3. Early origin and differentiation capacity of the neural crest

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This chapter is devoted to the early developmental stages of neural crest cells (NCCs), a fascinating population of migratory cells generated early in development and endowed with a remarkable differentiation potential. These cells contribute to essentially all organs and systems in the body because they play a major role in the formation of the peripheral nervous system. Additionally, they contribute to many other derivatives including: bone, cartilage, tooth forming cells, pigmented cells, muscle, and endocrine cells. The origin, migration, and differentiation potential of NCCs have prompted considerable scientific interest ever since their original description in 1868 by Wilhelm His [1]. Their vast differentiation potential and experimental amenability has made NCCs a great model to study general mechanisms of cell induction, specification, potential, migration, and differentiation. Despite constant interest and the large number of studies focused on neural crest cell development, many enigmas remain unresolved. This chapter provides a brief historical perspective on their discovery, followed by an overview of their early development and relevance to various disciplines. Next, an account of NCC derivatives and of their differentiation potential is provided. Herein will be discussed the perceived segregation and differentiation potential of neural crest cells relative to their axial position in the embryo; this will also include studies that have challenged NCC potential in vivo and in vitro. The next section provides a depiction of the
embryonic origin of the neural crest, and the relation of early neurulation to neural crest development. An account of the difficulties to understand the origin and formation of neural crest cells is provided by acknowledging the complexities of the neural plate border, by presenting models for their cellular and molecular induction, and by exposing the tools available to identify and investigate the early development of the neural crest.

Introduction

The amazing neural crest is a multipotent population of cells that originates at the border of the neural epithelium during early development in vertebrates. Later on, NCCs undergo an epithelial to mesenchymal transition (EMT) and delaminate from either 1) the open neural folds (amphibians and mammals); 2) an ectodermal thickening at the neural plate-epidermis boundary (fish); or 3) the closed neural tube (birds). From this departure point, NCCs follow stereotypic migratory pathways permeating throughout (essentially) the entire vertebrate body. The formation of neural crest cells, and their EMT and migration, are carefully orchestrated and proceed sequentially in a rostro-caudal wave closely linked to the rostro-caudal development of the neural epithelia. Due to largely unknown signals, NCCs stop migrating at their final destinations, and in several cases they condense and differentiate; however, some evidence indicates that these cells might initiate differentiation prior to the cessation of migration.

During development, critical events endow daughter cells with different potentials, which are modulated by specific environmental signals. In order to understand how NCCs are able to execute their remarkable migration and differentiation potentials, it seems critical to unveil their cellular origin, and the possible environmental effectors, which clearly depend on the time and regions where NCC precursors appear.

Discovery and early history of the neural crest

The story of the neural crest begins in the hands of the talented Swiss scientist, Wilhelm His (1831-1904). His instrumental work in developing the neuron doctrine that established neurons as individual units is well recognized [2]; however, his reputation was gained prior to this, as His challenged the dominant evolutionary perspective of the time by promoting the study of embryology in its own right. With this, His ignited the movement of experimental embryology by calling for the need to understand the mechanisms underlying the transformation of the fertilized egg into an organism. He provided some of the earliest embryological tools by developing methods to hold, move and cut thin sections of samples with
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precision (innovations that have been cited as the origin of the microtome). In a landmark study, His collected chick embryos during the first 2 days of development and generated accurate serial sections that allowed for an analysis of whole embryos for the first time in a systematic way [1].

In this initial study by His of chick embryo sections, neural crest cells are identified for the first time. However, His does not refer to them as neural crest cells. Rather, he identifies a specific set of cells as a middle furrow or groove ("zwischenrinne") surrounding the neural plate in early stages, and as a middle cord or thread ("zwischengstrang") of tissue in between the neural tube and the epidermis in more advanced stages of development [1]. His suggested that these zwischenrinne and zwischenstrang cells are the same, and that they migrate from their original position to generate the cranial and spinal cord ganglia. This proposal endured many years of dedicated research and controversy (see [3] for an animated discussion) to yield our current understanding of the origin, formation and extent of the contributions of the neural crest. His’ proposal that the neural crest generated the cranial and spinal cord ganglia was eventually rectified (cranial ganglia actually have a dual origin, made of placodal and neural crest derivatives), and extended to incorporate NCCs as the source of mesenchymal tissues of the head [4-7] all cited in [8]), and the melanocytes of the skin [9-11].

