Published in final edited form as:

Nature. 2015 April 23; 520(7548): 474–482. doi:10.1038/nature14436.

Evolution of vertebrates: a view from the crest

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Abstract

The origin of vertebrates was accompanied by the advent of a novel cell type: the neural crest. Emerging from the central nervous system, these cells migrate to diverse locations and differentiate into numerous derivatives. By coupling morphological and gene regulatory information from vertebrates and other chordates, we describe how addition of the neural crest specification program may have enabled cells at the neural plate border to acquire multipotency and migratory ability. Analyzing the topology of the neural crest gene regulatory network can serve as a useful template for understanding vertebrate evolution, including elaboration of neural crest derivatives.

Preface

The vertebrate body plan emerged in concert with extensive changes to anterior chordate morphology, including assembly of a craniofacial skeleton, expansion of the anterior neuroepithelium into a brain, reorganization of the pharynx, and appearance of novel sensory systems[1-3]. The genesis of this vertebrate “New Head”[1] has been fundamentally linked to emergence of two cell types, neural crest cells and ectodermal placodes. The neural crest is a transient vertebrate cell type, characterized by its site of origin within the central nervous system (CNS), multipotency, and ability to migrate and differentiate into numerous derivatives, as diverse as cartilage, bone, melanocytes, peripheral neurons and glia[4]. Together with ectodermal placodes that give rise to the sense organs of the head (see[5,6] for discussion of placode evolution), neural crest cells have contributed to the remarkable array of novel anatomies that make vertebrates unique.

Neural crest cells are unlike any other cell type, and advent of this progenitor cell population impacted chordate evolution in an unprecedented manner. Although cells with subsets of neural crest characteristics are present in invertebrate chordates, only vertebrates have a bona fide neural crest that gives rise to structural elements of the head, glia, pigment cells, and neurons. Imbued with broad developmental potential and extensive migratory ability, neural crest cells have gained developmental roles at nearly all axial levels and extensively interact with many other tissues. For these reasons, the neural crest is often referred to as the “fourth

Competing Financial Interests

The authors declare no competing financial interests.

novel germ layer"[7], associated with the emergence and elaboration of the vertebrate body plan[1,8,9].

In this review, we examine the morphological and genetic features that distinguish vertebrates from other chordates, focusing on cells and tissues derived from the neural crest. We place special emphasis on contributions that resulted in assembly of the vertebrate head, which has played a crucial role in establishment and diversification of vertebrates. We discuss the gene regulatory network underlying formation of early neural crest cells common to all vertebrates. We then use this network together with morphological criteria to discuss how neural crest cells may have emerged from putative homologues present in invertebrate chordates, highlighting how addition of the neural crest specification program may have enabled cells at the central nervous system (CNS) border to acquire multipotency and migratory ability. In this context, we examine how studies of neural crest gene regulatory networks may clarify patterns of morphological evolution within vertebrates, including expansion of neural crest derivatives during diversification of vertebrate taxa. Taken together, the data paint a picture of the neural crest as a malleable population that has continued to imbue the vertebrate body with novel features.

Neural crest-related innovations in early jawed and jawless vertebrates

Emergence of the vertebrate lineage was accompanied by acquisition of the neural crest and its novel derivatives. All vertebrates have neural crest cells that: 1) arise from the dorsal portion of the central nervous system, 2) exhibit multipotency by contributing to diverse derivatives, 3) undergo an epithelial to mesenchymal transition (EMT); and 4) have extensive migratory ability. 'Premigratory' neural crest cells initially reside in the dorsal neural tube, the newly formed CNS, of all vertebrates[10]. These cells undergo EMT to exit the CNS and migrate to numerous sites throughout the body, where they eventually contribute to their characteristic derivatives[4] (Fig. 1A). Cell lineage analyses have shown that many individual neural crest precursors can contribute to multiple cell types *in vivo*[11-13] and *in vitro*[14,15], and are thus "multipotent" stem or progenitor cells.

Comparisons between the two major groups of living vertebrates, the jawed vertebrates (gnathostomes) and their sister group the cyclostomes (agnathans)[16], identify many shared, derived traits likely to have been present in the neural crest of early vertebrates[17-20]. These include pigment cells, cellular pharyngeal cartilage and specialized pharyngeal musculature, an enteric nervous system, chromaffin cells, and perhaps cardiac valves[17,21]. Recent work has identified a new neural crest derivative, pillar cells[22] that support vertebrate gill epithelia (see Box 1). Because neural crest cells interact with many other tissues, they have a broad impact by modifying neuroepithelial patterning, craniofacial patterning, and cranial musculoskeletal development (See Box 2).

Many early vertebrate innovations are unique to jawed vertebrates and absent in cyclostomes. Some of these traits are likely to have arisen in stem gnathostomes, the early fishes leading to the jawed vertebrates. The best documented is the appearance of jaws, through modification of anterior pharyngeal arches. Other major gnathostome innovations include odontoblasts that produce dentine (See Box 1), paravertebral sympathetic chain

ganglia[23] (See Box 3), and exoskeletal armor. While exoskeletal armor might have arisen from neural crest at cranial levels, it is likely that trunk armor instead arose from mesoderm (See Box 4).

One central question in the early evolution of neural crest is the extent to which neural crest cell types are evolutionary novelties, rather than cell types (and regulatory programs) coopted from other tissues. There are clearly some novel neural crest-derived cell types, including pillar cells and odontoblasts, but many neural crest cell types are similar to cells in related chordates[24,25]. These cell types might either be homologous, representing a cell lineage that was coopted and incorporated into the neural crest, or they might have arisen by convergent evolution. In particular, a genetic program specifying pharyngeal cellular cartilage is likely to have coopted from a cellular cartilage seen in the oral region of cephalochordates[26]. Assessment of cooption or novelty depends in large part on evaluation of gene regulatory networks that govern their formation.

