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▶ To cite this version:

Elisabeth Dupin, Giordano Calloni, Juliana Coelho-Aguiar, Nicole Le Douarin. The issue of the multipotency of the neural crest cells. Developmental Biology, Elsevier, In press, 10.1016/j.ydbio.2018.03.024. hal-01772740

HAL Id: hal-01772740
https://hal.sorbonne-universite.fr/hal-01772740
Submitted on 20 Apr 2018

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Review article

The issue of the multipotency of the neural crest cells

Elisabeth Dupin¹, Giordano W. Calloni², Juliana M. Coelho-Aguiar³ and Nicole M. Le Douarin¹,₄

Corresponding authors
Elisabeth Dupin: elisabeth.dupin@inserm.fr
Nicole M. Le Douarin: nicoleledouarin@gmail.com
Phone: 33+153 462 537; Telecopy: 33+153 462 600

Affiliations
¹Sorbonne Universités, UPMC Univ Paris 06, INSERM, CNRS, Institut de la Vision, 17 rue Moreau, 75012 Paris, France
²Universidade Federal de Santa Catarina, Centro de Ciências Biológicas, Departamento de Biologia Celular, Embriologia e Genética, Laboratório de Plasticidade e Diferenciação de Células da Crista Neural; Florianópolis, Santa Catarina, Brasil. Tel: 55 48 3721 4581 giordano.calloni@ufsc.br
³Laboratório de Biomedicina do Cérebro, Instituto Estadual do Cérebro Paulo Niemeyer, Secretaria de Estado de Saúde do Rio de Janeiro - SES/RJ, Rio de Janeiro, Brasil”. Tel: 55 21 22779393 Email: jumcoelho@gmail.com
⁴Collège de France, 3 rue d’Ulm, 75005 Paris, France

Keywords:
near crest; peripheral nervous system; ectomesenchyme; stem cells; multipotency; cellular plasticity;

Running title: Neural crest cell multipotency

Abbreviations:
CNS, central nervous system; E, embryonic day; EDN, endothelin; EMT, epithelial-to-mesenchymal transition; ENS, enteric nervous system; Mab, monoclonal antibody; NC, neural crest; NCC, neural crest cells; PNS, peripheral nervous system; Shh, Sonic hedgehog.

Manuscript details: Text, 39 pages (93272 characters including spaces), 5 Figures
Abstract
In the neural primordium of vertebrate embryos, the neural crest (NC) displays a unique character: the capacity of its component cells to leave the neural primordium, migrate along definite (and, for long, not identified) routes in the developing embryo and invade virtually all tissues and organs, while producing a large array of differentiated cell types. The most striking diversity of the NC derivatives is found in its cephalic domain that produces, not only melanocytes and peripheral nerves and ganglia, but also various mesenchymal derivatives (connective tissues, bones, cartilages...) which, in other parts of the body, are mesoderm-derived. The aim of this article was to review the large amount of work that has been devoted to solving the problem of the differentiation capacities of individual NC cells (NCC) arising from both the cephalic and trunk levels of the neural axis. A variety of experimental designs applied to NCC either in vivo or in vitro are evaluated, including the possibility to culture them in crestospheres, a technique previously designed for cells of the CNS, and which reinforces the notion, previously put forward, of the existence of NC stem cells. At the trunk level, the developmental potentialities of the NCC are more restricted than in their cephalic counterparts, but, in addition to the neural-melanocytic fate that they exclusively express in vivo, it was clearly shown that they harbor mesenchymal capacities that can be revealed in vitro. Finally, a large amount of evidence has been obtained that, during the migration process, most of the NCC are multipotent with a variable array of potentialities among the cells considered. Investigations carried out in adults have shown that multipotent NC stem cells persist in the various sites of the body occupied by NCC. Enlightening new developments concerning the invasive capacity of NCC, the growing peripheral nerves were revealed as migration routes for NCC travelling to distant ventrolateral regions of the body. Designated “Schwann cell precursors” in the mouse embryo, these NCC can leave the nerves and are able to convert to a novel fate. The convertibility of the NC-derived cells, particularly evident in the Schwann cell-melanocyte lineage transition, has also been demonstrated for neuroendocrine cells of the adult carotid body and for the differentiation of parasympathetic neurons of ganglia distant from their origin, the NC. All these new developments attest the vitality of the research on the NC, a field that characterizes vertebrate development and for which the interest has constantly increased during the last decades.
Keywords
neural crest; peripheral nervous system; ectomesenchyme; stem cells; multipotency; cellular plasticity

Introduction
The neural crest (NC) has long been designated as a “fourth germ layer” from which multipotent cells migrate and invade the developing embryo. Apart from ectoderm, mesoderm and endoderm, the foundation of the vertebrate body involves this transitory ectodermal structure, which yields neural and mesenchymal cell types, otherwise derived from either the ectodermal or mesodermal germ layers, respectively (for references, Le Douarin, 1982; Le Douarin and Kalcheim, 1999; Hall, 2009; Dupin and le Douarin, 2014). This view has recently been reinforced by the work of Buitrago-Delgado et al. (2015), which shows that regulatory genes Snail1 and Sox5 are expressed both in the pluripotent blastula cells of amphibian embryos and in NC. In both cell types, they are required for embryonic cell competence to respond to instructive cues and therefore to keep functional multipotency. These findings led the authors to consider the NC a “neotenic” structure, in which part of the ectoderm has retained, over gastrulation, subsets of phenotypes characteristic of the early blastula, which is in sharp contrast with previous studies that favored a molecular model in which NC cells (NCC) reacquire multipotency through instructive signals like Wnt, Fgf and Bmp.
In addition to neurons and glial cells of the peripheral nervous system (PNS), melanocytes and some endocrine derivatives, the NC has the striking capacity of producing a large diversity of mesenchymal cell types in the head and neck, designated as mesectoderm or ectomesenchyme (for references, Le Douarin and Kalcheim, 1999; Le Douarin and Dupin, 2012, 2016). Such multipotency of the NC challenged the widely accepted von Baer’s germ layer theory, according to which mesenchymal cells endowed with skeletal and connective tissue capacities were exclusively produced, all over the body, by the mesodermal germ layer. The production of facial and skull bones from the ectodermally-derived NCC raised the question of the capacity of single NCC to generate both skeletal and neural cell types, or whether this capacity stands for the structure as a whole rather than for its constitutive cells, as proposed by some authors (Weston and Thiery, 2015).
This problem has prompted us, and several others, to systematically explore the extent of single NCC differentiation abilities, at various stages of their development. The approach of these studies was inspired by the in vitro clonal assays previously devised to address the developmental capacities of murine bone marrow progenitors (Metcalf, 2007 for references), which were instrumental for the understanding of blood cell lineage segregation.

This article will consider the work that has been done over the last decades in our and others’ laboratories to enlighten this issue, which included in vitro single NCC cultures and in vivo analysis of the fate of NCC individually labeled during the course of their migration from the neural folds. In these lineage experiments, the technologies used to isolate or target single cells and to identify their clonal progeny are critical; these technological issues are reviewed in the first section of this article. The problem of the interpretation of the behavior of cells in vitro versus in vivo will also be considered. The multipotency of the early NCC has been fully evidenced through this large body of work, which extended from the seminal and early contribution of Cohen and Konigsberg (1975) up to now. In the last decade, it was shown that, in the various locations of the body where the migratory NCC have settled and differentiated during embryonic life, NC-derived multipotent progenitors (or stem cells) persist in adult tissues.