Although neural crest cells initially fell under the umbrella of zwischenrinne and zwischenstrang, the actual term “neural crest” was first coined by Marshall in 1879 [12], and adopted soon after by the rest of the community [13,14]. Marshall referred to the borders of the neural plate as “neural ridges” (on either side of the neural plate at its rostro-caudal axis). As the neural plate closes to form a neural tube, the neural ridges on either side fuse with each other, generating a mass of cells separate from the neural plate and the overlying ectoderm. Initially, Marshall called this mass a neural ridge, but in a 1879 paper he opted for a term to differentiate between the two neural ridges lateral to the neural plate and the neural ridge above the neural tube, baptizing the latter as “neural crest”. Both the neural ridge and the neural crest correspond closely to the zwischenrinne and zwischenstrang identified by His. Thus, the developmental biology community has embraced the term Neural Crest (NC) to refer to the transient cell population found at the edge of the neural plate and dorsal neural tube, which then migrates to contribute to varied derivatives throughout the vertebrate body.

Relevance of neural crest development

Neural Crest cells (NCCs) constitute a fascinating population of multipotent migratory cells that contributes to a wide range of derivatives of
the vertebrate embryo. NCC derivatives include the neurons and supportive cells of the peripheral nervous system, melanocytes, and endocrine cells. In addition, NCCs also generate a set of derivatives collectively known as mesectoderm, which includes a large portion of the head skeleton (both bone and cartilage). The astonishing capacity of neural crest cells to generate such a broad spectrum of derivatives, specifically those traditionally seen as mesodermally derived, collides with our preconceptions of the potential of the three germ lines. Perhaps the early ectoderm has an extended capacity which is lost in epidermal and neural tissues, but not in the neural crest. An alternative proposal is that a fourth germ layer—constituted by NCCs—generates both ectodermal and mesodermal derivatives [15,16]. The semantic debate about the germ layers and their capabilities aside, the extensive differentiation potential of neural crest cells is fascinating, and offers a great model for the study of stem cell biology, pluripotency and differentiation. Additionally, the contribution of NCCs to the head of vertebrates (craniates) has prompted researchers to suggest an intimate link between the appearance of this cell population and vertebrate evolution [17-19].

The diverse differentiation capacity of neural crest cells is suggestive of stem cell like properties. Furthermore, true stem cells derived from this cell population have been isolated from regions colonized by differentiated NCCs (in embryos, newborns and even adults). The isolation of neural crest stem cells was initially achieved from embryonic dorsal root ganglia [20], a tissue derived from the neural crest. More recently, neural crest stem cells have been identified from varied sources in both infants and adults, including the carotid body, the dental papillae, the skin, and the hair follicles [21]. These cells can be propagated in vitro, retaining cell renewal capacities, and are able to differentiate into various neural crest derivatives upon proper stimulation.

As a consequence of the robust contribution of NCCs to many specific cells, organs and systems during development, this cell population is implicated in a large number of human pathologies [22-24]. Birth defects resulting from aberrations in NCC development include craniofacial malformations (cleft lip/cleft palate, fetal alcohol syndrome), congenital malformations of the cardiac outflow tract, and Hirschsprung’s disease, amongst other disorders. Additionally, neural crest related tumors include melanoma, neuroblastoma, neurofibromatosis, and pheochromocytoma. Thus, studying the basic developmental biology of NCCs is a critical step to improve our understanding of these conditions, and to generate diagnostic and therapeutic strategies. The interest of the scientific community in NCCs can be easily appreciated through the extensive literature available on the subject: more than 150 reviews on different aspects of neural crest development and differentiation potential were published from 1998 to 2008.
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(PubMed “neural crest”[TITLE] Limits: Publication Date from 1997/01/01 to 2008/08/01, Review), and three books devoted solely to NCC development have been published and revisited in subsequent editions or updated commentaries [16,25-28]. In the past 140 years, a large number of studies have addressed the origin, migration, and differentiation potential of the neural crest in various model organisms. Recently, within the last 20 years, great progress has also been made regarding the tissue and molecular events leading to crest induction.

