest cultures increases synthesis of melanin but not expression of *Dct, Tyrp1*, or *Mitf*.[44]

In addition to the Wnts and BMPs, *Pax3* is also expressed very early in the dorsal neural tube and neural crest[13,15] and is required for development of several neural crest derivatives. *Pax3^{sp}*/+ animals have belly spots and *Pax3^{sp}* homozygous mouse embryos are embryonic lethal and exhibit reduced numbers of melanoblasts suggesting that Pax3 may be important for early expansion, survival, and/or migration of melanoblasts.[45] Pax3 may be required both cell and noncell autonomously, since transplantation of *Pax3^{Sp}/Pax3^{Sp}* neural tubes onto neural tubes of chick embryos results in normal neural crest cell migration.[46]

Similar to Wnt signaling, Pax3 also promotes pigment cell differentiation. Pax3 transactivates *Tyrp1* and acts synergistically with Sox10 to activate *Mitf*.[24,47-49] In addition, a recent study detailed Pax3 expression in the bulge region of hair follicles, and identified dual roles for Pax3 in the activation and repression of pigment genes throughout their development.[24] Lang et al found that in addition to its role in transactivating pigment genes, such as *Mitf*, Pax3 can also inhibit Mitf-mediated expression of *Dct*. This Pax3-mediated repression may be abolished by expression of β-catenin. Together these results suggest that Pax3 may function in both developing pigment cells and in the regulation of pigment stem cell maintenance and differentiation.

Pigment Cell Specification, Differentiation and Survival

Several transcription factors regulate pigment cell specification and differentiation. These include Foxd3, Slug/Snail, Sox9, Sox10, Ap-2, and Mitf. Work from a number of organisms has helped to elucidate the complex relationships among these proteins. Although a genetic hierarchy is apparent, it is not linear and almost certainly involves positive and negative feedback loops to promote development of the pigment cells.

Foxd3 may function to inhibit pigment cell development. Expression studies in chick, mice, *Xenopus*, and zebrafish have shown that Foxd3 is expressed at high levels in the dorsal neural tubes and in premigratory and migratory neural crest.[50-54] In addition, Dottori et. al. also showed that Foxd3 is expressed at lower levels in pigment cells. Consistent with a role in inhibiting pigment cell development, Foxd3 depletion from chick neural tubes causes formation of ectopic pigment cells, while over-expression inhibits pigment cell formation and migration along the dorso-lateral pathway.[52] Additional studies will be necessary to delineate the specific role of Foxd3 in regulating pigment cell development.

Members of the Snail family of transcription factors, which include Snail and Slug, regulate the early specification and differentiation of pigment cells. Depending upon the organism, different members of the family are present and are expressed in premigratory neural crest of zebrafish (*snail2*), *Xenopus* (*snail* and *slug*), chick (*slug*) and mouse (*Snail*).[55-58] Over-expression studies in *Xenopus* have indicated that *slug* is sufficient to promote pigment cell formation.[59] In addition, expression of a dominant negative form of *slug* in *Xenopus* reduces expression of *sox10*, which marks neural crest and pigment precursor cells.[1] Since these studies also showed that over-expression of *sox10* induces ectopic *slug* expression, in *Xenopus slug* and *sox10* regulate each other's expression. In support of this complex relationship, Sox10 transactivates *Mitf*, which in turn transactivates the *SLUG* promotor in vitro.[48,60] Since some *snail2* homozygous mutant mice exhibit belly spots, it seems that the function of Snail and Slug may be conserved.[55,57]

Sox9 is another transcription factor that is thought to act early in pigment cell development.[61] Sox9 is a member of a family of SRY box-containing transcription factors that is expressed in the pre and post-migratory neural crest.[62,63] Studies in chick, *Xenopus*, and mice suggest that *Sox9* acts upstream of the neural crest marker, *sox10*. In chick, *Sox9* can induce *Sox10* expression.[1,63,64] In zebrafish it has been shown that *sox9* acts upstream of *foxd3* and *snail2b*.[65] In support of a role of sox9 in pigment cell specification and/or migration, electroporation of *sox9*-GFP into neural crest cells of chick embryo explants induces GFP positive cells that only migrate along the dorso-lateral pathway.[62] Consistent with this, Zebrafish harboring mutations in both orthologs of *sox9*, *sox9a* and *b*, have reduced numbers of iridophores but not other pigment cell types.[65] Taken together, these data suggest that Sox9 family members may promote pigment cell differentiation and possibly their migration.

Sox10 is another SRY containing transcription factor that is essential for pigment cell differentiation and survival. Sox10 is expressed in early premigratory and migratory neural crest in humans, mice, chick, zebrafish, and *Xenopus* and is detectable in developing pigment cells of mice.[1,14,66-72]

The first studies to indicate a requirement for Sox10 in pigment cell development came from analysis of *Sox10^{Dom}* heterozygous mouse mutants that have a premature truncation of the protein.[72,73] Heterozygous mice have belly spots and embryos contain fewer melanoblasts than wild-type embryos and younger embryos exhibit reduced *Dct* expression.[74] Homozygous mutant embryos also exhibit increased apoptosis of neural crest as assessed by TUNEL staining, suggesting that *Sox10* is essential for neural crest cell survival.[72,75] These mice also have defects in enteric ganglia resulting in megacolon, a condition associated with Hirschsprung's disease in man. Studies in mouse neural crest cultures confirmed that Sox10 acts intrinsically to the melanoblasts.[76]

Studies in zebrafish and *Xenopus* have corroborated those in mice and additionally helped to elucidate the relationship between Sox10 and other transcription factors. As mentioned previously, studies from *Xenopus* have shown that *sox10* acts downstream of *wnt1*, *sox9, and slug*.[1,77] These studies also demonstrated that over-expression of *sox10* induced ectopic pigment cells, while depletion of *sox10* in *Xenopus* led to a decrease in cell proliferation and an increase in apoptosis. *Sox10* zebrafish mutants and morpholinos treated embryos exhibit increased apoptosis and failure to differentiate, as assessed by reduced *mitf* expression.[69] Together these studies suggest a requirement for Sox10 in pigment cell differentiation and

survival. In support of this, in vitro studies have shown that Sox10 transactivates expression of *Mitf* and *Dct*.[48,74,78-81]

Downstream of Sox10 is another transcription factor, AP-2α, that promotes pigment cell development. In mice, AP-2α is expressed in the premigratory and migratory neural crest around E8.5 (although its expression is thought to be lost in differentiating neural crest).[61,82] A conditional knockout of *Ap-2α* in neural crest, using *Wnt1*-Cre mediated recombination, results in mice with belly spots and demonstrates a requirement for AP-2α in pigment cell development.[12] Similarly, zebrafish *ap-2α* mutants, called *lockjaw*/ *mont blanc*, and embryos injected with *ap-2α* morpholinos have reduced numbers of early melanophores and iridophores.[50,83-86] These studies demonstrate a role for AP-2α neural crest development that may be confined to pigment cells since the zebrafish embryos exhibited normal expression of the pan-neural crest markers *snail2*, *foxd3* and *sox10*, but reduced expression of *dct*, *mitf*, and *kit*.[50,84,86]

As mentioned above, Sox10 activates expression of another transcription factor, *Mitf*, that itself has been referred to as the pigment cell master regulatory gene.[87,88] *Mitf* directly regulates the expression of the melanogenic genes *Pmel17*, *Melan-a*, and *melastatin* (TRPM1), and the enzymes, *Tyr*, *Tyrp1*, and *Dct*[63,89-92,93] In vitro studies show Mitf and Sox10 act synergistically to activate Dct expression.[94,114] There are nine isoforms of *Mitf* with Mitf-M being the main isoform thought to regulate pigment cell development. Murine *Mitf-M* is expressed in developing neural crest-derived pigment cells and in the neuroepithelium-derived retinal pigment epithelium (RPE) of the eye beginning at E10.[17] At least twenty-three different murine mutant alleles of *Mitf* have been identified, displaying phenotypes ranging from moderate spotting to complete absence of mature pigment cells.[25,95]

Mitf homologs also regulate pigment cell development in zebrafish and *Xenopus*. A zebrafish *mitf* ortholog, *nacre* or *mitfa*, is required for melanophore development, and misexpression of *nacre* produces ectopic melanophores and abnormal eye development.[33] Recent work has demonstrated the existence of two *Xenopus mitf* isoforms, *XIMitfα* and *XIMitfβ*, which appear homologous to Mitf-M and Mitf-A, respectively. *XIMitfα* is expressed in presumptive pigment cells and the RPE.[96] In addition to a role for MITF in regulating pigment cell differentiation, *Mitf* also functions in cell survival by directly regulating *Bcl2* in pigment cells and osteoclasts.[97] Recent studies have shown that *Mitf* also activates INK4A and p21-Cip1 expression, suggesting *Mitf* coregulates differentiation and cell cycle exit in pigment cell precursors.[98-100] Thus Mitf may act as the pigment cell master gene by a combination of controlling differentiation and cell cycle progression.

Pigment Cell Migration and Development: Cell Adhesion and Signaling

Concurrent with induction, expansion and differentiation of the pigment cell population is their migration. Specified pigment cells are the only known neural crest derivative to migrate along the dorso-lateral pathway, in contrast to other neural crest derivatives that migrate ventrally relative to the neural tube and somites.[101,102] As mentioned, the timing of their migration differs amongst organisms. There is evidence that a long distance cue from emerging dermis could stimulate their dorso-lateral migration since dermal grafts in chick neural tube cultures can induce migration from a distance.[103] One possible candidate is Kit ligand, to be discussed below, since it is expressed in the dermamyotome.[104,105]

Questions remain concerning the timing of migration and specification. Neural crest cells may need to be initially specified to the pigment cell lineage thereby allowing their migration along the dorso-lateral pathway, or alternatively only those cells that migrate along the dorso-lateral pathway are subsequently specified as pigment cells. In support of the first theory, Erickson and Goens showed that specified cultured pigment cells transplanted back into chick neural tube explants, migrated only along the dorso-lateral pathway while non pigment cells failed to migrate along this pathway.[101] The expression of pigment cell specific markers, *Kit*,

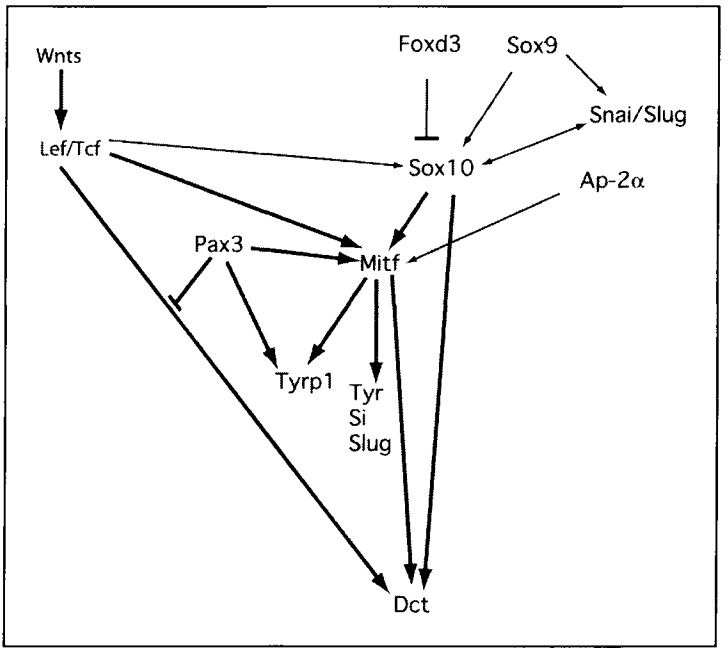

Figure 2. A schematic diagram that represents the transcription factor hierarchy of pigment cell development. Shown are the genetic relationships amongst transcription factors that regulate pigment cell development, based upon work in many vertebrates. Dark lines indicate direct relationships that have been characterized biochemically.

Mitf, and *Dct* adjacent to the neural tube is also consistent with this theory (Fig. 2). However, some other neural crest derivatives have been observed along the dorso-lateral migration pathway but may be eliminated by apoptosis.[102]

Inhibitory proteins within the dermamyotome may prevent nonpigment cells from migrating along the dorso-lateral pathway. In support of this, removal of dermamyotome in chick causes neural crest to migrate precociously along the dorso-lateral pathway.[106] Inhibitory factors may include components of the extracellular matrix. For example, proteins such as F-spondin, are required to block early neural crest migration along the dorso-lateral pathway.[107] Peanut agglutinin lectin-binding activity and chrondroitin-6-sulfate are also good candidates for inhibition of neural crest migration since they are expressed in dermamyotome.[108]

Not surprisingly, specific cell adhesion molecules Integrins and Cadherins are expressed in neural crest derived pigment cells and contribute to defining appropriate migration pathways. Integrins α-2, 3, and 5, which interact with extracellular matrix proteins are expressed in pigment cells.[109] Evidence supporting *Integrin β-1* involvement in dorso-lateral migration comes from analysis of null ES cells transplanted onto avian neural tubes.[110] These *Integrin β-1* deficient ES cells fail to migrate along the dorso-lateral pathway but do migrate ventrally. Conditional knockout studies in mice are necessary to examine the roles of the various integrins in melanoblast development.

Cadherins, which exhibit homophilic interactions between cells, also exhibit a dynamic expression pattern during pigment cell development and are involved in regulating the timing and path of migration. Immunohistochemical studies in mice have revealed that as neural crest initiate migration, E-cadherin and P-cadherin expression is high in these and surrounding cells.[111] As the melanoblast population migrates from the dermis to the epidermis to the hair

follicle, the expression pattern changes. Epidermal melanoblasts express primarily E-cadherin, while those in the dermis and hair follicles express primarily N and P cadherins, respectively. Interestingly, over-expression of *N-cadherin* and *Cadherin 7* in chick inhibits migration of melanocytes out of the neural tube.[31] This suggests that the expression level and type of cadherin expressed regulates pigment cell migration.

Santiago and Erickson also showed using chick explants that Ephrin B, a receptor tyrosine kinase, has dual requirements in regulating pigment cell migration.[112] In Chick, Ephrin B receptors are expressed in melanoblasts and the ligands are expressed along the dorso-lateral pathway throughout melanoblast migration.[113] Blocking ephrin signaling in stage 12 chick explants leads to increased neural crest migration but inhibits migration in stage 18 explants, when pigment cells normally initiate migration. While this analysis revealed an important function for ephrins in chick neural crest, there may be redundant genes in mice since the knockout of murine *ephrin B2* and 3 does not affect neural crest migration.[113,114]

Another receptor tyrosine kinase pathway that is required for both pigment cell survival and migration is Kit. Kit is expressed coincident with Mitf in both premigratory and migratory pigment cells throughout their development.[16,19,20] It has been reported that Kit expressing neural crest cells give rise to only pigment cells,[20,115] however in mouse neural crest cultures not all Kit positive cells are committed to the melanocyte lineage.[41] The ligand for Kit (Kitl) is expressed in a complementary pattern in the dermamyotome, dermis and hair follicles.[116,117] There are two alternatively spliced isoforms of Kit ligand. One generates a protein that is proteolytically cleaved to make a soluble protein while the other generates a protein thought to be primarily membrane bound.

The requirement for Kit signaling in pigment cell development was first identified from analysis of spontaneous mouse mutants, Kit^{W} and $Kitl^{Sl}$. Heterozygous mice have belly spots and in the case of $Kitl^{Sl}$ are hypopigmented on their ventrum. The temporal requirement for Kit in pigment cell development was explored by injection of Kit antibody into pregnant mice at various stages of gestation.[118,119] These studies showed that there are Kit dependent and independent times during development. Kit is required at the times coincident with migration in the dermis, proliferation and survival in the epidermis, however not for proliferation in the hair follicles during development.

Embryonic analyses of Kit mutants support a role for this pathway in multiple steps of melanoblast development. Kit^{h}/Kit^{h} and Kit^{Wv}/Kit^{Wv} embryos have reduced numbers of melanoblasts by E12.5, suggesting a requirement in melanoblast survival but not for migration and differentiation.[120,121] Similarly, $Kitl^{SD}$ mutant embryos, which lack the membrane form of Kitl, also have reduced numbers of melanoblasts; however, they are more evenly distributed across the embryo as compared to Kit mutants. This suggests that the membrane form is required for melanoblast survival and perhaps less so for melanoblast migration.[105] *Sparse* zebrafish mutants that lack Kit have increased numbers of dorsal melanophores but decreased pigment cells ventrally, demonstrating that Kit function is conserved.[122]

The specific requirement of Kit in migration was examined using *Kit* embryos that contain a mutation in *Nf1*, a gene that blocks apoptosis of melanoblasts (and other cells).[123] These embryos exhibit migration defects suggesting that the requirement for Kit in migration is distinct from its requirement in survival. It is worth noting that addition of exogenous Kitl bound to a bead in embryonic skin cultures is sufficient to promote migration of pigment cells into hair follicles but does not act as a chemoattractant.[124] In addition, analysis of various *kit* mutant alleles in zebrafish that distinctly affect pigment cell survival and migration suggests that Kit signaling promotes both processes but that they are separable.[125]

In vitro studies also support a role for Kit in pigment cell differentiation. Addition of Kitl to neural crest cultures increases the number of Dct-positive cells.[18] Kit^{lacZ} mutant neural crest cultures retain expression of some genes, including *Mitf*, but not all pigment cell markers.[126] Kit may promote pigment cell differentiation by post-translational modifications, since Kit signaling can lead to Mitf phosphorylation.[127]

The seven transmembrane domain, G protein-coupled endothelin receptor-B (*Ednrb*) is also required for normal development of pigment cells.[128] Ednrb is expressed in developing neural crest cells, pigment cells, and enteric ganglia in mouse embryos, while the ligand, Edn3, is expressed in tissues associated with *Ednrb*-expressing cells.[128-132] Two orthologs of *Ednrb*, *Ednrb1* (classically referred to as Ednrb) and *Ednrb2*, are present in chick and quail. The two Edrnb homologues in chick may have different functions since at the onset of pigment cell migration, Ednrb1 expression decreases and Ednrb2 expression increases.[133] This function may be conserved, since Ednrc, which is very similar to quail Ednrb2, was isolated from Xenopus melanophores.[134]

Studies in mice have established that *Ednrb* signaling is not necessary for the specification of pigment cells but is involved in the proliferation and migration of pigment cells.[21,128,132,135] Experiments using a conditional *Ednrb* knockout with inducible expression and repression demonstrated Ednrb is required between E10 and E12.5, when melanoblasts migrate and proliferate along the dorso-lateral pathway.[136] In zebrafish, mutation of *ednrb1* causes the *rose* mutant. Zebrafish *ednrb1* is expressed in embryonic melanophore, iridophore and xanthophore precursors. It continues to be expressed after melanophore and iridophore differentiation but its expression decreases during xanthophore differentiation. While *rose* mutants display normal larval pigmentation patterns, adults are missing a subset of pigment cells, including late stripe melanophores, and also display reduced iridophores.[137]

In addition to migration and proliferation, in vitro studies indicate that the Ednrb pathway also promotes pigment cell differentiation and survival.[18,131,132,138,139] Tissue recombination experiments using *Ednrb*-null neural crest cell cultures showed that *Ednrb* performs sequential cell-autonomous and nonautonomous actions during pigment cell differentiation.[139] Ednrb is not required for the generation of early neural crest-derived pigment cells but is required for the later expression of *tyrosinase*, via noncell autonomous production of Kitl. Taken together, these studies suggest that both Ednrb and Kit function during multiple steps of pigment cell development.

Concluding Thoughts

This chapter reveals that the genetic pathways that regulate how pigment cells form, differentiate, and migrate are highly conserved. While we have some understanding of how the various transcription factors, receptors and signaling proteins are related, there are still many questions. What are the downstream targets of these pathways? How are the genes regulated so that they can function at several steps during neural crest and pigment cell development? And ultimately, how do mutation in these genes cause human disease? Continued work of many laboratories in the above model organisms will provide additional insight into the complex and intriguing process of pigment cell development.

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CHAPTER 10

Specification of Sensory Neuron Cell Fate from the Neural Crest

David W. Raible* and Josette M. Ungos

Abstract

How distinct cell fates are generated from initially homogeneous cell populations is a driving question in developmental biology. The neural crest is one such cell population that is capable of producing an incredible array of derivatives.[1] Cells as different in function and form as the pigment cells in the skin or the neurons and glia of the peripheral nervous system are all derived from neural crest. How do these cells choose to migrate along distinct routes, populate defined regions of the embryo and differentiate into specific cell types?

This chapter focuses on the development of one particular neural crest derivative, sensory neurons, as a model for studying these questions of cell fate specification. In the head, sensory neurons reside in the trigeminal and epibranchial ganglia, while in the trunk they form the spinal or dorsal root ganglia (DRG). The development of the DRG will be the main focus of this review. The neurons and glia of the DRG derive from trunk neural crest cells that coalesce at the lateral edge of the spinal cord (Fig. 1). These neural crest cells migrate along the same routes as neural crest cells that populate the autonomic sympathetic ganglia located along the dorsal aorta. Somehow DRG precursors must make the decision to stop and adopt a sensory fate adjacent to the spinal cord rather than continuing on to become part of the autonomic ganglia. Moreover, once the DRG precursors aggregate in their final positions there are still a number of fate choices to be made. The mature DRG is composed of many neurons with different morphologies and distinct biochemical properties as well as glial cells that support these neurons.[2]

The Form and Function of Dorsal Root Ganglia Neurons

Beyond its value as a rich system for studying questions of cell fate specification, how the DRG develops is of interest given its important role in the functioning of vertebrate organisms. All of the somatosensory information from the body (excluding cranial regions) is transmitted through the DRG neurons. This includes four major sensory modalities: sense of touch, perception of pain and irritation (nociception), the sense of position and movement of the limbs and body (proprioception) and temperature sense. The DRG are organized in a metameric pattern along the ventrolateral edges of the spinal cord (Fig. 1). There is a single ganglion in register with each somite along the entire rostrocaudal axis. DRG neurons send out peripheral processes that innervate the skin, muscles and joints of the limbs and trunk. DRG neurons also send out central projections that extend dorsally, entering the spinal cord through the dorsal root. These central processes have branches that terminate either in the spinal cord or ascend to nuclei in the brain stem. In this way, DRG neurons transmit peripheral information either

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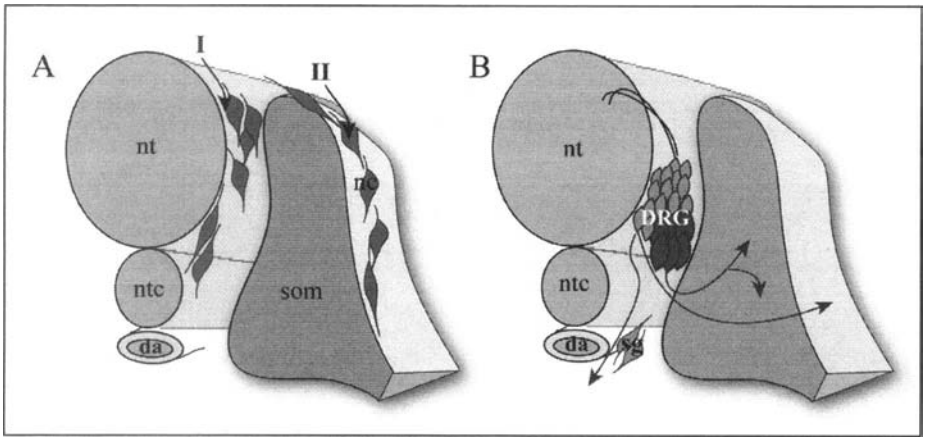

Figure 1. Illustration of neural crest migration pathways (A) and schematic diagram showing DRG projections (B) in a generic vertebrate embryo. A) Neural crest cells giving rise to DRG migrate along the medial pathway (I, A) along with precursors for sympathetic ganglia, glial cells, etc. Only pigment cell precursors migrate along the lateral pathway (II, A). B) DRG neurons project to skin, muscle and viscera to convey touch, thermal and pain sensation and muscle proprioception. Abbreviations: nt, neural tube; ntc, notochord; da, dorsal aorta; som, somite; nc, neural crest; DRG, dorsal root ganglia; sg, sympathetic ganglia.

locally to activate local reflex circuits or to the brain where peripheral information is then translated into our different modes of perception.