A Neural Crest Gene Regulatory Network is conserved across vertebrates

From a gene regulatory perspective, the body plan of all metazoans is encoded in the genome. During embryonic development, this code emerges as a complex gene regulatory network (GRN) formed by transcription factors and cis-regulatory elements, that cooperate with noncoding RNAs and epigenetic factors to pattern the body and drive development of individual elements and cell types[27]. According to this framework, the body plan modifications observed during evolution are a direct consequence of changes in the developmental regulatory program[28].

Neural crest cells are characterized by site of origin, migratory behavior and multipotency. Importantly, they also share a molecular signature, expressing a suite of transcription factors, including *tfAP2*[29], *Snai1/2*[30], *FoxD3*[31-33] and *SoxE*[34,35] genes. In particular *FoxD3* and *SoxE* are characteristic of premigratory and early migratory neural crest cells and *SoxE* genes are critical upstream regulators of all neural crest lineages. These transcription factors are part of the regulatory machinery that controls transcription of numerous effector genes, which together endow the neural crest with its unique properties. Interactions between transcription factors and their targets generate a GRN that controls neural crest formation, from induction at the neural plate border to differentiation into distinct cell types[36-39] (Fig. 1B).

The architecture of the neural crest GRN is thought to underlie the features observed in this cell population, such as multipotency and migratory capability. Functional experiments suggest that the neural crest GRN is comprised of distinct hierarchical levels[36,38]. First, signaling events (GRN Signaling Module) initiate the specification process, by inducing coexpression of transcription factors that comprise the ‘Neural Plate Border Module’. This in turn leads to specification of bona fide neural crest cells (Neural Crest Specification Module), their migration from the CNS to diverse sites (Neural Crest Migration Module), and finally to diversification into different derivatives through the deployment of distinct Differentiation Gene Batteries[36-39] (Fig. 1B). Each level of the neural crest GRN corresponds to a regulatory state that not only defines cell identity and behavior at a given

time point, but also drives transition to the next module of the network[40]. From an evolutionary perspective, assessing conservation of different levels of the neural crest GRN helps to identify the origin of each subcircuit and reconstruct the evolutionary history of neural crest cells[27,28]. As a result, the neural crest GRN provides a useful platform for understanding the molecular underpinnings of vertebrate evolution and how these cells may have participated in modifying vertebrate embryonic development. Neural crest GRN studies have indeed provided important clues regarding the establishment of the vertebrate lineage and its diversification[40-42].

Extensive work performed in amniotes, frogs, teleosts, and cyclostomes has revealed remarkable similarities in the overall structure of the neural crest GRN, demonstrating that it is virtually the same from amniotes to cyclostomes (Fig. 1B)[8,10,19,43]. Some important species-specific differences exist, but they are likely to reflect the continuous restructuring of the GRN in individual clades. Nevertheless, expression patterns and epistatic interactions between *FoxD3*, *SoxE*, *Snai1/2* and *Pax3/7* transcription factors points to a very conserved module of neural crest specification[38]. The overall conservation of the neural crest GRN correlates with conservation of morphology, migratory behavior, and differentiation into multiple derivatives, establishing the neural crest as an ancient vertebrate cell type. Superimposed upon the conserved basic structure of the neural crest GRN is adaptability and flexibility. During the course of evolution, differentiation modules that encode for novel derivatives, such as jaws and sympathetic ganglia, have been added to the neural crest repertoire and thus must have been added as “plug-ins” to the GRN.

While the core elements are highly conserved, adaptations, additions, and potentially losses, have occurred between species. Indeed, while it is clear that the specification module of the neural crest GRN is strongly conserved within vertebrates, there are important gene regulatory differences between jawless and jawed vertebrates that might provide interesting hints regarding the molecular roots of vertebrate morphological diversification. Extensive analysis of the lamprey neural crest GRN has revealed the notable absence of transcription factors *Ets-1* and *Twist* in the premigratory neural crest[10]. This is intriguing since *Ets-1* has been shown to be essential for cranial neural crest specification in gnathostomes[34]. Instead, in the lamprey, it is expressed much later in the neural crest derived portion of the branchial arches and dorsal root ganglia. One possibility is that *Ets-1* was added to the gnathostome neural crest specification, representing an example of cooption of a transcription factor present from distal to more proximal levels of the network. However, it is also possible that it may have been selectively lost in the lamprey neural crest. Examining expression of *Ets-1* in other cyclostomes and further functional experiments in lamprey may help clarify this point. Other GRN components that play critical functions in teleosts and amphibians may have been lost or replaced in amniotes. For example, while *Snai1/2* and *Twist* appear to be critical for neural crest formation in frogs[44,45], they are dispensable in the mouse[46] perhaps due to redundant functions with other EMT factors such as *Sip1*[47].

Taken together, these studies reveal that the topology of the neural crest GRN, with cells progressing through successive regulatory states from induction to differentiation, forms a useful template for understanding vertebrate evolution[36]. This GRN also can be useful for

assessing the likelihood that similar cell types in other animals might be homologous to the neural crest.

Do invertebrate chordates have neural crest cells?

Deciphering how the neural crest arose as a cell type is crucial for furthering our understanding of vertebrate evolution. Tackling this problem requires deeper knowledge of deuterostome embryonic development in multiple species, with particular attention to neural crest-like cell types in other chordates. In this regard, recent studies have described intriguing embryonic cell populations in ascidians that have some, but not all, neural crest characteristics. For example, the trunk lateral cells in the colonial tunicate *Ecteinascidia turbinata* are derived from the A7.6 lineage, which originates in the vicinity of the neural tube, undergoes migration and gives rise to pigmented cell types[48]. Similarly, in *Ciona intestinalis*, Abitua and colleagues show that the cell lineage a9.49 originates from the neural plate border and gives rise to the pigmented sensory cells of the otolith and the ocellus[49]. These cells normally translocate only a few cell diameters, whereas misexpression of Twist in this lineage results in acquisition of mesenchymal morphology and long range migration[49]. In cephalochordates, there have been many proposed homologs of neural crest (See [50] for discussion), most notably a bipotential neuroepithelial precursor to pigment cells of the ocellus[50]. Further assessment of this homology will require additional analyses of Amphioxus ocellus development. Cephalochordates also have an ependymal cell in the neural tube that expresses *Snail*, a neural crest specifier gene in vertebrates, but this cell appears to be non-migratory[51,52].