1- Technical considerations about in vitro analysis of NCC differentiation potentials

The NC is a fast evolving structure and important considerations concern when and how it is removed from the embryo, which procedure is used to ensure the clonality of NCC cultures, and what are the in vitro culture conditions appropriate for the growth and differentiation of individual NCC. We briefly describe here the different techniques applied for the preparation of cranial and trunk NCC in in vitro cultures.

1.1. The “classical” bi-dimensional culture method for trunk and cranial NCC

Initially devised for the quail NC by Cohen and Konigsberg (1975) and, later on by Sieber-Blum and Cohen (1980), the “classical” NC culture procedure takes advantage of the property of the NCC to undergo delamination from the neural epithelium at the onset of their emigration, a process that takes place in vitro from isolated neural tubes.
or neural folds. This general method is now widely used to isolate NCC from avian and rodent embryos and was recently adapted to human embryos (for a technical review, Etchevers, 2011).

At the trunk level, this technique is applied to avian (quail or chick) embryos at 2 days of incubation (E2); NCC can be recovered after a period of 15 to 24 hr of in vitro migration (Lahav et al., 1998; Trentin et al., 2004; Coelho-Aguiar et al., 2013). Similar methods for trunk NCC isolation were applied to E10.5 rat and E9 mouse embryos (Stemple and Anderson, 1992; Ito et al., 1993), and, more recently, to human embryos (Thomas et al., 2008). At the cranial level, the NCC can be recovered by several methods, which differ essentially according to the stage considered. One is an adaptation from the classical technique described above for trunk NCC, using quail mesencephalon and rhombencephalon (Sieber-Blum et al., 1993; Trentin et al., 2004; Calloni et al., 2007, 2009). Another is to remove mesencephalic NCC in the course of their early migration under the superficial ectoderm (Ziller et al., 1983, Baroffio et al., 1988). Finally, some authors surgically remove the cranial neural folds; in that case, the migratory NCC are produced in vitro (e.g., Abzhanov et al., 2003; McGonnell and Graham, 2002; Ito and Morita, 1995).

1.2. Control of clonality and culture conditions for the growth of single NCC in vitro

The in vitro clonal assays using dissociated NCC suspensions have been initially carried out either by the limit dilution method (e.g., Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989, 1991; Sieber-Blum et al., 1993). In our laboratory, we chose a direct cloning procedure, which consisted in selecting single cells with a micropipette, followed by their plating individually, under microscopic control, which ensured true clonal seeding of virtually all the cultures (Baroffio et al., 1988, 1991; Dupin et al., 1990; Lahav et al., 1998; Trentin et al., 2004; Calloni et al., 2007, 2009; Coelho-Aguiar et al., 2013).

Culture medium and substrate conditions have been optimized for the development of single NCC progeny in two-dimensional cultures. Our experiments were regularly performed by plating single quail NCC on a feeder-layer of growth-arrested mouse 3T3 fibroblasts, according to the procedure devised by Barrandon and Green (1985) to generate colonies from single human keratinocytes. The presence of this feeder-layer, together with a "clonal culture medium" enriched with serum, chick embryo extract and
a cocktail of hormones and growth factors (Baroffio et al., 1988, 1991; Dupin et al., 1990), strongly enhanced single NCC survival and proliferation, while allowing their differentiation along a large array of neural and non-neural NC lineages. However, for serial subcloning of NCC cultures, the 3T3 feeder-layer was replaced by a collagen1-coated substrate (Trentin et al., 2004). Collagens, fibronectin and other extracellular matrix (ECM) components were also routinely employed as a substratum for in vitro cultures of avian and mammalian adherent NCC (reviewed by Dupin and Sommer, 2012; Dupin and Coelho-Aguiar, 2013).

1.3. The new “crestosphere” procedure: growing early NCC in tri-dimensional cultures

Recently, Keruoso et al. (2015) devised a method for the in vitro long-term growth of early neuroepithelial NCC in free-floating spheres, the so-called “crestospheres”. Sphere-forming assays have been previously instrumental to identify stem cells in many organs, since the initial report of adult brain-derived neurospheres (Reynolds and Weiss, 1992; for a review, Pastrana et al., 2011). Keruoso et al. (2015) implemented such assays to the early NCC, obtained directly from the chick embryo by dissociation of the cranial dorsal neural tube cells and after in vitro derivation from human embryonic stem (ES) cells. On non-adhesive substrate, spherical clusters formed from single NCC and could be serially replated, allowing long-term propagation of premigratory NCC that retained expression of early NC genes and self-renewal ability. When transferred to adhesion- and differentiation-promoting conditions, cells of the “crestospheres” underwent EMT while migrating onto the substratum and they differentiated into neural, melanocytic, myofibroblastic and skeletogenic phenotypes. This clonal sphere assay thus provided evidence of cranial NCC multipotency at single cell resolution (Keruoso et al., 2015). The “crestospheres” therefore represent a valuable tool for further investigations on the regulation of NC stem cell properties, as recently exemplified by the role of c-myc in controlling the size of the multipotent NCC pool (Keruoso et al., 2016).

1.4. Identification of NC-derived phenotypes in vitro

In the pioneer work by Cohen and Konisgberg (1975), the analysis of single NCC progeny relied only upon the microscopic detection of melanin, the natural marker of pigment cells, which led to the first evidence for bipotent NCC, generating both
pigmented and unpigmented cells. Later on, the characterization of catecholaminergic cells within subsets of NCC colonies (Sieber-Blum and Cohen, 1980), paved the way towards a broader analysis of NC-derived phenotypes with cell type-specific markers. From the late 1980ies, the production of monoclonal antibodies (Mab) directed against antigens expressed by NCC and NC derivatives, has provided an increasing number of tools to identify cell types in the descent of isolated NCC.

One of the main technical issues in order to decipher the developmental repertoire of individual NCC was the choice and combination of markers, technically compatible for the analysis of a large diversity of phenotypes in each NC colony. With the aim of investigating the main neural and mesenchymal NC lineages, we examined as many as six distinct phenotypes in quail NC-derived clones (Calloni et al., 2009; See next section). The cells of the glial and pigment cell lineages were labeled using Mabs against the Schwann cell Myelin Protein (SMP) (Dulac et al., 1988) and the MelEM (Nataf et al., 1993) antigens respectively, whereas immunodetection of neurofilament proteins, β3-tubulin, and tyrosine hydroxylase, a marker for catecholaminergic cells identified neuronal cells. Immunoreactivity to α-smooth muscle actin (αSMA) defined smooth muscle/myofibroblastic cells. Chondrocytes and adipocytes were evidenced based on several morphological criteria (Calloni et al., 2007; Billon et al., 2007; Coelho-Aguiar et al., 2013) and bone cell identification relied on the expression of Runx2, a master gene of osteogenesis (Ducy et al., 1997; Otto et al., 1997), detected by in situ hybridization (Calloni et al., 2009; Coelho-Aguiar et al., 2013). We also used the HNK1/NC1 Mab to detect NCC subsets that did not express the aforementioned lineage markers. HNK1 is considered as a reliable marker of a large subset of migratory NCC in several, including avian, rat (not mouse) and zebrafish species. In the avian embryo, HNK1 labels the majority of undifferentiated migratory NCC and, later on, is retained in the peripheral nervous system (PNS) but lost in melanocytes and mesenchymal NC derivatives (Vincent et al., 1983; Tucker et al., 1984).

