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Neural crest progenitors and stem cells

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Abstract

In the vertebrate embryo, multiple cell types originate from a common structure, the neural crest (NC), which forms at the dorsal tips of the neural epithelium. The NC gives rise to migratory cells that colonise a wide range of embryonic tissues and later differentiate into neurones and glial cells of the peripheral nervous system (PNS), pigment cells (melanocytes) in the skin and endocrine cells in the adrenal and thyroid glands. In the head and the neck, the NC also yields mesenchymal cells that form craniofacial cartilages, bones, dermis, adipose tissue, and vascular smooth muscle cells. The NC is therefore a model system to study cell diversification during embryogenesis and phenotype maintenance in the adult.

By analysing the developmental potentials of quail NC cells in clonal cultures, we have shown that the migratory NC is a collection of heterogeneous progenitors, including various types of intermediate precursors and highly multipotent cells, some of which being endowed of self-renewal capacity. We also have identified common progenitors for mesenchymal derivatives and neural/melanocytic cells in the cephalic NC. These results are consistent with a hierarchical model of lineage segregation wherein environmental cytokines control the fate of progenitors and stem cells. One of these cytokines, the endothelin3 peptide, promotes the survival, proliferation, and self-renewal capacity of common progenitors for glial cells and melanocytes. At post-migratory stages, when they have already differentiated, NC-derived cells exhibit phenotypic plasticity. Epidermal pigment cells and Schwann cells from peripheral nerves in single-cell culture are able to reverse into multipotent NC-like progenitors endowed with self-renewal.

Therefore, stem cell properties are expressed by a variety of NC progenitors and can be re-acquired by differentiated cells of NC origin, suggesting potential function for repair. ***To cite this article: E. Dupin et al., C. R. Biologies 330 (2007).***

Résumé

Progéniteurs et cellules souches de la crête neurale. Chez l'embryon de Vertébrés, différents types cellulaires ont une origine commune dans une structure transitoire, la crête neurale, qui se forme aux bords dorsaux de l'épithélium neural. La crête neurale donne naissance à des cellules migratrices, qui colonisent un grand nombre de tissus et, plus tard, se différencient en neurones et cellules gliales dans le système nerveux périphérique, en cellules pigmentaires (mélanocytes) dans la peau et en cellules endocrines dans les glandes surrénales et la thyroïde. Dans la tête, la crête neurale fournit également des cellules mésenchymateuses, qui

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formeront les cartilages et les os du crâne et de la face, ainsi que du derme, du tissu adipeux et des cellules musculaires de la paroi des vaisseaux. La crête neurale constitue donc un modèle pour l'étude des mécanismes de la diversification cellulaire au cours du développement et du maintien des phénotypes cellulaires chez l'adulte.

En réalisant une analyse in vitro des potentiels de développement des cellules de la crête neurale de caille cultivées en cultures clonales, nous avons montré que les cellules de la crête neurale en migration constituent une collection de progéniteurs, où sont présents des cellules multipotentes, des précurseurs déjà déterminés et différents types de précurseurs oligopotents intermédiaires. La crête neurale céphalique comprend notamment des précurseurs communs aux cellules mésenchymateuses (chondrocytes, cellules musculaires vasculaires) et aux cellules nerveuses et pigmentaires. Certains de ces précurseurs peuvent se propager in vitro après sous-clonages successifs, et possèdent donc la capacité de s'autorenouveler, caractéristique des cellules souches. Ces résultats suggèrent un modèle hiérarchique dans lequel les lignages dérivés de la crête neurale sont ségrégés au cours des restrictions progressives des potentialités des cellules souches et progéniteurs oligopotents, sous l'influence de facteurs environnementaux. L'un de ces facteurs, le peptide endothéline3, est nécessaire au développement des mélanocytes dans la peau, et favorise la survie, la prolifération et l'autorenouvellement in vitro des précurseurs donnant naissance aux cellules pigmentaires et gliales.