Progress in understanding the differentiation of DRG sensory neurons will have an impact on human health. Sensory neuronopathies result from degeneration of DRG neurons, and are often associated with gait ataxia (see ref. 3 for review). Neuron degeneration is caused by autoimmune pathogenesis, often in response to cancer, or in response to neurotoxins, including chemotherapeutics such as cisplatin and carboplatin. Sensory loss and pain also result from DRG damage associated with diabetes (reviewed by ref. 4). Understanding sensory neuron development may eventually help in the design of therapies for recovery of function in patients affected with these diseases. Research on the molecular differentiation of sensory neuron subsets may also shed light on the different sensory pathways that process painful stimuli.[5,6]

Neural Crest Origin of the Sensory Ganglia

His recognized the contribution of neural crest cells to spinal ganglia in his original description of the neural crest.[7,8] Ablation, transplantation and vital dye labeling experiments in amphibians by Harrison, Detwiler, Raven and others confirmed these initial observations (reviewed in refs. 8,9). Neural crest cells migrate ventrally between the somite and neural tube to coalesce into segmentally reiterated DRG, contributing both neurons and glia. In contrast, the trigeminal ganglion has a dual origin, with neuronal contributions from both neural crest and placode,[10-12] while the epibrancihial ganglion neurons are thought to be solely of placodal origin.[13] Recently, a second population of neural crest-derived cells, the boundary cap cells, has been shown to contribute to sensory ganglia.[14] These cells first migrate ventrally and become associated with dorsal and ventral roots, and then subsequently enter into the DRG to form neurons and glia. A ventral neural tube-derived migratory population of cells ("neural trough",[15] "VENT" cells,[16]) has also been suggested to contribute to sensory ganglia (reviewed in ref. 17). The relationship between boundary cap cells and VENT cells remains to be determined.

Sensory Neuron Cell Types

Historically, sensory neurons have been recognized to fall into two broad classes based on cell body size. Although there are additional subclasses of neurons within the DRG, (e.g.,

refs.18-20) cumulative evidence suggests two classes of neurons are morphologically and functionally distinguishable. The large neurons correspond to large diameter myelinated fibers that convey fine touch and proprioceptive information directly to the dorsal column nuclei in the brainstem, and small neurons correspond to the small diameter unmyelinated fibers that convey pain information and terminate in the dorsal horn (see ref. 2 for review). Large and small neurons differ in expression of cytoskeletal components[21,22] and sodium channels.[23] Large and small neurons differentially express neuropeptides such as substance P and CGRP[24,25] and receptors such as vanilloid receptors,[26-28] P2X receptors[29,30] and G-protein coupled receptors.[31-34] In avian embryos, these neurons have distinct locations within the DRG, with large cells positioned ventrolaterally and small cells positioned dorsomedially.[35]

Trk Receptor Expression in Sensory Neuron Cell Types

Large and small neurons are also distinguishable by the expression of different neurotrophin receptors. These transmembrane tyrosine kinases serve as receptors for the neurotrophins NGF and NT-3, respectively. Study of the functions of the neurotrophins in DRG development has a long history: NGF was initially identified by Levi-Montalcini as a survival factor for sensory neurons. Originally thought to act mainly as target-derived cues involved in pruning of excess neurons, the functions of neurotrophins are now recognized to be more complex, playing roles in the specification of sensory neuron cell type and axonal outgrowth to targets.

Analysis of the expression of neurotrophin receptors has revealed that they are expressed in the two distinct classes of sensory neuronal subtypes. mRNA for *TrkA* is expressed in small diameter neurons[19,36] and overlaps with expression of the neuropeptide genes *CGRP* and *PPT*,[37] indicating that these cells are nociceptors. In contrast, TrkC is expressed in large diameter neurons[19,36] consistent with these cells acting as proprioceptors. Analysis of Trk expression in conjunction with neuron projections revealed that TrkA and TrkC are found in distinct populations.[38] Several studies have found that TrkC is expressed very early in DRG development,[39,40] suggesting that neurotrophins may play may play roles in the initial specification of neuron subtype or axon outgrowth.

Genetic analysis has confirmed that neurotrophins and their receptors differentially influence sensory neuron development. Targeted inactivation of Trk receptors and their ligands confirmed that they function in specific DRG neuron subtypes. When TrkC receptors or NT3 were functionally inactivated, large proprioceptors were selectively lost.[41-45] In contrast, when TrkA receptors or NGF were inactivated, smaller nociceptors were lost, including all cells expressing the neuropeptide CGRP.[45-48] However, these studies could not distinguish whether deficits were due to disruption in neuron differentiation or survival of neuron subtypes. To overcome this complication, sensory neuron phenotypes were analyzed where the cell death regulator BAX was also inactivated.[49-53] In these mice, neurons survive despite knockout of neurotrophins or their receptors, and thus the effects of these signaling molecules on neuron phenotype can be analyzed. Inactivation of neurotrophins or their receptors resulted in loss of some subtype specific markers and specific axon pathfinding defects. These results suggest that neurotrophins play a role in sensory neuron cell type specification independent of their effects on survival.

Specification of Sensory Neurons

While the neurotrophins are part of the puzzle of how sensory neurons are specified, it is clear that many additional factors play a role in specification of neuron subtypes and are important in distinguishing neuronal precursors from glial precursors in the DRG. While the signaling pathways that determine these cell fate decisions and the genetic pathways they regulate are still not understood, there has been substantial progress.

Several studies have addressed whether cell lineage is important in generating specific cell types in the DRG. Clonal analysis in avian embryos in vivo, by vital dye injection[54] or retroviral marking[55] suggests that some neural crest cells give rise to both neurons and glia in the DRG. These results suggest that interactions among precursor cells will be important for establishing different cell types. The Notch signaling pathway would be a good candidate for promoting such interactions (see below). Thus, these results not only support a role for early

fate restrictions in generating sensory precursors, but they also indicate the existence of at least two distinct classes of sensory precursor in the neural crest.

Tissue culture analysis has also detected the presence of both multipotent and restricted precursors. Clonal analysis of avian embryonic DRG cells detected precursors that could form pigment cells, sensory neurons and autonomic neurons.[56] Similarly, some mammalian DRG cells act as stem cells, generating neurons, glia and smooth muscle cells.[57] An early-differentiating sensory precursor has been identified in tissue culture[58-60] that gives rise only to neurons with proprioceptive and not nociceptive phenotypes.[61] Taken together, these results suggest that the embryonic DRG is heterogeneous, with multiple precursor types present.

Developmental Timing of Sensory Neuron Specification

DRG growth in avian and mammalian species occurs in two waves. Thymidine birthdating studies in avian[62] and mammalian[63] embryos have identified two periods of DRG growth. First, a subpopulation of large neurons is born, followed by a period where both large and small neurons are generated. Retrograde labeling studies have confirmed that large neurons that project to the brainstem are formed in the first wave of differentiation.[64] Several lines of evidence suggest that these two waves of differentiation result from two different precursor pools.[65] Retroviral lineage studies provide evidence for an early developing DRG precursor that forms only large neurons and a later developing precursor that forms both large and small neurons.[55] Early-differentiating sensory precursors identified in tissue culture[58-61] are likely to correspond to the cells responsible for the first wave of DRG development.

Transcriptional Regulation of Sensory Neuron Specification

The development of these two waves of DRG neurogenesis have been shown to be regulated by the Neurogenin basic helix-loop-helix transcription factors (Fig. 2).[65] Targeted knockouts of mouse *ngn1* and *ngn2* demonstrate that each *ngn* gene is necessary for development of a subset of sensory neurons.[65-67] In the absence of Ngn1, most of the later generated small diameter neurons are eliminated and the larger diameter neurons are reduced in number. In *ngn2* mutants, there is a delayed onset of appearance of TrkB and TrkC expressing cells, but by later stages DRG appear normal. However, in *ngn1-/-; ngn2-/-* embryos, DRG neurons are completely absent. Ngn1 is thought to compensate for the loss of Ngn2 by either generating or maintaining more TrkB and TrkC positive neurons.[65] These results suggest that the two different precursors are differentially regulated by neurogenins: a precursor that generates the early-born large neurons requires *ngn2*, and a precursor that generates later-born large and small neurons that requires *ngn1*. In zebrafish, a single neurogenin gene, *ngn1*, is required for sensory neuron specification.[68]

Neurogenin genes are sufficient to produce ectopic neurons when overexpressed in Xenopus[69] or zebrafish.[70] Retroviral-mediated mis-expression of *ngn* genes in chick premigratory neural crest has been shown to bias cells to localize to the sensory ganglia and forces ectopic expression of sensory specific markers. Furthermore, ectopic expression of *ngn* was also capable of driving expression of sensory neuronal markers in the dermamyotome.[71] Combined with the early expression of the chick *ngns* in migrating neural crest,[71] these results suggest that Neurogenins drive neural crest cells toward a sensory fate early in their development. Similarly, Greenwood et al[61] identified dividing precursors in rat neural tube explants that expressed *ngn2* and appeared committed to a sensory fate. Even when challenged with BMP2, a potent inducer of autonomic differentiation,[72] these cells continued to generate sensory neurons.

However, several studies suggest that expression of neurogenin alone is not sufficient to distinguish cells as sensory neurons. Although introduction of Neurogenin into neural crest cells promoted neurogenesis, cells could form sensory or sympathetic neurons depending upon the addition of exogenous factors.[73] Similarly, recombination of *neurogenin* into the *mash1* locus allows autonomic neurogenesis.[74] Thus neurogenin is not sufficient to specify sensory neuron cell type, and must work in combination with other factors.

The POU domain transcription factor Brn3a (Pou4f1- Mouse Genome Informatics) is necessary for the correct development and survival of sensory neurons in the trigeminal and

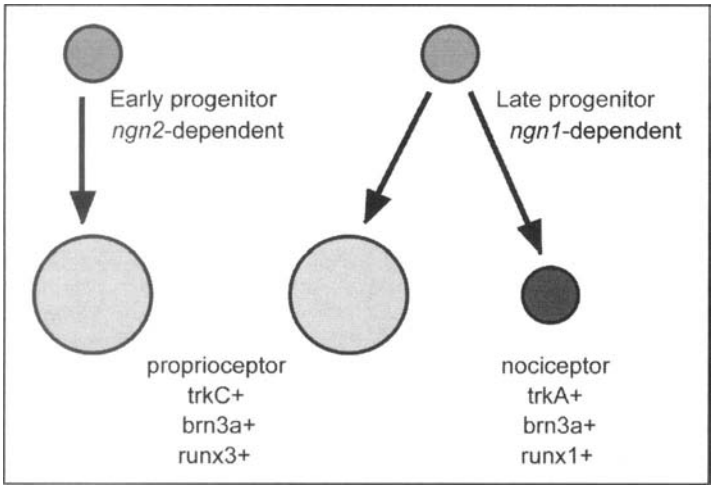

Figure 2. Two different neurogenic progenitors are distinguished by their neurogenin dependence.[65] An early progenitor, dependent on *ngn2* function, generates neurons with large cell bodies that include proprioceptors. A later progenitor dependent on *ngn1* produces both large and small neurons, including nociceptors. See text for more details.

dorsal root ganglia. Brn3a expression begins in migrating sensory precursors just undergoing their final cell division.[75] In culture, *ngn2* expression precedes Brn3a, and may regulate its expression.[61] Sensory neurons are specifically lost after targeted inactivation of Brn3a in mouse.[76,77] Both large and small diameter neurons are affected. Increased cell death in mutant animals is preceded by a decrease in trk expression[78] and altered axon outgrowth.[79] Extensive transcriptional profiling of wildtype and mutant animals has revealed that Brn3a regulates neurotransmitter systems, ion channels and neuronal structural components.[80]

The runx transcription factors are related to the Drosophila pair-rule gene runt (see ref. 81 for review). The mammalian homologue, Runx3 is specifically expressed in large diameter TrkC+ neurons in the DRG, and loss of function results in disrupted development of proprioceptors and severe ataxia.[82,83] While there are differences between trigeminal and DRG sensory neurons and also between mutations on different genetic backgrounds, in the most severely affected animals proprioceptive neurons are affected as early as they can be distinguished. The presence of runx binding sites in the *trkC* 5' genomic region is consistent with a regulatory role for *runx* in *trkC* expression,[82] but this idea has not yet been tested directly. Interestingly, *runx1* is expressed in small diameter sensory neurons, suggesting it might play a similar role in trkA+ nociceptive neurons, however its function in the DRG has not been tested since *runx1* knockout mice die early from blocked blood development.[84] Binding sites for Brn3a are found upstream of both *runx1* and *runx3*,[82,85,86] and runx1 expression is reduced in Brn3a knockout mice,[80] suggesting that runx genes might be directly regulated by Brn3a.

Understanding the regulation of trk gene expression may reveal clues about the specification of different sensory neuron subtypes. A defined enhancer sequence upstream of exon 1 of trkA directs specific expression of a lacZ reporter to DRG neurons.[87] The transcription factors Brn3a and Klf7 bind to this enhancer, and their binding sites are necessary for enhancer-directed expression in vivo.[87-89] Loss of Brn3a results in decreased TrkA expression, even when cell death is prevented by BAX inactivation.[88] TrkC expression is also dependent on the activity of Brn3a;[78] however, less is known about the regulation of TrkC expression, since no regulatory sequences have yet been identified. Both Brn3a and Klf7 are expressed in TrkA and TrkC positive cells.[78,89] The specific expression of trk receptors therefore cannot be

simply explained by the distribution of their known transcriptional regulators. The function of these proteins can be regulated by cofactors: Brn3a by HIPK2[90] and Klf7 by MoKa.[91] It seems likely that specification of neuronal subtype will involve multiple levels of transcriptional regulation.

Other genetic cascades regulate later events in sensory neuron differentiation. Development of proper synaptic connections is critical for sensory neuron function (see ref. 92 for review). The ETS family transcription factors Er81 and Pea3 play a role in defining specific central targeting of proprioceptors. The two transcription factors are expressed in subsets of sensory neurons that innervate distinct muscles; moreover motor neurons that innervate the same muscles express the same ETS gene as the corresponding sensory neurons.[93] Targeted inactivation of Er81 results in abnormal central projections of proprioceptive neurons, but initial development of these neurons is not affected.[94] In contrast, knockout of Pea3 affects motor neurons and not sensory neurons.[95] The exact roles of ETS transcription factors in specifying the stretch reflex circuit remain to be determined.

Signals Involved in Sensory Neuron Specification

Wnt/β-catenin signaling plays an instructive role in specifying mouse sensory neurons. Tissue-specific inactivation of β-catenin in neural crest cells results in specific loss of DRG sensory neurons,[96] and constitutive activation of β-catenin in neural crest cells promotes their differentiation into sensory neurons[97] and constitutive activation of β-catenin promotes ngn2 expression. Treatment of mammalian neural crest stem cells in vitro also promotes neuron formation.[97] Together, these studies provide strong evidence for Wnt signals playing an early role in mammalian sensory neuron specification, upstream of ngn expression. Studies in zebrafish[98] and in avians[99] suggest that in some contexts Wnts may inhibit neuronal differentiation from neural crest. While it is possible that there are species-specific differences in the roles of Wnts in sensory neuron development, it is likely that the developmental context in which Wnts function will determine the results of activating the Wnt pathway. Wnts play multiple roles in the development of neural crest cells,[100,101] and understanding the specificity of this signaling pathway remains a major challenge.

Notch signaling plays an important role in determining whether neural crest cells that migrate to the DRG form neurons or glia. Notch1 is expressed in proliferating cells in the DRG, while the Notch ligand Delta1 is expressed in differentiating DRG neurons.[102] Activation of Notch signaling in neural crest cells in vitro and in vivo prevents differentiation of sensory neurons and promotes glial cell differentiation.[102,103] Numb, a negative regulator of Notch signaling, is asymmetrically localized in dividing DRG cells[102] and its expression correlates with sensitivity of neural crest cells to Delta-mediated signals.[104] Targeted inactivation of Numb results in specific loss of DRG neurons, while other neural crest-derived neurons are not affected.[105] As for Wnts, Notch signals are likely to play multiple roles in neural crest development.[106-108]

Sonic hedgehog (Shh) signaling is required for proper development of zebrafish DRG neurons.[109] DRG sensory neurons do not form in zebrafish embryos carrying mutations in the Shh signaling pathway or in embryos treated with cyclopamine, a specific inhibitor of the pathway. Transplantation experiments demonstrate that the Shh signaling pathway must be intact in DRG precursors for neuron differentiation, suggesting that the requirement for Shh is direct. Expression of ngn is not initiated in Shh signaling mutants, and timed exposure of embryos to cyclopamine suggests that the critical period for Shh signaling occurs just before ngn expression normally begins. Although a role for Shh in DRG development has not been reported in other species, there is some evidence to suggest that Shh affects trigeminal sensory neuron development. In mouse cranial neural crest cultures, Shh promotes expression of ngn1, but not ngn2, and increased expression of Brn3a.[110] Manipulation of Shh expression alters the migration and differentiation of trigeminal precursors in vivo.[111] As with the signals discussed above, Shh signaling also regulates other aspects of neural crest development.[112-114]

Plasticity of Sensory Cell Fate Specification

Although it is common to think of allocation of cell fate as a process with sharp transitions, it is becoming increasingly clear that there is much flexibility in neural crest cell fate decisions. Recent studies demonstrate that as neural crest cells begin to express the genes involved in specification of sensory neuron precursors, they retain the ability to generate nonneuronal derivatives. Zirlinger et al[115] placed cre recombinase into the *ngn2* locus through homologous recombination. When introduced into the rosa-lacZ line, Ngn2-cre irreversibly marks cells and their progeny that express Ngn2 even transiently. When compared to wnt1-cre, which more broadly marks neural crest cells, Ngn2-cre was much more likely to mark DRG precursors. Surprisingly, however, both neurons and glia were labeled. This study demonstrated that although neurogenin promotes neuron formation and even biases cells towards contributing to sensory ganglia, expression of neurogenin does not irreversibly commit cells to become sensory neurons. Luo et al[116] examined the fates of trkC cells by performing lineage analysis after injection with vital dyes. A subset of neural crest cells recognized by surface labeling with trkC antibodies could subsequently generate both neurons and glia. However, when trkC cells were labeled later in culture, they generated only neurons, suggested that they had undergone a change in developmental potential.

Several studies also suggest that while some neural crest cells may be pleuripotent, they have intrinsic biases to generate distinct cell types. Study of neural crest stem cells from different sources or from different stages of development demonstrated that these cells can undergo self renewing divisions in culture and be instructed to differentiate as multiple cell types; however when transplanted back into the embryo they show distinct biases.[117-119] Taken together, these studies suggest a model where neural crest cells become biased to generate specific cell types yet retain a flexibility to respond to local cues to alter their developmental trajectory.

Future Challenges

Although there has been significant progress in understanding the molecular regulators involved in sensory neuron specification, many questions remain. It remains to be determined how the different signaling pathways are integrated and how they coincide with the known transcription factors to regulate the development of DRG sensory neurons. Future studies will likely focus on the combinatorial role of different transcription factors and the upstream regulation of these factors by Wnt, Notch and Shh signaling. In addition, although the transcription factors that regulate sensory neuron cell type are beginning to be identified, the signaling factors that distinguish neuron subtype remain unknown. Finally, there are many diverse types of neurons within the DRG, not just the major two classes discussed in this review. For example, different classes of nociceptive neurons have distinct receptors, transmitter types and target projections.[5,6] The regulatory mechanisms that are used to generate this diversity are largely unknown.

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CHAPTER 11

Neural Crest and the Development of the Enteric Nervous System

Richard B. Anderson, Donald F. Newgreen and Heather M. Young*

Abstract

The formation of the enteric nervous system (ENS) is a particularly interesting example of the migratory ability of the neural crest and of the complexity of structures to which neural crest cells contribute. The distance that neural crest cells migrate to colonize the entire length of the gastrointestinal tract exceeds that of any other neural crest cell population. Furthermore, this migration takes a long time—over 25% of the gestation period for mice and around 3 weeks in humans. After colonizing the gut, neural crest-derived cells within the gut wall then differentiate into glial cells plus many different types of neurons, and generate the most complex part of the peripheral nervous system.

The Enteric Nervous System

The ENS contains more neurons than the spinal cord.[1,2] There are many different classes of enteric neurons that differ in their neurotransmitters, electrophysiological properties, targets (circular muscle, longitudinal muscle, other neurons, blood vessels, epithelium etc), inputs and the direction along the gut in which their axons project.[3,4] The neurons are grouped into ganglia, and each ganglion contains many different types of neurons. Myenteric ganglia are located between the circular and longitudinal muscle layers, and submucosal ganglia are located internal to the circular muscle layer. The ENS plays a critical role in mediating motility reflexes, as well as regulating blood flow within the gut wall and water and electrolyte transport across the mucosal epithelium. The fact that some regions of the gastrointestinal tract can function autonomously (without CNS input),[5] and because of its high degree of complexity, the ENS has been termed "the second brain".[2]

Origins of the ENS

Although neural crest cells arise along the entire length of the body axis, studies by Yntema and Hammond (1954) and later by Le Douarin and Teillet (1973) showed that the ENS is derived from crest cells that originate from two specific regions of the neuraxis—the vagal (defined as post-otic hindbrain adjacent to somites 1-7) and sacral (caudal to somite 28 in chick embryos and caudal to somite 24 in embryonic mice and humans) levels[6] (Fig. 1). The vagal region includes the transition zone between the head and neck.[7]

By ablating neural crest cells from the chick post-otic hindbrain, Yntema and Hammond (1954) showed that enteric ganglia were absent from the esophagus, stomach and small and large intestine. In a series of elegant chick-quail transplantation studies, Le Douarin and Teillet (1973) later showed that vagal neural crest cells adjacent to somites1-7 are the major source of

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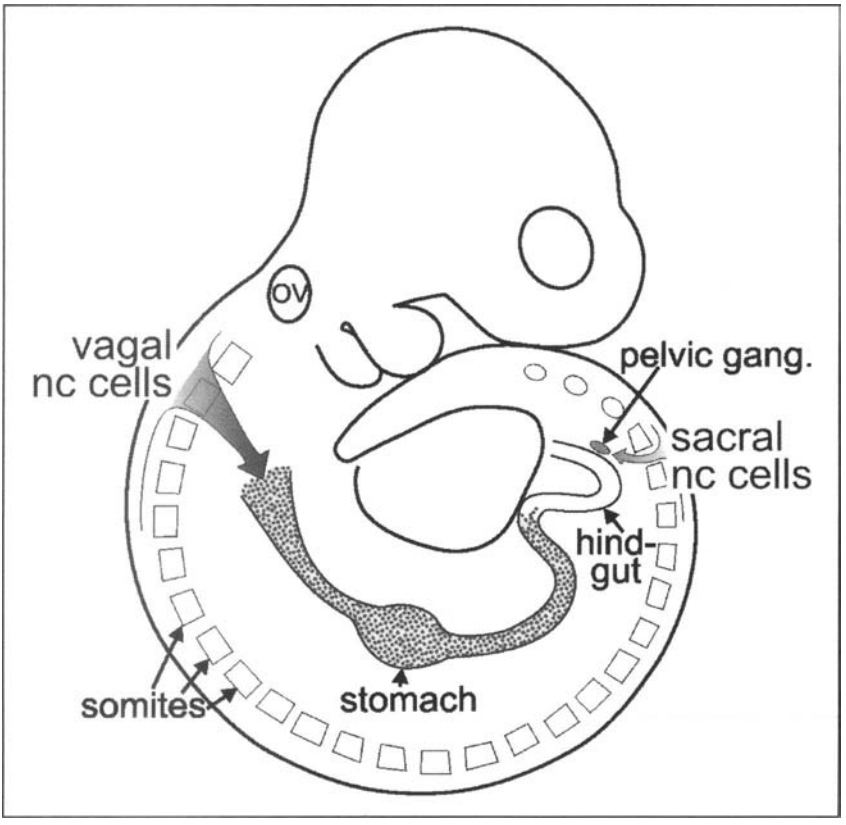

Figure 1. Diagram of an E10 embryo showing the origins of neural crest cells that colonize the developing gastrointestinal tract. nc—neural crest; OV—otic vesicle; pelvic gang.—pelvic ganglion primordia (note that pelvic ganglia are bilateral).

enteric neuron precursors, and that sacral level neural crest cells also contribute to the ENS, mostly in the hindgut. A sub-population of vagal neural crest cells also gives rise to major elements in the cardiac outflow tracts, as well as to neurons and support cells in intrinsic cardiac ganglia.[8-10]

More recent studies have further fine tuned the axial origins of the ENS. In chick embryos, vagal neural crest cells adjacent to somites 1-2 contribute predominantly to the esophagus and stomach; those adjacent to somites 3-6 contribute to the entire length of the gut; while neural crest cells adjacent to somites 6-7 are located mainly in the hindgut.[11-15] In mice, neural crest cell adjacent to somites 1-5 contribute enteric neurons along the entire length of the gut, while those adjacent to somites 6-7 give rise to cells only in the esophagus.[16] There therefore appears to be some species differences in the origin of esophageal neurons.