2- Individual cranial NCC give rise to both mesenchymal/ skeletogenic and neural phenotypes in vitro
Transplantation experiments in avian embryos based on the quail-chick chimera system, and, more recently, genetic fate mapping in the mouse, have shown that ectomesenchymal derivatives of the cranial NC in amniotes include chondrocytes and bone cells, smooth muscle cells and pericytes, tendon, dermal and other connective tissue cells, adipocytes, stromal and endothelial cells of the cornea, and, in mammals, odontoblasts (for references, Le Douarin and Kalcheim, 1999; Minoux and Rijli, 2010; Le Douarin and Dupin, 2012, 2016). The problem was thus raised as to whether, at the time they start migrating, the NCC of the cephalic area were capable to yield a large array of different cell types (neural cells, melanocytes and mesenchymal cells), or if they could comprise mesenchymal restricted progenitors, distinct from those yielding the neural-melanocytic derivatives.

2.1. In vitro clonal analysis of quail cephalic NCC to tackle the cellular origin of neural, melanocytic, myofibroblastic and chondrocytic cells

In vitro clonal cultures experiments carried out by ours and Maya Sieber-Blum’s laboratories first showed very diverse proliferation and differentiation potentials in cephalic quail NCC during the early migratory phase (Baroffio et al., 1988, 1991; Dupin et al., 1990; Ito and Sieber-Blum, 1991; Sieber-Blum et al., 1993; Sieber-Blum and Ito, 1995; Trentin et al., 2004). Initial investigations on mesencephalic migratory NCC isolated at 10-somite-stage (10ss), led us to identify multipotent progenitors for Schwann cells, adrenergic neurons, myofibroblasts and/or melanocytes. Noticeably, 2.5% of 842 clone-forming NCC produced both chondrocytes and glial cells, together with neurons, melanocytes and/or myofibroblasts (Baroffio et al., 1988, 1991; Dupin et al., 1990; Trentin et al., 2004; Le Douarin et al., 2004). Moreover, by in vitro clonal assays, Ito and Sieber-Blum (1991) found that 17% (13/74) of rhombencephalic NCC could generate chondrocytes together with sensory neurons and/or pigment cells. At that time, culture conditions and technical limitations to the analysis of multiple phenotypes in the colonies did not permit a more comprehensive characterization of mesenchymal NC progenitors, particularly those forming bones. More efficient production of skeletogenic cell types and terminal differentiation of cartilage and bone cells were obtained in vitro from cephalic quail NCC isolated after 15 to 17 hr of migration from the explanted mesencephalon primordium (Calloni et al., 2007, 2009; Dupin et al., 2010). Using these settings, Calloni et al. (2007) found that,
after 10 days of clonal culture, 14.5% of the cephalic NCC yielded chondrocytes together with neural and/or melanocytic cells. In particular, 6.6% of the clones were derived from a highly multipotent type of NCC, which differentiated into glia (G), neurons (N), melanocytes (M), myofibroblasts (F) and chondrocytes (C) (i.e., GNMFC progenitor). Furthermore, NCC were responsive to Sonic Hedgehog (Shh), a morphogen previously shown to be crucial for cranial NCC survival and formation of the craniofacial skeleton (Chiang et al., 1996; Ahlgren and Bronner-Fraser, 1999; Cordero et al., 2004; Jeong et al., 2004; Brito et al., 2006, 2008). In the presence of Shh, chondrocytes-containing clones showed a three-fold increase in frequency and were all derived from multipotent (i.e., at least, bipotent) NCC founders (43% and 14.5% of treated and untreated clones, respectively). Thus, the proportion of GNMFC progenitors reached 18.5% of clone-forming NCC after exposure to Shh. Therefore, neural-melanocytic and chondrocytic NC phenotypes arise from common progenitors that are highly frequent in the cranial NCC isolated at early migratory stages (Calloni et al., 2007; Le Douarin et al., 2008).

In these experiments, the colonies contained from about a few hundred to more than ten thousands of cells in both Shh-treated and untreated conditions. By scoring the total number of cells in the phenotypically distinct categories of clones, we found that the extent of NCC potentialities did not correlate with the total cell number in their progeny (Calloni et al., 2007 and unpublished data). For example, the total number of cells in the clones arising from highest multipotent GNMFC progenitors varied (Figure 1). Likewise, large-sized colonies (of about 5,000 to 20,000 cells) originated from tetra- and quintapotent progenitors as well as from more restricted NCC yielding neural and melanocytic cells only (e.g., GNM and GM). These findings indicate that acquisition of multiple fates in the progeny of single NCC is not dependent upon extensive proliferative activity.

In vitro clonal analysis allows to uncovering the developmental potentials of a cell a posteriori, through the phenotypic composition of its progeny, but one drawback of this approach is that the identity of distinct founders remains unknown. In attempt to further define different subsets of cephalic NCC, we took advantage of the HNK1 Mab, which recognizes a surface carbohydrate epitope expressed by the majority, but not all, of early migratory NCC (Vincent et al., 1983; Tucker et al., 1984). At later stages, this marker is not expressed in melanocytic and ectomesenchymal lineages while present in most PNS cells. Hence, the developmental potentials of the early NCC that express HNK1
remained elusive, particularly regarding their capacity to yield mesenchymal derivatives. In order to know whether HNK1-expressing NCC possess either a restricted or a full array of the differentiation capacities of the NC, we isolated mesencephalic quail NCC as described above, and we immunolabeled them with HNK1. The HNK1+ cells were then selected under the control of fluorescence microscopy for clonal plating in the presence of Shh (Calloni et al., 2007 and unpublished data). After 10 days, 45% of the plated HNK1+ cells had generated a clone. Of these clones, 27.5% contained glial, neuronal, melanocytic, myofibroblastic and chondrocytic cell types, therefore retrospectively identifying highly multipotent GNMFC progenitors (Figure 2). The remaining colonies lacked chondrocytes but contained glia, neurons, melanocytes and myofibroblasts in various combinations. Although preliminary, these data show that cephalic NCC bearing the HNK1 marker comprised a subset of multipotent progenitors capable to produce chondrocytes together with neural-melanocytic cells.