Aux stades les plus tardifs du développement, les types cellulaires dérivés de la crête neurale montrent une plasticité phénotypique remarquable. Ainsi, est-il possible, en culture in vitro, d'obtenir l'interconversion de cellules pigmentaires et de cellules gliales différenciées, isolées respectivement de l'épiderme et des nerfs périphériques de l'embryon. Dans le cas des cellules pigmentaires, nous avons montré que, sous l'influence mitogénique de l'endothéline3, la reprogrammation phénotypique s'accomplit en culture après dédifférenciation et retour à l'état de cellule souche multipotente.

Il apparaît donc qu'au cours du développement, des propriétés caractéristiques de cellules souches sont présentes dans une diversité de progéniteurs dérivés de la crête neurale. En outre, ces propriétés peuvent être ré-exprimées par des cellules différenciées, suggérant un potentiel de réparation chez l'adulte. ***Pour citer cet article : E. Dupin et al., C. R. Biologies 330 (2007).***

Keywords: Neural crest; Quail embryo; Stem cell; Self-renewal; Clonal culture; Endothelin3; Melanocyte; Reprogramming

Mots-clés : Crête neurale ; Embryon de caille ; Cellule souche ; Autorenouvellement ; Culture clonale ; Endothéline3 ; Mélanocyte ; Reprogrammation

1. Introduction

The neural crest (NC) is a group of cells originating from the dorsal margins of the neural folds during the development of vertebrates. NC cells undergo an epithelio-mesenchymal transition and, after a phase of extensive migration, they become widely distributed within the embryo. Induction of the NC at the neural plate border is regulated by signals emanating from the surrounding non-neural ectoderm and mesoderm; the interplay of BMP, Notch, Wnt and FGF signalling in NC induction and specification has been reviewed elsewhere (for references, see [1,2]). The NC is remarkably multipotent, giving rise to a variety of neural and non-neural cell types in the adult [3,4] (Table 1). The NC cells yield pigment cells (melanocytes), neurones, and glial cells in the sensory and autonomic ganglia of the peripheral nervous system (PNS) and some endocrine cells. In addition, mesenchymal cells capable of differentiating into connective tissue cells, tendons, cartilage, bone, and vascular smooth muscles are produced by the NC. Such mesectodermal capacity is widespread along the whole NC in teleosts, while in higher vertebrates it is limited to the cephalic domain of the neural axis, from mid-diencephalon down to the somite-4 level [4]. Tissue-specific contribution of the cephalic NC-derived mesenchyme has been precisely defined in birds thanks to quail-chick chimera [5,6], and recently confirmed in mammals, using genetic fate mapping in the mouse [7,8]. In Gnathostomes, the NC forms most structures of the skull and the entire facial and visceral skeleton. In addition, the mesectoderm provides

Table 1
Main cell types derived from the neural crest

• Neurones
Peripheral nervous system (PNS): sensory, sympathetic and enteric ganglia
• Glial cells
PNS satellite glial cells and Schwann cells of peripheral nerves
• Pigment cells (skin melanocytes)
• Endocrine cells
C cells of the thyroid
Catecholaminergic cells of the adrenal gland
• Mesenchymal cells in head and neck
Cartilages and bones
Odondoblasts
Dermis
Connective tissues in muscles and glands
Meninges of the forebrain
Vascular smooth muscle cells
Adipocytes

the connective tissue cells in head muscles and glands as well as the vascular smooth muscle cells associated with the vessels derived from the aortic arches and those irrigating the face and forebrain (reviewed in [9]).

Therefore, the NC-derived cells are very diverse and widely distributed in the adult organs and tissues; such diversity of locations and functions of NC-derived cell types is related to the large array of human pathologies, including multiple neoplasia, various skeletal syndromes, neurocristopathies (e.g., Hirschsprung's disease) and pigment disorders (e.g., Waardenburg syndrome), which are known to be associated with abnormal development or function of the NC cells.