Vagal Neural Crest

The emigration of vagal level neural crest cells from the dorsal neural tube commences about the 7-10 somite stage. In avians, most vagal level neural crest cells migrate in a ventral route through the rostral half of somites 3, 4 and 5,[12] with some passing through branchial arch 6.[17,18] Chick vagal neural crest cells then migrate further ventrally to form the circumpharyngeal crest,[19,20] which is a complex pattern of crest cells that gives rise to a range of

different derivatives including the ENS.[21] The first vagal neural crest cells reach the vicinity of the chick foregut within 9 hours, at the 13 somite stage.[22] In mice, vagal neural crest cells migrate underneath the epithelium, through the somites, between the somites, and between the somites and the neural tube.[23] Neural crest cell that emigrate adjacent to somites 1-4 migrate around the pharynx along a pathway that is later followed by the vagus nerve, while neural crest cells adjacent to somites 4-7 form a continuous stream of cells that migrate ventrally beyond the dorsal aortae.[16,24] Near the foregut, neural crest cells form left and right strings along the route that is later followed by vagal axons from the hindbrain.[17,25] Vagal neural crest cells enter the nearby foregut mesenchyme at the 20-25 somite stage.[24]

Once in the foregut, vagal neural crest cells migrate caudally along the entire length of the gut (Figs. 1,2). Neural crest cells have been reported to migrate at speeds of between 35-40 μm/h in both birds and in explants from embryonic mice.[26,27] In chick, vagal neural crest cells enter the foregut around E2.5-3, reach the level of the umbilicus at E5, the cecal region at E6 and the colorectum at E7.5. The entire length of the chick gut is completely colonized by E8.5.[13] In mice (Fig. 2A-C), the colonization of the gut by vagal neural crest cells takes around 5 days; crest cells enter the foregut around E9.5; reach the cecal region at E11.5 (Fig. 2B); and completely colonize the gut by E14.5.[28] In humans, vagal neural crest cells enter the foregut at week 4 and reach the terminal hindgut by week 7.[29] Some of the crest cells that colonize the foregut emigrate out into the lung buds and give rise to ganglia within the airways,[30] and some of the crest-derived cells in the small intestine emigrate into the pancreas and give rise to pancreatic ganglia.[31,32]

In humans, birds and mice, vagal neural crest cells initially migrate in the outer half of the fore and midgut mesenchyme, which comprises an initially uniform population of cells that lie between the endoderm tube and the squamous epithelial serosal layer that surrounds the gut externally.[21,29] After each fore and midgut region is colonized, vagal neural crest cells then form a narrow layer in close proximity to the serosa, where myenteric ganglia will later form.[26,33] By the time vagal neural crest cells have reached the proximal hindgut in birds, the circular muscle layer has begun to develop, and the cells migrate internal to the circular muscle in the region where the submucosal plexus will form; vagal crest cells then undergo a secondary migration that gives rise to the myenteric plexus.[13] However in the hindgut of mice and humans, vagal crest cells migrate in the outer part of the mesenchyme and aggregrate in the region where myenteric ganglia will form (as they do in the fore and midgut); the submucosal plexus arises several days later from a secondary migration of neural crest cells from the myenteric plexus.[33,34] In mice, the netrin family of guidance molecules and their receptors has been shown to be required for the secondary migration of vagal crest cells to form the submucosal plexus.[32]

Sacral Neural Crest

Sacral neural crest cells migrate ventrally through the rostral halves of the adjacent somatic sclerotomes and congregate near the dorsal wall of the hindgut where they formed extramural ganglia (i.e., paired pelvic ganglia and, additionally in birds, the nerve of Remak)[13,24,35] (Fig. 2C). The avian-specific nerve of Remak is a large ganglionated chain running along the mesentery adjacent to the hindgut and distal midgut. Sacral neural crest cells form the nerve of Remak at E3.5 and remain in this structure until E7, when axons from neurons in the nerve of Remak project into the hindgut. Sacral neural crest cells then migrate into the hindgut along these extrinsic axons and colonize the hindgut in larger number from E10.[13] In mice, sacral neural crest cells form the pelvic plexus (Fig. 2C) adjacent to the distal hindgut from around E10.5 and do not enter the hindgut until E14.5.[24,35] In both chick and mice, sacral neural crest cells directly adjacent to the gut undergo a "waiting period" and only colonize the hindgut after it has been fully colonized by vagal neural crest cells. It was initially suggested that sacral neural crest cells might require the presence of vagal neural crest cell in the hindgut, in order for them to enter. However, this has subsequently been shown not to be the case. Following ablation of the vagal level neural tube in chick, the migration of sacral neural crest cells into the hindgut was

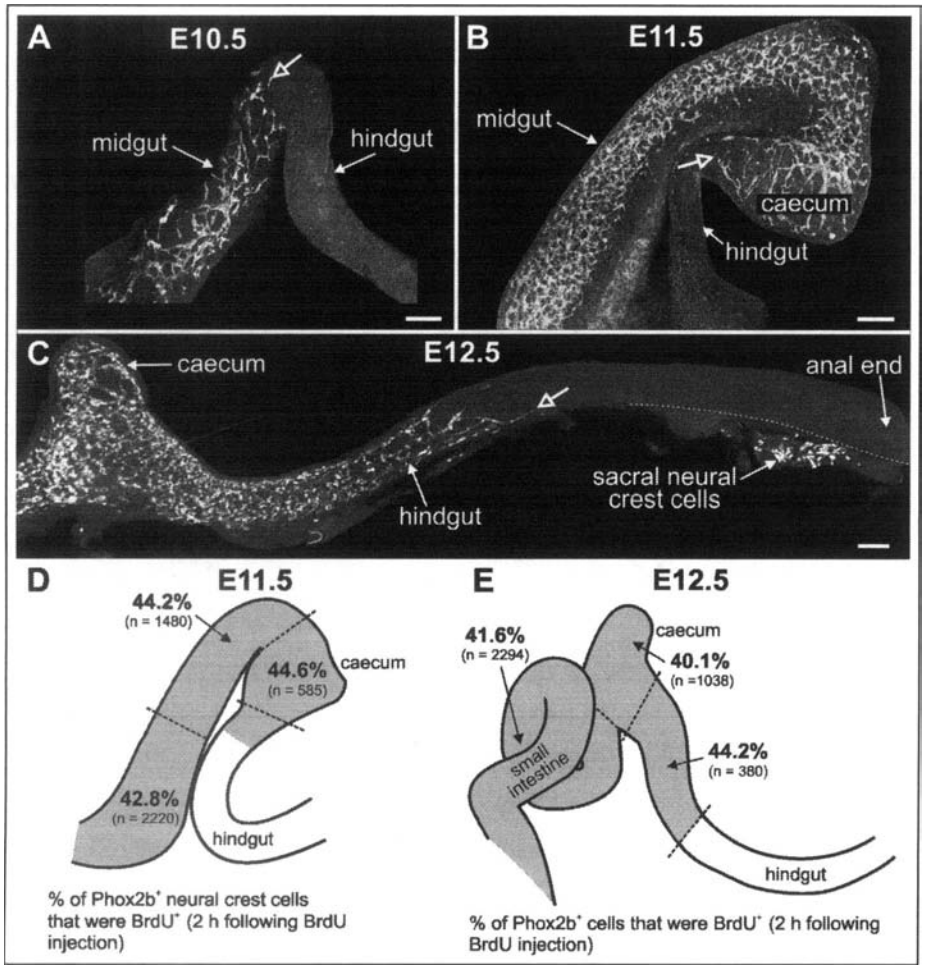

Figure 2. A-C. Micrographs of wholemount preparations of gut from E10.5 (A), E11.5 (B) and E12.5 (C) mice. The E10.5 and E11.5 (AB) preparations include the mid- and hindgut, whereas the E12.5 preparation (C) includes only the caecum and post-caecal hindgut. The preparations had been processed for immunohistochemistry using antisera to p75 (A,B) or Hu (C) to reveal neural crest-derived cells. The most caudal vagal crest-derived cell in each preparation is indicated with an open arrow. At E10.5, vagal neural crest cells have colonized most of the midgut, by E11.5, they have colonized the caecum and are entering the post-caecal hindgut, and at E12.5, crest cells have colonized the rostral half of the post-caecal hindgut. Sacral neural crest cells are present close to, but outside, the distal hindgut (C, dotted line indicates the border of gut). Scale bars: 100 μm. D, E. Diagrams of gut from E11.5 (G) and E12.5 (H) mice showing pooled data of the percentage of neural crest ($Phox2b^+$) cells that were also $BrdU^+$ from 6-8 embryos (E11.5) and 6 embryos (E12.5); BrdU had been administered to the mother two hours prior to sacrifice. The "n"s refer to the total number of crest-derived cells examined in each region (pooled from the different embryos). The regions of the gut that have been colonized by vagal crest-derived cells at these stages are shown in grey[54,141] (see also micrographs of E11.5 and E12.5 preparations above).

found to be unaffected,[14] and combination grafts of aneural hindgut with sacral cells, with and without vagal neural crest cells, have reported the same effect.[36] Similar finding have also been reported in mice, using *Wnt1-lacZ* transgene expression as an early marker of neural crest cells.[35]

The sacral "waiting period" could be due to an absence of attractive molecules (or their receptors on the sacral neural crest cells) and/or the transient expression of repulsive molecules in the distal hindgut. The chemorepulsive molecule Sema3A (formerly known as Collapsin-1) is expressed transiently in the distal hindgut of the embryonic chick, and is responsible for the delay in the ingrowth of axons from neurons in the nearby nerve of Remak.[37] Since sacral neural crest cells migrate into the hindgut along the axons of neurons in the nerve of Remak,[13] it is possible that the expression of Sema3A, either directly or indirectly, regulates the time of entry of the sacral neural crest cells into the hindgut.

Once sacral neural crest cells have entered the hindgut, they give rise to both neurons and glial cells.[13] The contribution of chick sacral neural crest cells was found to be confined predominantly to the distal hindgut, where they comprised up to 17% of myenteric neurons, and far fewer submucosal neurons.[13] Thus, even in the rectum, the vast majority of enteric neurons arise from precursors that originate in the hindbrain (vagal crest cells). In mice, the quantitative contribution of sacral neural crest cells to the ENS is still unclear. Mice in which the genes encoding members of the glial cell line-derived neurotrophic factor (GDNF) signaling pathway (GDNF, Ret or GFRα1) have been knocked out, lack enteric neurons in the gut caudal to the stomach[16,38] (Fig. 3). However, a small number of enteric neurons have been reported in the distal hindgut of these mice, which are almost certainly derived from sacral neural crest cells.[16,24,38] The small number of enteric neurons present in the hindgut of these mice is likely to be due to the fact that many of the sacral crest cells that normally enter the hindgut are dependent on the GDNF signaling pathway, but it is also possible that in mice, sacral neural crest cells contribute only a small number of enteric neurons.

Migratory Behavior of Neural Crest Cells

Images of fixed samples, and time-lapse imaging of living enteric crest-derived cells, have both shown that enteric crest cells migrate in chains, indicating the presence of cell-cell adhesive interactions between migrating neural crest cells.[28,39,40] The cell adhesion molecule, L1, is expressed by migratory and post-migratory crest cells in the embryonic mouse gut,[41,42] and in cultured gut explants, perturbing L1 activity with function blocking antibodies retards the rate of crest cell migration and increases the number of solitary cells (cells not in chains) near the migratory wavefront.[42] Other cell adhesion molecules, in addition to L1, are almost certainly involved in adhesive interactions between enteric crest-derived cells, as perturbing L1 function does not noticeably disrupt cell-cell contacts between post-migratory cells.[42] The gap junction protein, connexin 43, is also expressed by crest-derived cells in the embryonic mouse gut.[43] Direct cell-cell communication via gap junctions has been shown to be important for the migration and survival of trunk neural crest cells in culture,[44,45] but the role of gap junctions in enteric crest cells has yet to be determined.

Cell number can also influence the migratory ability of vagal crest cells. The partial ablation of vagal crest cells in chick embryos, results in aganglionosis of the distal gut.[11,14,46] Furthermore, when a small number of crest cells at the migratory wavefront in explants of embryonic mouse gut is isolated from the crest cells behind them, the isolated cells migrate more slowly.[40] These studies have lead to the idea of "population pressure" as a major driving force behind the colonization of the gut by crest-derived cells, and that high cell density and/or cell-cell contact stimulates migration and colonization.[47]

The migratory behaviour of crest-derived cells in the gut is also influenced by molecules expressed by the gut mesenchyme that could either promote or inhibit the motility of enteric crest-derived cells nondirectionally, or act as chemoattractive or chemorepulsive cues. GDNF, which is expressed by the gut mesenchyme, is chemoattractive to enteric crest-derived cells and appears to induce vagal cells to enter the gut and may also promote their rostrocaudal migration along the gut (see below).

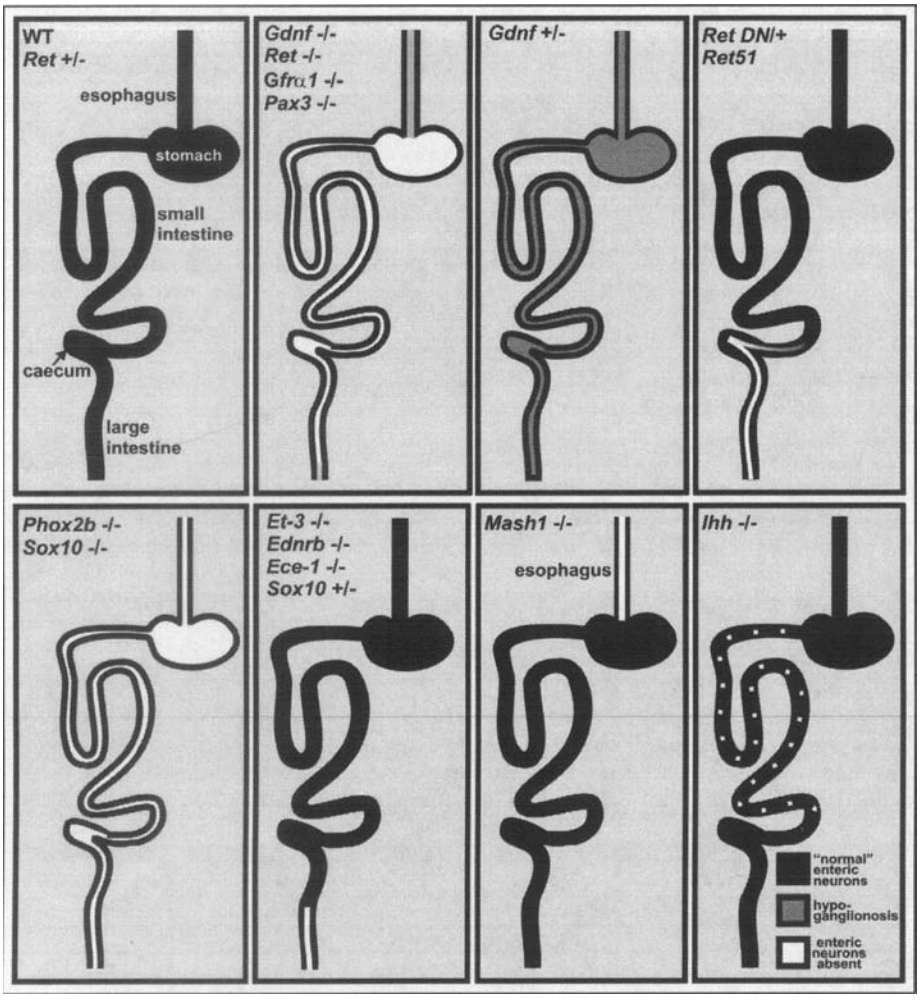

Figure 3. Diagram showing the gross phenotype of the best characterized genetically modified and naturally occurring mutant mice with enteric nervous system phenotypes. See text for details and references. *DN*—dominant negative; *Et-3*—endothelin-3; *Ednrb*—endothelin receptor B; *Gdnf*—glial cell line-derived neurotrophic factor; *Ihh*—Indian hedgehog.

Hirschsprung's Disease

Hirschsprung's disease (HSCR) is a congenital birth defect, with an incidence of around 1 in 5000 live births, in which enteric neurons are absent from variable lengths of the distal-most gut. Studies of mouse models have shown that HSCR is almost certainly caused by a failure of neural crest cells to colonize the distal regions of the gut, rather than a failure of crest cells to survive in the affected regions.[21,48] As enteric neurons are essential for propulsive activity in the gut, HSCR patients suffer from intestinal obstruction or severe constipation. The region lacking enteric neurons, termed the "aganglionic" zone, is usually contracted and devoid of contents. Although the region proximal to the aganglionic zone contains enteric neurons, it is distended (forming a "mega-colon") due to an accumulation of faecal contents. HSCR is treated by surgical resection of the aganglionic gut, although functional problems often persist. The

genetics of HSCR is complex.[48-51] Eleven "HSCR susceptibility genes" have been identified in humans, but mutations in known genes account for only about 50% of HSCR cases.[48,52,53] Many of the known susceptibility genes encode members of the RET- or EDNRB-signaling pathways or transcription factors that regulate *RET* or *EDNRB* expression. The importance of many of the known HSCR genes for normal ENS development is discussed below.

Proliferation, Cell Death and Differentiation in the Developing ENS

Proliferation

It is thought that somewhere in the order of 1000-2000 vagal neural crest cells enter the foregut of embryonic chicks and mice.[54] In small intestine and colon of adult mice, there are over one million neurons plus an equal number or higher number of glial cells.[55] Thus, crest-derived cells must undergo extensive proliferation in the gut during development. In fact, 2 hours following the administration of the thymidine analogue, BrdU, to pregnant female mice, over 40% of crest-derived cells in the gut of their E11.5 or E12.5 progeny are BrdU-labelled (Fig. 2C,D).[56] The expression of molecules that can influence the rate of crest cell proliferation (such as GDNF and endothelin-3) are higher in the caecum than in other regions of the developing gut. This has lead to the suggestion that the caecum may be a proliferative zone in which sufficient precursors to form the ENS of the colon are generated. However, a recent study has shown that the rate of proliferation of crest-derived cells does not vary in different regions of the embryonic mouse small and large intestine including the caecum, or at different distances from the migratory wavefront[56] (Fig. 2C,D). On the other hand, there does appear to be a lower rate of proliferation of crest-derived cells in the stomach of E12.5 mice compared to the midgut.[57]

Apoptosis

In most parts of the developing nervous system, excess neurons are generated, and then surplus neurons, usually those that have not projected to a target and have insufficient access to survival factors, are removed by programmed cell death (apoptosis). Surprisingly, studies of embryonic mouse and rat gut have failed to detect any evidence for apoptosis in the developing ENS.[55,58] Thus enteric neuron number appears to be regulated largely by the rate of proliferation.

Neuronal and Glial Differentiation

When crest-derived cells enter the mouse foregut at E10, a sub-population express pan-neuronal proteins (such as neurofilament, PGP9.5 and Hu).[25,59] By E14.5, around 50% of crest-derived cells in the small intestine express neuronal proteins, and the percentage of crest-derived cells that can be classified as neurons does not change much at later developmental stages.[60] Early differentiating neurons project their axons in the same direction (caudally) and along the same pathway through the mesenchyme as the migrating vagal cells.[61] Neural cells commonly migrate in close association with axons, for example in the development of peripheral nerves.[62] In the lateral line of the zebrafish, axons are the source of instructive cue(s) that guide migrating neural crest cells.[63] However, in the developing gut, it is still unknown whether axons are a source of guidance cue for migrating crest cells or vice versa, and hence whether crest cell migration can occur without axon growth.

The expression of molecules characteristic of particular neuron types (such as neurotransmitter synthesizing enzymes, etc) commences after the expression of pan neuronal proteins, and different neuron classes develop at very different developmental stages.[60] For example, in mice nitric oxide neurons develop at E12.5,[64] whereas cholinergic neurons do not develop in significant numbers until after birth.[65] In embryonic mice, cells expressing glial cell precursor markers are not detected until a day or so after crest cells have colonized a particular region of the gut, whereas in embryonic chicks, cells expressing glial markers are present close to the migratory wavefront.[39,60] Little is known about the molecular control of the differentiation of glial cells and different types of neurons from undifferentiated crest cells, but the transcription

factor, Sox10, appears to be required for the differentiation of enteric glial cells,[66] and another transcription factor, Mash1, is required for the differentiation of serotonin neurons.[67]

Molecules and Signaling Pathways Involved in ENS Development

A variety of signaling pathways and molecules have been shown to be required for normal enteric neural crest cell survival, proliferation, migration and differentiation. These genes and molecules have been identified from both basic research and from molecular genetic studies of patients with HSCR. Recent genetic and cell biological studies have shown that there are complex interactions between the different signaling pathways.[68-72]

GDNF Family

GDNF/Ret-GFRα1

GDNF is a secreted protein that is a distant member of the TGF-β superfamily. GDNF signals predominantly through the Ret receptor tyrosine kinase, but Ret is only activated if GDNF is bound to another protein, GFRα1, which is attached to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor.[73] GDNF is expressed by the gut mesenchyme prior to the entry of neural crest cells; Ret is expressed exclusively by neural crest-derived cells; and GFRα1 is expressed by both crest-derived cells and the gut mesenchyme.[74-77] GDNF-, GFRα1- or Ret-null mice die within 24 hours of birth, and lack neurons from most of the gastrointestinal tract, although they do have some neurons (but in greatly reduced numbers) in the esophagus, stomach and distal hindgut[78] (Fig. 3). *Gdnf*$^{+/-}$ mice have reduced numbers of enteric neurons throughout the gastrointestinal tract and motility defects.[79] Although *Ret*$^{+/-}$ mice have normal numbers of enteric neurons,[55] humans that are heterozygous for *RET* mutations can exhibit aganglionosis of the distal gut.[80]

There are two main isoforms of Ret, Ret9 and Ret51. Mice lacking Ret51 have neurons throughout the gut, while mice lacking Ret9 have no neurons in the colon (Fig. 3); this indicates that Ret9 is the most important isoform for the development of the ENS.[81] Recently, mice were generated that express a dominant negative mutation in Ret (*Ret*^{DN}) by inserting human cDNA encoding a mutant Ret9 (with two point mutations) into the first coding exon of the *Ret* gene. In contrast to *Ret*$^{+/-}$ mice, *Ret*$^{DN/+}$ mice have an aganglionic colon[82] (Fig. 3). Therefore, reduced Ret signaling in mice can be tolerated to some extent (as in *Ret*$^{+/-}$ mice), however a reduction in Ret signaling beyond a certain level results in defects in the ENS. The extent of aganglionosis is in all likelihood correlated with the degree to which Ret signaling is reduced. The developing ENS in humans appears to be more sensitive to reduced Ret signaling than the ENS of mice. In mice, it appears that excessive GDNF/Ret-GFRα1 signaling can also cause ENS defects. Mice lacking Sprouty2, an inhibitor of receptor tyrosine kinases, have ENS hyperplasia and intestinal pseudo-obstruction (delayed transit of intestinal contents) which can be corrected by inhibiting GDNF/Ret signaling.[83] Thus, the negative regulation of Ret by Sprouty2 appears to be important for maintaining appropriate levels of GDNF/Ret signaling during ENS development.

GDNF/Ret-GFRα1 signaling plays multiple and varied roles during ENS development. In mice lacking Ret, vagal neural crest cells die around the time at which they reach the foregut, suggesting that GDNF/Ret-GFRα1 signaling is required for survival.[16] Studies of enteric neural crest-derived cells grown in vitro have shown that GDNF also promotes their differentiation and proliferation.[76,84-87] Furthermore, in vitro assays have shown that GDNF is chemoattractive to migrating neural crest cells.[75,88] Therefore, GDNF expressed by the gut mesenchyme probably plays a role in inducing vagal neural crest cells to enter the gut, retaining them within the gut, and may also play a role in their caudally directed migration along the gut. Although GDNF/Ret-GFRα1 signaling plays multiple roles in ENS development, the circumstances under which Ret activation induces different processes (survival, proliferation, differentiation or migration) are unknown.

Other GDNF Family Members

In addition to GDNF, there are three other known members of the GDNF family—neurturin, artemin and persephin.[73] All GDNF family members signal through Ret, but bind to specific GFRα coreceptors. Persephin is not expressed outside of the CNS. Although artemin is expressed by the esophagus and stomach of embryonic rats,[89] its preferred coreceptor, GFRα3, is not expressed by enteric neurons,[90] and the sizes and numbers of enteric neurons in the small and large intestine of mice lacking artemin are no different from wildtype mice.[91] Neurturin is expressed by the mesenchyme of the developing gut, but at later developmental stages than GDNF, and its preferred coreceptor, GFRα2 is expressed by neural crest-derived cells within the gut.[90,92,93] In vitro studies have shown that neurturin promotes the survival, proliferation, differentiation, neurite outgrowth and migration of neural crest cells in the developing gut.[85,86,90] Mice lacking neurturin or GFRα2 are viable and fertile and have only minor differences in enteric neuron numbers from wild-type mice.[55,94,95] Thus, neurturin does not appear to be essential for the survival, proliferation or differentiation of enteric neural crest cells. However mice lacking neurturin or GFRα2 have smaller enteric neurons, reduced numbers of nerve fibres in the gut wall and some motility defects,[93] suggesting that neurturin may provide trophic support to enteric neurons.