2.2. In vitro analysis of the osteogenic potential of the cephalic NCC

Using Runx2 transcription factor as an early bone-specific marker (Ducy et al. 1997; Komori et al., 1997; Karsenty et al., 2009), we have further investigated the osteogenic potential of cephalic quail NCC in vitro (Calloni et al., 2009). We found that, after 5 days of in vitro culture, numerous NCC start to express Runx2 mRNA. Later on, large clusters of mineralizing bone cells differentiated in these cultures (Calloni et al., 2009). When grown as single cells, cephalic NCC gave rise to variable combinations of the six distinct recorded phenotypes, i.e., glial cells (G), neurons (N), melanocytes (M), myofibroblasts (F), chondrocytes (C) and osteoblasts (O). To our surprise, more than 90% of the clones contained both osteogenic and neural and or melanocytic cell types, and we reported the first evidence of common progenitors for the six cell types under scrutiny (i.e., GNMFCO progenitors) (Calloni et al., 2009; Dupin et al., 2010) (Figure 3). Treatment with Shh resulted in a higher proportion of the clones generated by hexa- and pentapotent progenitors (51.5% versus 23% in controls). While the vast majority of colonies contained osteoblasts but no chondrocytes in untreated NCC cultures, the frequency of osteo-chondrogenic progenitors increased by three-fold in the presence of Shh. Therefore, the skeletogenic cells derived from the cephalic NC, which rely upon Sox9
gene activity for development in the mouse (Mori-Akiyama et al., 2003), originated in vitro from Shh-responsive, highly multipotent progenitors.

In sum, the analyses of single NCC fate in vitro have revealed that large subsets of the early cephalic NCC are progenitors for both mesenchymal (including skeletogenic) and neural-melanocytic NC lineages. By providing an unequivocal demonstration of the origin of head chondrogenic and osteogenic cells from multipotent NCC, these findings further underline the broad multipotency of individual NCC, which thus are endowed with the striking ability to differentiate into both ectodermal and mesodermal lineages. A recent work reporting the generation and propagation of clonal spheres ("crestospheres") from chicken and human ES cell-derived NCC, further strengthens these conclusions by showing that premigratory cranial NCC are stem cells that exhibit self-renewal and can produce both mesenchymal and neural cell types (Keruoso et al., 2015). Together, these data obtained at single cell resolution contradict the contention that “cranial skeletal mesenchyme and other mesectodermal derivatives originate evolutionarily and developmentally from a distinct epithelial domain of the neural fold, the Metablast, rather than from the Neural crest” (Weston and Thiery, 2015). Nevertheless, a common cellular origin of mesectoderm and neural-melanocytic derivatives at the single NCC level does not preclude some heterogeneity in the neural folds. Along this line, distinct subdomains were recently evidenced in the chick midbrain NC regarding multiplex gene expression (Lignell et al., 2017), providing novel insights into the gene regulatory networks underlying early NCC heterogeneity.

3) Trunk NCC progenitors are multipotent and harbor dormant mesenchymal potentials

As opposed to cephalic NCC, the in vivo differentiation repertoire of trunk NCC in amniote vertebrates was so far considered to be limited to PNS neuronal and glial cells, melanocytes and adrenomedullary cells, until mouse transgenesis experiments by Joseph et al. (2004) led to the discovery that the NC, via Schwann cell precursors along the sciatic nerve, gave rise to a subset of endoneurial fibroblasts (Joseph et al., 2004). Using in vitro clonal cell culture approaches, we have shown that avian trunk NCC comprise various subsets of multipotent progenitors, including those endowed with the
capacity to clonally produce glia (G), neurons (N), melanocytes (M) and myofibroblasts (F) (i.e., GNMF progenitors), as well as more restricted NCC, such as glial-melanocytic (GM) and glial-myofibroblastic (GF) progenitors (Trentin et al., 2004). These bipotent GM and GF progenitors exhibited self-renewal capacity after serial in vitro subcloning in response to endothelin-3 (EDN3) and FGF2, respectively (Trentin et al., 2004; Bittencourt et al., 2013). The in vitro culture methods of avian NCC adapted to mammalian cells likewise led to identify multipotent progenitors in the mouse and rat trunk NC (Ito et al., 1993; Stemple and Anderson, 1992; Shah et al., 1994, 1996; Paratore et al., 2001). For the first time, Stemple and Anderson (1992) isolated precursors from the rat NC through their expression of p75 neurotrophin receptor, which gave rise to autonomic neurons, glial cells and myofibroblasts and exhibited self-renewal in vitro, thus deserving to be considered as NC stem cells. Furthermore, Anderson and co-workers identified NC stem cells of similar developmental capacities in the fetal rat sciatic nerve (Morrison et al., 1999). Several signaling pathways are at work to regulate the differentiation choice of these mammalian NC stem cells: neuregulin-1 and Delta-mediated Notch receptor activation triggered glial fate (Shah et al., 1994; Morrison et al., 2000), while BMP signaling promoted autonomic neuronal outcome at the expense of glial fate, and TGFβ drove NCC to adopt a myofibroblastic phenotype (Shah et al., 1996). In the mouse, Sommer and colleagues found that a combination of Wnt and BMP factors regulates the maintenance of multipotent NC stem cells (Kleber et al., 2005). In the absence of BMP, Wnt/β-catenin signaling exerts a temporal control on NCC fate: at premigratory stages, by promoting sensory neuron generation; and when activated later on, in migratory NCC, by triggering melanocyte production (Lee et al., 2004; Hari et al., 2002, 2012).

As already mentioned, the presence of myofibroblasts/smooth muscle cells was recurrently recorded in avian and mammalian trunk NCC in vitro progeny. In the recent years, it became clear that, besides myofibroblasts, trunk NCC in amniote vertebrates do have the capacity to give rise to diverse mesenchymal phenotypes, including skeletal cells. In appropriate culture conditions, avian trunk NCC differentiated into adipocytes (Billon et al., 2007; Coelho-Aguiar et al., 2013) and chondrocytes (McGonnell and Graham, 2002; Abzhanov et al., 2003; Calloni et al., 2007; Ramos-Hryb et al., 2013). As observed for the cephalic NCC, trunk NCC exposed in vitro to Shh exhibited markedly
increased chondrogenesis, and we could record a rare type of multipotent trunk NCC yielding glial cells, myofibroblasts and chondrocytes (Calloni et al., 2007; Le Douarin et al., 2008).

Recently, we further investigated the ability of trunk NCC to differentiate into bone and fat cells (Coelho-Aguiar et al., 2013). When using a cocktail of hormones and growth factors that enhanced terminal differentiation of adipogenic and osteogenic cells, quail trunk NCC isolated after 15-18 hr of migration from explanted neural tubes, exhibited a high ability to differentiate in vitro into chondrocytes, lipid-laden adipocytes and mineralizing osteoblasts (Figure 4 A-C). Strikingly, these mesenchymal cell types coexisted with PNS derivatives in trunk NCC clonal progeny (Coelho-Aguiar et al., 2013); for example, the majority of the colonies grown on 3T3 cell feeders comprised Runx2-positive osteoblasts (O) accompanied by autonomic neurons (N) and glial cells (G) (GNO; 57% of total clones) (Figure 4 D).

These data suggest that most of the trunk NCC, which adopt neural and melanocytic phenotypes in vivo, possess dormant potentials to differentiate into connective, osteogenic and adipogenic cells. Together with several reports that mammalian trunk NCC also displayed a limited ability to differentiate in vitro into skeletogenic cells (Ido and Ito 2006; John et al., 2011), these findings therefore show that, albeit reduced compared to cranial NCC, mesenchymal potentials are indeed present in the trunk NC. The reason for which these potentialities are not expressed in vivo remains unknown.