The multiple roles of the NC and the ubiquitous character of its derivatives are consistent with the NC as a stem-cell developmental model. Analogies between the hemopoietic system and the NC with respect to the mechanisms of cell diversification have been previously underlined [10,11]. Similarities between the two systems have been reinforced recently following the increasing number of reports showing the presence of still-undifferentiated NC cells in adult organs, which may provide new promise for regenerative medicine. Here we focus on the recent advances in characterizing the developmental potentials and stem cell properties of NC cells, which have given rise to a model of cell lineage segregation during NC ontogeny. We also discuss differences and similarities between trunk and cephalic NC, and provide some examples of the role of environmental cues in regulating phenotypic options in differentiating NC cells. The recent evidence for the persistence of NC progenitors and stem cells in postnatal stages and adult is discussed in the light of their possible use in repair after injury or disease. Finally, we discuss in vitro studies that have revealed the instability of NC-derived phenotypes, which support the idea that reprogramming and rejuvenation of differentiated cells may also account for the plasticity exhibited by NC cells.

2. Multipotent cells and intermediate progenitors in the early NC

Given the diversity of the derivatives generated by the NC in vivo, several attempts aimed at elucidating how and when the different NC-lineages become segregated during ontogeny, have been made by testing the developmental potential of individual NC cells in vitro and in vivo. Seminal experiments of in vitro clonal assays performed three decades ago by Cohen and collaborators unravelled that avian trunk NC cells migrating from cultured neural tubes are heterogeneous with respect to their potential to give rise to unpigmented and pigmented progeny [12,13]. The sound evidence for multipotency of NC cells in vivo was put forward by lineage tracing of the progeny of individual cells labelled following microinjection of vital fluorescent dye in the avian embryo [14,15]. These experiments identified premigratory and early migratory trunk NC-cell descendents of distinct types, including glia, sensory neurones, melanocytes, and adrenomedullary cells. However, the dilution of the dye with cell division precluded to follow long-term fate of the NC-derived cells.

The in vitro approach aimed at defining the whole spectrum of cell developmental options has highlighted the variety of progenitors present in the NC cell population and allowed characterizing the influence of extracellular signals on their development. In vitro clonal culture was later extended to NC cells freshly removed from the embryo at the cephalic level, and the phenotypic analysis of the colonies was improved by the use of various cell-type specific markers (Fig. 1). Experiments performed in our laboratory on quail early

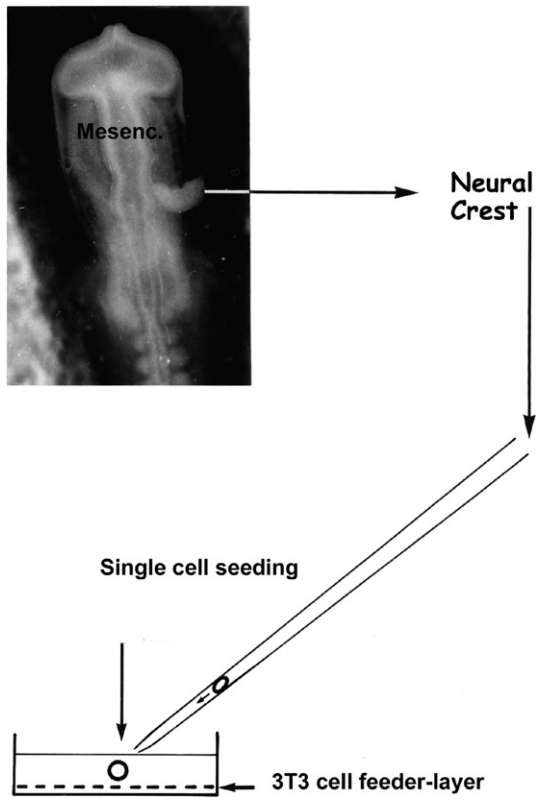

Fig. 1. Method for the cloning of quail NC cells. Cephalic NC cells were isolated when migrating from the mesencephalon of quail embryos and dissociated as single cells. Individual cells were then seeded under microscopic control by micromanipulation and grown on a previously established feeder-layer of mouse 3T3 fibroblasts.