Endothelin-3 (Et-3)/Endothelin Receptor B (Ednrb)

Et-3 is a secreted peptide that signals through a G-protein-coupled 7 trans-membrane spanning receptor, Ednrb. *Lethal spotted* (*ls*) and *piebald lethal* (s^l) are naturally occurring mutants for Et-3 and Ednrb respectively, and have been shown to lack enteric neurons in the distal bowel[96-98] (Fig. 3). Mutations in *ET-3* and *EDNRB* in humans can also result in Hirschsprung's disease.[80] Et-3 is expressed by the mesenchyme of the developing gut, with highest expression in the caecum.[69,99] Ednrb is expressed by both migrating neural crest cells and some mesenchymal cells.[69,100] Although enteric neurons are absent only from the distal colon of *Et-3* and *Ednrb*-null mice and rats, the migration of neural crest cells through the small intestine is also delayed.[58,69,100-102]

It is still unclear why perturbations in Et-3/Ednrb signaling result in delayed neural crest cell migration, and their failure to colonize the distal regions of the gastrointestinal tract. In vivo and in vitro studies have shown that Et-3 signaling does not effect the survival of enteric neural crest cells.[58,100,103] Different studies have reported variable effects of Et-3 on enteric crest cell proliferation,[58,69,84,87,103] and it appears that if Et-3 does influence proliferation, the effect is slight. Some in vitro studies have found that Et-3 inhibits neuronal differentiation induced by GDNF; it was therefore proposed that Ednrb signaling normally prevents the premature differentiation of crest cells into neurons, and thereby maintains sufficient numbers of undifferentiated migratory cells to colonize the entire gastrointestinal tract.[84,87] However, a recent study of Ednrb null embryonic rats has shown that the percentage of crest cells expressing neuronal markers was lower in the mutant rats than in wild-type animals.[58]

Other Secreted Factors

Neurotrophin 3 (NT-3) acting via TrkC receptors appears to be the only neurotrophin involved in the development of the ENS.[104] In vitro, NT-3 promotes the neuronal differentiation of crest-derived cells, and mice with null mutations in *Nt-3* or *Trk3* have reduced numbers of enteric neurons.[105] Interestingly, crest-derived cells respond to NT-3 at later developmental stages than they respond to GDNF.[76]

A number of studies have shown that BMP-2 and BMP-4 influence the differentiation of crest-derived cells in vitro, and mice that overexpress noggin, a BMP antagonist, have more enteric neurons.[58,106-108] Thus, BMPs seem to contribute to the regulation of enteric neuron number and differentiation. BMPs could also play a role in the concentric patterning of enteric ganglia within the gut wall and to ganglion size.[109,110]

Both Indian hedgehog (Ihh) and sonic hedgehog (Shh) have been shown to play a role in the development of the ENS.[109,111,112] Embryonic *Ihh*$^{-/-}$ mice are missing enteric neurons from some regions of the small intestine (Fig. 3), but the cellular and molecular mechanisms underlying the phenotype have not been elucidated.[111] In embryonic *Shh*$^{-/-}$ mice, some neurons are ectopically located under the epithelium instead of being restricted to myenteric and submucosal ganglia.[111] In vitro, Shh promotes the proliferation of enteric crest-derived cells directly, and inhibits GDNF-induced neuronal differentiation and migration.[112] Thus, the ectopic location of enteric neurons observed in the null mutants could be due to direct effects of Shh on the migratory behaviour of crest cells, or to influences of Shh on the mesenchyme and the concentric patterning of the gut wall.[109,111,112]

Transcription Factors

Gene knockout studies have revealed some of the transcription factors necessary for the development of the ENS. Mice lacking Phox2b or Sox10 lack enteric neurons throughout the entire gastrointestinal tract[113-115] (Fig. 3). Mice lacking Pax3 lack enteric neurons from the small and large intestine,[43] while mice lacking Mash1 lack enteric neurons in the esophagus[116] (Fig. 3). These null mutant mice all die during late embryogenesis or shortly after birth.

Phox2b is required for the expression of *Ret*.[115] Sox10 regulates the expression of a number of different genes; it can activate Ret directly by interacting with Pax3,[43,117] or indirectly by inducing Phox2b.[118] Sox10 also binds to the *Ednrb* gene and regulates the temporal and spatial expression of *Ednrb* in the gut.[72] Mash1 can also indirectly activate *Ret* by regulating Phox2a, a closely related protein to Phox2b.[119,120] Thus, Phox2b, Sox10, Pax3 and Mash1 seem to be required for normal ENS development because they regulate the expression of *Ret* and/or *Ednrb*.

In Sox10 null mutants, crest-derived cells die early.[113] Most Sox10 heterozygous mutant mice are viable, although some exhibit aganglionosis of the terminal colon[113,121] (Fig. 3). In *Sox10*$^{+/-}$ mice, the migration of crest cells through the gut is delayed and there is a reduction in the numbers of progenitor cells and an increase in the proportion of cells expressing neuronal markers.[66,121] These data show that Sox10 is required for the survival and correct cell fate of neural crest cells, in particular for the development of glial cells.[66] Sox10 is probably required for the early survival of enteric crest cells because it is necessary for *Ret* expression, and its role in glial development is probably due to interactions with other genes.[122] Sox8 is a transcription factor that is closely related to Sox10. Sox8 is expressed by enteric neural crest cells, but *Sox8*$^{-/-}$ mice are viable and fertile and show no ENS phenotype.[57] However, double heterozygous mice (*Sox8*$^{+/-}$, *Sox10*$^{+/-}$ mice) have a more severe ENS phenotype than *Sox10*$^{+/-}$ mice alone, suggesting that Sox8 functions as a modifier gene.[57]

SIP1 (smad-interacting protein-1), encoded by the *ZFHX1B* gene, is a transcription factor that appears to repress expression of target genes. Mutations in *ZFHX1B* have been implicated in HSCR-mental retardation syndrome, which is HSCR associated with multiple congenital anomalies.[80,123] In mice lacking *Zfhx1b*, the neural tube does not close and vagal neural crest cells fail to form.[124] Hence Sip1 appears to be required for the formation of the ENS because it is necessary for the formation of vagal neural crest cells in the neural tube.

Hox11L1 is expressed by enteric smooth muscle cells and by developing enteric neurons.[125] Mice with a null mutation in the Hox11L1 gene exhibit pseudo-obstruction (mega-colon),[125] and have been reported to have ENS hyperplasia in the colon.[126,127] However, a more recent study reported no detectable differences in the numbers of enteric neurons in the colon of mutant mice compared to wild-type mice.[125] Thus, it seems likely that the pseudo-obstruction phenotype observed in the Hox11L1 null mutant mice is due to myogenic, rather than ENS, defects.[128]

Other Molecules

Retinoic acids (RA) act at nuclear retinoic acid receptors to regulate the transcriptional activity of target genes. The main synthetic enzyme for retinoic acids during development is retinaldehyde dehydrogenase 2 (RALD2). *Raldh2*$^{-/-}$ mice die around E10 because of severe

cardiovascular defects, but maternal retinoic acid supplementation can prolong the survival of *Raldh2*$^{-/-}$ embryos until late gestational stages.[129] RA-rescued *Raldh2*$^{-/-}$ embryos lack an ENS, probably due to defects in the posterior pharyngeal arches and vagal level hindbrain, and consequent defects in vagal level crest cell migration.[129]

Conclusions

The development of the ENS from the neural crest encapsulates nearly all the major events of development, such as induction, cell migration, proliferation, stem cell replication and lineage restriction, cell associations, differentiation, neurite elaboration and connectivity. ENS development from the neural crest shows many desirable attributes both intrinsically and as a model of general developmental processes. This system appears to be highly conserved in vertebrates,[130-133] and many of these processes are of greater scale or duration, better defined and timetabled, and more accessible to experimental manipulation, compared to other early developmental systems. This is allied to a large number of genes implicated in ENS development, several serious clinical problems stemming from ENS dysgenesis (many already related to gene defects), and an array of informative animal mutant models (mostly mouse) (see above). In addition mathematical simulations of early ENS development are also being formulated.[134] Combined, these have placed the ENS at the forefront of research into clinically relevant, genetically complex developmental events.

ENS research is sufficiently detailed now to give an idea of what is not known; it drives home how complex development really is, and highlights the common difficulty in bridging the knowledge gap between genotype and phenotype. For the future, the discovery and isolation of ENS stem cells[107,135-138] and the recent use of neural stem cells in replenishing ENS neural deficits in animal models,[58,139] and even in providing some functional recovery[140] provides hope for clinical applications. But to reap these rewards requires even more exact basic research on ENS genes, molecules and cell biology.

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CHAPTER 12

Growth Factors Regulating Neural Crest Cell Fate Decisions

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Abstract

Because of its unique ability to generate a wide variety of both neural and nonneural derivatives, the neural crest is an ideal model system to study the factors regulating cell lineage decisions in stem and progenitor cells. The use of various cell culture techniques and in vivo functional assays, including cell type-specific gene manipulation in mouse, helped to identify signaling factors involved in this process. Moreover, it became apparent that the biological functions of growth factors acting on neural crest cells depend on the context provided by the extracellular microenvironment. Thus, signaling molecules have to be viewed as parts of complex networks that change with time and location. Neural crest cells have to integrate these signals to ensure the generation of appropriate numbers of differentiating progeny. It will be important to determine how such signaling networks are established and how they elicit multiple signaling responses in neural crest cells to activate appropriate genetic programs.

Plasticity of the Neural Crest

Neural crest cells give rise to multiple cell types, including neurons and glia of the peripheral and enteric nervous system, and nonneural cells such as pigment cells, smooth muscle cells in the outflow tract of the heart, and craniofacial bone and cartilage. To understand how such cellular diversity can be established during embryonic development has been an intriguing, yet largely still open question for many years. The issue has originally been addressed by transplantation of neural tube fragments from quail embryos to chick recipients,[1] which revealed that the neural crest from all levels along the neuraxis has a broad potential. These experiments also indicated that neural crest cells display a certain plasticity, which enables them to respond to local extracellular cues present during cellular migration or at sites of differentiation in the developing embryos.[2]

However, such transplantation experiments only reveal the potential of the neural crest as a cell population. In fact, a broad developmental potential at the population level could either reflect that neural crest cells at a given location or developmental stage consist of multipotent progenitor cells or that they are a mixture of lineage-restricted cells. To reveal multipotency of individual cells, it is therefore necessary to monitor the potential of single cells. In vivo, labeling of individual neural crest cells with either fluorescent dyes or retroviral vectors expressing marker proteins led to the identification of multipotent cells generating a diverse set of differentiated cell types.[2] To facilitate the analysis of cellular multipotency, clonal cell culture systems have been established. Using such systems, the fate and potential of single neural crest

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cells can be assessed by mapping individual cells immediately after isolation and determining the cell type composition of the clones generated by the mapped founder cells. Obviously, this requires that the founder cells can be prospectively identified by virtue of specific cell surface markers. For example, surface expression of the low-affinity neurotrophin receptor p75 allowed labeling of living cells and thus the prospective identification of multipotent neural crest cells either in clonogenic adhesive cultures[3,4] or upon fluorescence activated cell sorting.[5,6] Such experiments showed that progenitor cells able to generate multiple cell types are present in crest-derived tissues (including dorsal root and sympathetic ganglia, sciatic nerves, the gut, visceral arches, and the developing skin) and coexist with cells that are fate restricted in the chosen culture conditions.[1,7] The number of multipotent cells, however, decreases with age, although neural crest cells still displaying several potentials have been identified in the adult gut and skin.

Growth Factors Regulating Neural Crest Cell Fates in Culture

The availability of neural crest cell culture systems not only made it possible to demonstrate multipotency and self-renewal of neural crest cells (and thus to identify cells with stem cell features in the migratory and postmigratory neural crest),[3,5,8] in addition, it also allowed researchers to identify several growth factors able to promote differentiation of neural crest cells into specific lineages.[7,9] In the enteric nervous system, glial-derived neurotrophic factor (GDNF) promotes survival and differentiation of neural crest cells, while endothelin 3 (ET3) prevents their premature neuronal differentiation.[10] Melanocyte development, on the other hand is stimulated by ET3 and stem cell growth factor (SCF).

Using cell culture systems striking differences in factor responsiveness have been detected between cranial and trunk neural crest or between neural crest cells from different target structures of trunk neural crest.[11,12] For instance, members of the transforming growth factor (TGF)β superfamily promote smooth muscle formation and neurogenesis in trunk neural crest cells,[4,13,14] while they suppress these fates in cranial neural crest cell cultures.[11] Similarly, acutely isolated cranial but not trunk neural crest cells efficiently generate chondrocytes in the presence of fibroblast growth factor (FGF)2 and the growth factor sonic hedgehog (Shh). Competence of cranial neural crest cells to give rise to cartilage and bone is conferred by decreased or absent expression of Hox genes, encoding a family of homeodomain transcription factors.[15]

In principle, environmental signals that influence neural crest cell fates can act in two distinct manners: a growth factor could "instruct" a multipotent cell to adopt one particular fate at the expense of all other possible fates; alternatively, a signaling cue could "select" a specific cell lineage, either by supporting the survival of cells appropriate for a given stage and location or by specifically eliminating inappropriate cells (Fig. 1). These possibilities can be distinguished in clonogenic culture systems.[9] Single prospectively identified neural crest cells are mapped and then challenged with different growth factors; when all (or the majority) of the cells behave in the same manner and adopt a specific fate in response to a particular growth factor, the factor is acting instructively on these cells. Such experiments demonstrated the existence of several instructive growth factors regulating the fate of single neural crest stem cells (NCSCs) (Fig. 2).[4-6,12,13,16-18] For example, bone morphogenic protein 2 (BMP2) promotes autonomic neurogenesis[13] by upregulating the expression of the basic helix-loop-helix (bHLH) transcription factor Mash1, which is required for the differentiation of autonomic neurons.[19,20] To a lesser extent, BMP2 can also induce a smooth muscle-like fate in multipotent neural crest cells. Similarly, TGFβ promotes adoption of a nonneural, smooth muscle-like fate by single NCSCs derived from neural tube explant cultures, dissociated sciatic nerve, dorsal root ganglia, and gut.[13] Furthermore, individual NCSCs give rise to glia at the expense of other fates either upon Notch signal activation or upon exposure to neuregulin1 (NRG1). Interestingly, distinct NRG1 isoforms promote the generation of different peripheral glial sublineages. While the membrane-bound NRG1 isoform SMDF promotes Schwann cell development in migratory and postmigratory NCSCs,[21] the soluble NRG1 isoform glial growth factor (GGF) 2 induces the formation of satellite glia expressing the Ets domain transcription factor Erm.[22]

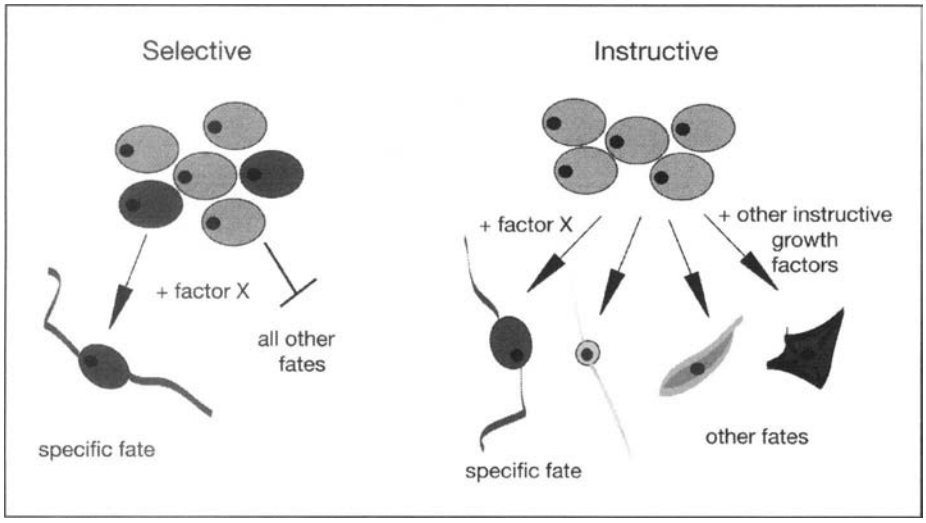

Figure 1. Selective vs. instructive effects of growth factors. Factors can influence the generation of specific cell types in different ways. Growth factors can act selectively on a heterogeneous population of lineage-restricted cells, selectively promoting proliferation or eliminating other cells. Alternatively, growth factors can have instructive effects on a homogeneous population of multipotent stem cells, promoting cell fate specification at the expense of all other possible fates.

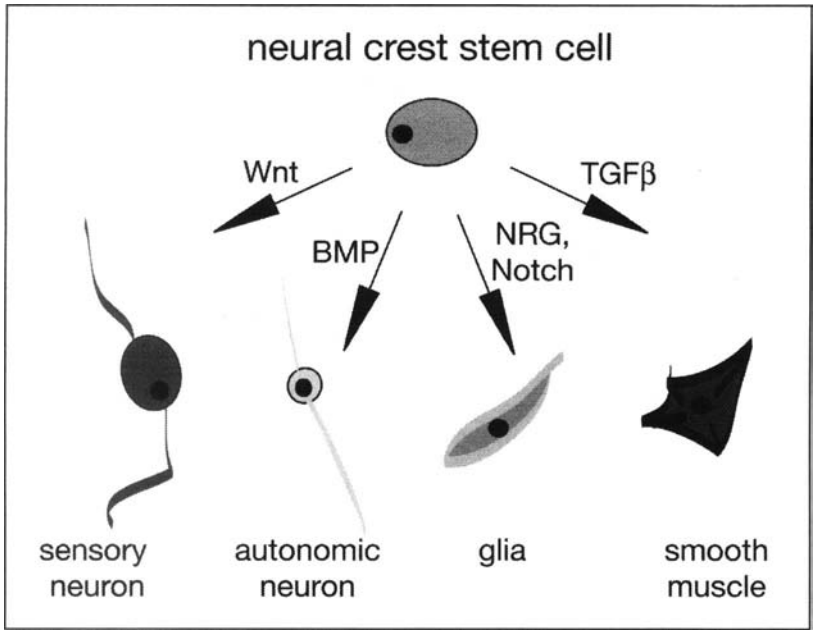

Figure 2. Instructive growth factors acting on neural crest stem cells. Clonal analysis of prospectively identified neural crest stem cells in culture led to the identification of Wnt signaling instructively promoting sensory neurogenesis,[18] BMP2 inducing autonomic neurogenesis,[13] NRG isoforms and Notch signaling promoting gliogenesis,[16,17,21,22] and TGFβ inducing a smooth muscle fate.[13]

Finally, in rodents canonical Wnt signaling, acting via the intracellular molecule β-catenin, instructs early migratory NCSCs (but not postmigratory cells) to adopt a sensory neuronal fate.[18] So far, the signals supporting NCSC maintenance and self-renewal have not been identified, although it has been possible to passage both migratory and postmigratory NCSCs for some generations in a nondefined medium.[3,12] Possibly, the combinatorial activity of multiple signals (rather than individual factors alone) might be involved in neural crest stem cell maintenance.[23] Maintenance of stem cell features is conceivably achieved by factors regulating the expression of the transcription factor Sox10. This hypothesis is based on the findings that Sox10 promotes maintenance of multipotency and growth factor responsiveness in cultured NCSCs[24] and is required for maintenance of enteric NCSCs in vivo.[25]

In the embryo, multipotent neural crest stem and progenitor cells encounter many different signals during migration and at sites of differentiation. Distinct signaling pathways might modulate, strengthen, or inhibit each other, and elicit biological responses in neural crest cells that are not obtained by the individual signals alone.[26] To address this issue, researchers have begun to study whether and how NCSCs can integrate different extracellular signals present in their environment. Culture experiments revealed that BMP2 and TGFβ are codominant in neural crest cells, while these TGFβ family factors suppress gliogenesis induced by NRG1.[27] In contrast, activated Notch signaling suppresses BMP2-dependent neurogenesis.[16] Furthermore, the biological activity of instructive growth factors can be modulated by short range cell-cell interactions termed community effects.[28] While single migratory and postmigratory NCSCs give rise to smooth muscle-like cells in response to TGFβ factors, cell communities generate autonomic neurons or—at slightly higher factor concentrations—are eliminated by apoptosis upon TGFβ factor treatment.[4,29] Thus, networks of synergistic and antagonstic signals are likely to regulate the development of neural crest derivatives in correct numbers at the proper time and embryonic localisation.

Factors Involved in Neural Crest Development in Vivo

Although cell culture experiments can provide valuable hints about the identity of factors involved in neural crest development and might give important insights into their mode of action, the function of candidate regulatory molecules has also to be addressed in vivo. Extracellular factors that influence neural crest cell fates in culture, such as Shh, BMP, TGFβ, NRG1 isoforms, Notch signaling components, and Wnt proteins are expressed in a manner consistent with a role in regulating fate decisions during neural crest development.[7,23] In chick embryos, BMP signaling is required for autonomic neurogenesis, as has been demonstrated by experiments with the BMP antagonist Noggin.[30] Moreover, virus-mediated BMP overexpression increases the size of sympathetic ganglia and causes the generation of ectopic sympathetic neurons.[14] In mice, mutations in NRG1 signaling components lead to reduced numbers of Schwann cells (but not of satellite glia), although it is unclear whether glial fate specification is indeed affected in these mutants.[31] While in the CNS, Notch signaling regulates stem cell maintenance and promotes gliogenesis,[32-35] the role of Notch in PNS development remains to be determined. To address the role of Shh, a function-blocking antibody has been injected into the chick cranial mesenchyme.[36] Similar to cranial neural crest ablation, perturbation of Shh signaling results in a significant reduction in head size due to increased cell death of cranial neural crest cells that normally contribute to the craniofacial skeleton.

