

Insights into neural crest development and evolution from genomic analysis

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The neural crest is an excellent model system for the study of cell type diversification during embryonic development due to its multipotency, motility, and ability to form a broad array of derivatives ranging from neurons and glia, to cartilage, bone, and melanocytes. As a uniquely vertebrate cell population, it also offers important clues regarding vertebrate origins. In the past 30 yr, introduction of recombinant DNA technology has facilitated the dissection of the genetic program controlling neural crest development and has provided important insights into gene regulatory mechanisms underlying cell migration and differentiation. More recently, new genomic approaches have provided a platform and tools that are changing the depth and breadth of our understanding of neural crest development at a “systems” level. Such advances provide an insightful view of the regulatory landscape of neural crest cells and offer a new perspective on developmental as well as stem cell and cancer biology.

The neural crest is an embryonic cell population with stem cell-like properties, including multipotency and the ability to self-renew. Unique to vertebrates, neural crest cells contribute to a wide variety of derivatives, including sensory and autonomic ganglia of the peripheral nervous system, adrenomedullary cells, cartilage and bone of the face, and pigmentation of the skin. Although similar cell types, such as pigment cells and sensory neurons, already exist in nonvertebrate chordates and other multicellular organisms, these derivatives arise *de novo* under the umbrella of the neural crest in the vertebrate lineage.

Since its discovery by His (1868), the neural crest has occupied a prominent place in developmental biology due to its extensive migratory properties and remarkable developmental potential. Interest in this cell population has been further fueled by its medical and evolutionary importance. For example, numerous congenital birth defects and neoplastic diseases are linked to abnormal development of the neural crest development and its derivatives (Hall 1999). Due to its inherent stem cell properties, there is great interest in using these cells in regenerative medicine to treat disorders like familial dysautonomia, cleft palate, and some heart conditions (Jones and Trainor 2004; Lee et al. 2009). Furthermore, as the neural crest gives rise to a number of vertebrate-specific traits, it is thought to have played an important role in chordate evolution (Gans and Northcutt 1983; Northcutt 2005).

The initial phases of neural crest formation include some of the most extensive morphogenetic movements observed during vertebrate embryonic development (Fig. 1). Initially the prospective neural crest cells reside in a territory known as the neural plate border, which is located at the edges of the neural plate, the embryonic region destined to form the central nervous system. Through a process called neurulation, the neural plate invaginates by elevation of the edges, or neural folds. The end result is the conversion of the flat neural plate into a cylindrical structure called the neural tube, which will later form the brain and spinal cord. During the process of neural tube closure, premigratory neural crest cells reside first within the neural folds as they converge

toward the midline and then in the dorsal aspect of the neural tube. Shortly thereafter, they lose their intercellular connections, undergo an epithelial to mesenchymal transition (EMT), and acquire mesenchymal, migratory characteristics that endow these cells with the ability to leave the neural tube (Gammill and Bronner-Fraser 2003; Sauka-Spengler and Bronner-Fraser 2008b).

Once they have emigrated from the neural tube, neural crest cells migrate in organized streams to populate different niches throughout the embryo. Depending upon their starting position along the body axis and the subsequent path taken, they give rise to different cell types and contribute to the formation of a variety of tissues and organs (Fig. 2). The cranial neural crest forms a large portion of the facial skeleton as well as cranial ganglia, smooth muscle, and pigment cells. The vagal neural crest has an important role in cardiac development since it contributes to the valves and septa of the heart and also forms the enteric nervous system that innervates the entire length of the gut. The trunk neural crest gives rise to dorsal root and sympathetic ganglia of the peripheral nervous system, as well as secretory cells and melanocytes. Finally, the neural crest formed at the sacral region cooperates with the vagal crest to form a small portion of the enteric nervous system (Le Douarin 1986; Le Douarin and Kalcheim 1999). Although neural crest cells appear to become progressively restricted as they reach particular targets, many early migrating cells are multipotent, with the ability to form several derivatives (Bronner-Fraser and Fraser 1988, 1989). Moreover, many neural crest-derived tissues appear to retain neural crest stem cells that are multipotent (Crane and Trainor 2006).

A number of classical experiments were fundamental for understanding the main characteristics of the neural crest. For example, early studies with amphibian embryos suggested that there was regionalization of neural crest populations along the body axis and broad developmental potential (Horstadius 1950). Importantly, the elegant quail-chick chimera experiments pioneered by Nicole Le Douarin in the 1960s and 1970s were central in defining the precise contributions of different populations of the neural crest to distinct derivatives in higher vertebrates (Le Douarin and Kalcheim 1999). Bronner-Fraser and Fraser (1988) tackled the question of the developmental potential of individual neural crest by labeling single premigratory cells with a vital dye. They showed that the progeny of a single neural crest cell included

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Article is online at <http://www.genome.org/cgi/doi/10.1101/gr.157586.113>.

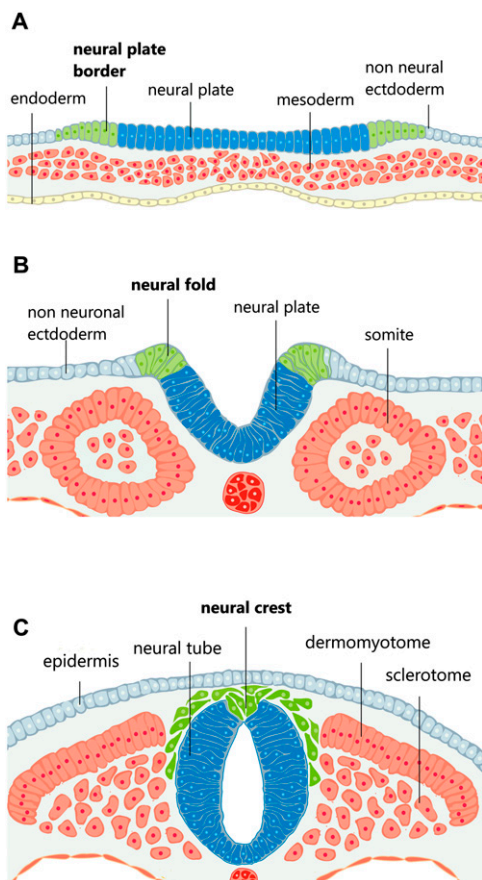


Figure 1. Morphogenetic movements during early neural crest development. (A) Schematic diagram of transverse sections through chick embryo during neurulation. Prospective neural crest cells reside in the neural plate border (green), a territory between the neural plate and the non-neural ectoderm. (B) As neurulation proceeds, the neural plate invaginates, resulting in the elevation of the neural folds, which contain neural crest precursors. (C) After neural tube closure, neural crest cells lose intercellular connections and undergo an epithelial to mesenchymal transition. Once they have delaminated from the neural tube, they migrate extensively to populate different niches throughout the embryo.

cell types as diverse as sensory neurons, presumptive pigment cells, ganglionic supportive cells, and neural tube cells (Bronner-Fraser and Fraser 1988). Clonal analysis of the neural crest in tissue culture further demonstrated multipotency of single neural crest cells to form multiple derivatives (Sieber-Blum and Cohen 1980; Trentin et al. 2004; Dupin et al. 2010) as well as their ability to self-renew (Stemple and Anderson 1992). However, the presence of some clones that formed single derivatives has been interpreted as suggesting a possible fate restriction in some subpopulations (Stemple and Anderson 1992). These and innumerable other experiments have characterized cellular properties of the neural crest that can now be tackled in molecular terms due to advances in molecular biology and genomics.