4) Evolutionary considerations on trunk versus cephalic NCC potentials

Taken together, the in vitro clonal assays have shown that, in both cephalic and trunk NCC, the mesenchymal phenotypes and the neural-melanocytic ones arise from common progenitors. In an evolutionary perspective, one attractive possibility is that such multipotent neural-melanocytic-mesenchymal progenitors, as recorded in the avian NC, might represent a primitive type of NCC. In early vertebrates, like extinct jawless fishes (Ostracoderms) of the Ordovician, a superficial armor of calcified dermal bone containing dentine covered the head and trunk. Since dentine is synthesized by NCC in all extant vertebrates (i.e., by the NC-derived odontoblasts in mammals), it has been assumed that both cranial and trunk skeleton of the early vertebrates originated from the NC (Janvier, 1996; Smith and Hall, 1990; Smith, 1991). During evolution, this
superficial skeleton regressed in the trunk while substituted for by an internal endochondral vertebral skeleton of mesoderm origin. The dermal skeleton in the distal part of the rays in dorsal and caudal fins of teleosts might be a remnant of the primitive superficial skeleton of extinct vertebrates, although its origin, from either the trunk NC or the somitic mesoderm in zebrafish is still debated (Smith et al., 1994; Kague et al., 2012; Lee et al., 2013a,b). In higher vertebrates, the NC skeletogenic capacity has been retained in the craniofacial region, whereas it vanished, but did not completely disappear in the trunk since it can be evidenced in appropriate in vitro culture conditions.

Much unknowns remain about the mechanisms and molecular determinants which underlie the differential expression of mesenchymal potencies between the cephalic and trunk NC. However, the expression domains of the genes belonging to the Hox clusters, which have the capacity to pattern the body of all bilateria, are remarkable in this respect: Hox genes are not expressed in the area where the vertebrate head skeleton develops (Couly et al., 1998) and, more strikingly, their ectopic expression in the cephalic region completely inhibits the development of the head skeleton (Grammatopoulos et al., 2000; Couly et al., 2002; Creuzet et al., 2002). In the mouse, similar severe defects of craniofacial structures resulted from conditional deletion in the NC of ezh2, which encodes a subunit of the Polycomb Repressive Complex 2 (PRC2) that catalyzes trimethylation of histone H3 at Lys27 (Schwarz et al., 2014). The craniofacial phenotype of ezh2 mutant embryos was correlated to de-repression of Hox genes in the cephalic but not the trunk NC, suggesting that epigenetic regulation of Hox genes controls head NC mesenchymal fate (Schwarz et al., 2014).

The role of intrinsic determinants in NC mesenchymal fate was further highlighted in a recent work, showing that a defined set of transcription factors (TFAP2b, Sox8 and Ets1) acts positively to establish the “cephalic character” of the NC (Simoes-Costa and Bronner, 2016). Expression of this set of genes was found to be enriched in the transcriptome of cranial versus trunk avian NCC (Simoes-Costa et al., 2014), while these two NCC populations were defined by, and isolated through, activation of distinct Sox10 enhancers at the onset of migration (Betancur et al., 2010). If co-electroporated in ovo at early stages of NC formation, TFAP2b, Sox8 and Ets1 conferred a cephalic NC-type identity to the trunk NCC, at least regarding their transcriptomic profile and their
capacity to produce chondrocytes after in vitro culture. Nevertheless, head mesenchymal tissue formation, which represents assessment of a complete trunk to cranial-like NCC conversion, was reported after grafting the reprogrammed trunk NCC in a cranial, favorable, location but was not achieved in situ in the trunk (Simoes-Costa and Bronner, 2016). Therefore, specific transcription factors act as positive regulators of the cranial NC fate and, together with yet undetermined environmental cues, can promote acquisition of cephalic NC features by the trunk NCC.

5) Multipotency versus restrictions of the early NCC in vivo

First attempts to tackle the pivotal question of NCC commitment and multipotency in vivo was reached through the development of methods to mark single premigratory NCC with a fluorescent intracellular dye allowing to trace their fate after migration in early embryos. Following the microinjection of individual cells in the dorsal neural tube of the chicken embryo with a lipophilic fluorescent dye, Bronner-Fraser and Fraser (1988) provided the first sound evidence of NCC multipotency in vivo. After 2 days of migration, about half of the labeled trunk NCC produced descendent cells that populated more than one NC derivative (e.g. skin, DRG, sympathetic ganglia, peripheral nerves and adrenal medulla) and adopted distinct fates, including glial cells, sympathetic and sensory neurons, melanocytes and adrenomedullary cells (Bronner-Fraser and Fraser, 1988, 1989). By similar experiments, they showed multipotency of subsets of individual trunk NCC in Xenopus (Collazo et al., 1993) and mouse (Serbedzija et al., 1994). These pioneer studies have revealed the heterogeneity of early NCC with respect to their developmental fate and uncovered the presence of multipotent as well as more restricted NCC in vivo before the onset of migration.

However, in zebrafish embryos, the labeling of individual premigratory cranial and trunk NCC, in most cases, gave rise to a lineage-restricted progeny homing to single locations (DRG, sympathetic ganglia, ventral root nerves and skin), suggesting earlier NCC fate restrictions in teleosts than in higher vertebrates (Raible and Eisen, 1994; Schilling and Kimmel, 1994). The hypothesis of an early determination of the chick NCC was put forwards recently by Krispin et al. (2010a,b), after microinjection of a fluorescent dye or discrete GFP plasmid electroporation into small numbers of trunk NCC, before their emigration from the dorsal midline. These experiments lend support to a model in which NCC fate could be predicted prior to delamination, depending on
their relative dorso-ventral position in the neural tube and their temporal order of exit from the neural tube; accordingly, the progeny of NCC labeled at an early emigration stage was restricted to sympathetic ganglia, whereas at the same level of the neural axis, later-emigrating NCC populated exclusively more dorsal derivatives and sequentially produced DRG cells and melanocytes (Krispin et al., 2010a, b).

It is presently unclear whether discrepancies between these data and the earlier in vivo experiments showing chick NCC multipotency (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991) are due to temporal and/or technical differences in single NCC labeling procedures. Recently, McKinney et al. (2013) further explored the dynamics of NCC migratory behavior using targeted photoconversion in single cells of the dorsal neural tube and time-lapse imaging in the chicken embryo in vivo and in slice cultures. In contrast to Krispin et al. (2010a,b) who found that NCC emigration was limited to the dorsal midline, they observed that NCC exited in a stochastic manner from the 25% dorsal most region of the neural tube. Moreover, except for the first NCC to emigrate, which preferentially populated the sympathetic ganglia, the remaining, later-emigrating NCC exhibited an unfixed fate, showing similar ability to contribute multiple NC derivatives, in the ventral root nerve, the DRG and the melanocytic subectodermal pathway (McKinney et al., 2013).