The neural crest GRN is particularly useful for understanding assessment of GRN conservation outside of vertebrates. The available molecular data obtained from embryonic cell types in tunicates and cephalochordates suggest that gene regulatory interactions that specify the neural plate border (Neural Plate Border Module) are deeply conserved throughout chordates[24,51] (Fig. 1C), and data from annelids suggests that this genetic program might be shared with protostomes, originating in stem bilaterians[53,54]. Similarly, the terminal differentiation programs (Differentiation Gene Batteries) that drive the neural crest to assume definitive fates are conserved, as exemplified by control of pigment cell differentiation. This is expected since most of the differentiation batteries are thought to be ancient subcircuits that were co-opted by different cell types[27]. Though they are integral parts of the neural crest GRN, these neural plate border and differentiation subcircuits do not fully define neural crest identity in vertebrates. Proximally in the program, the neural plate border contains other cell types (neural tube, placode) in addition to neural crest, and is important for the delimitation of the neural plate. Distally, other deuterostomes have some differentiated cell types that in vertebrates can arise from neural crest: melanocytes, ectomesenchyme, autonomic neurons, and glia. It has been proposed that during early vertebrate evolution, the neural crest specification module may have been assembled within the neural plate border cell lineage, interposed between the neural plate border and the distal differentiation modules of the network to endow these cells with a full “neural crest” phenotype.

Importantly, neural crest identity in all vertebrates is intrinsically linked to the Neural Crest Specification kernel of the GRN, which endows these cells with its defining features such as multipotency, ability to undergo EMT, and migratory capacity[40]. Important genes in the specification sub-circuit include *SoxE, FoxD* and *Snai1/2*, homologues of which are present in the genomes of invertebrate chordates[51,55]. For example, the amphioxus genome possesses all transcription factors identified in the neural crest specifier module of the vertebrate neural crest GRN. However, only *AmphiSnail* is expressed in the putative neural crest domain[56]. Therefore, a key question is whether the neural crest-like cells from tunicates possess this particular sub-circuit. Molecular analyses suggest that tunicates and amphioxus have the neural plate border subcircuit[24], and thus invertebrate neural crest-like cells may be homologous to neural plate border cells of vertebrates. However, while some neural plate specifier genes are expressed in these cells (e.g. *FoxD*[49]) other critical transcription factors, notably *SoxE* genes, appear to be absent. In ascidians, it is not yet clear whether epistatic interactions between the transcription factors expressed in putative neural crest cells are similar to those observed in the vertebrate neural crest GRN (Fig. 1C). This, together with the fact that cells of the a9.49 lineage have not yet been shown to be multipotent, or to have extensive migratory capabilities, makes it more difficult to determine whether they are true neural crest homologues. Further gene regulatory studies will be necessary to establish the relationship between these cells and the vertebrate neural crest.

As a cautionary note, there is inherent danger in assigning evolutionary relationships amongst cell types based on molecular similarity alone, since transcription factors are reused throughout development, and are neither lineage- nor cell type-specific. For instance, many bona fide neural crest transcription factors are expressed at the neural plate border, in later differentiation programs, and in other lineages. Thus, one cannot attribute homology or lineage relationships based on a few molecular markers. A more inclusive argument that includes morphological and behavioral information, expression data and, ideally, cis-regulatory studies[57] perhaps provides the most reliable means to establish conservation of developmental mechanisms and ascribe homology between cell populations.

Gene regulatory changes underlying the emergence of the neural crest

Radical changes of body plan, as those that took place in early vertebrate evolution, require substantial rearrangements in the structure of developmental GRNs[27]. The emergence of the neural crest was dependent upon the assembly of a specification subcircuit that allowed this cell population not only to exhibit its stereotypical behavior, but also to drive multiple differentiation programs, resulting in its multipotent state. Understanding how a novel, complex specification sub-circuit emerged during chordate evolution is a daunting task. However, observation of the neural crest GRN can provide important clues into vertebrate evolution and suggest likely scenarios for the creation of a novel cell type.

Given the deep conservation of the neural plate border specification program[24], it seems reasonable to assume that this circuit was critical for assembly of the vertebrate neural crest GRN. Since all of the neural crest specifier genes are present in the genomes of invertebrate chordates[58,59], it is likely that they were added to the GRN by deployment/cooption of transcription factors that were originally part of other developmental GRNs, such as the

neural plate border sub-circuit, mesodermal programs, and also from terminal differentiation modules. According to this view, changes in their cis-regulatory apparatus placed the neural crest specifier genes downstream of the neural plate border program and signaling systems. Such cis-regulatory changes might have facilitated redeployment of neural plate border (*Pax3/7, TFAP2*) and stem cell genes (*FoxD3*) in the specification module. For example, an amphioxus *FoxD* enhancer that recapitulates endogenous amphioxus *FoxD* expression in mesoderm and notochord[60] was able to drive similar expression when electroporated into chick embryos[51]. However, this enhancer failed to drive expression in the neural crest, suggesting that vertebrate transactivators were able to drive AmphiFoxD-mediated reporter expression in mesoderm but not in neural crest[51]. Similarly, co-option of EMT driver genes such as *Snail2*[30] and *Sip1*[47] may have allowed the neural crest to leave the neural plate border/neural folds. This was likely accompanied by co-option of mesenchymal gene circuits that allowed these cells to exhibit migratory behavior.