Because germ line deletion of genes encoding factors involved in neural crest cell fate regulation often results in embryonic lethality, stage- and lineage-specific gene ablation and gain-of-function experiments have to be performed to investigate the in vivo functions of these molecules. The system of choice for such conditional gene manipulation has turned out to be the *Cre/loxP* system (Fig. 3).[37] In order to generate NCSC-specific mutations, a so-called "floxed allele" of the gene of interest is generated by flanking genomic sequences essential for the function of the gene by *loxP* sites. These short DNA elements are recognized by an enzyme called Cre recombinase. Mice carrying a floxed allele are then mated with

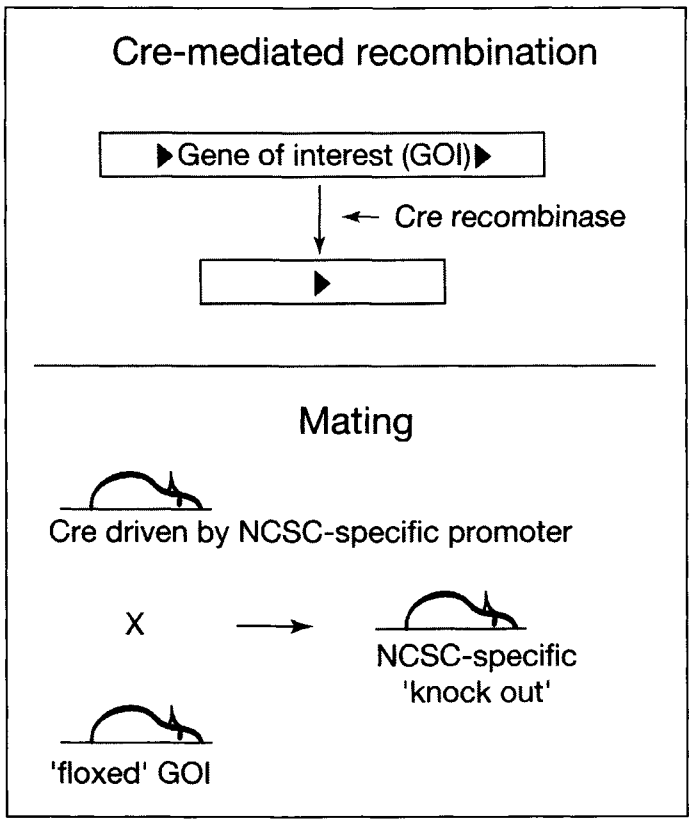

Figure 3. Conditional, cell type-specific gene ablation in mice. Conditional gene ablation is performed by using mice that carry a so-called 'floxed allele', i.e., an allele in which the gene of interest is flanked by loxP sites (triangle). Cell type-specific expression of Cre eliminates the gene of interest (GOI). Additionally, inducible forms of Cre recombinase are available,[63] allowing not only cell type-specific but also stage-specific gene manipulation. For neural crest stem cell (NCSC)-specific gene manipulation, mice that express Cre recombinase from a NCSC-specific promoter (*wnt1-Cre* mice[64] have often been used for this purpose[18,51,54]) are mated with a mouse carrying a floxed allele of the gene of interest.

transgenic mice expressing Cre recombinase in NCSCs. This leads to Cre-mediated deletion of the floxed allele and thus to cell type-specific manipulation of gene function. Such an approach has revealed that β-Catenin is crucial for sensory neurogenesis and melanoycte development.[38] β-catenin mediates both cadherin-dependent cell-cell interactions and canonical Wnt signaling.[39] While cadherins appear to influence neural crest specification and emigration from the neural tube,[40,41] experiments in *Xenopus*,chicken, mouse, and zebrafish revealed that Wnt signaling regulates neural crest induction, expansion, delamination, and melanocyte development.[42-46] In mice, *β-catenin* is efficiently ablated in virtually all NCSCs using the *Cre/loxP* system.[38,47] Both in vivo and in culture, mutant NCSCs emigrate and proliferate normally but fail to undergo sensory neurogenesis.[38] In particular, mutants do not express the bHLH transcription factors ngn1 and ngn2, which control sensory lineage specification in the emigrating neural crest and sensory neurogenesis in aggregating dorsal root ganglia.[48] In complementary gain-of-function experiments, canonical Wnt signal activation in NCSCs promoted sensory fate decisions.[18] In particular, NCSCs expressing constitutively active β-catenin produce sensory neurons at the expense of virtually all other crest

derivatives, as also shown by in vivo fate mapping of mutant cells. Some of the sensory ganglia in these mutants are even found at ectopic cranial locations of the embryo that are usually devoid of neural derivatives of the neural crest. Similarly, sensory rather than sympathetic neurons are generated at sites of normal sympathetic ganglia formation. As in the loss-of-function experiments,[38] NCSCs expressing sustained β-catenin activity migrate and proliferate normally in culture.[18] However, the mutant cells give rise to sensory neurons, while stem cell marker expression is suppressed. Together with the above-mentioned cell culture studies in which NCSCs have been exposed to Wnt protein, the combined data demonstrate that canonical Wnt signaling promotes sensory fate decision in multipotent NCSCs. This is in contrast to the role of Wnt in other types of stem cells, in which it controls proliferation and stem cell expansion.[23] These data also raise the question of how Wnt signaling can elicit so many different responses during neural crest development, ranging from neural crest induction, delamination, and NCSC expansion to melanocyte formation and neurogenesis.[18,42,45,46] Possibly, neural crest cells might acquire intrinsic changes over time that influence Wnt responsiveness in a developmental stage-dependent manner.[49] This assumption has been supported by experiments performed in zebrafish, in which blocking of Wnt signaling at various time points indicated its reiterated but distinct roles in neural crest development.[50]

Cre-mediated gene deletion was also used to further address the role of Shh in neural crest development in vivo.[51] Comparable to facial abnormalities observed in human *SHH* mutants, mice with neural crest cells lacking *shh* display failures in craniofacial skeleton and nonskeletal structures developing from neural crest-derived cranial mesenchyme. In contrast, peripheral neural tissues are normal in the mutant. Intriguingly, lack of Shh does not interfere with neural crest migration or survival but rather affected the development of postmigratory cranial neural crest cells. This process is conceivably mediated by the activity of transcription factors of the forkhead family, which are regulated by Shh and have also been implicated in head skeleton formation.[52,53]

Craniofacial anomalies are also observed upon neural crest-specific deletion of the *TGFβ receptor type II (TβRII).*[54] In addition, inactivation of TGFβ signaling in neural crest cells also results in cardiovascular defects and the formation of hypoplastic or aplastic thymic and parathyroid glands. All these affected tissues derive from the pharyngeal apparatus and contain neural crest-derived cells. Strikingly, the *TβRII* -mutants display all morphological features found in patients suffering from DiGeorge syndrome, the most common microdeletion syndrome in humans.[55] Moreover, neural crest-specific inactivation of the type I BMP receptor Alk2 also results in cardiac outflow tract malformations (but not in other tissue anomalies associated with DiGeorge syndrome), further supporting the role of TGFβ family factors in the development of nonneural tissues derived from the neural crest.[56] Cardiovascular defects and some other symptoms characteristic for DiGeorge syndrome also develop by manipulations that interfere with neural crest cell migration into the pharyngeal apparatus and/or their survival, as seen for instance upon cardiac neural crest ablation in chicken or neural crest-specific deletion of *FGF8* in mouse.[57-60] In contrast, *TβRII* -mutant neural crest cells migrate normally into the pharyngeal apparatus, but fail to express early markers of the smooth muscle and osteochondrocyte lineages and are unable to acquire nonneural cell fates.[54] This is consistent with the previously mentioned role of TGFβ in promoting nonneural lineages in cultured NCSCs.[13] Furthermore, TGFβ signaling in neural crest cells of the pharyngeal apparatus is both sufficient and required for phosphorylation of CrkL, a signal adaptor protein implicated in DiGeorge syndrome.[61] Thus, TGFβ signal modulation in neural crest cells is possibly involved in the development of this disease. In support of this hypothesis, human patients with mutations in *TβRI* and *TβRII* also develop cardiovascular and craniofacial deficiencies.[62] This underlines the usefulness of conditional mouse mutants as model systems to study the etiology of human diseases associated with aberrant neural crest development.

Conclusions

It becomes more and more evident that during neural crest development, a complex network of signaling cascades rather than individual signaling pathways controls cell proliferation, lineage specification, and differentiation. The combination of signals involved likely changes with time and space, which makes it a challenge for the researcher to identify the crucial knots in the signaling network that determine neural crest cell fates. In the future, functional genomics and proteomics might provide several candidate molecules involved. Because the activities of several factors can be tested in defined but changeable contexts, cell culture experiments are helpful to elucidate the function of such candidate factors and factor combinations. However, it is also necessary to complement such approaches by functional in vivo studies, including animal models carrying cell type-specific and inducible mutations.

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CHAPTER 13

Neural Crest Stem Cells

Lu Teng and Patricia A. Labosky*

Abstract

Stem cells are defined by their ability to both self-renew and give rise to multiple lineages in vivo and/or in vitro. As discussed in other chapters in this volume, the embryonic neural crest is a multipotent tissue that gives rise to a plethora of differentiated cell types in the adult organism and is unique to vertebrate embryos. From the point of view of stem cell biology, the neural crest is an ideal source for multipotent adult stem cells. Significant advances have been made in the past few years isolating neural crest stem cell lines that can be maintained in vitro and can give rise to many neural crest derivatives either in vitro or when placed back into the context of an embryo. The initial work identifying these stem cells was carried out with premigratory neural crest from the embryonic neural tube. Later, neural crest stem cells were isolated from postmigratory neural crest, presumably more restricted in developmental potential. More recently it has been demonstrated that neural crest stem cell progenitors persist in the adult in at least two differentiated tissues, the enteric nervous system of the gut and the whisker follicles of the facial skin. In all cases, the properties of the stem cells derived reflect their tissue of origin and the potential of the progenitors becomes more restricted with age. In this chapter we will review this work and speculate on future possibilities with respect to combining our knowledge of neural crest gene function in the embryo and the manipulation of adult neural crest stem cells in vitro and eventually in vivo.

Neural Crest Stem Cells

At the onset of migration, neural crest (NC) cells are a heterogeneous mixture of cell populations with extensive proliferative and developmental potential. Later, the postmigratory crest appears either fate-restricted, highly committed or unipotent. However, there are some limited number of long-term pluripotent progenitor cells derived from the embryonic NC that persist in the adult. These rare cells have the capacity for both self-renewal and generation of multiple differentiated progenies in vitro, and are therefore bona fide stem cells. They exist in several NC-derived tissues in both the embryo and postnatal animal and represent an enticing source of stem cells with potential for therapeutic applications. Figure 1 illustrates the location of these progenitors at different stages and in specific locations of the developing mouse embryo that will be referred to throughout.

Identification of Neural Crest Stem Cells (NCSCs)

Self-renewing multipotent NCSCs were originally isolated by Stemple and Anderson from premigratory neural crest by taking advantage of expression of the low affinity nerve growth

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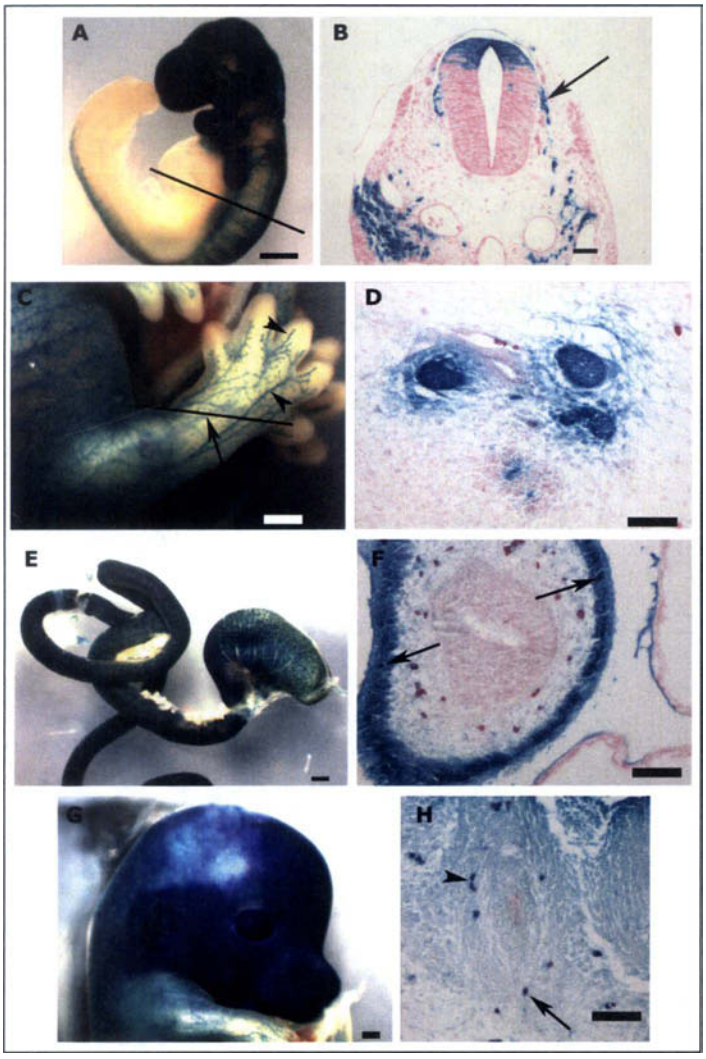

Figure 1. *Wnt1-Cre; Rosa26R lacZ* positive cells during different stages of mouse embryogenesis illustrate the various NC lineages from which NCSCs have been generated. A,B) At 9.5 dpc early migrating NC is exiting the neural tube and populating the branchial arches. Cells such as those indicated with the arrow in panel B are the ones that Stemple and Anderson isolated in vitro to first culture NCSCs.[1] C) At 14.5 dpc the peripheral nervous system has extended well into the developing limbs. The developing sciatic nerve is internal and indicated by the arrow. Arrowheads indicate superficial peripheral nerves. D) A section through the hindlimb of a 14.5 dpc embryo counterstained with eosin shows a developing sciatic nerve similar to that which Morrison et al isolated NCSCs.[5] E,F) At 17.5 dpc the entire digestive tract is populated with NC-derived enteric nervous system. This is the region from which gut derived NCSCs are isolated.[25] A section from a 14.5 dpc gut in F shows the location of the NC in the outer layers of the myenteric plexus and not in the gut epithelium. G,H) The 14.5 dpc facial skin has a very high contribution of NC cells. Some NC derived cells are found near the bulge region of the whisker follicles (arrowhead in H) while some are found near the dermal papilla (arrow in H). There are many other NC-derived cells scattered throughout the skin. These cells are the progenitors for NC-derived facial SKPS and eNCSCs.[26,31] Scale bars are equal to 0.5mm in A, C, E and G and 0.05 mm in panels B, D, F and H.

factor receptor (also known as p75 or p75NTR) and nestin in a limited population of cells.[1] The p75 neurotrophin receptor binds several related growth factors: nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4. This receptor has been demonstrated to play an important role in modulating the susceptibility of specific cell populations to programmed cell death (reviewed in ref. 2). Nestin, an intermediate filament expressed in neuroepithelial stem cells is often expressed in multi-lineage progenitor cells and differentiation of the cells is associated with loss of immunoreactivity to nestin. Embryonic rat neural tubes (E10.5) were placed in culture and cells that migrated away from the neural tube (presumably the premigratory NC initiating migration) were isolated either by fluorescence-activated cell sorting (FACS) for p75 expression or manually. Serial subcloning of cells and extended culture times demonstrated that these cells underwent self-renewal in vitro. Prolonged time in culture revealed that NCSCs were multipotent based on their ability to differentiate into peripheral neurons, Schwann cells and smooth muscle cells as assessed by expression of lineage specific markers.[1,3]

Molecular Control of NCSC Differentiation

Multiple factors affect either the self-renewal and/or the multipotency of NCSCs; addition of fetal bovine serum (FBS), containing a myriad of uncharacterized growth factors, causes NCSCs to differentiate. Alterations in the substrate molecules fibronectin (FN) and poly-D-lysine (pDL) influence cell fate. The addition of bone morphogenic proteins 2 and 4 (Bmp2 and 4) induces neurogenesis in NCSCs, transforming growth factor beta molecules (Tgfβ1, 2 and 3) promote smooth muscle differentiation, and glial fates are observed by adding Neuregulin I (NrgI; also called glial growth factor 2) to the culture medium.[1,3,4]

While these initial experiments demonstrated that premigratory NC contained multipotent progenitors, later work by Sean Morrison in Anderson's laboratory showed that the postmigratory embryonic NC also contained progenitors with similar potential.[5] Sciatic nerves from late gestation rat embryos (up to E17.5) contained NCSCs that gave rise to neurons, glia (Schwann cells) and myofibroblasts, but the potential of the progenitor cells decreased with increasing embryonic age (E14.5 compared with E17.5). Expression of p75 and the lack of expression of P_0, a peripheral myelin protein normally expressed by Schwann cells, was used to enrich the NCSC progenitors, although the populations are still not pure and the precise in vivo location of the progenitors is still not identified.

Strikingly, however, NCSCs isolated from sciatic nerve respond differently to Bmp2 than do premigratory NCSCs from the neural tube, presumable reflecting potency changes in the cells.[6] Other non-NC stem cell populations require Tgfβ related molecules to remain undifferentiated and self-renewing: Bmp4 is sufficient to derive embryonic stem cells (ES cells) and Nodal and/or Activin is required to derive trophoblast stem cells (TS cells),[7-10] but in the case of NCSCs it appears that Bmp2 and/or Bmp4 promote neuronal differentiation of progenitors. *Bmp2-/-* embryos contain fewer NC cells in vivo than wild type,[11] however, NCSCs have not yet been isolated from *Bmp2-/-* embryos to determine if they have defects in neurogenesis. Genetic evidence from mouse embryos carrying a mutation in *Bmpr1a*, a Bmp2/4 type I receptor normally expressed in the dorsal neural tube, showed a requirement for Bmp signaling in the NC cells that form the outflow tract of the heart,[12] but again, the role of this receptor has not been examined specifically in NCSCs.

This observation that NCSCs from different sources respond differently to growth factors was reinforced by Morrison's observations that NCSCs isolated from embryonic gut (the enteric nervous system) versus sciatic nerve respond differently to Bmp4; both types of NCSCs form neurons when exposed to Bmp, but gut NCSCs were 5 to 10 fold more responsive to Bmp4 than NCSCs from the sciatic nerve.[13] However, gut NCSCs are not simply more sensitive to all growth factors. Gut NCSCs were less likely than sciatic nerve NCSCs to form glia at lower concentrations of the Notch ligand Delta. A similar response was seen with Neuregulin, which activates members of the Epidermal Growth Factor Receptor/ErbB family of

proto-oncogenes. This is perhaps expected, since progenitor cells in the sciatic nerve would be primarily involved in forming glial cells while NC progenitors of the enteric nervous system are more likely to form neurons, but it is important to note this bias of NCSCs from different regions of the embryo to differentiate into terminal fates. In fact, it is known that between 10.5dpc and 14.5dpc, neural crest stem cells (NCSCs) become increasingly sensitive to Delta.[14] In vivo evidence also supports the observation that not all NCSCs are equal; when NCSCs from either gut or sciatic nerve were transplanted into the neural crest migratory stream of the chick embryo, gut NCSCs gave rise primarily to neurons while sciatic nerve NCSCs gave rise exclusively to glial cells.[13] These data support the hypothesis that postmigratory NCSCs are not equivalent and that both the stem cell niche and age of the cells influence the multipotency of these progenitor cells. However, these data do not distinguish whether these effects are cell autonomous and/or if the qualities of the NCSC niche are changing over time.

There is considerable interest in the molecules that regulate stem cell maintenance and inhibit differentiation. Microarray analysis of gut-derived NCSCs has generated a list of many transcription factors, secreted ligands and cell surface receptors that are certainly involved in these processes.[15] One of these is the HMG transcription factor Sox10. *Sox10* mRNA was almost 13 times more abundant in gut NCSCs than in the fetus as a whole. Sox10 is expressed in migrating NC cells, then maintained in the glial lineage while downregulated in NC-derived neurons and required for the differentiation of peripheral glial cells.[16] Sox10 plays multiple roles in NC development and it is suggested that it may be especially important for NCSC maintenance. Anderson's lab has shown that Sox10 can override the effect of soluble lineage restricting growth factors on NCSC; both neurogenic and gliogenic differentiation capacity is maintained despite exposure to Bmp2 and/or Tgfβ. At the same time, higher levels of Sox10 inhibit both neuronal and smooth muscle differentiation, thereby maintaining multipotency of the cells.[17] Another transcription factor, Erm, a member of the Pea3 subfamily of Ets domain proteins, is expressed in multipotent neural crest progenitor cells and glial cells. Blocking Erm function in NCSCs did not disrupt differentiation or survival but it severely impeded proliferation of the cells.[18] The basic helix-loop-helix protein Twist is required within the first branchial arch for the proper migration of NC cells into the arch. Later, Twist is also required for normal differentiation of first arch derivatives into bone, muscle, and teeth, but it has not been investigated if this protein is similarly required for differentiation of NCSCs.[19] Mouse mutants in both Snail and Foxd3, two proteins expressed in early NC cells that have been shown to play a role in NC formation in Xenopus and chick, die before the NC can be specified so tissue-specific deletions will be required to address the role of these transcription factors in the NC and in NCSCs.[20,21] Curiously, a mutation in the Snail-related transcription factor Slug has no phenotype in the NC and it is likely that the expression of Snail may compensate for the mutated *Slug* gene.[22]

As discussed above, NCSCs have different responses to growth factors based on the location and age of the progenitor cells. However, like central nervous system neural progenitors, NCSCs require bFGF to remain undifferentiated. Although the stem cell culture medium is fairly well defined (no serum is present) there is a high percentage (15%) of chicken embryo extract added that provides many undefined factors. Therefore, the minimally required factors to maintain the stem cell characteristics of NCSCs are still unknown. Many other stem cell populations utilize Wnt signaling to control proliferation, thereby expanding the stem cell pool and maintaining the transit amplifying pool of progenitors. However, NCSCs are unlike these stem cell populations in their requirement for the Wnt signaling pathway. Wnt signaling in migrating NC cells does not affect the population size; instead, β-catenin activation by Wnt signaling promotes differentiation of sensory neurons at the expense of other neural crest derivatives.[23] However, Wnt1, in combination with Bmp2, maintains p75 and Sox10 expression in NCSC cultures and inhibits differentiation of the cells.[24] Therefore, much is left to explain in terms of the minimal requirements for secreted signals, and it is likely to be a combination of factors that will change with respect to the location of the progenitor cells.

Postnatal NCSCs, Disease and Cell Therapy

Further work from Morrison's group showed that postnatally, sciatic nerve derived progenitors are either not present or at least not receptive to the same culture conditions. However, gut-derived NCSCs progenitors exist in the gut as late as postnatal day 110 in the rat.[25] These gut NCSC progenitor cells change their cell surface markers over time; E14.5 cells are p75+, α4integrin+, while the postnatal gut progenitors are largely p75+, α4integrin-. But more importantly, their ability to self-renew and differentiate into tyrosinase hydroxylase (TH) or dopamine-β-hyroxylase (DβH) expressing neurons decreases substantially with age.[25]

The link between stem cell function in vivo and stem cell dysfunction in disease is well illustrated in the case of NCSC defects in Hirschsprung's disease which is caused by a failure of the enteric nervous system to innervate the gut properly. Microarray analysis of gut NCSCs revealed elevated expression of several of the genes linked to Hirschsprung's disease including *Ednrb*, *Gfra-1*, *Ret*, and *Sox10*. NCSCs from *Ret-/-* mouse embryos were examined and it was confirmed that the anterior-most NCSCs in the esophagus were normal while the more posterior NCSC progenitors in the stomach and intestine were reduced in number.[15] The ability to isolate and characterize NCSCs from other mouse models of NC disorders may reveal more about the genes required to maintain this progenitor pool in the embryo and control their differentiation in the adult. So far it appears that the stem cell potential for postnatal NC-derived tissues is far less than that of embryonic tissue, but this raises the question of why these progenitors exist at all in the postnatal setting. Perhaps they are an accessible source of progenitor cells to repair tissues subject to either injury or degeneration, or perhaps they are simply remnants of embryogenesis. It is also possible that these progenitors are the stem cells of origin for neuroblastomas, one of the most common pediatric tumors that originates from the neural crest.

The most potent NCSCs discussed so far originate from internal tissue sources and embryonic and/or early postnatal ages, making them relatively impractical for cell therapeutic approaches. Embryonically derived stem cells are difficult to obtain and are fraught with ethical and political controversies and restrictions, so the development of adult sources for stem cells is ideal for therapy. The most accessible tissue of the body and one in which most patients easily tolerate biopsy is the skin. Freda Miller's group demonstrated that it was possible to culture self-renewing, multipotent cells from the dermis of rats, mice and human.[26-28] These SKP cells (for **sk**in derived **p**recursor cells) are different from previously described NCSCs; they are p75$^-$, they require Fgf2 and Egf in the culture medium, and they grow as nonadherent spheres similar to central nervous system-derived neural stem cells. However, many of their characteristics are similar to both NC and NCSCs; they can differentiate into neurons, glia and smooth muscle, are more easily generated from embryonic versus adult skin and express a number of NC specific molecular markers: *Slug*, *Snail*, *Twist*, *Pax3* and *Sox9*. The SKP progenitors were not present in embryonic skin before embryonic day 14, coincident with the time that the NC derived peripheral nerves populate the skin. Although all regions of the skin have progenitors that generate SKPs, Miller's group showed conclusively that SKPs isolated from facial dermis are derived from the NC.[26] This was accomplished by indelibly marking the NC with *lacZ* using a *Wnt1-Cre* transgene in combination with the *R26R* reporter allele activated by Cre expression.[29,30] SKPs derived from facial dermis expressed beta-galactosidase and are therefore NC-derived. Miller's group went on to show that cells in or near the dermal papilla of both hair and whisker follicles in the face contain the NC-derived cells that are presumably the SKP progenitors.[26] However, Sieber-Blum and colleagues demonstrated that similar NC-derived progenitor cells give rise to what they termed eNCSC (for epidermal NCSC) and reside in the bulge area of the hair and whisker follicles.[31] It is not apparent what the differences are between these two populations, but it is clear that both groups are studying NC derived adult stem cells. SKPs have been derived from both adult human scalp samples and juvenile foreskin samples,[27,28] and the human SKPs can generate neurons, glia and smooth muscle, and maintain a normal karyotype for over a year in culture. It is intriguing to imagine the possibility of using these multipotent human NCSCs to generate differentiated cells for therapeutic uses.

The discovery of NCSCs in postnatal skin raises the possibility that other NC niches might contain progenitors that could be readily available for isolation, manipulation and potential therapeutic uses. One tantalizing and very accessible postnatal niche is the tooth. Progenitor cells have been isolated from human teeth; these so-called SHED cells (for stem cells from human exfoliated deciduous teeth) grow as multipotent spheres in vitro. SHED cells express markers common to mesenchymal stem cells (Stro-1 and CD146) and can differentiate not only into odontoblasts in vitro and in tumor models, but also into neurons and glia in vitro and when transplanted into the dentate gyrus of a mouse hippocampus.[32] Lineage labeling using the *Wnt1-Cre*; *Rosa26R* mice, as described above, revealed that the dental pulp originates from the NC, so it is possible that SHED cells also derive from NC.[29] This remains to be tested, but it is possible that such an accessible source of postnatal stem cells would be readily adaptable for cell therapies. Our knowledge of the factors that control the specification, migration, patterning and multipotency of the embryonic NC, when combined with the experience of stem cell biologists should lead to promising avenues of exploration and hopefully, to the development of effective stem cell therapies in the future.