In the last three decades, advances in recombinant DNA technology and molecular genetics techniques have made it possible to start examining the genetic program controlling neural crest development. Identification of a number of tissue-specific transcription factors allowed for the description of the first regulatory interactions necessary for establishment of neural crest identity. Studies in several model organisms have provided

a wealth of molecular data that has helped to formulate our views on how complex processes such as induction, specification, differentiation, and migration are regulated. This extensive body of work has established the neural crest as a prime model for the exploration of questions central to developmental biology—such as how complex and diverse tissues arise from a seemingly homogeneous population of cells.

Currently, genomic approaches are being used to address essential aspects of neural crest development. The extensive work done on the signaling and transcriptional control of neural crest development culminated with the assembly of a gene regulatory network that attempts to explain, in molecular terms, the process of neural crest formation. Sequencing of chordate genomes has allowed for identification of novel genes involved in neural crest formation and provided numerous tools to scrutinize the regulatory mechanisms underlying this process. Furthermore, comparative genomic analyses are providing important insights about the evolution of the neural crest and the origins of the vertebrate clade. Here, we will present a synopsis of the current state of the field of neural crest biology from a gene regulatory perspective and will also discuss how recent technological advances can help shape future research.

Overview of the current state of the neural crest gene regulatory network (GRN)

The regulatory machinery controlling neural crest formation and diversification is comprised of an intricate array of transcription factors and signaling molecules that act in concert to provide this cell population with its defining features (Sauka-Spengler and Bronner-Fraser 2008b). In order to interrogate such a complex regulatory program, we and others have assembled a multistep neural crest GRN that integrates transcriptional inputs and diverse environmental signals (Meulemans and Bronner-Fraser 2004; Betancur et al. 2010a). A simplified version of the cranial neural crest GRN is represented in Figure 3. It is composed of a series of regulatory steps arranged hierarchically, which include induction of the prospective neural crest, specification of the neural plate border, specification of the bona fide neural crest cells, and the diversification of the neural crest cells through the action of neural crest effector genes (Sauka-Spengler and Bronner-Fraser 2008b). Remarkably, these regulatory stages not only define cell identity and behavior at a given time point, but also drive seamless transitions to the next regulatory state.

The first level of the neural crest GRN is comprised of induction events that lead to the formation of the neural plate border. This process is dependent upon the interplay of different signaling pathways such as Wnt, Fgf, BMP, and Notch/Delta. Secreted signaling molecules are produced by adjacent tissues and result in the activation of a particular set of genes in the edges of the neural plate. These *neural plate border specifier genes* (e.g., *Msx*, *Pax3/7*, *Zic1*, *Dlx3/5*) initiate expression prior to the traditional neural crest marker genes and often are down-regulated once neural crest identity is established. Their region of overlap at the neural plate border defines a broad territory of cells competent to respond to neural crest specifying signals and later form migrating neural crest cells (Meulemans and Bronner-Fraser 2004).

The next step in the neural crest GRN is initiated when neural plate border specifiers in combination with signaling molecules activate transcription of *neural crest specifier genes*. These include transcription factors like *Snai1/2*, *Tfap2a*, *Foxd3*, *Twist*, *Id*, *Myc*, and *Sox9/10*. Expression of these factors reflects the specification

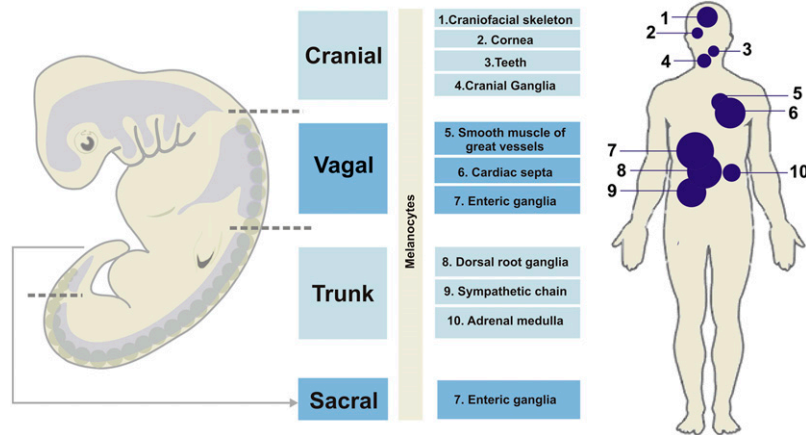


Figure 2. Contributions of different neural crest cell populations to adult tissues and organs. Depending on their axial level of origin and migratory pathway followed, neural crest cells adopt different fates and contribute to distinct tissues and organs. Cranial neural crest forms a large portion of the facial skeleton as well as cranial ganglia, most of the dental tissues, and the cornea. Vagal neural crest contributes to the valves and septa of the heart, the smooth muscle of the great vessels, and the enteric nervous system. Trunk neural crest gives rise to dorsal root and sympathetic ganglia of the peripheral nervous system and the chromaffin cells of the adrenal gland. Most caudally, the neural crest formed at the sacral region contributes to a small portion of the enteric nervous system. Melanocytes of the skin and integuments are derived from neural crest at all axial levels.

state of the neural crest population and endows them with its defining features, like the ability to undergo EMT and become migratory. Activation of the neural crest specifiers can be quite complex and depends upon various inputs from all levels of the neural crest GRN. For example, *Snai2* activation requires cooperation between elements of the Wnt pathway, *Zic1*, and *Pax3/7* (Sato et al. 2005). In addition, *SoxE* transcription factors are necessary for the maintenance of *snai2* expression (Honoré et al. 2003). Thus, factors from different hierarchical levels of the neural crest gene regulatory network operate in concert to establish and maintain the neural crest transcriptional state (Sauka-Spengler and Bronner-Fraser 2008b).

The neural crest specifier genes, in turn, regulate expression of effector genes involved in cell cycle control, epithelial to mesenchymal transition, and migration. *Neural crest effector genes* jumpstart a number of gene batteries that instruct the behavior of the newly formed neural crest cells, allowing them to delaminate from the neural tube, proliferate and maintain population size, migrate along different pathways, and finally differentiate into a wide variety of derivatives (Meulemans and Bronner-Fraser 2004; Sauka-Spengler and Bronner-Fraser 2008b). At the same time, the effector genes activate the expression of receptors and signaling molecules that equip the cells with the capacity to respond to environmental cues. This molecular toolkit also allows cell–cell interactions that influence not only other neural crest cells, but also numerous embryonic tissues with which they interact during migration. For example, neural crest cells instruct somite cells to differentiate into muscle precursors (Rios et al. 2011).