A key feature of the neural crest is its ability to form numerous derivatives, i.e. multipotency. Mechanistically, this implies that neural crest cells are capable of deploying a variety of differentiation gene batteries depending upon environmental interactions during migration and their final site of localization. Neural crest specifier genes from the *SoxE* family play a crucial part in activating differentiation programs that lead to multiple derivatives, as diverse as neurons, Schwann cells, pigment cells, and cartilage[38]. Thus, a likely scenario was that a variety of differentiation gene batteries were placed downstream of the Neural Crest Specification Module by gain of function cis-regulatory changes, which placed *differentiation driver genes* (e.g. *Mitf, Ascl1, Phox2b*) under the control of neural crest specifier genes. Again, examples of redeployment of such ancient differentiation gene batteries by different cell types have been described in different contexts, and are thought to be a common feature in GRN evolution[27,61]. Indeed, a study by Jandzik and colleagues[26] suggest that cis-regulatory changes in ancestral pro-chondrocytic genes allowed for their activation in the neural crest by factors such as *SoxE* and *Tfap2*, allowing for the establishment of the vertebrate head skeleton. Thus, it is possible the emergence of the neural crest specifier module served as a platform for the re-deployment of multiple, pre-existing genetic sub-circuits that endowed the neural crest with its defining features.

While cis-regulatory changes were probably the most important events in emergence of the neural crest specification module, it is also likely that changes in protein sequence played an important role therein. Neural crest cells employ a large repertoire of adhesion molecules, receptors and signaling molecules, and gene diversification and neofunctionalization might have enabled acquisition of complex cell behaviors exhibited by the neural crest. Furthermore, recent data suggest that neofunctionalization of neural crest specifier genes like *FoxD3* was important for emergence of this cell type[62], perhaps by mediating new protein-protein interactions and allowing for the assembly of novel, vertebrate specific transcriptional complexes.

A role for gene duplications in early neural crest evolution

The extensive changes in gene regulation required for the evolution of the neural crest as a cell type might have been facilitated by large-scale genome duplications that took place

early in the vertebrate lineage. It has long been suspected that rare, large-scale genomic rearrangements and genome-wide duplications in stem vertebrates played a key role in elaborating the vertebrate body plan[54,63-65] and increasing vertebrate complexity[66,67]. The presence of multiple homologous Hox clusters and conserved syntenic paralogy regions among jawed vertebrate chromosomes are usually taken to support the contention that there were two rounds of genome duplication during early vertebrate evolution[66]. Recent analysis of the genome of the sea lamprey (*Petromyzon marinus)* suggested that ancestors of lamprey (and hagfish) diverged from vertebrates after these two rounds of duplication[68-70], but this is still controversial, and an alternate model suggests there was only a single round of duplication in stem vertebrates, followed by lineage-specific segmental duplications in jawed vertebrates and cyclostomes[71]. Analysis of genomic sequence in the Japanese Lamprey (*Lethenteron japonicum*) suggests they might have two additional Hox clusters, raising the possibility that cyclostomes might have gone through a third, lineage-specific genome duplication[72] (See Fig. 2). Regardless of the precise number and timing of genome duplications, vertebrates have certainly undergone additional gene duplications relative to invertebrates, and these increases in gene number may have facilitated evolution of vertebrate regulatory and anatomic complexity[63], potentially impacting the formation of the many novel cell types in vertebrates.

A full assessment of the extent to which gene and genome duplications have affected early vertebrate evolution remains incomplete, and is somewhat controversial[73]. One way to approach this question is to determine whether the timing of acquisition of particular traits compares with inferred timing of gene duplications. Many traits were thought to arise in the vertebrate stem: these include key innovations such as the addition of neural crest-derived pharyngeal cartilages, modification of cranial muscles, the development of segmented and Hox-patterned hindbrain, and perhaps the beginnings of peripheral nervous organization (See Fig. 2). These distinct vertebrate characters are rooted in invertebrate chordates but appear to have been fundamentally transformed by the innovation of neural crest cells and their interactions with other cell types. Thus, the timing of acquisition of these traits correlates nicely with inferred instances of genome duplication, although one cannot distinguish cause from effect.

Ultimately, the fundamental question is how genomic duplications impacted the organization of developmental GRNs. As discussed by Ohno[54], such duplications may cause important shifts in gene regulatory mechanisms during vertebrate evolution. Indeed it is possible that large-scale genome duplications may have facilitated extensive changes in the cis-regulatory apparatus controlling transcription of neural crest genes[74], leading to their co-option and assembly into the Neural Crest Specification Module. Such events might have enabled the deployment of novel genes, like *SoxE* transcription factors, in the neural crest specification module. Depending on the species, *Sox8*, *Sox9,* and *Sox10* have early and sometimes overlapping functions in neural crest specification, with different paralogs deployed at different times depending upon the species. However, expressing at least one of the *SoxE* paralogs appears critical for maintenance of neural crest identity. Interestingly, it has recently been shown that *Sox10* alone is sufficient to reprogram fibroblast cells to a neural crest fate, highlighting the importance of *SoxE* genes in neural crest specification[75]. Furthermore, acquisition of migratory ability by the neural crest may have been fostered by

diversification of receptors and ligands that enabled chemotactic behavior. Genome-wide analysis shows that vertebrates have a much more complex arsenal of such molecules than do invertebrate chordates[58,76]. Thus, while the role of whole-genome duplications in neural crest evolution still is not fully understood, it is likely these duplications provided the neural crest with the molecular toolkit necessary for its complex behavior.

Evolution of Different Neural Crest Populations along the Rostrocaudal Axis

Neural crest cells arising from different levels of the neural axis are endowed with distinct developmental potentials and behavior. For example, the cranial neural crest of gnathostomes gives rise to ectomesenchymal derivatives (e.g. bone and cartilage of the face) in addition to melanocytes, glia and a subset of cranial sensory neurons. In contrast, the trunk neural crest is not able to contribute to cartilage and bone *in vivo*. Rather, these cells form melanocytes, dorsal root and sympathetic ganglia and chromaffin cells. Although the gene regulatory interactions underlying these differences remain unknown, they likely reflect disparities in the mechanisms of specification observed amongst neural crest subpopulations[33].