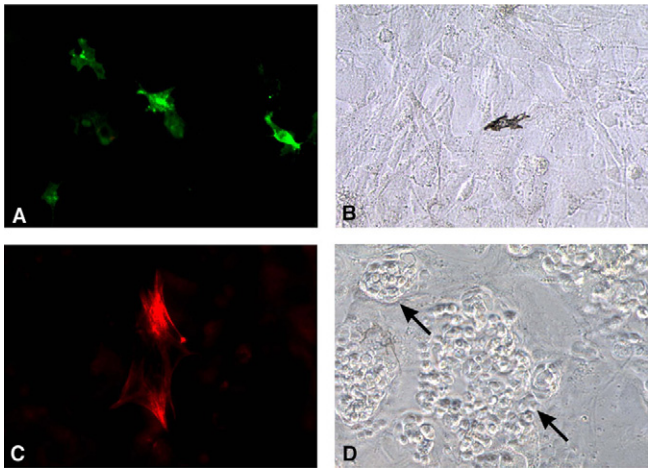

Fig. 2. Skeletogenic progenitors in the cephalic NC. Single mesencephalic NC cells yield multiphenotypic clones that contain glial cells (A, SMP marker), pigment cells (B, phase-contrast), myofibroblasts (C, α smooth muscle actin), and tridimensional nodules of chondrocytes (D, arrows). From [19].

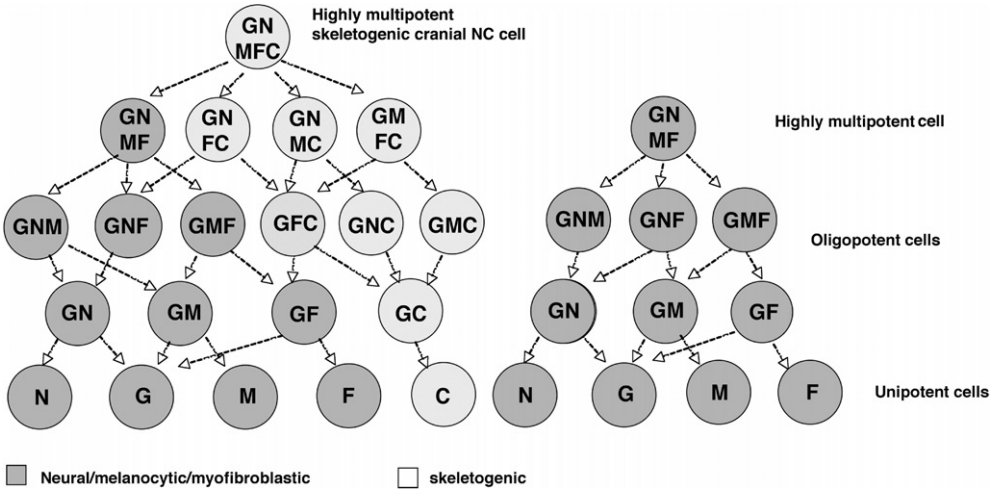

Fig. 3. Model for the diversification of NC-derived lineages in the NC from head (left) and trunk (right) levels. The progenitors identified in the quail cephalic NC by in vitro clonal cultures are classified according to their number of potentialities. Data support a hierarchical model for lineage segregation, according to which progressive restrictions of a highly multipotent stem cell-like species gives rise to oligopotent progenitors and finally to precursors committed to each of the main NC-derived phenotypes, namely, glia (G), neurones (N), melanocytes (M), myofibroblasts (F) and cartilage (C). Skeletogenic progenitors able to yield cartilage were found in the cephalic NC-derived progeny (left). The trunk NC cells are heterogeneous and comprise neural-melanocytic and myofibroblastic progenitors only, which lie downstream of a highly multipotent cell (right).

migratory NC cells have provided compiling evidence for the presence of several types of multipotent cells (able to yield several combinations of three or more NC derivatives) (Fig. 2), as well as more restricted NC cells such as bipotent glial-neuronal progenitors. Unipotent precursors for neurones, glial cells or melanocytes accounted for small proportion of the clonogenic cells grown on 3T3 cell feeder-layers [16–19]. From the analysis of thousands of colonies, a hierarchical model of NC lineage diversification emerges (Fig. 3). Highly multipotent cells able to yield a heterogeneous progeny consisting of all, including mesenchymal cells (chondrocytes), NC-derived phenotypes, are present in the migratory cephalic NC at low frequency. These cells can be considered as putative NC stem cells lying upstream in the NC cell hierarchy, although their self-renewal property still remains to be assessed. The existence of common precursors for chondrocytes and other non-mesenchymal NC lineages also argues against the idea that the mesectoderm is segregated from other NC lineages in the neuroepithelium. Most of the other progenitors recorded exhibit various combinations of NC phe-

notypes. It is noticeable that all these oligopotent cells were found to be endowed with the potential to give rise to glial cells in addition to one, two or three other options (among the neuronal, melanocytic, myofibroblastic, and chondrocytic ones) (Fig. 3 left). These data thus suggest that gliogenic potential may be a common signature to all NC cells before they become fully committed.