**Neural crest differentiation potential**

**Variety.** The derivatives generated by NCCs are astonishing due to their variety and number. NCCs generate most of the neurons (sensory, cholinergic and adrenergic) and glia of the peripheral nervous system, all the pigmented cells in the skin (melanocytes), and endocrine cells of the thyroid and adrenal gland. Perhaps the most striking derivative of the NCCs is the mesectoderm, a special mesenchyme from ectodermal origin capable of generating derivatives once thought to be made exclusively by mesoderm. NCC-derived mesectoderm is capable of forming cartilage, endochondral bones, dermal or intramembranous bones, teeth, dermis, smooth muscle, and other connective tissues.

**Number.** The NCC derivatives referred to above, are only subtypes which represent many different cell types, and which contribute to several tissues and organs throughout the vertebrate body. For example, more than 20 different cranial bones are made by neural crest cells. Furthermore, many different sensory neurons expressing a different array of molecules and employing characteristic neurotransmitters are made by neural crest cells in specific locations of the embryo. The same is true for the other neuronal subtypes (parasympathetic, sympathetic, and enteric).

**Regional contributions of NCCs in vivo.** Fate map studies generated via ablation or grafting strategies have provided a very detailed picture of the specific contribution of neural crest cells from different regions of the avian embryo. Neural crest cells from the cranial region, which are subdivided into prosencephalon, mesencephalon and rhombencephalon groups, generate mesectoderm and pigmented cells. The prosencephalon makes no other crest contributions, while NCCs from a slightly more caudal location (the mesencephalon) generate, in addition to neurons and glia of the parasympathetic and sensory ganglia, mesectoderm and pigmented cells. Yet, in the caudal portion of the rhombencephalon, sensory ganglia, enteric
ganglia and endocrine cells accompany the mesectoderm and pigmented cells as NCC derivatives. In the rest of the embryo (caudal to the head), a similar display of specific derivatives has been described. The cervical spinal cord, thoracic spinal cord, and lumbosacral spinal cord regions generate a variety of derivatives according to specific locations within each territory. However, no mesectoderm or parasympathetic ganglia are generated in these more posterior locations; instead, posterior NCCs contribute to sympathetic and sensory ganglia, and pigmented cells. In addition, the anterior cervical spinal cord and the lumbosacral region (but not the interceding territories) also contribute to the enteric ganglia. Similarly, only the caudal portion of the cervical, and the anterior half of the thoracic spinal cord generate endocrine cells [28,29].

NCC-derived mesectoderm contributes to the formation of the dorsal fin in the trunk region of lower vertebrates [30,31], but in higher vertebrates the capacity to generate mesectodermal derivatives is restricted to the head region where they generate most of the cranium, dermis, odontoblasts, adipocytes, and muscle cells [28]. However, recent studies in the turtle suggest that perhaps the bones of the shell also receive trunk NCC-derived mesectoderm [32], and in mouse embryos two separate studies have recently demonstrated that trunk NCCs generate mesenchymal stem cells which are thought to be able to generate derivatives similar to those made by mesectoderm [33,34].

**Experimental in vivo differentiation potential of NCCs.** Beyond the normal in vivo fate of NCCs, their differentiation potential has been tested by heterochronic and heterotopic grafting experiments that placed neural crest cells at earlier or later stages of development, and in different locations of the embryo. It was found that NCCs hold an extensive “regulative” capacity that allows them to modify their differentiation, adapting to external conditions. These cells respond to “new” environments by modulating their expected fate according to the new conditions. Collectively, this research demonstrates that NCCs can generate a wide range of derivatives - much larger than the expected in vivo fate for a given region [35-42]. For example, NCCs from the medial trunk region that normally do not contribute to the enteric ganglia readily do so when grafted into the vagal region [42,43].