Classic heterotopic grafting experiments in the chick demonstrate that the trunk neural crest has a restricted developmental potential compared with the cranial population (reviewed in[4]). Cranial neural crest cells transplanted to the trunk not only can give rise to all trunk neural crest derivatives, but also form ectopic cartilage nodules characteristic of their site of origin[77,78]. In contrast, trunk neural crest transplanted to the head fail to contribute to facial bone and cartilage, although they can form sensory neurons and glia[79]. These results indicate that there are cell-autonomous differences between neural crest subpopulations established during specification. This is consistent with cis-regulatory analysis of neural crest specifier genes, which show that expression of both *FoxD3* and *Sox10* in the neural crest is controlled by separate enhancers in the head versus trunk[33,34]. Furthermore, activity of these enhancers depends upon axial-specific inputs, suggesting that specification of the cranial and trunk neural crest cells relies on different genetic programs[33,38].

The potential of the trunk neural crest has important implications for vertebrate phylogeny. For instance, it has been suggested that the neural crest played a central role in gnathostome evolution by giving rise to the exoskeleton of early vertebrates such as ostracoderms (armored fishes)[41]. According to this scenario, at some point during vertebrate evolution the trunk neural crest was endowed with ectomesenchymal potential, which was subsequently lost in extant vertebrates. This hypothesis is based primarily on the fact that the skeletal plates that form the exoskeleton armored fishes were composed of dentine, a bona fide neural crest derivative[80,81]. Furthermore, studies in different model organisms suggest that the trunk neural crest exhibits at least some ectomesenchymal potential. For example, fate map studies in zebrafish and frog performed with vital dyes indicate that trunk neural crest contributes to the mesenchyme of the fins[81,82]. Finally, *in vitro* clonal analysis of avian trunk neural crest cells has shown that some clones exhibit expression of genes characteristic of cartilage and bone[83], suggesting that these cells might possess a latent ectomesenchymal

potential, which can be unlocked by environmental signals[84]. These studies suggest the trunk neural crest might have some residual capacity to form ectomesenchyme, consistent with the hypothesis that the trunk neural crest gave rise to the exoskeleton of basal gnathostomes.

Recently, however, this view has been challenged by a number of studies that employ genetic fate mapping and cell transplantation analysis to define neural crest contributions in teleost fishes (See Box 4). These data show that mesenchyme-derived structures formerly attributed to the trunk neural crest lineage, such as the fin osteoblast, fin mesenchyme and mineral forming cells of the scales, are in fact of mesodermal origin[85-88]. Taken together, these studies indicate that the trunk neural crest of teleosts has the same developmental restrictions observed in amniotes, calling to question the neural crest origin of the exoskeleton in armored fishes. While further studies in other model organisms are necessary for a pan-vertebrate view of trunk neural crest potential, these results indicate that trunk neural crest has been devoid of skeletogenic potential throughout its evolutionary history. These findings suggest that alternate hypotheses for the evolution of the neural crest subpopulations require consideration.

In a second scenario, it is proposed that the cranial neural crest was endowed with gene regulatory mechanisms that are absent from the trunk and may have been “added on” early in vertebrate evolution. To date, a few developmentally important cranial specific regulators have been identified. In gnathostomes, for example, *Ets1*[89] and *Id2*[90] are enriched in cranial crest cells and are crucial neural crest specifier genes for this subpopulation, but their expression is absent from the trunk. This raises the intriguing possibility that the genetic circuits underlying ectomesenchymal potential were added to an ancestral, trunk-like neural crest GRN. According to this view, the ectomesenchymal machinery was either co-opted from the mesoderm[26] or assembled de novo in the cranial region. This scenario implies that the trunk neural crest cells have a simpler GRN topology than the cranial neural crest, an experimentally tractable hypothesis that can be addressed by comparative studies. This view is supported by the large number of transcriptional regulators that are shared amongst all neural crest populations, consistent with a common origin.

However, a complication is that transcription of genes like *Sox10* and *FoxD3* are activated uniformly along the entire neural axis but by distinct enhancers with differential inputs in the trunk versus cranial regions[33,34]. A third scenario proposes that neural crest subpopulations may have segregated early in vertebrate evolution and possess different gene regulatory network topology. Consistent with enhancer analysis, this hypothesis suggests that many ancestral neural crest GRN connections have been rewired during evolution and that these changes in topology resulted in two populations that have multiple differences in potential and behavior, despite sharing a similar genetic toolbox. This scenario implies that the trunk and cranial neural crest GRNs have substantial differences, and predicts that that pan neural crest genes are generally controlled by distinct, axial-specific enhancers. Importantly, the hypotheses discussed above can be tested by in depth analysis of the genetic pathways controlling neural crest formation at different axial levels. In particular, elucidating the circuits controlling ectomesenchymal differentiation of the neural crest will have great impact on how we interpret the evolution of this cell population. Furthermore, additional

neural crest subpopulations exist, including vagal and sacral subtypes, which have distinct migratory pathways and contribute to different derivatives. A more inclusive gene regulatory view of these subpopulations might clarify how the developmental potential of the neural crest is established at the regulatory level, and impact our views on the evolution of the vertebrate body plan.

Adult neural crest stem cells and post-embryonic growth

Like many invertebrates, the earliest vertebrate fossils show a small body size[91]. Only later did vertebrates begin to attain larger sizes, presumably through a process that involved extending the duration of post-embryonic growth. Extended growth requires coordinated development of many cell types, possibly including the establishment of stem cell-niches that govern the growth and regeneration of novel tissues.