The neural crest GRN integrates >20 yr of work from many research groups performed in different model organisms, and provides a conceptual framework for the study of the neural crest genetic program. It is, however, not yet complete and will greatly benefit from the high-throughput genomic approaches that are currently part of the scientific repertoire. For instance, macroarray screens have uncovered numerous new molecules that are up-regulated in the neural crest (Gammill and Bronner-Fraser 2002;

Adams et al. 2008). Transcriptome analysis of pure neural crest populations has uncovered a variety of new specific transcription factors, nuclear receptors and signaling molecules that are expressed during different stages of development (M Simões-Costa and ME Bronner, unpubl.). Furthermore, novel techniques for genome-wide chromatin profiling allow genome-wide identification of active and poised *cis*-regulatory modules (Rada-Iglesias et al. 2012) and facilitate functional analysis for rapid characterization of epistatic interactions. Such approaches will increase the complexity of the neural crest GRN by providing a more accurate and complete representation of the different regulatory modules and their interactions.

The current version of the neural crest GRN is grounded in interactions between transcription factors and signaling molecules, and does not include extra levels of regulation such as those imparted by epigenetic, post-transcriptional and post-translational control. Nevertheless, there is increasing evidence pointing

to an important role for chromatin modification in the development of the neural crest. For example, the histone demethylase *JMJD2A* directly regulates a number of neural crest specifier genes, thus controlling the time of onset of their expression in the neural folds (Strobl-Mazzulla et al. 2010). Furthermore, the neural crest specifier, *SNAI2*, forms a complex with histone deacetylases (HDACS) to silence genes such as *CAD6B*, thus allowing neural crest delamination (Strobl-Mazzulla and Bronner 2012). Epigenetic silencing is also crucial for the initial demarcation of the neural crest territory. Hu and colleagues have shown that DNA methyltransferase3A promotes neural crest specification by repressing neural genes *SOX2* and *SOX3*, thus acting as a fate switch in the cells of the neuroectoderm (Hu et al. 2012). Recent results also emphasize the role of post-translational modifications in neural crest regulation. Lee and colleagues have shown that SUMOylation of *SoxE* factors causes recruitment of the repressor Grg4 (also known as Tle4) instead of its usual coactivator partners. This study highlights how post-translational regulation can alter gene function and drive the same factor to play opposing roles in a context-dependent manner (Lee et al. 2012).

At the present time, the neural crest GRN (Meulemans and Bronner-Fraser 2004; Betancur et al. 2010a) primarily focuses on the regulatory interactions of the cranial neural crest. Since important differences exist between distinct subpopulations of the neural crest along the body axis (Le Douarin et al. 2004), it would be naïve to assume that such interactions are common to all neural crest populations. Indeed, there are likely to be important modifications that help explain regional differences in neural crest cell migratory properties and cell fates (Simões-Costa et al. 2012). Thus a more inclusive version of the GRN should include the molecular circuits that are particular to each of the neural crest subpopulations. Studies scrutinizing the regulatory states of these distinct populations will reshape the GRN and provide important clues about the plasticity of the neural crest. Ultimately and optimally, the GRN will have predictive value, which will help anticipate the molecular outcome of particular pertur-

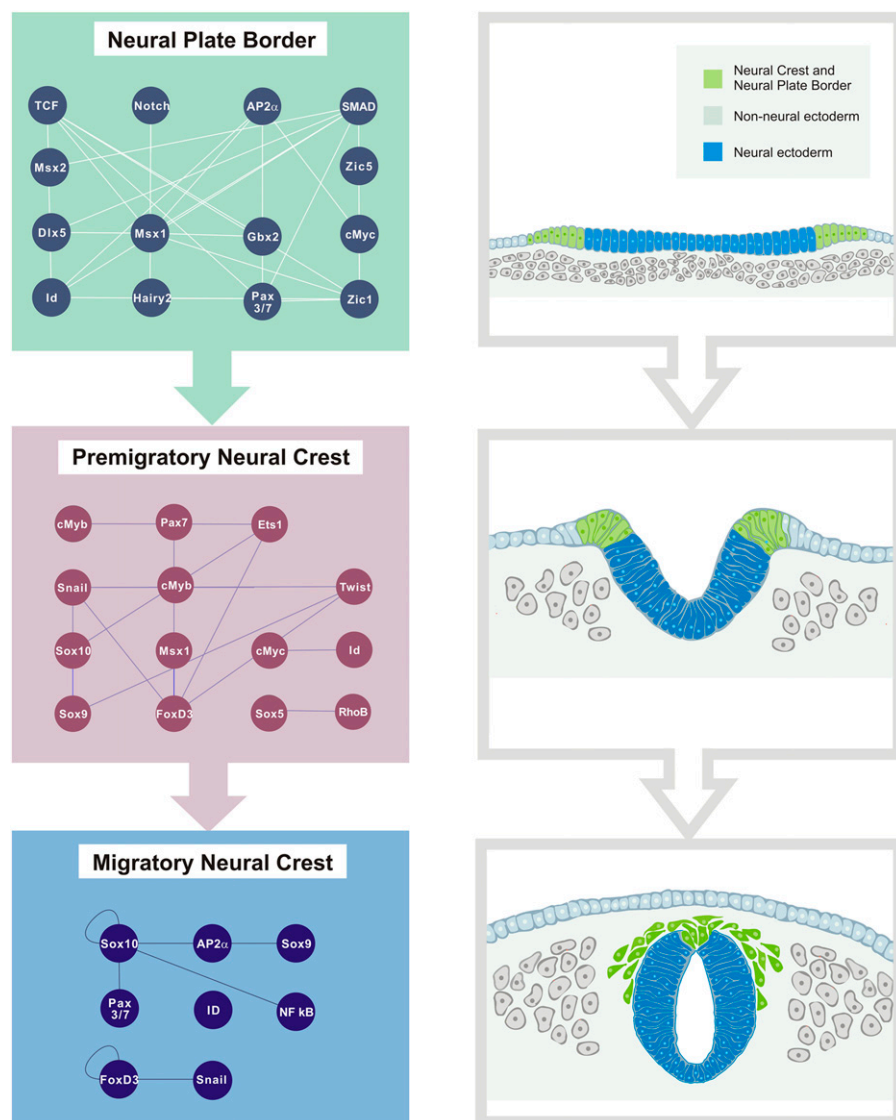


Figure 3. Cranial neural crest gene regulatory network (GRN). The neural crest gene GRN is composed of different regulatory modules arranged hierarchically. Each regulatory state defines cell identity and behavior at a given time and also drives the transition to the next level of the network. This is a simplified representation of the cranial neural crest GRN (Betancur et al. 2010a).

bations. This will be useful for etiological studies, of particular importance due to the large number of neural crest-related birth defects. A complete neural crest GRN will also inform upon genetic reprogramming of induced pluripotent stem (iPS) cells or embryonic stem (ES) cells into neural crest cells, which will have significant implications for regenerative medicine and cancer.

Cis-regulatory analysis of neural crest enhancers

The first version of the neural crest gene regulatory network was based on data assembled from a combination of gene expression patterns and functional studies obtained from several model organisms (Meulemans and Bronner-Fraser 2004). Although this approach provided a conceptual framework for investigating neural crest identity, further resolution of the GRN requires the identification of direct epistatic interactions among its players.