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CHAPTER 14

Molecular Bases of Human Neurocristopathies

Heather C. Etchevers,* Jeanne Amiel and Stanislas Lyonnet

Introduction

Neural crest cells (NCC) form in the human embryo during the third to fifth weeks of pregnancy, within the neural folds that delineate the neural plate from the ectoderm. During the fusion of the neural folds, which ultimately yields a tube that will become the central nervous system (CNS), NCC detach and become mesenchymal. They migrate throughout the body, integrating nearly every organ.

NCC derivatives include the neurons and support cells of the entire peripheral nervous system (sensory and autonomic), adrenergic and other endocrine cells, and all pigment cells except those arising from the retina (reviewed by Le Douarin and Kalcheim[1]). In the head, in addition to the cell types mentioned above, NCC differentiate into connective and structural tissues such as dermis,[2,3] bones and cartilage of most of the skull[3,4] and muscle tendons.[5,6] They also infiltrate and are essential for the function of glandular and vascular elements such as the thymus, the thyroid and parathyroid glands, the conotruncal region of the heart and the entire branchial vascular sector,[7-9] giving rise to connective, adipose and smooth muscle cells.

The astonishing diversity of NCC derivatives has led to this population being nicknamed the "fourth embryonic germ layer." The fact that NCC were known to exist only in the embryo precluded their being perceived as a true stem cell type. Recently, however, it was demonstrated that the enteric nervous system of the adult rat contains neural crest stem cells that self-renew and remain oligopotent.[10] Avian melanocytes are able to transdifferentiate into glial cells, neurons or smooth muscle-like cells in vitro, also implying the long-term existence of multipotent progenitors.[11] It is thought that NCC derivatives are generated through progressive restriction of developmental potential.[12,13]

The ultimate choice in phenotype made at a given site of differentiation is the result of a combination of extrinsic factors in the embryonic microenvironment and cell-intrinsic properties that modify its responsiveness to these external influences. The former have been documented through observation of the disruption of neurotrophic growth factors and receptor genes that result in deficiencies of selected subsets of NCC derivatives.[14,15] Both their migration pathways and fate are imposed on NCC by surrounding tissues as they leave the neural primordium; these are not dependent on intrinsic properties regionally distributed along the neuraxis, as had initially been presumed. For instance, truncal NCC transplanted at the vagal level colonize the gut and differentiate into enteric ganglia in which neurons synthesize acetylcholine rather than catecholamines, as they would have done normally at the truncal level.[5] A notable exception, the cephalic NCC contributing to the branchial arch-derived facial skeleton has some intrinsic positional information and commitment,[16,17] apparently imparted by the rostral endoderm before and during their emigration from the neural folds.[18,19]

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Definition of a Neurocristopathy

Abnormal migration, differentiation, division or survival of NCC lead to organ and tissue dysplasias with highly diverse clinical and pathological features. Referring to their proposed common embryological origin, Bolande first introduced the concept of "neurocristopathy" in order to highlight the potential for shared pathogenetic mechanisms.[20]

Nuanced definitions of a "neurocristopathy" have been made due to the great diversity in NCC derivatives and the organs in which they play an integral functional role over time. One can attempt to remain "purist," including only those clinical entities where direct derivatives of the neural crest are affected. However, in order to be useful, the term neurocristopathy over the last thirty years has also been applied to include entities where abnormal NCC affect the development of other tissues not themselves solely derived from NCC, such as the heart or the thyroid gland. Unfortunately, there have been inconsistencies in the inclusion (neurofibromatosis type II) or exclusion (craniostenoses) of many pathologies. At the time any given disease has been termed a neurocristopathy, its classification was based on a corpus of knowledge that has been subject to enormous change with the advent and renewal of experimental molecular embryology.

The situation has hardly been clarified by molecular studies. In those rare cases where truly only NCC derivatives are affected and the responsible gene is identified, it usually turns out to be an evolutionarily conserved gene with distinct known functions in separate organ systems (or "fields") in humans or animals. Early developmental genes seem to affect multiple germ layers and moments in cellular existence through an evolutionary sort of functional recycling known as "cooption" (reviewed in ref. 21). Why are NCC more vulnerable than other cell types? Like many other embryonic cell types, they undergo all the processes of epithelial-mesenchymal transition, proliferation, migration, a drawn-out period of differentiation with maintenance of plasticity, niche occupation and apoptosis. Perhaps the adaptability of the evolutionarily recent NCC,[22] useful for making wildly diverse head and body appendices,[23] is its Achilles' heel with respect to pathogenesis.

Certain disorders have not been included as neurocristopathies below. In particular, we have excluded those that arise from functional deficiencies in differentiated NCC. Thus, although oculocutanous albinism does indeed affect neural crest-derived melanocytes, it is less directly a result of their *development* but rather of their final *metabolic function* (to synthesize melanin). We have also, with more difficulty, left aside those clinical entities largely involving fields in which NCC play no part during development. These would comprise the limb, kidney, and liver, all of which can be associated with defects in NCC-derived tissues (e.g., von Hippel-Lindau syndrome with pheochromocytoma; tuberous sclerosis syndrome with hypopigmented or café-au-lait macules). The aim is to restrict the definition to those entities in which a NCC defect is causative, excluding secondary phenomena. Simple craniostenoses (craniosynostoses), for example, have not been included because they appear to represent defects in osteoblast function and are also associated, in syndromic forms, with bone problems in the limbs, ribs or vertebrae, none of which have a NCC component. One exception, craniofrontonasal syndrome, will be mentioned among the gene cascades involved in NCC migration.

Here, we attempt to describe which entities we consider to be neurocristopathies and why, based on recent advances in basic and clinical research.

Clinical Appreciation of Neurocristopathies

When considering a patient with multiple congenital birth defects, it is clearly useful to take an embryological point of view in order to find an underlying common cause. NCC colonize four compartments unequal in size: the skin, the peripheral nervous system, some of the endocrine system and a pharyngocephalic pole. Anomalies affecting any of these compartments can be considered to arise from one initial field, and warrant closer examination of the other compartments. Table 1 presents isolated versus syndromic neurocristopathies on one axis

Table 1. Examples of neurocristopathies according to compartment and type

	Skin	Peripheral Nervous System	Endocrine	Pharyngocephalic
Cancer:				
Isolated	·Melanoma ·Merkel cell carcinoma	·Neuroblastoma ·Schwannoma ·Paraganglioma	·Pheochromocytoma ·Familial or sporadic medullary thyroid carcinoma ·Chromaffin paraganglioma ·Carcinoid tumors	·Hemangiocytoma ·Non-chromaffin paraganglioma (ear)
Syndromic	·Neurofibromatosis I[#] ·Neurocutaneous melanosis	·Hirschsprung+neuroblastoma	·Multiple endocrine neoplasias 2A,B	·Congenital central hypoventilation
Malformation:				
Isolated	·Congenital giant nevus ·Piebaldism	·Hirschsprung		·Cerebrodural arteriovenous malformations ·Cleft palate/lip ·Moya-moya ·Isolated conotruncal cardiopathies ·Aplasia of lacrymal and salivary glands
Syndromic	·Sturge-Weber	·Waardenburg ·Familial dysautonomia type 2	·Allgrove ·Bamforth-Lazarus	·CHARGE* ·DiGeorge ·Pierre Robin ·Holoprosencephaly*[#] ·Kallmann* ·Rieger* ·Binder* ·Mœbius* ·Johanson-Blizzard* ·Treacher-Collins-Franceschetti ·Craniofrontonasal[#] ·Goldenhar*[#] ·Oro-facial-digital*[#]

The endocrine component of the syndromes marked with an asterisk (*) may be imputed to non-endocrine neural crest cells that are necessary for the formation of the pituitary gland. Conditions that combine a neurocristopathy with non-neurocristopathic malformations are indicated by a number sign (#).

and the useful distinction between cancers or malformations on the other. Naturally, the syndromic neurocristopathies may have both cancerous and malformative components. It is truly rare among these rare diseases, to see a "pure" syndromic neurocristopathy. However, the concept of "neurocristopathy" is useful to the clinician in orienting their approach to the patient by grouping apparently divergent and unrelated signs under one umbrella.

Isolated Neurocristopathic Tumors

Although the following tumors occur sporadically in many cases, all have been demonstrated to be transmitted in families to some extent. They also all occur in syndromic forms, with one another or with the neurocristopathic malformations. In particular, neuroblastoma and medullary thyroid carcinoma occur in conjunction with Hirschsprung disease, as discussed below.

Neuroblastoma (MIM 256700) is the most common extracranial childhood solid tumor, and the one with the highest rate of remission (around 90%; reviewed in ref. 24). Neuroblastomas, derived from sympathetic components, as schwannomas are from sensory components, possess phenotypic markers common to components of the peripheral nervous system. Histologically, they consist of cells that resemble undifferentiated NCC mesenchyme and, upon tumor regression, acquire nonmalignant characteristics resembling neurofibromas or ganglioneuromas.

Medullary thyroid carcinomas (MTC, MIM 155240), derived from calcitonin-producing C cells of the thyroid gland, exist in both sporadic (~75%) and familial forms (~25%). MTC cells inappropriately (over) express peptides such as serotonin, vasoactive intestinal peptide or calcitonin, leading to hypercalcemia among other effects. Histologically, they resemble carcinoid tumors, which occur usually in endodermally-derived organs such as the digestive or pulmonary tracts and are diagnosed at all ages.

Pheochromocytomas (MIM 171300) are derived from chromaffin cells of the adrenal medulla and also cause systemic effects such as sweating, tachycardia and hypertension (with subsequent effects on, for example, the retinal or cerebral vasculature), arising from an increase in epinephrine and norepinephrine production. Both sporadic and familial forms exist. Pheochromocytoma is a hallmark of a number of neurocristopathic syndromes discussed below, as well as of von Hippel-Lindau syndrome (VHL; MIM 193300), in some forms in conjunction with renal carcinomas. Paragangliomas (MIM 168000) arise in the complementary, nonchromaffin chemoreceptors of the head and neck region. Tumors of the glomus jugulare and carotid body are often seen in this heterogeneous familial neurocristopathy with complex inheritance—at least one of the genes is subject to imprinting and is only transmitted from the paternal line.[25]

The cutaneous NCC cancers, melanoma (MIM 155600) and Merkel cell (MC) carcinoma, are both locally and systemically aggressive in behavior. Malignant melanoma arises in melanocytes that are one of the latest-differentiating and most widely disseminated NCC phenotypes, and can occur in any part of the skin, even those never exposed to the sun (for instance, the nasal epithelium or the genitals). Childhood melanoma, while rare, carries one of the highest rates of distant metastasis among cancers. In contrast, around half of MC carcinomas are localized in the head and neck region, with mostly limited spread,[26] and they arise preferentially in the dermis of elderly adult patients.[27] MC carcinomas are occasionally associated with neurofibromatosis type 1[28] or breast and ovarian adenocarcinomas, and more frequently with squamous cell carcinomas.[26] Recently, the controversial hypothesis that mammalian MCs, commonly found in the basal epidermis, are in fact NCC derivatives was supported by experimental evidence.[29] MC cells, in synaptic-like contact with sensory nerve terminals in the skin, appear to have a local neuroendocrine or mechanosensory function, much as melanocytes are postulated to have had earlier in evolution.[30]

Isolated Neurocristopathic Malformations

These include many of the most frequent birth defects such as Hirschsprung disease (1 in 5000 births), cleft lip and/or palate (1 in 1000 births), conotruncal heart malformations involving (or not) the great arteries (1 in 500 births), and congenital nevi (1 in 100 births).

Indeed, such malformations overlap to some extent with neural tube closure defects (1 in 1000 births) by virtue of a segmentally defective specification or fusion of the neural folds from which NCC emigrate. Isolated defects in neural tube closure are not considered to be neurocristopathies as they are in and of themselves a very heterogeneous group of disorders that derive from an embryological event preceding that of NCC specification.[31] Nonetheless, as will be discussed in the section devoted to genetic cascades, common molecular origins can be at the root of both neural tube closure defects and various neurocristopathies.

Hirschsprung disease (HSCR), or aganglionic megacolon, is a congenital malformation characterized by the absence of enteric ganglia along a variable length of the intestine. It was first reported by Harald Hirschsprung in 1886. The enteric ganglia, components of the autonomic nervous system, are organized in two concentric rings throughout the gut wall: the outer myenteric plexus (Auerbach plexus) and the inner submucosal plexus (Meissner plexus). The neurons making up these ganglia include: sensorineurons detecting information from the gut, interneurons processing the sensory information, and motor neurons that provide innervation to smooth muscles regulating the contractility of the gut as well as the secretory activity of glands. In the late 1940's, a surgical procedure was developed in which the aganglionic segment of the bowel is resected by an abdomino-anal pull-through (reviewed in ref. 32). This previously fatal disorder became surgically treatable and enabled the survival of patients and the discovery of familial transmission of HSCR.[33]

Orofacial clefting arises from defects in the fusion of the palatal shelves, derived from the maxillary portion of the first pharyngeal arch. Mesenchymal NCC derived from the mid- and hindbrain migrate toward the endoderm and into the five bilaterally paired arches. The mesenchyme surrounds transitory pairs of aortic arch arteries that circumvent the pharynx and act as organizing centers for the structures of the lower face and neck.

The heart is essentially a mesodermal derivative. However, the NCC that enter the most caudal pharyngeal arches encase both the arteries (the left part of the fourth pair will persist as the aorta) and the cardiac tube in its conotruncal extremity.[3] Within the wall of the outflow tract, thickenings of this mesenchyme will lead to separation of the aorta from the pulmonary trunk, formation of the semilunar valves and completion of ventricular septation. Experimental removal of NCC lead to defects in all of these processes[8] as well as atrophy of the thymus and parathyroid glands.[7] Clonal expansion of a single defective NCC precursor in the cardiac region may lead to the individualized, spatially restricted phenotypes.

A similar mechanism could explain how congenital melanocytic nevi arise in any area of the skin at a frequency decreasing inversely proportionally to surface, from 1 in 100 births for the smallest lesions, to an estimated 1 in 20,000 for surfaces over 100 cm.[2] All melanocytic nevi are characterized by an abnormally high and localized concentration of melanocytes, and are often associated with hyperpilosity and variable coloration. The giant congenital melanocytic nevi (GCMN, OMIM 137550) can also present with nodules, absent or deficient skin annexes and abnormal dermis.

Complex Tumor Predisposition Syndromes

These include neurofibromatosis I (NF1), the multiple endocrine neoplasias (MEN) type 2A and 2B, familial medullary thyroid carcinoma (FMTC), Sturge-Weber syndrome and neurocutaneous melanosis.

NF1, also known as Recklinghausen disease (MIM 162200), is one of the most common neurocristopathies with a prevalence of 1/2000 to 1/3000 live births. Both Schwann cells and melanocytes are affected, undergoing abnormal proliferations that give rise to neurofibromas and "café au lait" spots. Neurofibrosarcomas as well as leukemia, and Wilms tumors in tissues not themselves derived from NCC, can also occur in patients. NF1 is an autosomal dominant disorder with high penetrance. However, since the disease is expressed in a broadly variable manner, clinical criteria for diagnosis need to be searched for carefully. Lisch nodules, visible as spots on the iris (colored by NCC-derived melanocytes), are one of the most penetrant features after the age of 20.

Familial medullary thyroid carcinoma (FMTC, MIM 155240), MEN type 2A (MEN2A, MIM 171200) and type 2B (MEN2B, MIM 162300) are also transmitted with autosomal dominant inheritance. MEN2A is characterized by hyperplasia of the calcitonin-producing parafollicular cells of the thyroid with subsequent neoplastic progression to medullary thyroid carcinoma (MTC) as described earlier, but also pheochromocytoma and parathyroid hyperplasia. Patients affected with MEN2B can also present with oral neuromas, marfanoid habitus and hyperganglionosis of the hindgut (contrasting with HSCR), although the clinical presentation can be similar to functional intestinal obstruction syndromes. The penetrance of MEN2 is age-related; only about 70% of MEN2A gene carriers will present with MTC by the age of 70 years, but precursor C-cell hyperplasia is detectable in almost all carriers prior to 40 years of age.

Sturge-Weber syndrome (around 1 in 50000 births, MIM 185300) merits discussion as it is not usually included as an example of a neurocristopathy, yet it is the only one of the classical "phakomatoses" (a term invented by Van der Hœve in 1921, not yet fallen into complete disuse) that from an embryological viewpoint can be unequivocally attributed to a simple NCC defect. Symptoms include a facial capillary hemangioma along the trigeminal nerve, with soft tissue or skeletal hypertrophy beneath it; leptomeningeal angioma with occasional calcification in the underlying cortex and ensuing epilepsy or hemiparesis; ocular choroid angioma with ipsilateral glaucoma due to permeable blood vessels. It is now understood that one discrete segment of cephalic NCC gives rise to vascular wall components within all affected areas,[9,34] and a single event in a clonal precursor could find itself expressed in a somatically mosaic manner.[35] The same mechanism may be responsible for the association of neurocutaneous melanosis (melanocytic tumor of the meninges) with congenital giant nevus of the skin and, more infrequently, with malignant melanoma.

Complex NCC-Related Malformative Syndromes

Waardenburg syndrome (WS), a genetically heterogeneous condition, combines pigmentary anomalies and sensorineural deafness. At a frequency of 1 in 50,000 live births, WS accounts for 2-5% of all congenital deafness. The condition results from the absence or reduction of melanocytes in both the skin and the stria vascularis of the cochlea.[10] WS is clinically and genetically heterogeneous (OMIM 193500, 148820 and 193510). The combination of HSCR with WS defines the WS4 type (Shah-Waardenburg syndrome, OMIM 277580). The addition to of a Peripheral demyelinating neuropathy and a Central dysmyelinating leukodystrophy to Waardenburg and Hirschsprung disease (WS4) define the PCWH syndrome[36] (MIM 609136). The close lineage relationship between glial cells and melanocytes[11] also justifies grouping these diverse clinical entities together on a continuum, pointing at the defective specification of a single NCC precursor type.

CCHS (Ondine's curse, MIM 209880) is a life-threatening disorder primarily manifesting as sleep-associated respiratory insufficiency and markedly impaired ventilatory responses to hypercapnia and hypoxaemia.[37] The autonomic nervous system is affected: from the level of its central control in the hindbrain through the vagus nerve and out to the peripheral, NCC-derived target ganglia. In addition, HSCR or tumors such as neuroblastoma, ganglioneuroblastoma and ganglioneuroma are noted in 20-30% and 5-10% of CCHS patients respectively (e.g., see ref. 38).

Most of the other malformative syndromes markedly affect the cardio-cephalic pole (Table 1). Facial dysmorphy is a distinguishing feature, as expected from the enormous contribution of NCC to the face and brain vault. However, it has only recently been acknowledged that pituitary development, like that of the heart outflow tract, depends on the presence and participation of cephalic NCC,[39] although the final contribution of these cells to the gland is negligible. Thus, syndromes that entail growth or genital abnormalities in addition to more obvious effects on NCC derivatives should be considered as potential neurocristopathies. One example is that of Allgrove syndrome (MIM 231550), an autosomal recessive disorder also known as AAA syndrome for alacrima, achalasia of the esophagus due to neurological impairment and adreno-corticotropic hormone (ACTH)-resistant adrenal failure. Bamforth-Lazarus

syndrome (MIM 241850) associates cleft palate and choanal atresia with thyroid agenesis.[40] A NCC contribution to the connective tissue of the thyroid gland has been recognized for decades[3,7] but is not commonly evoked.

Gene Cascades Implicated in Human Neurocristopathies

Breaking down the timeline of NCC development into stages is one approach to considering the abundant molecules that are now known to be involved in or suspected in pathogenesis.

Neural Crest Induction

Specification of the neural folds is the subject of many excellent reviews[41-43] and ongoing research,[44-47] going beyond the scope of this chapter. To resume, the roles of signaling molecules such as members of the Wnt,[45,46,48,49] fibroblast growth factor (FGF), bone morphogenetic protein (BMP) or hedgehog families and their control by gradients of retinoic acid and *Hox* transcription factor expression have been underlined. Several transcription factors are also expressed during early induction of the vertebrate neural crest lineage in the neural folds or surrounding tissues such as members of the highly conserved families *Slug/Snail*, *Sox*, *Fox*, and *Pax*.

To date, germline mutations of the *PAX3*,[50] *MITF*,[51] *SNAI2*[52] and *SOX10*[53] genes have been demonstrated to be directly involved in pure syndromic neurocristopathies (WS1-4 respectively), suggesting that impairment of the other pathways may be lethal or, as yet, unrecognized. The spontaneous mouse mutation in *Pax3*,[54] known as Splotch for its heterozygous coat color defect, leads to hindbrain exencephaly in homozygous mice; it was somewhat of a surprise to find that its later role in glial/melanocyte lineage determination was predominant in human disease, rather than its earlier one in NCC specification (similar findings for *SNAI2* and *SOX10*, see below). Although no PAX3 mutations have been demonstrated in isolated neural tube closure defects to date, some WS1 patients do have spinal neural tube defects and WS3 patients, skeletal muscular abnormalities.[55] *SNAI2*, also known as *SLUG*, is mutated in WS2D[52] and piebaldism[56] but also is possibly an oncogene in an activated form.[57] Expressed in premigratory and migrating NCC throughout the vertebrate subclass, *Snail* or *Slug* homologues are also found in the gastrulating mesoderm and are generally implicated in the epitheliomesenchymal transition.[58]

NCC Migration

The term migration describes a long period in NCC existence: from the epithelio-mesenchymal transition and delamination from the neural folds, to reorganisation of the cytoskeleton, to migration along pathways constrained by anatomy and the local extracellular matrix (ECM) in order to encounter appropriate orientation and differentiation cues. The mechanism of migration has an intrinsic component, represented by the capacity of a given NCC to respond to its environment by physical changes and motility, and an extrinsic component, represented by the extracellular matrix or cellular/anatomical environment through which NCC migrate. It is the latter component that may explain the neurocristopathic contribution to other malformation syndromes (e.g., basal cell nevus syndrome), in particular those involving the cardio-cephalic pole and its abundant NCC-derived mesenchyme.