Because of the large number and variety of derivatives generated by neural crest cells, it seems valid to question whether these derivatives are generated from a heterogeneous population of cells, each with a more modest or restricted differentiation capacity, or whether single cells actually have the differentiation potential to generate the full repertoire of neural crest derivatives.
Clonal analysis of the differentiation potential of NCCs in vitro: Experiments launched in the late 1970’s in the laboratory of Alan M. Cohen addressed the individual potential of clonally cultured quail NCCs [44,45]. These studies clearly demonstrated that single NCCs generate multiple derivatives in vitro, and that progeny of these clonally obtained NCCs, once injected back into normal NC migratory routes, were able to contribute to various derivatives in the embryo [46]. Several other clonal studies have contributed to our current perspective supporting the existence of multipotent NCC precursors in avians [47-52] and mammals [20,53]. The laboratory of Nicole Le Dourain has been instrumental in the identification and characterization of the potential to differentiate and to self renew of the various multipotent NCC precursors. It was only in 2007 that a long awaited NCC precursor, able to generate neurons, glia, melanocytes, myofibroblasts and cartilage (NGMFC) was identified [54]. This study detected a frequency of 6.5% for this NGMFC precursor in clonal cultures of cranial NCCs, which was increased to 18.5% in the presence of Sonic Hedgehog. Today’s prevailing model ratifies the sequential restriction of potential differentiation of NCCs, such that precursors with a wide differentiation potential give rise to other precursors with more restricted potential. In general, it is believed that as NCCs emigrate from their original location, they are conformed by a heterogeneous population of cells, some of which hold a wide differentiation potential, others with more restricted potential, and yet some others with a unique potential.

Single cell labeling and the in vivo progeny of individual NCCs. In agreement with the in vitro clonal studies described above, in vivo studies have demonstrated the existence of premigratory and migratory NCC progenitors endowed with multiple differentiation potentials. Single cells were labeled with vital fluorescent dye (lysinated rhodamine dextrans), and their fate determined after further embryonic development through morphological, antigenic and positional analysis. For example, melanocytes, dorsal root and sympathetic ganglia cells were found to be derived from a single labeling event [55,56]. Complementing these experiments, single NCCs were labeled by viral infection and their progeny determined. The results obtained confirmed the existence of premigratory NCCs with multipotential capacities to differentiate into various derivatives, and also supported the existence of some other precursors that seem to be restricted and give rise exclusively to one type of derivative [57]. Single cell analysis of NCC derivatives performed in mammal and amphibian embryos [58,59] corroborate these avian results. In zebrafish, NCC potential seems to be restricted in the cranial region at the time of migration, while in the trunk the
existence of both multipotent and restricted precursors have been recorded [60,61].

**Embryonic origin of the neural crest cells**

NCCs were identified by His as being adjacent to the neural plate of chick embryos. Since then, NCCs have been identified in a similar territory flanking the neural plate in other species, from urodels to mammals. Walter Vogt generated the first fate maps of vertebrates by applying vital dyes to salamander embryos in 1929 [62]. Vogt’s maps identified a clear border between the neural plate and the prospective epidermis, and further showed some contribution of this border to NCC-derived peripheral ganglia. Vogt’s fate map was later modified by Harris to properly display the position of neural crest precursors at this neural plate/epidermis border [63]. In the chick embryo however, it was not until 1981 that Rosenquist provided definitive evidence that the origin of the neural crest was at this border [64].

To date, cell labeling techniques and grafting experiments have been used to map the precursors of the neural crest to the border of the presumptive neural plate in all vertebrate embryos examined. However, the precise identity, and location of this border region has been the subject of debate over the years [65]. A recent study has generated a map suggesting a slightly new shape for the early neural plate. More importantly, this map incorporates several molecular markers previously suggested to label neural or epidermal prospective cells [66]. The results of this study suggest that none of these molecular markers (Ganf, Plato, Sox2, Otx2 and Dlx5) match precisely the shape of the neural plate at early stages; instead, a range of possible combinations might define it. Thus, the molecular nature of the early border region remains unclear.

**Neurulation and early NCC development.** The neural plate appears early in development after the ectoderm receives signals from the embryonic node and underlying mesoderm. These signals trigger a thickening of the ectoderm that will generate the neural plate. In tetrapods, the neural plate deepens centrally while the lateral edges (neural folds) appear to elevate until they touch each other and fuse in the middle/dorsal portion of the embryo. This is the standard primary neurulation common to most vertebrates.

The appearance of the neural plate proceeds in a rostral to caudal wave, such that while neural folds are fusing in the anterior regions of the embryo, the neural plate is just being formed in more posterior territories. It is therefore believed that as new neural plate is formed, new border territories emerge that include new neural crest precursors.
The complex neural plate border

One could imagine the border of the neural plate as being a sharp line between the thin prospective epidermis and the thick neural plate. However, the border territory is a transition zone between both tissues, and is characterized by a gradual shift from thin to thick epithelium. Traditionally, it has been proposed that the neural crest appears at the neural plate/epidermis border, and this border is a consequence of, or secondary event to, the formation of the neural plate. Initial experiments monitoring the second axis induction by node grafts suggested that neural plate border markers appeared after neural plate markers [67]. More recent experiments, however, have shown that border markers can appear in the absence of neural markers [68].