This can only be accomplished through systematic identification and characterization of the *cis*-regulatory apparatus that controls expression of specific components of the neural crest GRN (Fig. 4).

Tissue-specific enhancers have been invaluable for investigation of the genetic program underlying neural crest formation and can be exploited in several different contexts. This is exemplified by numerous studies that have taken advantage of the mouse *Wnt1* enhancer to address the contribution, potential, and genetic regulation of the neural crest. This enhancer region, which is a 5.5-kb element at the 3' region of the *Wnt1* locus, activates transcription in the dorsal neural tube and migrating neural crest cells of murine embryos (Echelard et al. 1994). It has been employed extensively in mapping the contribution of different neural crest populations (Jiang et al. 2000; Nakamura et al. 2006; Barraud et al. 2010) through the use of the *Rosa26* system (Soriano 1999). It has also been employed for targeted genetic manipulation through conditional knockout animals (Ito et al. 2003; Akiyama et al. 2004; Brewer et al. 2004; Dudas et al. 2004; Nakamura et al. 2006) that have genes inactivated specifically in the neural crest. This has allowed for the functional characterization of a number of genes in a neural crest cell-autonomous manner.

The initial approach for the identification of enhancers of neural crest genes was done by screening noncoding DNA isolated from the locus of the gene of interest (Fig. 4; Echelard et al. 1994). Fragments from the noncoding DNA were cloned upstream of a minimal promoter plus reporter sequence and tested *in vivo* or *in vitro*. The sheer size of the genetic loci and number of subregions that had to be tested made this approach laborious and time consuming, particularly given

the time and expense of using mice. New genomic tools have since emerged and revolutionized the way *cis*-regulatory modules are identified. Importantly, the sequencing of complete genomes has greatly benefited the search for conserved *cis*-regulatory elements (Cooper and Sidow 2003; Uchikawa et al. 2004). Comparison of the noncoding regions between multiple species has proved an efficient approach for uncovering evolutionarily conserved regions (ECRs) that may regulate transcription of neighboring genes (Uchikawa et al. 2003; Betancur et al. 2010b; Simões-Costa et al. 2012). The activity of the ECRs subsequently can be investigated by stable or transient transgenesis. In this context, the chicken embryo has proved to be an excellent amniote model for *cis*-regulatory analysis in higher vertebrates, due to highly efficient transient transgenesis accomplished through electroporation techniques (Sauka-Spengler and Barembaum 2008; Takemoto et al. 2011). Similarly, *tol2*-mediated transgenesis in zebrafish is an ef-

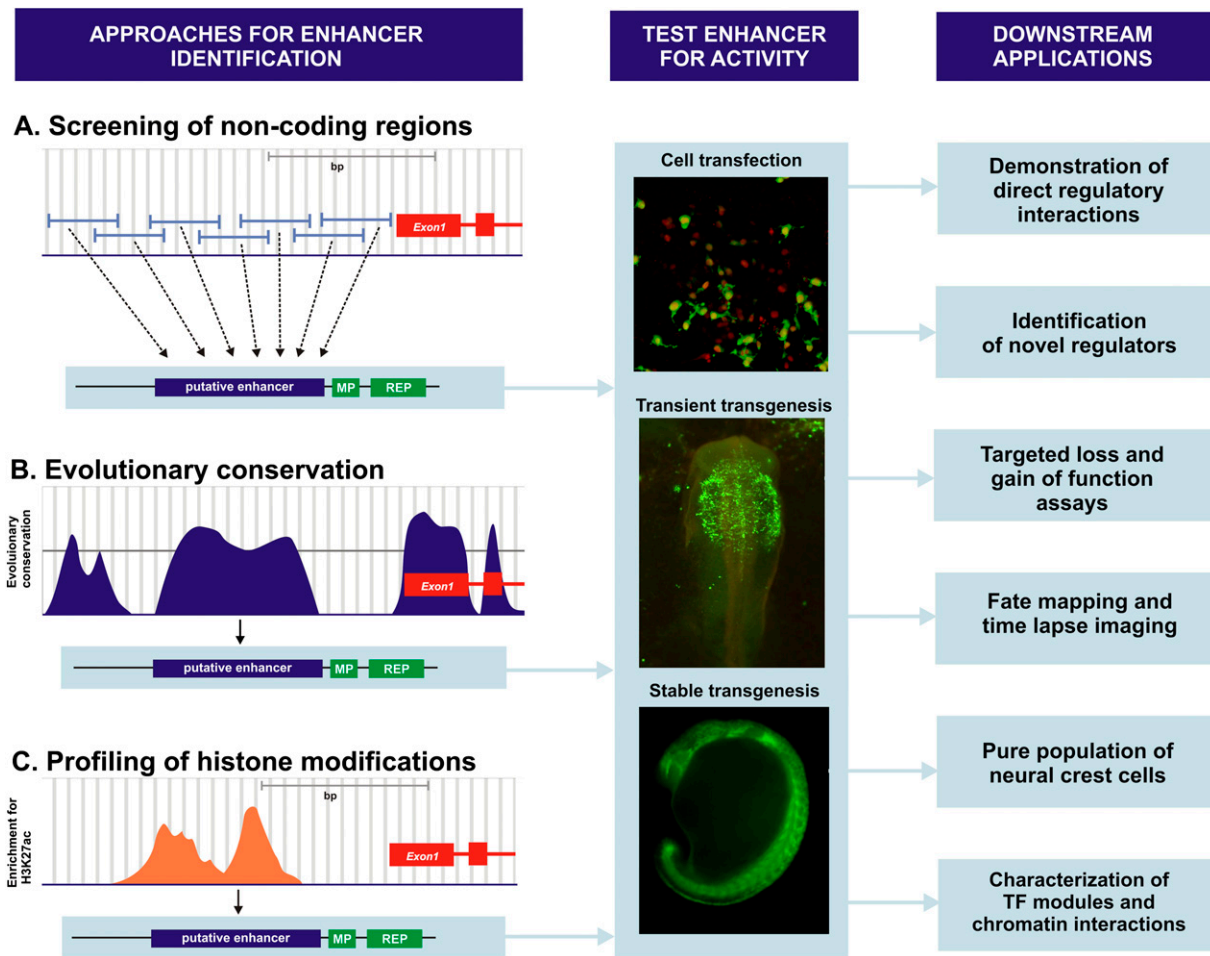


Figure 4. Strategies for *cis*-regulatory analysis of the neural crest. (A) Early approaches for identifying enhancers of neural crest genes included screening long stretches of noncoding DNA. Fragments from the locus of the gene of interest were cloned upstream of a minimal promoter and a reporter gene and tested *in vivo* by cell transfection or transgenesis. (B) Sequencing of vertebrate genomes facilitated the search for *cis*-regulatory modules. Computational comparison between different species reveals evolutionary conserved regions (ECRs) that are putative regulators of nearby genes. The ECRs are subsequently tested for activity by stable or transient transgenesis in different model organisms. (C) Novel approaches allow for genome-wide analysis of the *cis*-regulatory modules. Profiling of histone modification through ChIP-seq allows mapping of chromatin mark patterns and identification of active and poised enhancers. This method was used to annotate enhancers that are active in human neural crest cells induced from human embryonic stem cells (Rada-Iglesias et al. 2012). Once enhancers are validated *in vivo*, they become valuable tools that can be exploited in different contexts. (MP) Minimal promoter; (REP) reporter gene; (TF) transcription factor.

ficient way to test enhancers, including those that work across distant species (Fisher et al. 2006; Rada-Iglesias et al. 2012).