The noncanonical Wnt signaling pathway, exemplified physiologically by Wnt11 and its receptor Fz7 in mouse,[46] the EphB-ephrinB,[59] neuregulin-ErbB[60] and endothelin-endothelin receptor[61] families of ligands and their receptors, are known to be involved in initiating and maintaining migratory behavior of NCC. The transcription factors Sox9, then Slug/Snail, FoxD3 and Sox10 appear to control the cell-autonomous acquisition of a migratory phenotype.[47,62] The composition of the ECM in fibronectin[63,64] and vitronectin[65] and appropriate collagens[66,67] and laminins[68,69] is certainly relevant to the direction of integrin-bearing NCC as they distance themselves from the neural tube and enter either the dorsoventral or the dorsolateral compartment pathways. Integrins, like both Eph receptors and their ephrin ligands, effect changes in the cytoskeleton upon binding their substrates. Their specificity is

Table 2. Known genes or loci in neurocristopathies

Pathology	MIM	Transmission	Gene	Chromosomal Localization	Spontaneous Mouse Model or Transgenic (+)
Piebaldism	172800	AD	*KIT*	4q12	*White-spotting, +*
Melanoma	155600	AD		1p36	
Melanoma	155600	S	*NRAS*	1p13.1	+
Melanoma	155600	S	*BRAF*	7q34	+
Melanoma	155600	AD, S	*CDKN2A*	9p21	+
Melanoma	155600	AR, S	*MC1R*	16q24.3	*Tawny, recessive yellow, tobacco-darkening, sombre, +*
Melanoma	155600	AD, S	*CDK4*	12q14	+
WS1	193500	AD	*PAX3*	2q35	*Splotch, +*
WS3	193500	AR	*PAX3*	2q35	*Splotch, +*
WS2A	193510	AD	*MITF*	3p14	*Microphthalmia, vitiligo, white, red eyed white, brownish, +*
WS2B	600193	AD		1p	
WS2C	606662	?		8p23	
WS2D	608890	AR	*SNAI2*	8q11	+
WS4	277580	AR	*EDNRB*	13q22	*Piebald, piebald lethal, +*
WS4	277580	AR	*EDN3*	20q13	*Lethal spotting,+*
WS4	277580	AD	*SOX10*	22q13	*Dominant megacolon, +*
HSCR	142623	CI*	*RET*	10q11.2	+
HSCR	142623	CI	*GDNF*	5p13.2	+
HSCR	142623	CI	*ARAF*	Xp11.3	+
HSCR	142623	CI	*NTN*	19p13.3	+
HSCR	142623	CI	*EDNRB*	13q22	*Piebald, piebald lethal, +*
HSCR	142623	CI	*EDN3*	20q13	*Lethal spotting,+*
HSCR	142623	AD	*PHOX2B*	4p12	+
HSCR	142623			3p12	
HSCR	142623			19q13	
HSCR	142623	AD	*L1CAM*	Xq28	+

continued on next page

Table 2. Continued

Pathology	MIM	Transmission	Gene	Chromosomal Localization	Spontaneous Mouse Model or Transgenic (+)
NB	256700	AD, S	*PHOX2B*	4p12	+
Haddad	209880	AD, S	*PHOX2B*	4p12	+
CCHS	209880	AD, S	*PHOX2B*	4p12	+
Pheochromocytoma	171300	AD	*RET*	10q12	+
Pheochromocytoma	171300	AD	*SDHB*	1p36	
Paraganglioma 4	115310	AD	*SDHB*	1p36	
Paraganglioma 2	601650	AD		11q13.1	
Paraganglioma 3	605373	AD	*SDHC*	1q21	
Paraganglioma 1	168000	AD	*SDHD*	11q23	+
Pheochromocytoma	171300	AD	*SDHD*	11q23	+
MC carcinoma	602690.0019	S	*SDHD*	11q23	+
MEN2A	171400	AD	*RET*	10q12	+
MEN2B	162300	AD	*RET*	10q12	+
MTC	155240	AD	*RET*	10q12	+
Familial dysautonomia 2	256800	AR	*NTRK1*	1q21	+
NF1	162200	AD	*NF1*	17q11	+
DiGeorge	188400	AD	*TBX1*	22q11.2	+
CHARGE	214800	AD	*CHD7*	8q12	+
Goldenhar	164210	AD		14q32	
Moebius	157900	AD		13q12.2-q13	
Bamforth-Lazarus	241850	AR	*FOXE1*	9q22	+
Rieger 1	180500	AD	*PITX2*	4q25	+
Rieger 2	601499	AD	*FOXC1*	13q14	*Congenital hydrocephalus*, +
Craniofrontonasal	304110	XLD	*EFNB1*	Xq13.1	+
Allgrove	231550	AR	*ALADIN*	12q13	

AD or R, autosomal dominant/recessive; XL, X-linked; CI, complex inheritance; * major locus; S, somatic mutation.

partly conferred by the numerous alpha integrin subunits, of which alpha-1, alpha-4, alpha-5 and alpha-v appear to be widely expressed, like the beta-1 subunit, on emigrating NCC;[63-65,67,68,70] alpha-6 and alpha-7 appear on subsets of cranial[71] or trunk[72] NCC respectively. Some alterations in ECM molecules have been observed in HSCR patients,[66] although their distribution may be a secondary effect of changes in intestinal architecture rather than causative. In mouse and chick models, the alpha-4 subunit and its specific binding site to fibronectin are needed for normal NCC emigration from the neural tube to occur.[64]

If the recently identified role of ephrin signaling is a typical example, mutations in any of these adhesion molecules should affect NCC derivatives but are likely to lead to broader polymalformative syndromes. Binding of ephrinB to its receptor, EphB, leads to contact-mediated repulsion by the bilateral activation of protease activity and the rapid release of adhesion via the integrins.[73,74] Human and mouse mutations in the *EPHRB* (ephrinB) gene lead to severe clinical effects on NCC derivatives such as the skull (coronal craniosynostosis), the palate and the face, but also lead to effects on the chest and limb skeleton and fingernails; this association is known as craniofrontonasal syndrome).[75,76]

Differentiation of NCC

The restriction of the ability of NCC to develop into the various lineages begins before migration. In vivo, cranial NCC are distinct in their potential from trunk-level NCC. The cranial crest lineages include mesectoderm derivatives such as bone, cartilage, teeth, adipocytes, dermis, glandular and vascular connective tissue and smooth muscle, as well as the possibility to become all other derivatives of trunk NCC if transplanted into the appropriate location in the body.[77] Only after a long sojourn in vitro and apparent reprogramming can trunk NCC acquire some chondrogenic ability.[78]

The subsequent refinement of the different classes of NCC derivatives is done over time in different locations in the body. We have judged it convenient to address the involvement of the three major gene groups sequentially. However, it is important to keep in mind that any individual NCC is subject to interactions between these gene cascades as it differentiates and its increasingly fate-restricted progeny proliferate. Genes affecting pigmentation, neural differentiation or the establishment of cardiocephalic structures are grouped separately simply for the purpose of discussion.

The Pigmentation Gene Cascade

In vitro, embryonic NCC will give rise over successive generations to a mix of stem-like cells with varying degrees of potential to differentiate into all or only some NCC derivatives.[13] In particular, the bipotent glia-melanocyte precursor[79] as well as a tripotent melanocyte-neural-adrenergic precursor[80] have been shown to proliferate and expand preferentially in the presence of endothelin-3 (EDN3). The three known endothelins are peptides, first identified by their potent vasoconstrictive activity on vascular smooth muscle, but soon understood to be mitogens for melanocytes.[81]

Deletion of the mouse endothelin type B receptor (*Ednrb*) gene produces an autosomal recessive phenotype of white spotting and megacolon, bringing to light the critical role of endothelins in melanocytic and enteric development.[82] Mutations in human *EDNRB* lead to HSCR isolated or syndromic (WS4), according to the type and copy number of the mutation.[83-85] A patient with HSCR, heterozygous for weak hypomorphic mutations in both the *RET* receptor gene (see the "neural selector" group below) and *EDNRB*, has recently been reported.[86] Each mutation was inherited from a healthy parent. Interestingly, overexpression of EDNRB is sufficient to direct NCC-like migration in vivo and melanocytic differentiation in noncommitted embryonic stem cells.[61]

Although EDNRB can bind all three EDNs, only EDN3 is a physiologically relevant ligand in the enteric environment.[87] Homozygous EDN3 mutations have also been found in patients with WS4.[88] Mutant heterozygotes in one of these families were either unaffected or had mild

pigmentary anomalies, as did other untested family members. In addition, an EDN3 frameshift mutation has been identified in a patient with CCHS and chronic constipation.[89] Thus, distinct human tissues—melanocytes versus components of the autonomic nervous system—appear to have different sensitivities to EDN3/EDNRB dosage.

EDNRB, like many other growth factor receptors, transduces its activation to nuclear targets via the Ras signaling cascade. N-ras is a direct component of the MAPK/Erk pathway but also can activate phosphatidylinositol-3-kinase and its targets.[90] Ras molecules in general activate the mitogen-activated protein (MAP) kinase pathway to induce proliferation; EDNRB binding also counters apoptosis through the parallel activation of phosphatidylinositol-3-kinase. Two important molecules in the formation of nevi appear to be N-RAS and the next effector in the MAP kinase pathway, B-RAF. An early indication of *B-raf* function came from the initial knockout mice in which endothelial cells underwent abnormal differentiation and did not organize into mature, functional blood vessels,[91] as happens in numerous models with mutated growth factor receptors. However, it was upon the recent demonstration that reproducible mutations in *BRAF* were involved in highly diverse cancers, malignant melanoma in particular,[92,93] and, surprisingly, in a number of clinically benign nevi,[94] that a more specific role of this molecule in the development of NCC derivatives began to be explored.

The activating mutation of *BRAF* found most frequently in humans was specifically expressed in zebrafish melanophores experimentally. While wild-type *B-Raf* did not change the pigmentation of the zebrafish, the activated form of the gene led to the appearance of nevus-like clusters of pigment covering large areas, up to 40% of the body surface.[95] This was the first animal model of nevus formation. Crossing these fish to those deficient in the tumor suppressor p53 (product of the *CDKN2A* gene) led to the development of aggressively invasive melanoma in which the MAPK/Erk pathway was unduly active.

Dominant mutations of the *PAX3* transcription factor and the *MITF* (microphthalmia-associated transcription factor) genes have been reported in WS1, WS2 or Tietz syndrome[96] (MIM 103500). In the mouse, *Pax3* is critical for skeletal muscle development as well as the development of the dorsal neural tube and the NCC that migrate from it.[97] *Mitf*, *Trp-1* (tyrosinase-related protein-1) and the tyrosine kinase receptor *c-met*, all expressed in melanocytes, appear to be transcriptional targets of Pax3.[51,98,99]

WS4 patients can also carry mutations of the Sry-type HMG box family transcription factor member, *SOX10*. This implies some interaction between endothelin signaling and Sox10 transcriptional effects. Indeed, *Sox10* is expressed in premigratory NCC, then in both melanocytes and enteric ganglia, as well as in glia of both CNS and PNS origin.[100] It is involved in cell lineage determination and is capable of transactivating *MITF* synergistically with *PAX3*.[101,102] *SOX10* mutations can cause either WS4[53] or PCWH:[36] Translated, mutant Sox10 proteins, whose mRNAs are *not* degraded by the nonsense-mediated decay pathway, lead to the more severe form associated with peripheral neuropathy. Mere haploinsufficiency through the proper degradation of mutant *SOX10* mRNAs leads to WS4.[36] Combined with the observed lack of *Ednrb* transcripts in mice with a truncating mutation of *Sox10*,[103] one might postulate that yet unidentified genes regulate the different response of melanocytes[104] or glia versus precursor NCC to *Sox10* dosage, and that *Ednrb* lies downstream of both *Sox10* and these other genes. Indeed, additional modifying loci for aganglionosis have been identified recently in mice.[105,106] Misregulated control of mRNA degradation is likely to be found with increasing frequency in human pathology and offers an additional explanation for how different mutations in the same gene can result in very distinct diseases, especially for master regulatory transcription factors of development.

The Neural Selector Gene Cascade

The *Drosophila achaete-scute* complex is a cluster of four proneural genes coding for basic helix-loop-helix domain-containing transcription factors achaete, scute, lethal of scute and asense.[107] The complex controls early development of both the central and peripheral nervous

system in the insect by instructing the differentiation of neuroblasts from ectodermal precursors. In the mouse, two homologues of the *achaete-scute* complex (ash) are known as *Mash-1* and *Mash-2*.[108] *Mash-2* is an imprinted gene expressed only in the trophoblast lineage.[109] In contrast, *Mash-1* expression is restricted to the CNS and PNS within a restricted spatiotemporal window.[110] The entire ventricular zone of the CNS expresses *Mash-1* at one point or another during the proliferation of neurectodermal precursors. The enteric and sympathetic neurons of the PNS also express *Mash-1*.[111] Homozygous *Mash-1* knockout mice die within the day following birth due to respiratory failure and inability to suckle.[112] The PNS is dramatically affected with an absence of the sympathetic and parasympathetic ganglia as well as the esophageal enteric neurons. Mash-1, like its evolutionary forerunner, has proneural properties.[113,114]

The human homologue to Mash1, *HASH1*, is a small, two-exon gene localized at 12q24. It is involved in a feedback loop with the PHOX2 (paired homeobox 2) homeodomain-containing transcription factors. *PHOX2A* and *PHOX2B* are very similar in their homeodomains but divergent in their promoters. Both are expressed in all CNS or PNS neurons that embark upon the noradrenergic synthesis pathway, be it temporarily or permanently.[115] They are both found in the branchiomotor and visceromotor neurons and the motor tracts of cranial nerves VII, IX and X. Only *PHOX2A* is expressed in the nuclei corresponding to cranial nerves III and IV. In the autonomic nervous system, *Phox2b* has been shown to act upstream of *Mash1*[116] and *Phox2a*,[115,117] but the feedback control of *Phox* genes by *Mash1* is not direct.[118]

Mutations in *PHOX2B* have recently been shown to be responsible for CCHS[37] but, according to the type of mutation, also can predispose to neuroblastoma or HSCR.[38,119] Phox2b promotes cell cycle exit and neuronal differentiation in the sympatho-adrenal lineage (ref). Downstream, *Phox2b* directly binds the promoter of the dopamine beta-hydroxylase (*DBH*) gene (the key enzyme of noradrenaline synthesis)[120] and, indirectly, activates *TH* (tyrosine hydroxylase)[121] and the *Ret* receptor tyrosine kinase, which is the major gene involved in HSCR, MEN2 syndromes and isolated pheochromocytoma (see below). *Phox2b+/-* mice present a dysfunction of their respiratory system that is similar to the one observed in CCHS patients, although it remains mild and transient.[122,123] They do display a temporarily altered response to hypoxia and hypercapnia[122] as well as sleep-disordered breathing (apnea and hypoventilation).[123] Ventilatory changes induced by hypoxia are mediated by afferents from carotid body glomus cells to the nucleus of the solitary tract (nTS) via the IXth cranial ganglion, where tyrosine hydroxylase (TH) expression is significantly decreased during the same period. All of these structures express *Phox2b* in mice[115,121] and humans[37] and fail to form or degenerate in *Phox2b-/-* mouse mutants.[116]

Most patients with *RET* mutations have HSCR only, but some develop pheochromocytoma or MEN2. A multiplicative oligogenic model with three loci has been proposed for isolated, nonsyndromic HSCR, with *RET* being the major susceptibility gene and the two other genes remaining to be identified.[124] The RET mutations identified in HSCR are unique and occur throughout the gene. This is in contrast to MEN2A, for which RET mutations occur in a cluster of six cysteines,[125-127] and MEN2B, which is uniquely associated with an M918T mutation.[128,129] Remarkably, some HSCR mutations occur at the same cysteines as the ones involved in MEN2A. *RET* mutations in MEN2 are activating mutations which constitutively dimerize the receptor, leading to transformation,[130] while those in HSCR are generally inactivating mutations which lead to misfolding or failure to transport the protein to the cell surface. The identification of HSCR patients with *RET* deletions[131] argues in favor of haploinsufficiency as a mechanism for pathogenesis. Consequently, although a MEN2A/MTC-type activating mutation has been observed in HSCR, haploinsufficiency may have occurred due to inefficient transport to the cell surface[132] at a key period in enteric development.

In mice, the Ret signaling pathway is implicated in the development of all noradrenergic derivatives[133,134] as well as the kidney, an organ rarely affected in human mutations of *RET*.[135] Likewise, a major ligand for RET, GDNF (glial-derived neurotrophic factor), is exceptionally responsible for HSCR,[136] but in mice it is essential for both enteric nervous system and renal development.[134] GDNF, being a TGF-β related protein, is unusual in activating RET only in

the presence of a glycosylphosphatidylinositol (GPI)-linked coreceptor, GFRA1. No mutations in GFRA1 have been discovered despite a careful search for variants in HSCR patients with similar phenotypes to Gfra1$^{-/-}$ mice.[137-139] Four structurally related GPI-linked coreceptors, GFRA1-4, and four related soluble growth factors, GDNF, neurturin (NTN), persephin (PSPN) and artemin, have been identified to date (reviewed in ref. 140). Of these, one family with a putative *NTN* mutation, in conjunction with a *RET* mutation, has been identified.[141] Like GDNF, NTN does not appear to have a major effect on HSCR and probably can only exert its mutational effect in conjunction with other disruptions of RET signaling.

Genetic interactions between *RET* and *EDNRB/EDN3* have been demonstrated in both humans and mice for the HSCR phenotype.[142,143] Indeed, similar interactions of many of the molecules in the pigmentation group with those of the neural selector group lead to the conclusion that a wide and complex cascade of events fine-tunes NCC differentiation. For instance, while human *BRAF* and *NRAS* mutations are causal in nevogenesis and formation of melanoma as mentioned above, *A-Raf* null mice have megacolon.[144] However, *A-raf*-deficient fibroblasts are able to maintain normal signalling through the MAPK/Erk pathway via increased activity in *B-raf* and *Raf-1*,[145] implicating target genes in the appropriate differentiation of neurons, glia and melanocytes from their common progenitor.

The Cardio-Cephalic Gene Cascade

In the 1980's and 1990's, experimental embryologists demonstrated that ablation of most or all posterior cephalic NCC phenocopies many of the aspects of the 22q11.2 deletion syndromes (DiGeorge; MIM 188400).[7,8,146] For many years, these results were interpreted to mean that NCC deficiency was directly responsible for outflow tract and caudal pharyngeal arch anomalies, especially since the affected structures had long been known to have a significant NCC component (although transient in the heart[3]). However, it was recently discovered that the pharyngeal endoderm is responsible for the survival and patterning of cephalic NCC.[19] This nonautonomous effect may initially be due to early mesendodermal production of retinoic acid[147,148] and subsequent activation of transcription factors regulating rostrocaudal identity, the *Hox* genes, in nested domains within surrounding tissues (reviewed in ref. 149), and finally the production of secreted signaling molecules such as Sonic hedgehog (Shh) or FGFs.[150,151]

FGF8 (one of >23 different FGFs), on chromosome 10q24, is transcribed during neurulation by the paraxial mesoderm and, later, by defined regions of the forebrain and cerebellar primordia, facial ectoderm and pharyngeal endoderm.[151] In animal models, a second wave of retinoic acid synthesis in these latter epithelia is responsible for the spatiotemporal coordination of Fgf8 localization with Shh.[150] Mutations in *Shh*, in genes coding for enzymes responsible for its biosynthesis, or in components of its intracellular signaling cascade lead to holoprosencephaly and fusion of the retinal fields (from hypotelorism to cyclopia, reviewed in ref. 152). Absence of cephalic NCC, exposure to high levels of maternal ethanol or maternal diabetes can also lead to a holoprosencephalic phenotype.[153-155]

Among its many roles, Fgf8 both induces and maintains proliferation of cephalic NCC.[45,156] *Fgf8* haploinsufficiency in mice gives rise to an intriguing spectrum of malformations also recalling that of 22q11.2 chromosomal deletions in humans.[151,157] All mutants, with *Fgf8* levels intermediate between a half and full dose, have micrognathia, many have cleft palate, otic ossicle and external ear anomalies and all mutants have central nervous system malformations, including hypoplasia or aplasia of the cerebellum and olfactory bulbs. Nearly all have outflow tract defects of the heart, including persistent truncus arteriosus, and hypoplasic or aplasic thymus and parathyroid glands.

Fgf10, another member of this large family of growth factors, signals through a different splice isoform of the same receptor as Fgf8, and is present in many organs where epithelia bud into *Fgf10*-expressing mesenchyme: lung, spleen, teeth, pituitary, salivary and lachrymal glands. Interestingly, in the developing limb bud, pharyngeal arches and heart, and pituitary gland, *Fgf10* and *Fgf8* lie within an autoregulatory loop controlling each other's expression.[157,158]

Tbx1, a T-box and homeodomain-containing transcription factor directly produced by the pharyngeal endoderm, is also expressed in the mesenchyme of the branchial arches and rostral head, in discrete areas of the outflow tract of the heart, in the ventral otocyst and sclerotome in both mouse[159-161] and human embryos (HCE, unpublished observations). *Tbx1* is a target of Shh signaling in the pharyngeal endoderm.[162]

Given that it is physically located in the 22q11.2 critical interval for DiGeorge syndrome, it had long been thought to be the best candidate gene. Recent efforts have identified *TBX1* mutations in DiGeorge patients.[163,164] *Tbx1* haploinsufficiency in mice affects the remodeling of the definitive aorta at the level of the fourth aortic arch and conotruncal septation in the heart.[165] These mice also have malformed, tiny external ears as in DiGeorge syndrome.[159]

Recently, it was demonstrated that *Tbx1* affects morphogenesis of the great vessels of the heart in a non cell-autonomous manner, meaning that its normal transcription affects the function of another, secreted mediator of its activity.[166] Tbx1 directly upregulates the transcription of *Fgf10*, and *Tbx1*$^{-/-}$ mice lack *Fgf10* expression specifically in the mesoderm of the secondary heart field.[165,166] Fgf10 normally maintains the proliferation and incorporation of myocytes from the splanchnic mesoderm into the outflow tract region of the growing cardiac tube.[167] Likewise, *Tbx1*$^{-/-}$ mice lack pharyngeal endodermal expression of *Fgf8*, probably similarly responsible for the proliferation of the rhombencephalic neural crest cells necessary for colonization of the outflow tract and subsequent mediation of correct septation.

Neurocristopathies affecting the cardiocephalic pole often leave their most visible mark on the face, since most facial tissues (bone, cartilage, teeth, vascular walls, and dermis) are direct NCC derivatives. In addition, when cephalic NCC are reduced in number or completely ablated in the embryonic chicken, other non NCC elements such as the forebrain, the pituitary gland, the thymus or facial muscles are themselves severely hypoplasic or absent.[7,153] Compromised pituitary function or holoprosencephaly can therefore be considered to be part of the spectrum of cephalic neurocristopathies, in the way that malformations of the heart outflow tract have long been admitted to be. Indeed, numerous forebrain and premaxillary nasofrontal malformations are frequently reported in association with human pituitary deficiencies (reviewed in ref. 39). The NCC surrounding the forebrain are supported in their proliferation and survival by a localized source of Fgf8 at the anterior neural ridge and, in return, maintain that source for forebrain outgrowth to take place (ref. 156 and S. Creuzet, personal communication).

Part of the patterning activity of cephalic NCC on non NCC head elements can be accounted for by the secretion of proteins belonging to other well-known signaling cascades as well. Cephalic NCC synthesize the BMP antagonists Gremlin and Noggin[168] and the Wnt antagonist Frzb.[49] Frzb acts by inhibiting the canonical Wnt signaling pathway via beta-catenin, which is normally activated by the local expression of *Wnt-3a* by the dorsal neural tube and/or *Wnt-2b/Wnt-13* by the facial ectoderm.[168] Recently, it has been demonstrated that induction of migratory behavior only takes place upon the reception of a noncanonical Wnt11 signal, again synthesized by the ectoderm, by Frizzled-7 receptors on NCC.[46]

The phenotypic spectrum of the murine *Fgf8* hypomorphs resembles CHARGE syndrome (MIM 214800) as much, if not more, than DiGeorge syndrome.[151] The acronym CHARGE refers to an association of congenital malformations first described by Hall et al[169] including ocular Coloboma, Heart outflow tract malformations, choanal Atresia, Retarded growth and mental development, Genital hypoplasia, Ear abnormalities and/or deafness. The *CHD7* gene has recently been shown to be mutated in 60% of CHARGE postnatal patients[170] and 100% of prenatally diagnosed patients.[171] *CHD7* belongs to a family of proteins thought to play a role in chromatin organization through their conserved chromodomain (reviewed in refs. 172,173). Chromodomain-containing proteins maintain a silencing, heterochromatin-like structure around such embryologically important genes as the *HOX* transcription factor clusters or tumor suppressors like *CDKN2A*.[174] In addition, CHD subfamily members also contain a helicase-ATPase domain that is directly involved in histone deacetylation.[175] Indirect targets of CHD7 activity in the cardiocephalic pole, given their phenotypes in mouse inactivation

models, are likely to include some of the genes previously investigated as candidates for CHARGE or DiGeorge syndrome such as *TBX1*, among others.

Discussion

Cooption of Developmental Gene Cascades during Evolution and Teratogens

Many syndromic neurocristopathies also affect tissues that are not directly derived from the neural crest. Nearly all genes identified as involved in seemingly isolated neurocristopathies are well known in the development of other, non NCC derivatives. The primary reason for these observations is that molecular cascades are conserved and coopted for new purposes during embryonic development of diverging species over the course of evolution. (The secondary reason has involved the historical inclusion or exclusion of given clinical entities in the definition of a neurocristopathy, as discussed earlier). Indeed, molecular inroads into the mechanisms of development are blurring the distinction between what is or is not a neurocristopathy so that it is probably a more useful term for clinicians than for embryologists or molecular biologists.

In various instances, neurocristopathies arise from embryonic exposure to teratogens such as vitamin A derivatives, high maternal blood glucose, cyclopamine or ethanol. Retinoic embryopathy MIM 243430, is a distinct clinical entity associating conotruncal heart or great artery defects, micrognathia and malformed external ears, and posterior fossa malformations. Known effects on the hedgehog signaling pathway by retinoic acid[147,148,150] or cyclopamine[176] contrast in their specificity with more general possible effects on mitochondrial respiration and oxygen management,[177-179] to which migrating, undifferentiated cells such as the NCC could be more vulnerable. Thus, environmental teratogens particularly affect NCC by coopting those genetic programs used to maintain plasticity and adaptation in NCC during the course of vertebrate evolution.

Neurocristopathy Genetics in Mouse and Man

Many mouse models for HSCR, carrying mutations in known HSCR or WS genes, do not transmit the phenotype in the same way as in humans. Full phenotypic penetrance is usually observed in the mouse models and generally not in humans, as there is enormous intra- and inter-familial variation in presentation. This observation can be explained by the fact that mouse models are made on homogeneous backgrounds, which is not the case within even a given human family. Thus, the expression of phenotype is subject to variations in genetic background. In addition, many of the mouse models are made with homozygous null mutations, whereas most HSCR patients have heterozygous mutations that range in potency from weak hypomorphic changes to complete loss of function. The conclusion that HSCR is a genetically heterogeneous, oligogenic disease is now uncontested. HSCR is the best studied neurocristopathy from a genetic point of view, but its complex inheritance is likely to apply to other neurocristopathies affecting the same subset of NCC derivatives.