In an ideal world, the precursors of the neural crest cells would be easily identifiable in the neural plate border, at the neural folds. However, life at the border is a lot more interesting than expected. This border is apparently composed of a heterogeneous mix of cells, intermingled and moving. Within this border reside epidermal cells laterally and prospective neural cells centrally; neural crest cells are adjacent to the neural plate from the midbrain level and downwards. In the head, cranial placodal cells also reside in the border region [69]. At the caudal end of the embryo, along the open neural plate, past the node, resides a stem zone of cells capable of generating mesoderm and neural cells [70]. This stem zone is flanked by the neural plate border containing prospective neural crest cells, which in turn are surrounded by prospective epidermis. At the most posterior end of the stem zone a gastrulating primitive streak can be recognized.

Given that the neural plate border is so complex, it is easy to appreciate the difficulties earlier scientists faced defining the precise location or origin of NCC precursors. This issue has never been fully solved, in part due to our incapacity to isolate neural crest versus neural plate, epidermal or placodal precursors. In fact, during early stages of development, it is unclear whether separate precursors for each lineage exist. Single cell labeling studies suggest that cells in the neural folds are capable of generating epidermal, neural and neural crest cells at early stages; at the time of neural tube closure, both neural and neural crest derivatives are also formed [71]. This latter result suggests that the neural and neural crest lineages do not separate. Adding to this complexity, grafting experiments suggest that neural cells placed in NCC migratory paths can behave like neural crest cells, and that migratory neural crest cells placed in the neural plate acquire central nervous system properties [72,73]. These results indicate that both cell types have an equivalent differentiation potential, and are capable of responding to alternative environments by making the derivatives dictated by the environment.
Collectively, this work supports the view of a shared precursor between neural and neural crest cells.

Specific gene expression has provided molecular markers that label prospective epidermal, neural, placodal, and neural crest cells. However, these markers are often shared amongst two or more of the cell precursors found at the border, and their possible participation on the formation or development of the other cell type(s) has not been fully characterized. Added difficulties emanate from changing patterns of expression, as well as the possible movements of cells in these territories. We can clearly identify neural crest cells after they have initiated their emigration from the lateral regions of the neuroepithelium, and we are also able to identify populations of cells harboring neural crest precursors; however, identifying exclusively early neural crest precursors at this border region is still an unattained goal.

**Cellular and molecular events responsible for neural crest formation**

The neural folds (where prospective neural crest cells reside) are surrounded by the neural plate medially and by non-neural ectoderm (prospective epidermis) laterally. Additionally, the ectoderm of the embryo is underlined by mesoderm. Specifically, axial mesoderm lays under the central most region of the embryo, and paraxial mesoderm under the more lateral sides of the embryo (beneath the neural folds). The location of these tissues has prompted investigators to suggest their involvement in the formation of the neural crest, and a considerable body of evidence based on juxtaposition experiments performed *in vivo* and *in vitro* supports a role of these tissues in NCC development. Juxtaposition of “naïve” intermediate neural tissue (ventral to the neural folds and dorsal to the ventral midline or prospective floor plate) against prospective epidermis (lateral non-neural ectoderm) triggers crest induction. Interestingly, these experiments have shown that epidermal and neural tissues signal to each other, and both tissues generate neural crest cells. Most attention has focused on the possible signal(s) from the epidermis that trigger neural crest induction in the neural tissue [71,74-80]. However, a similarly large body of experiments demonstrates that mesoderm is the source of induction activity, and it is this germ layer that directs the overlaying ectoderm to form the neural crest [71,80-86].