A number of neural crest-specific enhancers have been identified in the past few years in different model organisms. Comparative genomics was used to identify neural crest-specific enhancers for *Zic3*, *Sox9*, *Foxd3*, *Sox10*, *Snai2*, and *Twist*, among others (Smith et al. 1997; Bagheri-Fam et al. 2006; Betancur et al. 2010b; Garnett et al. 2012; Simões-Costa et al. 2012). For example, *cis*-regulatory analysis of murine *Pax3* revealed intriguing aspects of how neural crest genes are regulated. This analysis uncovered two distinct enhancers that are capable of driving very similar neural crest-specific expression. Mutation of either region by itself does not disrupt gene expression, and thus these enhancers constitute an interesting case of redundancy at the *cis*-regulatory level (Milewski et al. 2004; Degenhardt et al. 2010). The lack of similarity between the two regions also suggests that they require different inputs, highlighting not only the complexity of the regulatory code but also the necessity for thorough analysis of

the *cis*-regulatory apparatus (Degenhardt et al. 2010). Similar to the case in mice, a study in zebrafish by Garnett and colleagues shows that the zebrafish *pax3a* gene expression is also controlled by two distinct enhancers. Although manipulations in BMP, Wnt, and FGF signaling disrupt the activity of the individual enhancers, when combined in the same construct, they are less susceptible to environmental perturbations. Thus, synergistic activity of different regulatory modules can bring a level of robustness to transcriptional control, particularly in the case of genes downstream from signaling pathways (Garnett et al. 2012).

Tissue-specific enhancers for neural crest derivatives have also been identified. For example, evolutionarily conserved regions of the gene *Mef2c* (Agarwal et al. 2011), and tyrosinase-related family member *Tyrp1* (Murisier et al. 2006), can drive specific reporter expression in melanocytes. Interestingly, both enhancers seem to be regulated by the transcription factor Sox10, a key regulator of the melanocytic lineage. Similarly, a *Hoxb3* enhancer that is active in the vagal neural crest drives reporter expression in the enteric

nervous system (Chan et al. 2005). Such regulatory modules are useful tools for targeted functional studies, particularly in chicken and zebrafish embryos, given the new approaches for efficient and rapid integration of transgenes in these embryos (Fisher et al. 2006; Yokota et al. 2011).

Cis-regulatory analysis can also provide insights into how differences between distinct neural crest populations arise. The *cis*-regulatory analyses of both *SOX10* (Betancur et al. 2010b) and *FOXD3* (Simões-Costa et al. 2012) point to distinct genetic programs controlling gene expression at different axial levels of the chick embryo. Although both *SOX10* and *FOXD3* are pan-neural crest markers, different enhancers are responsible for driving transcription in the cranial versus trunk neural crest populations. Furthermore, cranial and trunk enhancers are activated by different inputs (Simões-Costa et al. 2012), which suggests an intrinsic heterogeneity in the neural crest population that precedes specification. For example, two enhancers, *NC1* and *NC2*, were uncovered, which collectively drive reporter expression that recapitulates the endogenous pattern of *FOXD3* in the neural crest but separately control expression in nonoverlapping neural crest populations. *NC1* drives initial expression in the cranial neural crest but is not active in the trunk region. In contrast, *NC2* mediates initial expression in trunk neural crest and only drives reporter expression later in migrating cranial crest cells that no longer have *NC1* activity. Whereas *PAX7* and *MSX1/2* are common inputs to both *NC1* and *NC2*, *ETS1* is a direct input into *NC1* and *ZIC1* into *NC2*. Consistent with axial level specific inputs, *ETS1* is a cranial-specific transcription factor, whereas *ZIC1* is expressed in a graded fashion, from posterior to anterior along the body axis (Simões-Costa et al. 2012). This differential regulation is consistent with the differences observed in the migratory patterns, potential, and behavior of the trunk versus cranial neural crest cells (Le Douarin and Kalchauer 1999). Further characterization of unique regulatory modules in distinct neural crest subpopulations hold the promise of providing important insights into neural crest heterogeneity and developmental potential.

Currently, an array of new technologies allows high-throughput identification of *cis*-regulatory modules. In the future, these will allow a systems biology-level analysis of gene regulation during neural crest formation. Importantly, novel approaches such as ChIP-seq allow for genome-wide analysis of the *cis*-regulatory apparatus at specific times and axial levels, promising to yield new insight into the regulatory events underlying neural crest development. This technique can be used to map the genome-wide occurrence of histone modifications as well as active binding sites for transcription factors and coactivators (Valouev et al. 2008; Creighton et al. 2010). In a remarkable effort to describe the epigenomic landscape of neural crest cells, Rada-Iglesias et al. (2012) mapped the chromatin mark patterns and transcription factor occupancy in induced neural crest cells produced from human embryonic stem cells. This study identified *TFAP2A* and nuclear receptors *NR2F1* and *NR2F2* as key players in neural crest development, as the regulators occupy active enhancers characterized by high levels of *EP300* and *H3K27ac* occupancy (Rada-Iglesias et al. 2012). It also revealed thousands of active enhancers functioning during neural crest development. Moreover, the resulting epigenomic annotation of these cells constitutes an invaluable resource for further investigation of the neural crest genetic program.

Such studies provide a wealth of information and highlight the extraordinary complexity of the regulatory events controlling neural crest formation. Not surprisingly, new paradigms are cur-

rently emerging in the field of gene regulation that may reshape our views of the neural crest gene regulatory network and bring an extra layer of complexity to the proposed regulation of this cell population. Increasing evidence indicates that a complete understanding of transcriptional events will require taking into consideration nuclear architecture and dynamics (Cremer and Cremer 2001; Mateos-Langerak et al. 2007). In the context of the neural crest, a recent study has shown that mutations of histone *H3.3* cause down-regulation of neural crest specifiers and loss of crest-derived ectomesenchyme (Cox et al. 2012). This is particularly interesting since the *H3.3* variant histones tend to be associated with permissive chromatin structures and have been described as regulators of transcription and specification of germ cells. The results highlight how chromatin rearrangements can impact the transcriptional state of the neural crest and their importance for its developmental potential (Cox et al. 2012). Interestingly, transcription factors also have been shown to interact with repressive molecules involved in epigenetic silencing (Wang et al. 2011; Strobl-Mazzulla and Bronner 2012). Thus, increasing evidence points to a new paradigm in which transcription factor complexes are constantly rearranging chromatin structure, resulting in dynamic shifts of the regulatory state.