Until recently there was little indication of how adult neural crest cell populations were maintained. Recent evidence suggests that amniotes have adult neural crest stem cell populations that maintain multipotency into adulthood, and which might enable the continuous replenishment of neural crest derived tissues[92,93], thus facilitating post-embryonic growth in concert with other tissues. These cells, called ‘Schwann cell precursors,’ reside on peripheral nerves and can produce multiple derivatives, including pigment cells and parasympathetic ganglia[94-97]. Whether the GRN underlying differentiation of these neural crest stem cells mirrors that of embryonic progenitor cells is an open and intriguing question that warrants further study. To date these cells have only been identified in amniotes (in mammals and avians), but there is an obvious need for cells that fill this requirement in other vertebrates, and it is likely that cells like these originated in early vertebrates.

These studies suggest that the influence of the neural crest in molding the vertebrate body plan may extend beyond embryonic development, perhaps influencing the increase in size observed in several vertebrate clades. As vertebrates continued to grow post-embryonically, they may have required the setting aside of a population of neural crest stem cells, in the form of Schwann cell precursors, that were retained to later stages. The degree to which these crest-derived stem cells contribute to derivatives of the adult is not yet known. Emerging data suggest that this cell population may form many derivatives classically attributed to the embryonic neural crest. Equally, they may represent the key to post-embryonic growth of the vertebrate body and therefore play a heretofore-unknown role in promoting vertebrate evolution.

Conclusion

Invention of the neural crest sets vertebrates apart from invertebrate chordates. Formation of this novel cell type was likely facilitate by addition of a new and uniquely vertebrate ‘specification’ kernel to the GRN, which in turn conferred multipotency and migratory ability to cells at the neural plate border/dorsal CNS. During the course of vertebrate evolution, ever more derivatives have been emerged under the umbrella of the neural crest (e.g. additional elements to the peripheral nervous system, elaboration of the jaw, formation

of the middle ear). Consolidation of key neural crest specifier genes like *FoxD3*, *SoxEs,* and *TFAP2* in the Neural Crest Specification module of its GRN may have facilitated evolution of this cell type, by allowing cooption of additional differentiation batteries under control of neural crest regulators. Arguably, this has made the neural crest one of the most rapidly changing cell types in the vertebrate embryo and perhaps contributed to the maintenance of neural crest stem cells in the adults.

Acknowledgements

We would like to thank Hugo Parker for comments on this manuscript. This work was supported by NIH grant R01NS086907. MS-C was funded by a fellowship from the Pew Foundation and by NIH grant 1K99DE024232.

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BOX 1

Neural crest derivatives and the vertebrate pharynx

Changes in pharyngeal patterning are central to the evolution and diversification of vertebrate groups[1,99]. Vertebrate pharyngeal arches have a similar general structure, characterized as a bilaterally symmetric series of endodermal evaginations that, with ectoderm, enclose a region of neural crest cells surrounding paraxial mesoderm[100,101]. Neural crest cells and paraxial mesoderm give rise to pharyngeal skeletal elements and musculature, respectively.

Some aspects of vertebrate pharyngeal patterning are integrated within or modified from features common to many deuterostomes. Pharyngeal segmentation is a trait of ancestral deuterostomes[102], and unambiguous pharyngeal arch homologues with similar genetic controls are present in hemichordates, cephalochordates, and adult urochordates[100,102], despite being secondarily lost in echinoderms[100,103]. Pharyngeal mesoderm also has a broad phylogenetic distribution, being present throughout chordates[104,105]. Neural crest derived cellular cartilage of vertebrates, rather than being a novelty of vertebrates[21], instead appears have been coopted from cellular cartilage homologous to that present within the oral cirri of Cephalochordates[26].

Though some vertebrate pharyngeal patterning stems from ancestral conditions, many novel elements arise from vertebrate neural crest cells. Modification of early neural crest development was important for generating the diversity of pharyngeal structures observed throughout vertebrates. For example, in vertebrate gills, epithelial surfaces are supported by novel neural crest-derived cells, pillar cells, which are ancestrally shared throughout vertebrates[22]. Additionally, in the transition from agnathans to gnathostomes, modifications to the anterior most pharyngeal arch cartilages and neural crest-modified musculature resulted in formation of the jaws, as well as formation of neck muscles[18,106-108].

Another vertebrate novelty associated with the pharynx and its integuments are odontodes: dental elements composed of mineral material and associated cells. In living jawed vertebrates, their formation is mediated by conserved gene regulatory sub-circuits, identified by coexpression of transcription factors including *runx2* and *eda/edar*, among others[109], and require the inductive influence of neural crest derived mesenchyme. Fossil evidence suggests that odontodes emerged during the evolution of stem gnathostomes, in external dermal armor[109-111], consistent with the ‘Outside-In’ model that odontodes emerged first as structural elements associated with external integument, and were later incorporated into the oral cavity and pharynx. Mineralized dental elements found in conodont fossils are considered nonhomologous to gnathostome teeth[110]. Both groups of living cyclostomes, lamprey and hagfish, have keratinized dental elements, but these are morphologically distinct from gnathostome teeth and are probably not homologous. Continued analysis of cyclostome dental elements might clarify whether neural crest cells played a role in their ontogeny.

BOX 2

Role of the neural crest in signaling

Brain and facial patterning

Increased complexity in vertebrate neuroanatomy might in part stem from interactions between neural crest cells and other cell types. An example of the important role of the neural crest in expansion of the head comes from recent experiments in amniotes[112]. Surgical removal of the neural crest at forebrain to rostral hindbrain levels results in the absence of facial and skull cartilages and bones, as well as severe brain defects including anencephaly[113]. These defects can be rescued by grafting small populations of premigratory neural crest from the same axial level, but not from more caudal regions with *Hox* gene expression. At a molecular level, this results from production of BMP inhibitors, Gremlin and Noggin, by the rostral neural crest that in turn lead to regulation of expression of FGF8 in the anterior neural ridge (ANR). Consistent with this, implantation of FGF8 beads after neural crest ablation rescues this phenotype to restore subsequent downstream signaling events and proper head development[101,114]. FGF signaling associated with an ANR-like signaling center is potentially present throughout deuterostomes[115,116], suggesting that that neural crest cells have adopted or coopted roles in regulation of neural/craniofacial patterning, at least in amniotes. Examination of additional vertebrate groups might clarify when this might have arisen.