The participation of both tissues in neural crest induction is possible; however, distinguishing between the real mode of induction *in vivo* and potential experimental artifacts is extremely difficult. The timing of events, which are normally perceived by the expression of specific markers, complicates things further. Inductive events occur at slightly different time
points across model organisms, and the appearance of markers that allows us to distinguish such events also varies. Furthermore, differences in the manner of neural crest induction between models challenges our capacity to resolve this problem. For example, experiments in mice and zebrafish, suggests that mesoderm is not required for NCC induction [87-89]. Similarly, in chick embryos, mesoderm-independent neural crest specification has been recognized at early gastrula stages [90-92]. In contrast to this, it has been shown that in Xenopus a dual role for mesodermally-derived signals involved in neural crest induction exists. Here, an early neural crest induction signal is generated by prospective mesoderm, and a later signal required for the maintenance of the induced state is supplied by the intermediate mesoderm that lies underneath the neural crest territory [93].

At the molecular level, several signals have been identified as neural crest inducers. The most prominent of these are BMP, FGF and Wnt. Studies from the Jessell laboratory were the first to identify, at the molecular level, a neural crest inducer. Members of the TGF-β family (Dorsalin-1, Activin, BMP2, 4, & 7) effectively induce neural crest formation from the naïve neural plate [77,94,95]. Together with studies in *Xenopus* [79,84,96,97] and zebrafish [98,99], this research suggested that BMP signals operate at intermediate concentrations during neural crest formation.

The participation of FGFs in neural crest induction has been better characterized in *Xenopus* embryos, where neuralized ectoderm can be specified to form neural crest by FGF [79,86,100,101]. It was once thought that FGFs could have an indirect role in NCC development, functioning through the induction of mesoderm and/or Wnt signals. However, it has now been shown that, in *Xenopus* embryos, FGF8 can directly induce neural crest in the absence of mesoderm [85].

In whole amphibian embryos, as well as in neuralized animal caps, Wnts are required for neural crest formation [86,102-107]. Furthermore, XWnt-8-mediated signals can establish a lateral neural plate domain, marked by Pax-3 and Msx-1, from which NCCs arise [82]. In zebrafish embryos, Wnt8 is also required for neural crest formation [108]. In chick embryos the requirement and sufficiency of Wnt signals to induce NCC formation has been demonstrated [109], and the role suggested for Wnt6 as a candidate inducer [109] was recently demonstrated [110].

In an attempt to assess the combined role of BMPs, Wnts and FGFs in neural crest cell formation, a recent study investigated the molecular interactions of these signals in *Xenopus*. This work suggested a multi-step process where BMP establishes the neural crest border, along with the expression of an early neural crest marker, Msx1. According to this model, FGF and Wnt signals operate after a BMP signal, and in various subsequent
steps during NCC development [85]. While the synergistic participation of several of these molecules has been corroborated in different organisms, this is not the case for all. Critical differences complicate generalizations about the exact mechanism of NCC induction across species. For example, while intermediate levels of BMP signaling are required for NCC induction in both fish and frog embryos, its role in amniote embryos remains unclear [111-114].

**Identification of neural crest cells**

**Histology, grafting, labeling and antibodies.** Identification of NCCs can only be performed once they have emigrated from the neural tube or its borders. This task was achieved initially on cell morphology grounds. Subsequently, scientists introduced grafting experiments [30] using amphibians, with different cell sizes or pigmentation to recognize donor from host tissues (reviewed [25]). In higher vertebrates, a major improvement was the use of grafts from a donor chick embryo “labeled” with tritiated thymidine into un-labeled chick hosts [115]. This technique was surpassed by the arrival of the “quail-chick chimera” [116], based on the ability to identify the quail nucleus after feulgen staining. It took almost fifteen years for the arrival of antibodies that allowed the identification of migrating NCCs (HNK-1 and NC-1 [117,118]). However, these antibodies recognize a sugar motif also expressed by other non-NCC cells in the embryo, preventing their wide use without additional tools. A valuable addition to this set of tools was the QCPN monoclonal antibody, which identifies perinuclear proteins exclusively in quail cells. This allowed for the identification of migratory NCCs of quail origin in chick embryo hosts. And while different labeling approaches and chimeras are available today, the quail-chick strategy still serves as a prime tool to investigate migration, specification, commitment, and differentiation of neural crest cells.