The development of novel techniques such as ChIA-PET, 5C, and Hi-C have allowed the identification of transcription factor modules that are comprised of *trans*-regulators complexed with different *cis*-regulatory regions and transcription start sites (Sanyal et al. 2011; Lan et al. 2012). These innovative approaches allow for the characterization of chromosome looping, interaction between different enhancers and promoter regions, and provide a three-dimensional assessment of the state of transcriptional regulation. Recent studies using chromosome conformation capture carbon copy (5C) in different human cell lines have pointed to a large number of long-range interactions between enhancers and promoters and indicate that only a minority of such interactions occur with the nearest gene (Sanyal et al. 2012). Furthermore, ChIA-PET analysis highlights a large number of promoter-promoter interactions between often-distant genes, suggesting the formation of multigene complexes that are coregulated by the same transcriptional machinery (Li et al. 2012). These findings infer a different model of gene regulation in which promoters and enhancers coalesce in transcription “factories” assembled at particular compartments in the nuclei (Sandhu et al. 2012). Such emerging concepts in eukaryotic gene regulation will surely impact how the neural crest genetic program is interpreted and scrutinized in the years to come.

Evolutionary conservation of the neural crest GRN

The neural crest gives rise to a number of vertebrate-specific features such as the craniofacial skeleton and peripheral nervous system. As a vertebrate synapomorphy, neural crest cells are thought to have been crucial for the early evolution of vertebrate body plan and predatory lifestyle. This idea was put forward in the “New Head” hypothesis by Gans and Northcutt, which postulated that the emergence of neural crest derivatives allowed for remodeling of the chordate head and resulted in a shift from filter feeding to active predation (Gans and Northcutt 1983). Although the main premise of the New Head hypothesis is still supported by evidence from the fossil record and developmental studies, tracing the origin of the neural crest to an invertebrate precursor cell type has proven to be a challenge, as invertebrate chordates lack migratory cells that are formed at the neural plate border (Northcutt 2005; Hall and Gillis 2013; Medeiros 2013). Assessment of the regulatory identity of the

neural crest and its putative evolutionary precursors holds the promise of shedding light on the evolutionary origins of the vertebrate neural crest.

Genomic studies have been key in informing scenarios that attempt to explain the origins of neural crest and its role in vertebrate evolution. Sequencing of chordate genomes allowed for the systematic characterization of expression and function of the neural crest GRN homologs in the chordate lineage (Yu et al. 2008). Studies from basal vertebrates such as the lamprey have offered interesting insights pertaining to the origin and evolution of the neural crest (Sauka-Spengler et al. 2007). Finally, whole genome phylogenetic analysis also has had considerable impact on how vertebrate evolution and neural crest evolution are surveyed. Molecular phylogenetic studies have placed tunicates as sister groups of vertebrates (Philippe et al. 2005; Delsuc et al. 2006) and also pointed to a potential role for genome-wide duplications in facilitating the origins of the neural crest (Green and Bronner 2013).

The use of the lamprey *Petromyzon marinus* as a model organism has provided important information about the ancestral state of the neural crest. Lampreys are basal vertebrates that lack several neural crest derivatives, including a neural crest-derived jaw and sympathetic ganglia (Nicol 1952; Häming et al. 2011). Nevertheless they possess a SoxE expressing population of migrating neural crest cells (McCauley and Bronner-Fraser 2006) that contribute to branchial arch cartilage, as well as the formation of pigment cells, cranial, and dorsal root ganglia. In an extensive analysis of the lamprey neural crest genetic program, Sauka-Spengler et al. (2007) described the expression patterns of orthologs of numerous genes occupying different hierarchical positions within the neural crest GRN. The results from this study definitively show conservation of most of the neural crest genetic program in this basal vertebrate with genes maintained in the same spatial and temporal relationships as those observed in jawed vertebrate models (Sauka-Spengler et al. 2007). This was corroborated by functional studies demonstrating that the epistatic relationships between neural plate border genes and neural crest specifiers are largely conserved (Nikitina et al. 2008). Although these results point to a strong conservation of the neural crest GRN across vertebrates, some intriguing differences have emerged from this work. For example, the neural crest specifier *Twist*, which is thought to be crucial in the acquisition of the mesenchymal state in higher vertebrates, is only expressed in lamprey neural crest cells at a later time, when they populate the branchial arches rather than within the “neural crest specifier” module. Similarly, *Ets1*, which acts as a critical neural crest specifier gene in jawed vertebrates, functioning directly upstream of both *Sox10* and *Foxd3*, is only employed in the effector module in the lamprey GRN (Sauka-Spengler et al. 2007; Sauka-Spengler and Bronner-Fraser 2008c). Such differences suggest that shifts in the circuitry of the specifier module within the neural crest GRN might result in species-specific traits.

Since comparative genomic analyses support the position of tunicates as the sister group of vertebrates, *Ciona intestinalis* or other ascidians now occupy a key phylogenetic position for examining the origins of vertebrate features (Schubert et al. 2006). Studies of the development of the ascidian, *Ecteinascidia turbinate*, led to the identification of pigmented cells from the A7.6 lineage that arises near the neural tube and undergoes migration (Jeffery et al. 2004). This cell lineage, which is known as the trunk lateral cells, expresses the HNK-1 epitope as well as the *Twist*, *FoxD*, *AP2*, and *Myc* orthologs (Jeffery 2006; Jeffery et al. 2008). Further studies are necessary

to determine unequivocally if these cells are derived from the neural plate border, neural tube, or other tissue (Hall and Gillis 2013). In an intriguing recent study, Abitua et al. (2012) showed that a lineage a9.49 in *Ciona intestinalis* expresses *ID*, *Snail*, *ETS*, and *FoxD*, as well as melanogenic genes like *MITF*, *TYR*, and *TYRP*. This lineage normally contributes to the formation of the otolith and the ocellus and does not migrate extensively. However, misexpression of *Twist* causes these cells to adopt a mesenchymal fate and exhibit long-range migration (Abitua et al. 2012). Because these lineages share some common gene signatures with vertebrate neural crest cells, they may hold important clues to neural crest origins.

The expression of neural crest orthologs also has been investigated in the amphioxus (*Branchiostoma floridae*) (Meulemans and Bronner-Fraser 2002; Meulemans et al. 2003; Yu et al. 2008), a cephalochordate. Although the amphioxus body plan shares important morphological traits with vertebrates, molecular phylogeny has now placed cephalochordates at the base of the chordate tree (Philippe et al. 2005). A systematic analysis of neural crest-related genes indicates conservation of specification mechanisms at the neural plate border between cephalochordates and vertebrates. The transcription factor *Snail* is expressed specifically in the lateral portion of the neural tube in the neurula, although no other neural crest-specifier genes are coexpressed at this location (Yu et al. 2008). Thus, it is tempting to speculate that this transient expression of *Snail* may reflect the beginnings of the assembly of a neural crest-specifier module.

Co-option of regulatory elements is likely to have played an important role in the origins of the neural crest in the vertebrate lineage. As an example, Manzanares et al. (2000) performed transgenesis experiments in which they expressed *cis*-regulatory regions encoding amphioxus *Hox* gene expression in vertebrates. Surprisingly, despite the fact that amphioxus lacks neural crest, these enhancers drove spatially localized expression in vertebrate neural crest and placode derivatives like cranial ganglia and branchial arches (Manzanares et al. 2000). This study demonstrates that *cis*-regulatory elements capable of driving gene expression in neural crest cells are already present in the cephalochordate genome. Such elements might have been co-opted to control expression of neural crest-specifier genes, thereby facilitating emergence of this cell type.