Malformations and Carcinogenesis

It has long been suspected that, depending on the nature of gene mutations or on the gene series involved, mutations of developmental pathways in humans may result in either malformative syndromes or cancer predispositions. The field of neurocristopathies has already been been rewarding for that hypothesis, with the following examples: (i) RET mutations in HSCR and in MEN2 syndromes; (ii) PHOX2B gene mutation in CCHS and neuroblastoma; (iii) BRAF and NRAS mutations in congenital naevi, lung and prostate cancers and melanoma; (iv) FOXC1 mutations in Rieger syndrome and rhabdomyosarcoma;[180] (v) *PAX3* mutations in WS and its fusion to *FOXO1*, also in rhabdomyosarcoma;[99,181] (vi) SNAI2 mutations in WS and a likely role in leukemia (reviewed in ref. 182). Clearly, the identification of new genes involved in rare congenital malformations will ultimately continue to bear fruit in the wide field of cancer research.

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CHAPTER 15

Evolution of the Neural Crest

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Abstract

The recent advances in studies of the neural crest in vertebrates and the analysis of basal chordates using molecular and embryological approaches have demonstrated that at least part of the genetic programs and the cellular behavior were in place in nonvertebrate chordates before the neural crest evolved. Nevertheless, both the missing aspects and the close similarities found could explain why basal chordates lack a bona fide neural crest population, even though some migratory neurons and pigment cells have been recently identified in ascidians and amphioxus.

Introduction: Was There Anything Like This Before?

The most interesting aspect of the neural crest, besides its amazing multipotency, is the pivotal role it has played in the evolution of vertebrates.[1] The neural crest is a vertebrate characteristic and indeed without it, the vertebrate head would look quite different. Together with the ectodermal placodes, the neural crest was crucial to the formation of paired sense organs and the transition towards a more active life style with complex behaviors. It is considered to be so important, that the neural crest has been termed the fourth germ layer. In this sense, the neural crest together with the ectoderm, endoderm and mesoderm would make the vertebrates quadroblastic animals.[2]

As a vertebrate innovation, the neural crest is considered as one of the important steps in the evolution of Chordates and in evolutionary terms, it can define how this Phylum developed. The Chordates are animals with a bilateral symmetry, a notochord (a hollow tube with support functions) and a dorsal tubular central nervous system. The most primitive Chordates, the ascidians (Urochordata = most basal chordates) regress in adulthood to the sessile filtering form having existed as a free-swimming larva (tadpole) that contains an axial notochord and a dorsal neural tube. The amphioxus or lancelets (Cephalochordata = chordates with head) preserve the notochord in the adult as an endoskeletal support, and have a regionalized pharyngeal endoderm. With respect to the basal vertebrates, the hagfish (Myxinoidea) develop a cranium that surrounds their primitive brain, and the lampreys (Agnatha = no jaws) have a vertebral column to support the body and protect the spinal cord. More importantly, they have a truly segmented neural crest, but no jaws. Finally, the jawed vertebrates (Gnatosthomata = mouth with jaws: fishes, reptiles, birds and mammals) depict all of the evolutionary novelties that gave rise to the vertebrate head.

It is important to establish when the neural crest arose in animal evolution and how this cell population acquired the capacity to migrate and form such a wide variety of structures, the two key characters of the neural crest cells. Did a latent neural crest exist in the more primitive chordates? It is certainly difficult to assume that the neural crest just suddenly appeared. Indeed, evidence is now accumulating to suggest that nonvertebrate chordates do develop a precursor

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neural crest population. Essentially, gene expression patterns and the identification of some cells with a migratory behavior indicate that part of the genetic and cellular programs related to neural crest development were definitely in place. However, as discussed by Stone and Hall,[3] they may still be considered as provocative observations awaiting hierarchical developmental evidence, not only at the cellular level but also at the tissue level. In relation to this, we will discuss here the evidence that has arisen in the last few years that may define different steps in the development and appearance of the neural crest.

Neural Crest and the Neural Tube: The Only Way Is Dorsal

Since the neural crest arises from the dorsalmost part of the neural tube, whether the dorso-ventral patterning of the neural tube is already in place in basal chordates has been examined. As a result, it seems that prototypes of the dorsal and ventral genes are indeed expressed similarly in amphioxus and ascidians. With respect to the ventral neural tube, both signaling molecule sonic hedgehog and transcription factor HNF3 expressions are conserved between vertebrates and amphioxus,[4,5] as is also the case for the ascidian HNF3beta ortholog.[6]

Similarly, the orthologs of genes expressed in the dorsal neural tube in vertebrates are conserved in basal chordates. With respect to the *Pax* family of transcription factors, a single *Pax3/7* gene in amphioxus and ascidians corresponds to the vertebrate *Pax3* and *Pax7* genes. Not only is *Pax3/7* expressed in the dorsal part of the neural tube in both *Ciona* and amphioxus,[7,8] but over-expression of Pax3/7 in ascidia leads to dorsalization.[9] Mutations in *Pax3* and *Pax7* in mice and humans have been related to neural crest defects,[10,11] highlighting the relevance of the appropriate dorsoventral patterning of the neural tube for neural crest development.

Several transcription factors of the Msx family are expressed in the dorsal neural tube and the neural crest, where they are crucial for craniofacial development.[12] Likewise, the single *AmphiMsx* gene is expressed in the lateral neural plate and later restricted to the dorsal part of the neural tube.[13] *Msx-a*, one of the two *Msx* genes found in ascidians, is expressed in the ectoderm and mesoderm at sites that are undergoing morphogenetic movements, such as the neural plate as it folds to form the neural tube.[14] The second, *Msx-b*, is expressed in the neural tube.[15]

Several members of the Zic family of transcription factors are involved in vertebrate neural development, and Zic2 seems to retain the cells at the border of the neural plate in an undifferentiated state, preventing them from differentiating into dorsal neurons. In this way, the development of alternative dorsal fates, such as neural crest, is favored.[16] Furthermore, other family members have been more directly implicated in neural crest development.[17,18] It is therefore noteworthy that, a Zic ortholog in amphioxus is also expressed at the neural plate border during early neurulation stages.[19]

In summary, the transcription factors involved in the dorsalization of the neural plate/neural tube in vertebrates have representatives in the nonvertebrate chordates, indicating that the dorsoventral patterning is conserved and established before the divergence of the vertebrate lineage.

Regarding the signaling pathways that induce dorsalization and neural crest formation in vertebrates, it is worth mentioning that triggered by the bone morphogenetic proteins (BMPs). A gradient of BMP activity has been described in *Xenopus* and zebrafish that influences cell fate.[20,21] In areas with intense BMP activity epidermis forms, whereas low levels of activity are permissive for neural development and in the intermediate regions, the neural crest forms. In amniotes, BMPs are clearly expressed in the nonneural ectoderm from where they can influence the development of the neural crest. Amphioxus has a single BMP2/4 ortholog that is also expressed in the nonneural ectoderm[22] and it could therefore play a role in determining the dorsal part of the neural tube.

Interestingly, one of the functions of BMP4 later in development highlights the evolutionary importance of the neural crest. Transplanting neural crest cells from duck into quail embryos and viceversa, resulted in the formation of a characteristic duck beak in a quail embryo and viceversa.[23] Hence, the morphology of the beak depends on the neural crest, this being one of the best examples of evolutionary adaptation to functional diversity in different birds. Indeed, differences

in the levels of BMP4 seem to be responsible for different beak morphologies, not only between chick and duck,[24] but also between closely related species such as the Galapagos finches.[25]

Cell Migration: A Whole Body to Populate

One of the key characteristics of the neural crest cells is its ability to migrate and populate all parts of the embryo. Once specified, they undergo an epithelium to mesenchyme transition (EMT) and migrate as individual cells. To study the origin of the neural crest in evolution, the basal chordate embryos have been scrutinized to search for migratory cells that might resemble a primitive version of the vertebrate crest.

In the amphioxus embryo, nonneural ectoderm cells at the neural plate border migrate towards the midline to cover the neural plate.[26] Unlike the true neural crest, these cells migrate as a continuous sheet rather than as individual cells because they do not undergo EMT. Interestingly, the single *Dlx* representative in amphioxus, *AmphiDll*, is expressed in those migrating ectodermal cells[26] while in vertebrates *Dlx* genes are expressed in the neural crest after the onset of migration. As discussed by Holland et al,[26] it is possible that the ancestor of craniates neurulated as amphioxus does, and that the migratory epidermal cells became integrated into the dorsal part of the neural tube in the transition to vertebrate neurulation. Thus, these cells may be the precursors of the neural crest. The acquisition of the ability to migrate as individual cells would have subsequently been acquired to permit delamination from the neural tube. If this were true, at least part of the neural crest would have a nonneural origin. In relation to this, James Weston has recently proposed a provocative theory that suggests that the nonneural ectoderm adjacent to the neural plate could produce cells that after undergoing EMT, would migrate to give rise to the head ectomesenchyme (cartilage and bone) which is generally believed to derive from the neural crest.[27] The proposal is that the crest would have a neural and a nonneural component. Interestingly, the Dlx-positive neural crest cells give rise to ectomesenchymal derivatives in the vertebrate head.[28] Thus, if the "nonneural" crest exists, the *Dll*-positive cells of amphioxus could be the precursors.

In addition to the movements of the nonneural ectodermal cells to cover the neural plate in amphioxus, it has recently been reported that some cells from the anterior part of the neural tube in the ascidian embryo have migratory capacities characteristic of neural crest cells.[29] These migratory cells were identified in *Ecteinascidia turbinata*, an ascidia species whose embryos are especially large, therefore facilitating cell labeling and tracing. Labeled cells from the anterior neural tube could be seen to migrate individually between the dorsal mesoderm and the epidermis. Most of these cells differentiated into pigmented cells and expressed neural crest specific markers such as HNK1 and the transcription factor Zic. This is possibly the best evidence that these cells are neural-crest-like in the sense discussed by Stone and Hall.[3] The cells are located at the appropriate place, express genes characteristic of vertebrate neural crest, and they delaminate and migrate individually generating one of the cell types known to be derived from the neural crest, pigment cells. From this finding, one may speculate that in a further step, other cells that arise in the neural tube could also migrate and differentiate into different cells types, such as peripheral neurons and glia.

Another example of individual ectodermal cells migrating through the mesenchyme comes from amphioxus.[30] Cells that migrate dorsolaterally from the ventral side of the embryo fit with the dorsolateral shift in the expression of the single amphioxus ortholog of the vertebrate *Trk* genes, the neurotrophin tyrosine kinase receptors. Trks are related to the sensory functions of structures derived from the neural crest and placodes in vertebrates.[31]

Members of the Snail-family of transcription factors are among the earliest genes expressed in the prospective neural crest at the neural plate border where they are essential for triggering the EMT.[32] In amphioxus and ascidia, *snail* genes are also expressed along the edges of the neural plate,[6,33] although, *snail*-expressing cells have not been seen to delaminate and migrate. It would be very interesting to determine whether the migratory cells recently described in ascidia express the *snail* ortholog.[29] Even considering the exciting discovery of these cells, the

production of migratory cells in nonvertebrate chordates is minimal when compared to that in vertebrates. Since *Snail* genes are crucial for triggering the EMT, they could represent the link between neural crest determination and migration. This raises the intriguing question of why so many *snail*-expressing cells do not delaminate and migrate in basal chordates. It could be that Snail needs partners to be able to fully induce the migratory phenotype and that these can only be recruited in vertebrates. The recruitment of downstream targets could also have occurred only in the vertebrate lineage. Alternatively, it is possible that the environment is not permissive even if *snail*-expressing cells in amphioxus could migrate. In addition, there is still no evidence that snail is active in the neural tube of ascidian and amphioxus embryos. The possibility that the Snail protein is not translated or is maintained inactive by post-translational mechanisms still cannot be excluded. To try to understand why the snail-expressing cells (or the majority of them) are retained in the neural tube, it would be extremely interesting to analyze whether ectopic expression of ascidian, amphioxus or vertebrate *Snail* genes could induce migratory behavior in amphioxus or ascidian embryos.

Consolidation of the Neural Crest Population: The More, the Merrier

As we have just mentioned, it seems possible that cells can migrate away from the neural tube in basal chordates. However, as far as we know, migration appears to be quite limited in terms of the number of cells. Thus, in order to develop a proper neural crest population, a further step had to be acquired during evolution: the capacity to produce migratory cells in significant numbers. In this sense, it has been shown that Sox2 expression in the neural plate is incompatible with neural crest formation in vertebrates, and that it is downregulated at the neural plate borders.[34] The only representative of the B sub-family of *Sox* genes in amphioxus is *sox 1/2/3* and it is not excluded from the neural plate borders.[35] As discussed by Meulemans and Bronner-Fraser,[37] the repression of *sox2* at this location may have been a necessary evolutionary step to permit the neural crest to form.

In addition to establishing permissive conditions, a mechanism to consolidate the potential population of neural crest cells is also necessary. Such a process could be fulfilled by *Id3*, a member of the helix-loop-helix inhibitors. Knock-down experiments in *Xenopus* revealed that Id3 is essential for the survival and cell cycle progression of neural crest progenitors at the neural plate border.[37] Moreover, forced expression of Id3 in migratory neural crest apparently maintains them in a progenitor state.[38] Although it remains unclear whether Id3 plays a role in fate determination in the prospective neural crest territory, the proposed functions of Id3 would contribute to the segregation of the neural crest from other dorsal derivatives and its consolidation as an undifferentiated precursor population. In relation to this, it is interesting to note that the single amphioxus *Id* gene is not expressed in the neural plate.[39] This is compatible with the idea that its expression in the dorsal neural tube could have helped to consolidate and expand the scarce population of migratory cells that constitute the evolutionary predecessor of the neural crest.

To Hox or not to Hox

The *Hox* genes provide some of the main influences that pattern the anteroposterior axis of the animal body from flies to humans. Their particular genomic organization in clusters has fascinated biologists since its discovery. Within each *Hox* cluster, the rostral limit of expression for each gene is directly related to its position in the cluster i.e.: those located towards the 3′ end of the cluster are expressed more anteriorly.[40,41] This property is known as colinearity and has important implications for the patterning of the hindbrain and the neural crest that streams out of the rhombomeres.[42,43] This colinear expression of *Hox* genes is also observed in the neural tube of amphioxus,[44] and interestingly, a regulatory region of the most "anterior" amphioxus *Hox* gene is able to drive expression in the migratory neural crest of transgenic mice and chicken embryos.[45] This finding implies that amphioxus has the regulatory machinery to express genes in the neural crest were it to develop and thus, that they just lack the final crest-inducing factor that appeared in the next step of evolution which shaped vertebrates.

Another peculiarity of *Hox* genes is that none of them are expressed in the most anterior stream of the hindbrain neural crest, which will contribute to the first branchial arch. In relation to this, it is interesting to note that Hoxa2 overexpression in neural crest cells that migrate to the first arch causes a transformation of the skeletal elements into a mirror image of those in the second arch.[46,47] Furthermore, in *Hoxa2* mutants, the skeletal elements of the second arch mimic those of the first arch.[48,49] These data indicate that the development of the structures derived from the first arch is not compatible with the presence of Hoxa2 and by extension, possibly with the expression of *Hox* genes. To investigate this suggestion further, the expression of *Hox* genes has been analyzed in lampreys, vertebrates without jaws (agnathans). In these vertebrates, a group *Hox6* gene is expressed in the first branchial arch,[50] which is compatible with the idea that its presence prevented the formation of the jaw. However, more recently the first pharyngeal arch of a different species of lamprey appeared not to express any *Hox* gene somewhat complicating this matter.[51] Hence, while the absence of Hox genes is compatible with the lack of jaws, it cannot be assumed that the presence of a *Hox* gene in the first arch is responsible for the lack of a mandible in lampreys. Nevertheless, we can still conclude that jaws are generally present when no *Hox* genes are expressed and thus, the absence of Hox may be a prerequisite for jaw formation, perhaps generating a permissive state.

Evolution's Toolbox I. Playing with Genes and Genomes: Two Is Better Than One

We cannot leave the topic of the Hox genes without talking about how evolution has affected the genome, these genes providing us with one of the clearest examples. One of the events that evolution has employed to produce diversity is the existence of massive gene or whole genome duplications.[52,53] In particular, it has been proposed that the evolutionary leap represented by the appearance of the craniates was aided by two whole genome duplications.[54] In support of this idea, many gene families composed of several members in vertebrates are represented in basal chordates by a single gene.[55] The *Hox* genes have undergone tandem gene duplications and have also been subjected to whole genome duplications. Among the most basal chordates, the tunicate *Hox* set is only partially clustered[56,57] and amphioxus has only one single *Hox* complex.[58] Vertebrates have four *Hox* clusters[59] and some ray-finned fish, like zebrafish, contain seven *hox* clusters.[60] This latter finding is in agreement with the extra-duplication proposed to have occurred in the teleost lineage.[61]

One of the current models that supports the genome duplication theory as a way to increase complexity and enhance evolution is the duplication-degeneration-complementation model (DDC).[62] This model explains the high rate of gene duplication and preservation. Right after the duplication event, the two duplicates are identical, yet since the native function can be fulfilled by only one of the copies, the other duplicate is left to mutate freely. Indeed, a rapid divergence of the duplicates may lead to the acquisition of a new function that conveys an adaptive advantage (neofunctionalization), while the other is forced to preserve the ancestral function. For pleiotropic genes, the partitioning of ancestral functions between copies (subfunctionalization) can also lead to positive selection and preservation of the duplicates.

The extra-duplication events believed to have occurred in the teleost lineage[61] enable the DDC model to be verified by comparing the expression patterns of the genes duplicated in zebrafish with that of the single-copy genes in other vertebrates. With respect to the neural crest, the duplications of the *snail1* and *sox9* genes represent good examples. The territories of expression of zebrafish *snail1a* and *snail1b* together (previously known as *snail1* and *snail2*, respectively)[63,64] are equivalent to those in which *Snail1* (previously *Snail*) expressed in the mouse. Of the two, only *snail1b* is expressed in the premigratory neural crest, a site of prominent *Snail1* expression in mammals. Thus, *snail1* has suffered subfunctionalization, which is compatible with our unpublished data showing that the knock-down of *snail1b*, but not of *snail1a*, leads to defects in neural crest development. Likewise, *sox9b* expression in the premigratory neural crest

is higher than that of *sox9a*.[65] *Sox9* is expressed very early during neural crest development in vertebrates and seems to be upstream of Snail genes in the network that regulates this process.[66] Within the framework of subfunctionalization, loss of *sox9b* but not *sox9a* function leads to defects in the neural crest that imply *snail1b* downregulation.[65]

Evolution's Toolbox II. Cooption: Take Genes from Your Neighbor Cells

In the context of the DDC model, neofunctionalization is exemplified by what has been called gene cooption, i.e.: when genes used in a particular tissue at a particular developmental stage, or for a particular process in the adult, are recruited to perform a new function. Cooption is a very valuable evolutionary tool and since the neural crest embodies an evolutionary novelty with significant implications for the formation of the vertebrate head, it could not escape from coopting genes already important in other tissues. The neural crest arises at the border between the neural plate and nonneural ectoderm, where complex networks of transcription factors interact throughout the processes from specification to differentiation. Furthermore, tissue interactions are also fundamental for neural crest formation since its development relies on signals from the ectoderm and the underlying mesoderm. Good examples of cooption can be seen by analyzing in nonvertebrate chordates several of the transcription factors involved in crest development in gnathostomes.[36]

The expression of the *Id* genes in mesoderm and endoderm is conserved between amphioxus and vertebrates. However, the expression in the dorsal neural tube is only observed in vertebrates,[39] compatible with *Id* being coopted to participate in the development of the nervous system. Indeed, as mentioned above, *Id3* seems to play an important role in consolidating the population of neural crest precursors in the vertebrate dorsal neural tube.[37,38] Hence, the lack of *Id* expression in the dorsal neural tube of nonvertebrate chordates may contribute to the absence of a consolidated neural crest population.

FoxD3 is one of the members of the wide fox (forkhead homeobox) gene family[67,68] that fulfills important functions in vertebrate neural crest development.[66,69-72] Both ascidians and amphioxus have a single *FoxD* gene whose expression is conserved in the mesoderm but not at the neural plate border.[73,74] Again, this is a case of cooption from the mesoderm that may have interesting implications in the process of neural crest development. Indeed, Cheung et al,[66] proposed that in the chick, FoxD3 participates in the alterations of cell-cell adhesion required for the neural crest to migrate.

Not only might cooption have been used to acquire the genes necessary during early steps of neural crest development, but it may also have been taken advantage of to obtain properties that may be beneficial at later stages. For instance, the AP-2alfa (tfpa2a) transcription factor is essential for the differentiation and survival of migratory neural crest cells, although it is expressed at the neural plate border from early stages.[75,76] The Amphioxus *AP2*, like the vertebrate *AP2* genes, is expressed in the nonneural ectoderm, but it is absent from the neural plate border.[77] Interestingly, AP2 family members are necessary for *Hoxa2* expression in the neural crest of mice,[78] and *AmphiAP2* and *AmphiHox2* expression overlaps in the preoral pit. In accordance with this, AP2 consensus binding sites have been found in the noncoding sequences of *AmphiHox2*.[77] Thus, the relationship between AP2 and Hox2 is conserved in amphioxus and the cooption of AP2 to play a role in the neural crest provided a new territory in which this gene network could interact.

Conclusions: So, What Now?

After reviewing some of the developmental and molecular aspects that might help to reveal the existence of neural crest precursors in nonvertebrate chordates, we can identify some of the factors involved that are either missing or misplaced in urochrodates and cephalochordates

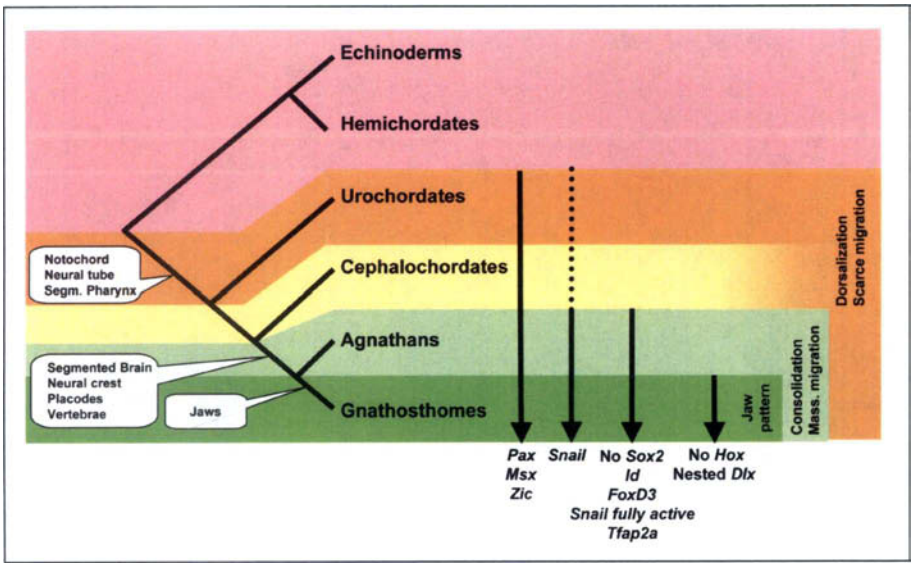

Figure 1. Scheme depicting the proposed staging towards the vertebrate state. Major features acquired in every evolutionary transition are indicated on the boxes on the left, while processes involved in neural crest formation are on the right and genes related to each process are cited below. In basal chordates, the dorsalization of the neural tube needed for the specification of the neural crest is already established, and the migration of some cells is observed; one *snail* gene is expressed at the appropriate location, but not linked to massive migratory behaviour (dotted line). The migratory neural crest population is consolidated in Agnathans. Gnathostomes add to this scenary the necessary machinery to pattern the jaw: absence of *Hox* expression in the first arch and nested expression of *Dlx* genes.

with respect to vertebrates, summarized in Figure 1. As such, the presence of Sox2 in the dorsal neural tube of basal chordates may have prevented the proper generation of the neural crest population and the expression of *Id* genes may be required to generate a solid neural crest population. The possibility of Snail being inactive or not fully active in the dorsal neural plate may be a further reason for cells not being able to migrate from the neural tube, and the absence of FoxD3 could prevent some of the necessary changes in cell-cell adhesion that favor migration. In the transition to the jawed vertebrates, the absence of *Hox* gene expression in the mandibular arch as a permissive state and the nested expression of *Dlx* genes allowed the formation and patterning of the jaw.[79]

Transgenic approaches in the mouse and ectopic expression studies in the chick have shown the ability of amphioxus sequences to drive expression in the vertebrate neural crest.[45] The possibility of microinjecting expression constructs in amphioxus[80] makes it extremely interesting to check whether the expression of the missing or misplaced genes could induce the formation of neural crest cells than might migrate from the neural tube and eventually differentiate.

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