The precise time and pattern of NCC diversification remain ill defined and vary according to the diverse NC sublineages. Yet altogether, the analyses of NCC fate in vivo have pointed to the presence of distinct progenitors in the early NC population, comprising several types of multipotent cells together with NCC generating a more limited repertoire of cell types. This is in agreement with the results obtained by in vitro clonal NC cultures. Some subpopulations of avian NCC can undergo lineage specification very early, at the onset of migration or a few hours after exiting the neuroepithelium, as observed in chick neural tube explant cultures (Henion and Weston, 1997). Early fate restriction of NCC was documented particularly for the sensory neuronal phenotype. At the neural fold stage or soon after the onset of migration, the avian cranial and trunk NC comprises a set of apparently committed precursors that can readily differentiate in vitro into sensory neurons (Ziller et al., 1983, 1987; Bader et al., 1983). In transgenic mice, expression of definite marker genes (e.g., neurogenin2, GDF7 and neuropilin2) in premigratory NCC subpopulations could be linked to a predictable sensory fate
(Ziringler et al., 2002; Lo et al., 2005; Lumb et al., 2014). However, it should be emphasized that, amongst the large diversity of sensory neuron subtypes, most of them arise from multipotent NCC (for references, Marmigere and Ernfors, 2007), as shown by in vitro and in vivo single cell studies (Sieber-Blum, 1989; Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991; Kleber et al, 2005). Collectively, these data illustrate the complex pattern whereby sequential waves of cell specification occur in the NCC, hence the challenging task to dissect how diversity of developmental lineages is created in the heterogenous population of the early NCC.

Providing a way to circumvent the inherent technical drawbacks of intracellular tracer injections or electroporation into single cells in vivo, novel genetic fate mapping approaches have been devised to trigger heritable multicolor labeling of many clones in the intact embryo (Livet et al., 2007; Snippert et al., 2010; Loulier et al., 2014; Weissman and Pan, 2015). In this respect, Baggiolini et al. (2015) have used the R26R-Confetti reporter combined with inducible Wnt1-Cre and Sox10-Cre mouse lines to drive multicolor reporter expression in single premigratory and early migratory trunk NCC, respectively. Quantitative phenotypic analysis of the labeled clones showed that, both before and during migration, the vast majority of trunk murine NCC are multipotent (Baggiolini et al., 2015). Meanwhile, several types of clones were also recorded, which attested to the presence of distinct multipotent progenitors in the mouse trunk NC, in accordance with the results from in vitro clonal cultures of avian NCC (Sieber-Blum et al., 1991; Trentin et al., 2004, Calloni et al., 2009; Dupin and Sommer, 2012).

6) Lineage promiscuity of Schwann cells and melanocytes

Adameyko and colleagues (2009) have discovered an unexpected important role of Schwann cell precursors in the development of pigment cells. In the course of Schwann cell differentiation, “Schwann cell precursors”, corresponding to an intermediate early step between NCC and “immature Schwann cells”, are defined in mammalian peripheral nerves as Sox10+ NCC located along developing nerves, which strictly depend for survival on nerve-derived factor neuregulin1, and which express early Schwann cell lineage markers (Dhh, PLP, P0 Protein) (Jessen et al., 2015). In chicken and mouse embryos, significant subsets of Schwann cell precursors were found to detach from the axons of peripheral nerves growing to the skin, lose glial traits while acquiring expression of the melanocytic lineage master gene MITF, then migrate to the skin and
eventually differentiate into pigment cells (Adameyko et al., 2009, 2012; Ernfors, 2010). This glial to melanocytic conversion was restricted to Schwann cell precursors and could occur at later stages of Schwann cell development only in experimental contexts of disrupted association between axons and Schwann cells like after nerve transection. The production of melanocytes from nerve-adjacent cells required downregulation of the expression of Foxd3, a transcription factor involved in maintenance of NCC multipotency and inhibition of melanogenesis (Mundell and Labosky, 2011; Thomas and Erickson, 2009; Nitzan et al., 2013a,b).

Besides the melanocytes that first differentiate from NCC that migrate dorso-laterally from the neural primordium to the skin, this second later source of pigment cells was estimated to contribute at least 50% of whole pigmentation in adult mouse hair follicles (Adameyko et al., 2009). Of note, a similar strategy of the deployment of nerve-associated NC progenitors seems to operate for the generation of colored stripes in the adult zebrafish (Singh et al., 2016).

The propensity by cells of the Schwann cell lineage to generate melanocytes was previously evidenced in vitro in Schwann cells isolated from embryonic quail sciatic nerves. In the presence of EDN3, a cytokine expressed in the embryonic skin, Schwann cells proliferated and reverted to early NCC-like multipotent progenitors that clonally produced melanocytes and myofibroblasts in addition to glial cells (Dupin et al., 2003; Real et al., 2005) (Figure 5). Schwann cells isolated from adult rat palatal ridges also behaved as multipotent NC-like cells in neurosphere cultures (Widera et al., 2011).

Strikingly, EDN3, which is highly mitogenic for avian melanocytic cells (Lahav et al., 1996, 1998), triggered the reverse in vitro conversion, from pigmented melanocytes to Schwann cells (Dupin et al., 2000; Real et al., 2006). When analyzed in single pigment cell cultures, this conversion took place by the proliferation and dedifferentiation of pigment cells into oligopotent, self-renewing NC-like stem cells, such as bipotent glial-melanocytic ones, which were previously identified in the early avian NC (Lahav et al, 1998; Trentin et al, 2004; Real et al., 2006) (Figure 5). The capacity of producing multipotent NCC in vitro, was also recently highlighted in rodent melanoblasts cocultured with stromal cells (Motohashi et al., 2009, 2011; Kunisada et al., 2014)
Therefore, peripheral glial cells and melanocytes have a common direct ancestor in the NCC hierarchy and are highly prone to dedifferentiating and recapitulating an early multipotent NC state.

7) Pre-glial NCC as a mean for the spreading of NC-like stem cells at postmigratory and adult stages

The first evidence that plasticity is retained at NCC postmigratory stages was provided by back-transplantations of PNS ganglia into the early NC migration path in quail-chick chimeras (for references, Le Douarin and Kalcheim, 1999). One of the most striking findings was that embryonic quail sensory ganglia contain precursors able to give rise to sympathetic neurons and glial cells. Such precursors belong to the non-neuronal lineage of PNS ganglia, as shown unequivocally by tracing the fate of transplanted chimeric nodose sensory ganglia, in which either only the placode-derived neurons or the NC-derived glial cells harbored the quail marker (Ayer-Le Lièvre and Le Douarin, 1982).