Although invertebrate cell lineages described above share some regulatory and behavioral traits with the neural crest, it is difficult to ascertain their position with respect to the evolutionary precursors of neural crest cells. If one defines the neural crest by virtue of its specification kernel, that is, the network module that comprises the neural crest specifier genes, then a bona fide neural crest must have factors that control multipotency, EMT, and diverse lineage specification. As highlighted by Davidson (2009), there are numerous instances in which a given regulatory gene functions in different levels of the GRN, often within the commitment and differentiation of the same cell type. This is observed repeatedly in neural crest development with genes such as *Foxd3*, *SoxE*, and *Msx1/2*, being employed early for specification purposes, and again later to activate differentiation of various cell types (e.g., melanocytes, cartilage, and neuronal cell types). Further work will be necessary to define whether the regulatory similarities observed between neural crest cells and intriguing lineages in invertebrate chordates are a consequence of conservation of ancient differentiation programs or if they indeed are part of the novel regulatory module that characterizes the neural crest.

Taken together, these studies indicate that the proximal levels of the neural crest gene regulatory network, such as induction of

the neural plate border, are primitive features shared amongst chordates, whereas the core neural crest specification modules of the network might have been added during early vertebrate evolution. This is expected since delimitation of the neural plate border is necessary for development of the central nervous system. The terminal differentiation programs of the neural crest GRN also are deeply conserved in both cephalochordates and tunicates, particularly the control of pigment cell differentiation (Yu et al. 2008; Davidson 2009). This is not surprising as most of the differentiation batteries are thought to be ancient (Davidson 2009). Placement of the origin of the specification kernel of the GRN still demands further studies, but it is clear that it has a high degree of conservation among vertebrates, as supported by studies in the lamprey (Sauka-Spengler et al. 2007; Sauka-Spengler and Bronner-Fraser 2008a).

Neural crest cells and disease

The above data show that scrutinizing the regulatory mechanisms controlling neural crest formation can expand our understanding of essential cellular processes such as delamination, migration, and differentiation, as well as shed light on important evolutionary questions pertaining to vertebrate origins and adaptation. Beyond its relevance for basic biological questions, the neural crest is also of remarkable medical importance for its role in birth defects and malignant diseases. Studies on the plasticity of the neural crest and its fate switches also offer clues for strategies for cell reprogramming and stem cell manipulation. In addition, the persistence of neural crest stem cells in adult tissues offers potential targets for therapies aiming to utilize endogenous progenitors for repair and regeneration.

Given the sheer number of cell types and derivatives formed by the neural crest progenitor cell population and the complexity of its developmental program, it is not surprising that a large fraction of congenital birth defects can be traced back to the neural crest (Hall 1999). Neural crest developmental anomalies, or neurocristopathies, are responsible for the vast majority of craniofacial malformations, and more than 700 different syndromes have been described (Trainor 2010). In addition to the skeletal elements of the face, abnormal neural crest development can also affect the heart, adrenal medulla, pigment cells, and the peripheral nervous system. Although treatment of neurocristopathies has improved considerably over the years, dissection of the molecular mechanisms underlying many of these disorders could bring considerable improvements to management and prevention of these conditions.

In the past few decades, the molecular basis of several neural crest-related syndromes has been investigated through genetic linkage as well as the use of animal models. One example is Treacher Collins disease, an autosomal dominant disorder that is marked by severe hypoplasia of skeletal elements of the face and irregularities in otic and ophthalmic development (The Treacher Collins Syndrome Collaborative Group 1996; Trainor 2010). Genetic mapping and gene expression analysis identified mutations in the *Tcof1* gene as the cause of the disease. Mapping of TCOF1 expression in mouse embryos shows that it is enriched in neural crest cells and facial mesenchyme, raising the intriguing possibility that the mutations could be interfering with neural crest development (Dixon et al. 2006). In fact, TCOF1 is important for ribosome biogenesis and disruption of this process results in TP53-mediated apoptosis, and analysis of *Tcof1* null mice reveals strong reduction in the migratory neural crest population as a conse-

quence of increased apoptosis and reduced proliferation of migrating cells (Dixon et al. 2006).

Genomic approaches have greatly benefited the dissection of mechanisms leading to congenital disease. Genome-wide association studies have been pivotal to the identification of a number of loci and genetic variants associated to craniofacial anomalies (Grant et al. 2009; Beaty et al. 2010; Mangold et al. 2010; Dixon et al. 2011) and also suggest genes linked to normal variation in facial structure (Liu et al. 2012). Similarly, recent studies using exome sequencing have been used to map the causes of craniofacial defects in conditions such as Miller syndrome (Ng et al. 2010) and Nager syndrome (Bernier et al. 2012). Current transcriptome and epigenomic analysis should accelerate the dissection of the genetic basis of neural crest-related syndromes through regulatory network comparisons between normal and disease states. Such studies promise to uncover more complex aspects of disease such as variable penetrance and multifactorial etiology. In this context, centralized databases of genomic information containing data sets of high-throughput studies such as Facebase and ENCODE will be key (The ENCODE Project Consortium 2004; Hochheiser et al. 2011). Such integrated databases and user-friendly bioinformatic software will become increasingly important in the neural crest field as it continues to shift toward genomic approaches.

The medical importance of neural crest cells is not restricted to congenital birth defects. This cell population has an important link to metastatic conditions due to its migratory and invasive properties. Neural crest-derived cancers, such as melanoma and neuroblastomas, tend to be particularly aggressive and prone to metastasis. This has led to the assumption that malignancy in neural crest-derived cell types involves the reactivation of regulatory circuits important for embryonic development but that are normally silenced once cell differentiation has occurred. Indeed, evidence from studies of malignant melanoma cell lines shows anomalous transcriptional reactivation of genes such as *Snai1*, *Twist*, *Sox10*, and *Myb*, all of which are important neural crest specifier genes in the early GRN (Shakhova et al. 2012; Shirley et al. 2012; Weiss et al. 2012). Interestingly, transplanting melanoma cells to chicken embryo causes “regulation” of some of the cells that migrate along neural crest pathways, although some do localize in ectopic sites (Kulesa et al. 2006). This suggests that melanoma cells can respond to cues in the embryonic environment similar to neural crest cells. Thus, the embryonic environment may be able to control or inhibit the metastatic state (Hendrix et al. 2007), at least under some circumstances. Although this is an intriguing possibility, further work that compares the genetic programs controlling neural crest and neural crest-derived tumor cells is needed to inform upon the link between neural crest development and metastatic behavior in malignant cells.