**Gene expression and Molecular markers.** In addition to the antibodies mentioned above, the arrival of molecular biology brought a whole new approach to the study of developmental biology. The expression of various genes became associated specifically to certain cells, allowing their use as markers. This also sparked an interest in the study of genes themselves to further understand the capacities they convey to the cells that express them. Because transcription factors and regulators play a central role in the modulation of expression of molecules that finally define the properties and capacities of cells, the identification of neural crest related transcription factors and modulators became of utmost relevance. Amongst the first neural crest markers to be identified is the gene Twist, a basic HLH protein involved
in neural crest migration [119]. During the last 15 years, considerable progress has been made towards the identification of additional neural crest-related transcription factors. This list includes, amongst many others: AP2, Zic, Msx1, Msx2, Pax3, Pax7, Snail1, Snail2, Sox8, Sox9, Sox10, FoxD3, cMyc, Ets1, and the proteins of the ID group.

In chick embryos, the transcription factors Ap2, Msx1 and Pax3 and Pax7 all participate in early NCC development. AP2 is expressed in most of the prospective epidermis in a wide, oval territory surrounding the center of the embryo where the prospective neural plate and the primitive streak are located [120]. This expression includes the neural plate border where NCC precursors reside, and excludes the most lateral prospective epidermis. Msx1 and Pax3 are expressed in a “Y” pattern on caudal portions of the neural plate border, as well as in the lateral caudal epiblast, caudal half of the primitive streak, and caudo-lateral mesoderm. Pax7 is co-expressed initially at the neural plate border with Msx1 and Pax3, but it is also expressed in the more rostral neural plate border (slightly more lateral than Pax3). In addition to these markers, the TGFβ signaling molecule BMP4 is coexpressed with Msx1, Pax3 and Pax7 in the posterior “Y” pattern, but the anterior expression is extended in an inverted “U” shape that surrounds the prospective neural plate completely. Later in development Snail2 and Sox9, two additional transcription factors, appear in the anterior neural folds. These markers are followed by the expression of FoxD3, cMyc, Msx2, RhoB, and Sox10 in later premigratory and early migrating stages. As NCCs emigrate, they express specific adhesion molecules (Cad7), and surface markers such as the sugar motif recognized by the HNK-1 antibody.

The chick expression data presented above serves as an example of the progression of NCC development, which is accepted to match closely with the order of events in other model organisms. However, some differences exist. For example, Snail1 appears early in frog and mouse NCC development, while in the chick, it is Snail2 that is expressed in early NCC development. The expression of Pax3 and Pax7 is also a good example of slight divergence between species, as in fish and frogs Pax3 is expressed earlier and more anteriorly than Pax7, while the reverse is true in chicks.

Genes required and/or sufficient for NCC development

Our understanding of the regulation, function, and interactions between the NCC molecular markers is limited. Most of our knowledge is based on the study of a few transcription factors expressed by early NCC precursors, or by both premigratory and migratory NCCs. The function of these transcription factors has been investigated through overexpression and
inhibition approaches in whole embryos, or in explanted tissues. These studies have identified transcription factors that are required for neural crest development (Ap2, FoxD3, Msx1, Pax3, Pax7, Snail1, Snail2, Sox9, Sox10, Zic2, etc.), some of which are able to promote an expansion of NCCs in relatively endogenous territories (FoxD3, Pax3, Snail1, Snail2, Sox9, Sox10, Zic1, Zic3, Zic5).

A more stringent test has been to challenge the inductive capacity of certain factors to trigger the formation of neural crest cells in naïve ectoderm. To date, only FoxD3 and Snail1 have been identified as having this capability. However, Snail and/or Snail2, Sox9 and FoxD3 are all vital to neural crest formation, and are considered by the neural crest community to be \textit{bona fide} neural crest markers. Therefore, the current understanding of the participation of these three factors in NCC development is addressed bellow:

\textbf{Snail.} Amongst the most prominent markers of neural crest development are the zinc finger transcription factors of the Snail family, Snai1 and Snail2 (formerly known as Slug [121]). These genes are expressed in neural crest cells, as well as in early mesodermal tissues and are associated with morphological changes and movements including those necessary for the epithelial to mesenchymal transition that neural crest cells undergo prior to their migration [122]. In the chick, Snail2 is expressed in the anterior neural folds before and after NCCs emigrate from the neural tube [123]. In \textit{Xenopus}, both Snail1 and Snail2 are expressed in premigratory and migratory NCCs, and Snail1 operates upstream of Snail2 [124]. In the mouse, Snail1 is expressed in premigratory and migratory NCCs, while Snail2 is only present in the latter [125]. In general the overexpression of Snail genes expands neural crest formation [126], while inhibition of Snail genes prevents neural crest formation and migration [123,127]. This suggests that these factors are required and sufficient for NCC development. However, in the mouse neither of these members seem to be required for NC development [128].