In the last two decades, genetic fate mapping studies have revealed that virtually any tissues and organs of the fetal and adult mice harbor NC-derived cells, which exhibit multipotency and stem cell properties when isolated in vitro (for references, Dupin and Sommer, 2012; Dupin and Coelho-Aguiar, 2013). Adult sources of these NC stem cells encompass NC derivatives (e.g., peripheral nerves, DRG, enteric ganglia, corneal stroma and dental pulp) as well as tissues that are not of NC origin but contain NC-derived cells, such as bone marrow and hair follicles (Sieber-Blum et al., 2004; Fernandes et al., 2004; Wong et al., 2006; Nagoshi et al., 2008, Glejzer et al., 2011; for recent reviews, see Motohashi and Kunisada, 2015; Liu and Cheung, 2016). In most of these tissues however, the precise localization, specific markers and renewal capacity of resident NC-like stem cells remained undefined, with the notable exception of the carotid body, a small organ located near carotid artery bifurcation, which contains catecholaminergic neuron-like cells responsive to variations of arterial oxygen pressure. Pardal et al. (2007) provided evidence that NC-derived stem cells in the adult rodent carotid body belong to the glial cell compartment and were multipotent in vitro; in response to chronic hypoxemia, these cells produced myofibroblasts and new neuroendocrine glomus cells in vivo.
A particular type of peripheral glial cells, the boundary cap (BC) cells, turned out to assume an unsuspected role in the production of several NC-derived differentiated cell types. BC cells, derived from late-emigrating NCC, form clusters at the limit between peripheral nerve roots and the neural tube (Niederlander and Lumsden, 1996; Golding and Cohen, 1997) and they exhibit NC stem cell properties in vitro (Hjerling-Leffler et al., 2005). Taking advantage of BC-specific Krox20 expression during early development, Maro et al. (2004) genetically traced the fate of BC cells and showed that they gave rise to sensory nociceptive neurons and satellite cells in the DRG, in addition to Schwann cells in the dorsal roots. Moreover in the adult mouse, BC-derived cells migrated as far as the dermis and produced Schwann cells, terminal glia and endoneurial fibroblasts in cutaneous nerves (Gresset et al., 2015).

By using selective ablation of cranial nerves and genetic lineage tracing in mice, two independent teams recently unveiled another neurogenic function of Schwann cell precursors, in the building of the parasympathetic ganglia (Espinosa-Medina et al., 2014; Dyachuk et al., 2014). Located at distal sites along growing visceral motor cranial nerve fibers, Schwann cell precursors assumed a dual glial-parasympathetic neuronal identity, before undergoing transition to differentiated neurons in the parasympathetic ganglia. Thus, pre-Schwann cells dwelling from cranial nerve VII contributed neurons in submandibular and pterygopalatine ganglia while formation of the otic ganglia required the presence of presynaptic cranial nerve IX and its associated glia.

Neurogenesis from nerve-associated cells also occurred in the murine enteric nervous system (ENS), wherein a late-differentiating subset of neurons originated from Schwann cell precursors that invaded the gut at perinatal stages alongside the terminals of extrinsic sympathetic nerve fibers (Uesaka et al., 2015; Heuckeroth and Schäfer, 2016). Moreover, in the oesophagus and stomach, enteric neurons developed from pre-Schwann cells that traveled along the vagus nerve (Espinosa-Medina et al., 2017). One of the remaining questions is how long Schwann cell precursors maintain their neurogenic capacity after a functional ENS has been established. At postnatal stages, when murine neurogenesis continues, multipotent NC-derived stem cells persist in the ENS (Kruger et al., 2002; Bondurand et al., 2003; Heanue and Pachnis, 2007). In the adult gut however, neurogenesis seems very limited and is still a matter of debate (Liu et al, 2009; Joseph et al., 2011; Laranjeira et al., 2011). Nevertheless, the ENS of adult mammals harbors glial
progenitors with latent neurogenic potential, which can be activated after in vitro culture and in vivo after injury (reviewed by Coelho-Aguiar et al., 2015).

The plasticity of PNS glial precursors remarkably extends to the production of endocrine chromaffin cells in the murine adrenal medulla (Furlan et al., 2017) and even to the generation of mesenchymal cell types. As already mentioned, nerve-associated mouse NCC generated endoneurial fibroblasts in vivo in the sciatic and cutaneous nerves (Joseph et al., 2004; Gresset et al., 2015). Furthermore, using the model of the continuously growing mouse incisor, Kauku et al. (2014) recently uncovered that Schwann cell precursors in nerve terminals at tooth apex were at the origin of dental pulp cells and odontoblasts. Moreover, these nerve-derived precursors displayed mesenchymal stem cell characteristics and were capable to regenerate dentine after trauma of the adult tooth.

Taken together, these data underline the remarkable plasticity of the Schwann cell lineage in various peripheral sites. They also suggest that, as in the CNS, in which radial glia and astrocytes function as neural stem cells in developmental and adult neurogenesis (Kriegstein and Alvarez-Buylla, 2009 for a review), NC-derived stem cells in the PNS assume a pre-glial identity. These pre-glial NC stem cells are transported via the peripheral innervation network, thus ensuring a nearly ubiquitous distribution in the body of NC-like progenitors endowed with multiple differentiation options.

**Concluding remarks**

The NC, a specific embryonic structure of vertebrates, has attracted the interest of many researchers in developmental and stem cell biology, due to the remarkable migration properties of its component cells and their large contribution to multiple cell types in various organs and tissues. Here, we have focused on the question of NCC multipotency and fate diversification.

Collectively, the available data show that, before and during migration from the neural folds, the NCC exhibit a certain level of heterogeneity regarding their differentiation capacities. This heterogeneity and the evidence of NCC multipotency in the premigratory NCC, both at the level of the population and of single cells, was an undisputable result drawn from several and complementary approaches.
Early multipotency may be lost rapidly in particular NCC subpopulations, such as in those giving rise to some sensory neuronal subtypes. However, multipotent cells are present in subsets of all migratory NCC, isolated in avian and mammalian species. NC-derived cells isolated at postmigratory stages still comprise oligopotent progenitors, such as bipotent progenitors for Schwann cells and pigment cells, which are functional in vivo during development and in regenerating hair follicles.

More generally, an unexpected and important function of peripheral nerves and nerve terminals is to provide a niche for multipotent NC-like stem cells. Activation of these nerve-associated NCC represents a parcimonious strategy at play to take over and amplify the role of NC stem cells. Nerve-associated NCC insure the production of diversified cell types, at late developmental stages and in remote sites relative to their initial source. In the adult, they can replenish NC derivatives in tissues with high cellular turnover (mouse incisor, carotid body) or during injury repair (e.g., in the teeth).

One future goal will consist in molecularly defining the heterogeneity and plasticity of NCC and NC-derived cells. Recent advances in single-cell technologies (for a review, Hoppe et al., 2014), such as single cell RNA-sequencing, will likely be beneficial to unveil distinct state identities and developmental lineage trajectories of the NCC, as shown recently in embryonic and neural stem cells (Klein et al., 2015; Dulken et al., 2017), and during the diversification of mesodermal tissues (Loh et al., 2016) and the segregation of neuro-mesodermal lineages (Gouti et al., 2017).