Cranial muscles and the neural crest

The vertebrate head includes muscles that control the movement of the eyes (extraocular muscle), face, jaws, throat, larynx, and tongue, collectively called branchiomeric muscles[117]. Derived from unsegmented paraxial mesoderm anterior to the otic vesicle, they form under control of a Pitx2c and Tcf21/MyoR regulatory sub-circuit that appears to be conserved at least throughout the bony fishes[118,119]. The neural crest is crucial for multiple stages of cranial mesoderm development, including defining the location, orientation, patterning, and differentiation state of muscle precursor cells[57,107,108,117]. Mesoderm cells follow migrating neural crest cells into the pharyngeal arches[87,117]. Branchiomeric muscles initially remain in a precursor state, repressed by signals emanating from the nearby neural tube and ectoderm. Neural crest cells secrete signals that derepress myogenesis, allowing formation of cranial myofibers[120]. These distinct myogenic regulatory subnetworks are thought to have arisen in early vertebrates concurrent with other cephalic modifications[118,120], but have also been compared to muscle precursors in the amphioxus atrium[105] and potentially with visceral musculature of protostomes[121]. Vertebrate cranial muscle patterning, differentiation, and organization might require regulatory control that arose from novel interactions with neural crest (See Fig. 2).

Box 3

Trunk peripheral nervous system

The peripheral nervous system, comprised of sensory and autonomic ganglia including the sympathetic chain ganglia, is a common feature of all jawed vertebrates. Sympathetic ganglion cells are responsible for regulating homeostatic functions of peripheral organs. They arise from neural crest cells that migrate ventrally from the trunk neural tube to positions adjacent to the dorsal aorta, and form under the control of a gene regulatory circuit including *Phox2*, *Hand2*, and *Ascl1*. These genes collaborate to promote the construction of a sympathetic neural phenotype, including production of norepinephrine. In bony fishes and tetrapods, sympathetic ganglia are connected along the anteroposterior axis via chains, but in extant Chondrichyans (sharks, rays, and skates) ganglia are largely separate. Cyclostomes do not appear to have a comparably organized sympathetic system, but very rare ganglion-like cells of unknown function have been identified[122]. In general, autonomic function in cyclostomes appears to be controlled directly by spinal neurons of the CNS[122], which is similar to the peripheral organization of amphioxus, and thus is likely to represent a primitive condition for chordates. Taken together, these data suggest that sympathetic ganglia likely evolved in stem gnathostomes, and were further elaborated in stem osteichthyes.

BOX 4

Dermal skeleton

A dermal skeleton derived from odontodes is present in many vertebrates, both fossil and living. Dermal skeletal elements among living vertebrates include fin rays (lepidotrichia) of ray-finned (Actinoptyerygian) fishes and scales, with multiple subtypes including placoid, ganoid, and elasmoid scales in various taxa. Dermal skeletal elements have been proposed to be neural crest derived[123] at both cranial and trunk levels. However, recent analyses indicate that osteoblasts responsible for the elasmoid integumentary scales and fin rays of zebrafish derive from mesenchyme of mesodermal origin[88] rather than neural crest[81,124]. Similarly, ossified turtle shells that had been hypothesized to originate from both mesoderm-derived (endochondral rib) and neural crest-–derived (dermal) osteocytes, instead appear to develop only from mesoderm[125]. These data raise the question of whether the extensive dermal armor of stem gnathostomes originated from mesoderm or neural crest. At trunk levels, these dermal plates may have originated from mesoderm rather than neural crest, though they do arise from neural crest at cranial levels. However, it remains possible that neural crest cells contribute to other scale types, including the placoid scales of cartilaginous fishes that some have argued are more similar to dermal armor[88].