\textbf{FoxD3.} The fork-head or winged-helix transcription factor FoxD3, is expressed in prospective and migrating NCCs in mice, frog, fish and chick embryos. Its earliest expression is concomitant with the expression of Snail genes. Overexpression experiments offer controversial results, in some contexts leading to an expansion of the neural crest territory. Furthermore, combinatorial experiments suggest that Snail genes and FoxD3 genes may act in parallel pathways leading to NCC development [129,130]. In the mouse, FoxD3 is required for implantation, and conditional removal of FoxD3 in NCCs leads to a dramatic reduction and loss of NC derivatives in cranial and trunk regions [131].
SoxE. SoxE genes (Sox8, Sox9 and Sox10) are a subgroup of transcription factors containing a high mobility group (HMG) DNA binding box. SoxE genes are also expressed by precursors and migratory NCCs, and their functions are required for different aspects of NCC development. Sox9 appears to be critical for early NCC development, and downregulation experiments demonstrate this requirement. In contrast, overexpression experiments suggest that Sox9 can trigger the formation of neural crest cells. Mice deficient for Sox9 display regional differences regarding the role of Sox9 in crest development. In the cranial region, NCCs apparently form and migrate normally and it is only later aspects of NCC differentiation into cartilage that display marked aberrations [132]. Instead, Sox9 deficient mice display increased cell death in trunk levels likely responsible for a reduction of NCCs and dorsal neural tube markers [130].

Neural crest genetic network

The expression, function and interaction of many neural crest markers has been incorporated into models as a cascade of gene regulation [133], or a neural crest gene regulatory network [111,134,135] that propose a logical progression of gene expression and function regulating neural crest development from their induction to eventual migration and differentiation.

Early induction events involve at least 5 signaling pathways triggered by BMP, FGF, Notch, RA and Wnt molecules. This induction triggers the expression of early neural crest markers, also called border specifiers (Zic genes, Msx1, Pax3/7, and Dlx), as they appear early in development flanking the neural plate. Later in development, it is proposed that the expression of these early neural crest marker genes, in association with more signaling pathways, leads to the expression of neural crest markers or neural crest specifiers (transcription factors Snail and Snail2, SoxE, FoxD3, Ap2, Id family members, etc). The expression of these neural crest marker or specifier genes, in turn, is thought to lead to the expression of late neural crest markers or effector genes, that regulate various aspects of the neural crest phenotype - including the epithelial to mesenchymal transition, the emigration from the neural plate or tube, the stereotypic migration, cessation of migration and differentiation into the various different cell derivatives (RhoB, Cad7, Col2a, cRet, Mitf, etc). It is thus apparent that signaling pathways are reiteratively used and participate in later stages of development in conjunction with corresponding sets of transcription factors to regulate later events in neural crest development.

The proposed neural crest cascade and gene regulatory network is a starting place that provides a useful framework to better understand and study...
neural crest development. It consolidates available data, and facilitates the segregation of different components (signals, transcription factors and other molecules) or processes. It also facilitates comparative studies between different species enabling phylogenetic analysis. However, the available information regarding the direct or indirect regulation of these components, the minimal understanding of the existing and participating splice variants, and the lack of knowledge regarding their protein-protein interactions offers a wide and fertile working environment to improve our understanding of NCC development.

Concluding remarks

Despite intense research surrounding neural crest cells, many questions remain to be answered. It will be critical to resolve the pending issues on the maintenance, segregation, and distribution of different multipotent precursors. It will also be important to increase our knowledge regarding the function and molecular interactions between the genes and proteins that have been identified as important players in NCC development. Additionally, we are likely to identify new players as partners of these molecules, or as new independent components relevant for neural crest development. It would not be surprising either to identify new NC derivatives and a broader presence of NCC stem cells in adult tissues. After nearly 150 years of neural crest research, we have learned a great deal about their capabilities, migratory paths, and origin, and we are beginning to unravel the molecular underpinnings of their formidable capacities. The near future promises a true understanding of neural crest biology, which will lead to diagnostic and therapeutic strategies addressing the large number of human conditions derived from NCC anomalies.

Bibliography

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