Acknowledgements

This work was supported by Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM) and Université Pierre et Marie Curie (UPMC). G.W.C. and J.M.C.-A. were supported by doctoral fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil).
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Figure Legends

Figure 1: Small- and medium-size clones from multipotent GNMFC progenitors identified in single mesencephalic NCC cultures. Colonies derived from single quail mesencephalic NCC cultures were analyzed after 10 days for the differentiation of glial cells (G), neurons (N), melanocytes (M), myofibroblasts (F) and chondrocytes (C) and for their total number of cells (see Calloni et al., 2007 for details). Multiphenotypic colonies such as those derived from GNMFC progenitor type exhibited various sizes regarding their number of cells. (A-B, same microscopic field) Micrographic views of a small-size (about 250 cells) GNMFC multiphenotypic colony that includes several dense nodules of cartilage cells (chondroitin sulfate, green in A and B), surrounded by a network of glial cells (HNK1+, green in A); this colony also contains neurons labeled with tyrosine hydroxylase (TH) and Tuj1 (red, thin arrow, in B), melanoblasts (MelEM+, in red, thick arrow in B) and myofibroblasts (expressing α-smooth muscle actin, in red, arrowhead in B). (C-F, same microscopic field) Micrographs of a medium-size (about 1000 cells) GNMFC clone showing the presence of a large cartilage nodule (chondroitin-sulfate green fluorescence in C-E and star in C-F), neurons (labeled with TH and Tuj1, red fluorescence in D and F, thin arrow in D), myofibroblasts (α-smooth muscle actin+, arrowhead in D), glial cells (HNK1+, in E) and melanoblasts (MelEM+, green fluorescence in F). Phenotypes were identified by immunocytochemistry. Cell nuclei were visualized by Hoechst staining (blue in A, C-F). Magnification, X140.

Figure 2: Single HNK1+ NCC give rise to both neural and skeletogenic cell types in vitro. Schematic representation of the types of progenitors identified in clonal cultures
of cephalic NCC (CNCC) using either the bulk population of CNCC (Calloni et al., 2007) or only CNCC immunoreactive to HNK1 surface epitope. For this purpose, CNCC suspension was labeled with HNK1 and then, under epifluorescence microscopy, individual fluorescent HNK1+ cells were isolated from the suspension using a micropipette and clonally seeded in culture medium supplemented with Shh. In both cases, the resulting colonies were analyzed after 10 days for differentiated cell types (G, glial cells; N, neurons; M, melanocytes; F, myofibroblasts; C, chondrocytes). Data reveal the presence of three main categories of progenitors, those producing only G, N, and/or M cell types (“neural only” progenitors shown in dark grey), those yielding only F and/or C mesenchymal cell types (“mesenchymal only” progenitors, shown in light grey) and the “neural-mesenchymal” progenitors that generate several combinations of both neural and mesenchymal cell types; the latter progenitors are splitted in two, chondrogenic (C+ in blue) and non-chondrogenic, subgroups (C- in yellow). The frequency (% of total clones) of each progenitor type is indicated. Noticeably, single plated HNK1+ CNCC (n=64) include multipotent GNMFC progenitors that were previously identified in the clones derived from the bulk CNCC population (n=150, Calloni et al., 2007), although the diversity of progenitors was reduced compared to CNCC, probably due to the lower number of plated HNK1+ cells.

**Figure 3: Cephalic NC progenitors for neural and chondro-osteogenic lineages are favored in clonal cultures treated with Shh.** Scheme of the diverse types of progenitors recorded in clonal cultures of mesencephalic NCC grown in the absence (control CNCC) and presence of the morphogen Shh (+Shh) and analyzed for the presence of six main NC phenotypes: glial cells (G), neurons (N), melanocytes (M), myofibroblasts (F), chondrocytes (C) and Runx2-expressing osteoblasts (O) (Data from Calloni et al., 2009). Progenitors (n=109 control and n=146 +Shh) are classified according to the number of the distinct cell types they clonally produced and their respective frequencies (percentage of total clones) are indicated; red asterisks indicate statistically significant differences in the clone type frequency between control and +Shh cultures ($P<0.05$). Cellular composition of the colonies identifies three main categories of progenitors as defined in legend of Fig. 2, i.e., “neural only” progenitors for G, N, M (dark grey), “mesenchymal only” progenitors (light grey) yielding only F, C and/or O cell types, and “neural-mesenchymal” progenitors producing both neural and mesenchymal
cell types. In both medium conditions, 93% of the clones arise from neural-mesenchymal progenitors; Shh significantly increases the frequency of multipotent NCC endowed with chondro-osteogenic potential (GNMFCO, GMFCO and GFCO progenitors). Of note, the bipotent GM and GF progenitors exhibited self-renewal capacity (indicated by a curved arrow) after serial in vitro subcloning (Trentin et al., 2004; Bittencourt et al., 2013). Adapted from Calloni et al. (2009).

**Figure 4: Skeletogenic and adipogenic in vitro differentiation capacity of trunk NC progenitors.** (A-C, same microscopic field) Quail trunk NCC (TNCC) cultured for 21 days under medium condition appropriate for mesenchymal cell terminal differentiation give rise to mineralized bone cells (A, alizarin red staining), mature chondrocytes (B, alcian blue staining) and lipid-storing adipocytes (C, oil red O staining). (D) Single TNCC cultures performed in various conditions were analyzed for the differentiation of glial cells (G), neurons (N), melanocytes (M), myofibroblasts (F), chondrocytes (C), osteoblasts (O) and adipocytes (A) (Data from Trentin et al., 2004; Calloni et al., 2007; Coelho-Aguiar et al., 2013). TNCC progenitors were classified according to the extent of their phenotypic repertoire. Most of the progenitor types yielded “neural only” colonies (containing G, N and/or M only) as well as various types of “neural-mesenchymal” clones, which in addition to “neural” cell types, contained one or two of the mesenchymal cell phenotypes (F, C, O, A). Skeletogenic cells and adipocytes thus arose in vitro from multipotent TNCC. Self-renewal capacity (indicated by a curved arrow) of bipotent GM and GF progenitors of the trunk NC was shown by serial in vitro subcloning (Trentin et al., 2004; Bittencourt et al., 2013). Adapted from Coelho-Aguiar et al. (2013).

**Figure 5: Reversion of differentiated Schwann cells and pigmented melanocytes to multipotent NC-like progenitors.** Schematic model for in vitro reprogramming of avian NC-derived Schwann cells and melanocytes. (Upper panel) Schwann cells from E10.5 quail sciatic nerves were purified by FACS using the Schwann cell Myelin Protein antibody and grown in single cell culture as described (Dupin et al., 2003); after 17 days in vitro, they generated heterogenous colonies containing Schwann cells, melanocytes and/or smooth muscle cells-myofibroblasts. Such in vitro transition of single Schwann cells to the melanocytic and fibroblastic lineages was promoted by exposure to EDN3 and TGFβ, respectively (Dupin et al., 2003; Real et al., 2005). (Bottom panel) Pigmented
melanocytes obtained from dissociated epidermal cells of the quail embryonic skin were individually seeded under microscopic control and cultured for 11 days as described (Real et al., 2006). Up to 90% of the pigment cells grown in the presence of EDN3 formed clones containing Schwann cells and/or fibroblasts in addition to melanocytes. Serial subcloning of melanocyte progeny showed that the in vitro reprogramming of melanocytes induced by EDN3 involved the maintenance of oligopotent NC-like stem cells endowed with self-renewal (curved arrow). (Adapted from Dupin et al., 2000; Real et al., 2006).