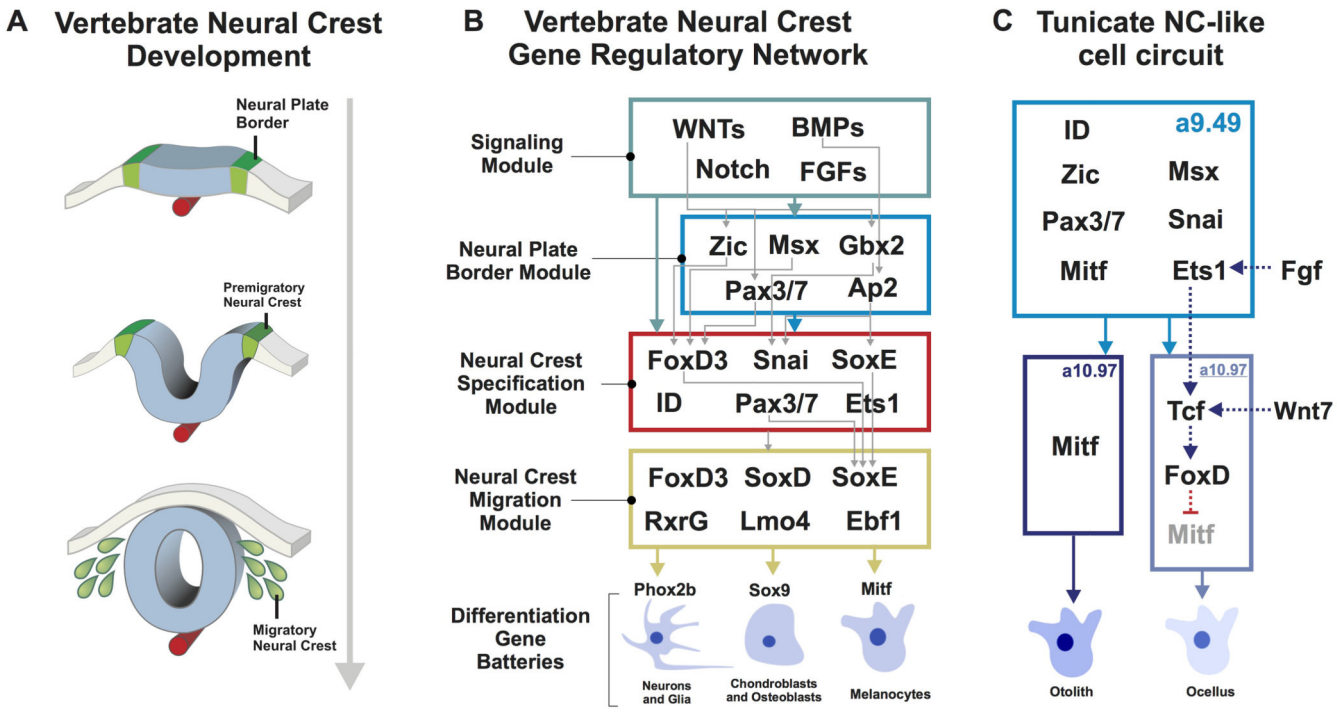

Figure 1.

Gene regulatory interactions controlling vertebrate neural crest formation and the tunicate a9.49 cell lineage. (A) Different stages in neural crest formation. Neural crest cells are defined by their origin at the neural plate border, epithelial to mesenchymal transition, migratory capacity and multipotency. (B) A neural crest gene regulatory network endows this cell population with its unique features. This GRN is comprised of different modules arranged hierarchically, which control each step of neural crest development[38]. Notably, the neural crest specification module, marked in red, appears to be missing from the neural plate border of invertebrate chordates. (C) Regulatory circuit of a tunicate neural crest-like pigmented cell precursor. Diagrams adapted from Simoes-Costa and Bronner[39] and based on the results of Abitua and colleagues[49].

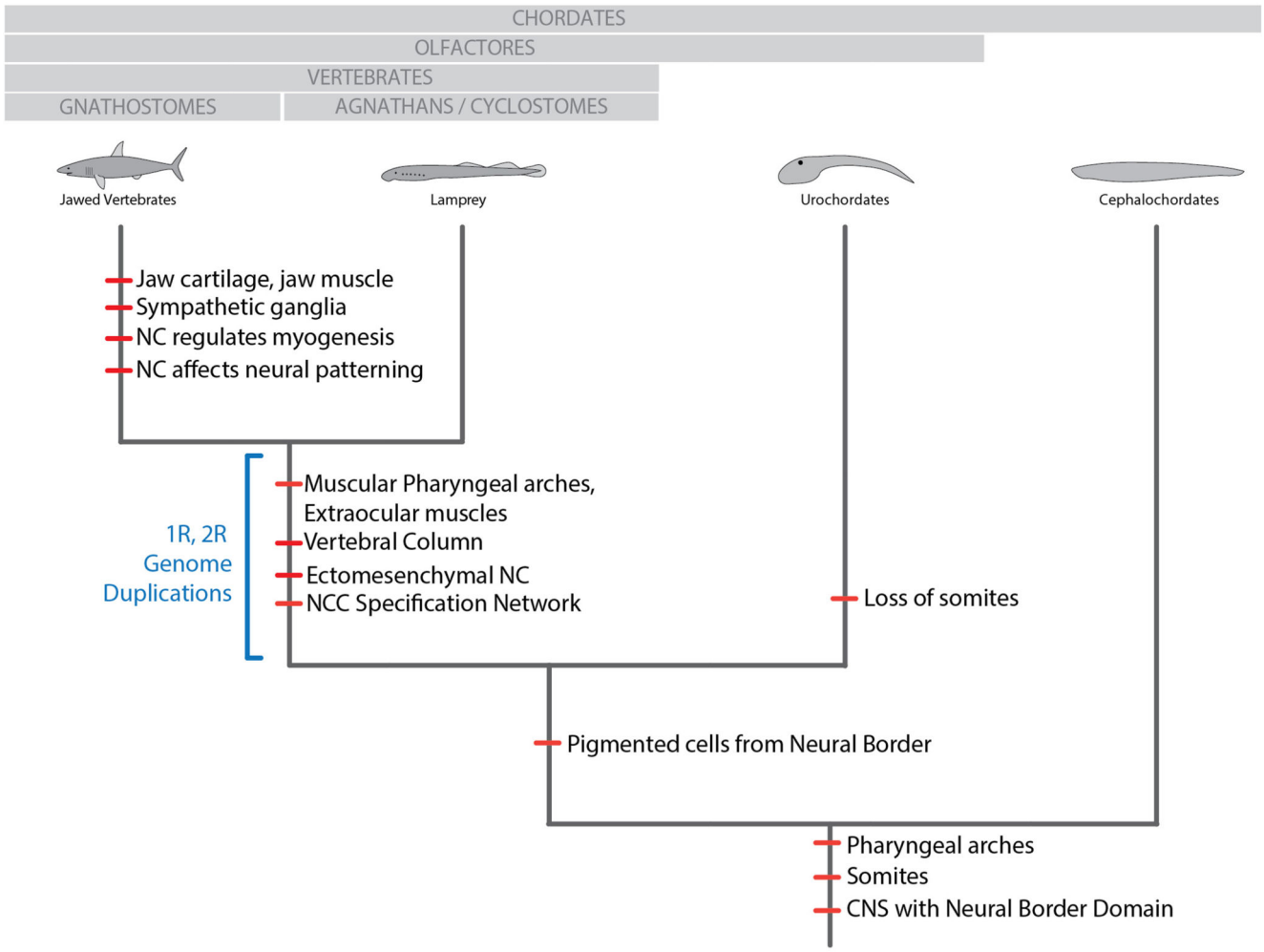

Figure 2.

Schematic cladogram of chordate features associated with neural crest cells or their derivatives. Labels at top indicate names of monophyletic groupings below. The timing of duplications is indicated in blue, while character changes are indicated by red lines. The order of character changes within a stem group is arbitrary. Adapted from Green and Bronner[98].