The neural crest has also been an important model for the study of EMT, which is central for metastatic processes. A wealth of knowledge regarding the genetics and cell biology underlying EMT has been obtained through the use of the neural crest as a model (Barrallo-Gimeno and Nieto 2005; Lee et al. 2006; Yang and Weinberg 2008; Acloque et al. 2009). Interestingly, recent evidence suggests that cells undergoing EMT seem to acquire stem-like properties. Induction of EMT in different cell lines has been shown to up-regulate totipotency markers such as POU5F1 (also known as OCT4) and NANOG in conjunction with transition to a mesenchymal state (Mani et al. 2008). Such findings highlight similarities between the metastatic process and neural crest development. They also point to interconnectivity between cell behavior and developmental potential. In fact, it is likely that advances in the

assembly of the neural crest regulatory network will highlight common nodes in the genetic circuits controlling EMT, plasticity, and migratory potential. Such events are likely to depend on the shared regulatory circuits that interconnect to define neural crest identity.

The question of plasticity has been central to neural crest biology, and interest in the topic of self-renewal and developmental potential has driven efforts to isolate neural crest stem cells. Work by several investigators has led to the identification and purification of neural crest stem cells—cells with the potential to self-renew and also to give rise to the diverse population of derivatives that are generated by the neural crest. The first neural crest progenitor cells were isolated in vitro by clonal analysis of quail cells and shown to be multipotent (Sieber-Blum and Cohen 1980). The ability of neural crest cells to self-renew was first demonstrated in elegant studies by Stemple and Anderson using clonal cultures of murine neural crest cells purified by cell sorting based on expression of the NGFR (p75) cell surface epitope (Stemple and Anderson 1992, 1993). These cells have the ability to self-renew and also to form neurons or glia, thus demonstrating true stem cell properties. More recently, clonal analysis of cranial neural crest in quail has revealed that this population has the potential to differentiate into osteoblasts (Calloni et al. 2009), and that the majority of the cranial neural crest is comprised of progenitors with both osteogenic and neural-melanocytic potential. Remarkably, this study also identified precursors that can give rise to all neural crest-derived phenotypes analyzed, which indicates that part of the migratory cranial crest remains multipotent (Calloni et al. 2009). Although isolated neural crest stem cells represent a good model for studies of plasticity and commitment, there are possible caveats. Because it is likely that in vitro culture conditions introduce changes that do not reflect in vivo behavior, embryological observations of these cells in their normal environment will be critical to uncovering the mechanism underlying maintenance and renewal of the latent neural crest stem cell population.

Neural crest-like cell populations have been derived from human embryonic stem cells by a number of different methods (Chimge and Bayarsaihan 2010). For instance, neural crest cells were isolated from neural rosettes through fluorescence activated cell sorting (FACS) using markers NGFR (p75) and B3GAT1 (previously HNK1,) and subsequently express neural crest specifiers, such as *SOX10* and *SNAI1* (*SNAIL*). These have been shown to differentiate into a wide range of neural crest derivatives, including sensory and autonomic neurons, Schwann cells, myofibroblasts, adipocytes, cartilage, and bone cells (Lee et al. 2007). Another study identified early migrating neural crest cells in neurospheres cultured on fibronectin plates. These cells express the neural crest specifier *SOX10* and clonal analysis indicates they are multipotent. Importantly, when these cells are injected in chick embryos, they migrate normally and contribute to the same derivatives as endogenous neural crest cells (Curchoe et al. 2010).

Induced neural crest cells (iNCCs) from biopsied human tissue may be useful in different contexts for clinical purposes. First, iNCCs can be used as a model for the study of human neurocristopathies, as cells from patients can be reprogrammed to adopt a neural crest fate (Lee et al. 2009). This approach essentially allows the observation of an embryonic cell population from reprogrammed adult cells, which can be used for transcriptomic, epigenomic, or behavioral investigations that can uncover molecular mechanisms of disease and possibly also expand our knowledge of the neural crest gene regulatory network. There is also the promise that induced neural crest cells could be used for cell therapy for

diseases that involve failure or impairment of neural crest-derived tissue (Crane and Trainor 2006; Barraud et al. 2010). For instance, transplantation of induced “neural crest-like cells” to diabetic mice resulted in improvement of the impaired nerve and vascular functions that result from diabetic neuropathy (Okawa et al. 2012). One would imagine that engineered neural crest cells could be used in such fashion to treat neurodegenerative disorders that affect peripheral nervous system, among others.

Although it is clear that iNCCs are a powerful tool that can reveal novel molecular mechanisms and hold promise in treatment of disease, it is important to bear in mind that neural crest development is intrinsically linked to the embryonic environment. In vitro conditions do not necessarily reflect the complexity and dynamism of a developing embryo and thus are unlikely to recapitulate in vivo conditions. This is especially true for the neural crest, which is continuously interacting with different cell types and embryonic environments. Therefore, in vivo approaches continue to represent the gold standard for neural crest biology, and findings obtained with isolated neural crest cells or iNCCs should be validated with model organisms when possible.

Conclusions

Over one hundred years of investigation of the neural crest has produced a remarkable body of knowledge about neural crest cell behavior, derivatives, and plasticity. The ingenuity and creativity of classical embryologists helped to frame the very questions that remain integral to the field of neural crest biology today. The introduction of recombinant DNA technology in the 1990s has finally allowed these questions to be addressed at the mechanistic level by analysis of the components of the genetic machinery that imbues the neural crest with its fascinating properties. In the last twenty years, several aspects of the genetic circuitry controlling these cells have been identified and functionally characterized, providing important insights into the logic of neural crest development.

Yet we are still at the “tip of the iceberg” in terms of understanding this intriguing cell population from an integrated genomic perspective. Further transcriptome and enhancosome analysis of neural crest cells at various stages and states of development will greatly increase our understanding of the neural crest GRN. Since regulatory states are dynamic and interconnected, this should be expanded to encompass more axial levels and time points of development, ranging from the neural plate border to the final differentiated state. The formulation of computational models that can predict outcomes within the GRN and account for species-specific differences across vertebrates hold the promise of greatly increasing our understanding of deuterostome evolution (Peter et al. 2012). Finally, additional levels of control have been shown to act in the control of neural crest behavior and identity, including epigenetic (Hu et al. 2012), post transcriptional (Jayasena and Bronner 2012), and post-translational regulation (Lee et al. 2012). Future research along these lines has the potential to reveal the secrets of the neural crest from its inception at the neural plate border to their acquisition of specific differentiated fates.

Another exciting feature of the neural crest is its position at the convergence of developmental, cancer, and stem cell biology. As such, neural crest cells serve as a model for the investigation of processes as diverse as the epithelial to mesenchymal transition, to cell motility and migration, and transition from stemness to the differentiated state. Genomic analyses provide tools and insights that are poised to reveal the complexities of these events with unprecedented depth and breadth. Given the inherent inter-

connections that exist between diverse biological processes, the neural crest represents an excellent model system for unbiased examination of the genetic basis of cellular identity and behavior. In turn, the results of these studies will undoubtedly have implications for other aspects of stem cell biology and metastatic transformation.

Acknowledgments

We would like to thank Tatiana Hochgreb, Sujata Bhattacharyya, and Stephen Green for critical reading of the manuscript and helpful suggestions. This work was supported by NIH grants HD037105 and DE16459 (M.E.B.), the Pew Fellows Program in the Biomedical Sciences (M.S.-C.), and a Caltech Cell Center fellowship (M.S.-C.)

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