Analysis of the in vitro progeny derived from trunk quail NC cells yielded similar conclusions as for the cranial NC, with the noticeable difference that no skeletogenic progenitors were identified as expected [19] (Fig. 3 right). Compiling data however indicate that the capacity to generate myofibroblasts (as defined by expression of the smooth muscle-specific isoform of actin) is expressed at high rate by cultured trunk NC cells, although only a small contribution to the perineurial fibroblasts in vivo has been deduced recently from transgenic mouse studies [20].

The differentiation potential of the cardiac NC has been also investigated. This region of the cephalic postotic NC has important contribution to heart development, by forming the aorticopulmonary septum and conotruncal cushions as well as by populating the aortic arch smooth muscle and the parasympathetic cardiac ganglia [5,7,21–23]. Ito and Sieber-Blum recorded melanocytes, myofibroblasts, connective tissue, chondrocytes, and sensory neurons in colonies derived from early migratory quail cardiac NC cells [24]. They identified multipotent cells as well as more restricted precursors for melanocytes and smooth muscle cells. Similar results were raised recently in mammals [25].

Taken together, the lineage relationships found between the diverse NC progenitors are consistent with the role of environmental signals in instructing the differentiation choice of multipotent cells as well as in selecting for specific restricted precursors in order to ensure the final development of appropriate cell types in the various NC target tissues. The opportunity to treat individual NC cells in culture with soluble growth factors has led to characterize the action of several cytokines on the in vitro behaviour of particular NC progenitor subsets.

3. Environmental cytokines regulate the fate and stem cell properties of NC progenitors

Transplantation experiments performed in the 1970s and 1980s in the avian embryo have established a fate map of the various NC derivatives along the neural axis and revealed that the environment into which NC cells home at the term of their migration strongly influences their fate (for references, see [3,4]). One striking example is the ability to give rise to enteric cholinergic neurones and to catecholaminergic cells of the adrenal medullary gland. Although regionalized along the antero-posterior NC, these fates were not determined in the neuroepithelium itself and could be elicited from any region of the trunk NC, provided that this region becomes exposed to the appropriate signals after heterotopic transplantation. The paramount influence of external factors on NC cell differentiation has since been underlined by a number of in vitro culture studies on NC cell populations which, however rarely, could identify which types of progenitors these environmental factors were targeting on. This gap began to be filled in the recent years thanks to the analysis of single NC cell cultures performed in avian and mammals.

David Anderson and collaborators have devised a method to isolate multipotent cells from trunk NC cells in the rat embryo, by using fluorescence-activated cell sorting of the early migratory NC cells that express low-affinity NGF receptor p75 [26]. These cells are multipotent progenitors for glia, autonomic neurones, and myofibroblasts and are capable to self-renew in vitro. They have been extensively studied to decipher the molecular pathways involved in controlling their differentiation. Glial growth factor Neuregulin 1 [27] as well as Notch activation [28] have been shown to play crucial role in glial determination, whereas TGFβ has been suggested to instruct multipotent NC cells to adopt a myofibroblast fate. BMP2 signalling promotes neuronal autonomic development at the expense of glial fate, by activating the bHLH transcription factor *Mash1* [29]. How the multipotent trunk NC cells integrate these different signals is not yet fully understood [30], although Notch proved to be dominant over BMP2 signalling by triggering irreversible switch from neurogenesis to gliogenesis [31]. Interestingly, these cells were not capable to generate sensory neurones, in support to previous data suggesting an early segregation of sensory and autonomic NC lineages [32–34]. Sensory neurones were found to develop from cultures of the mammalian neural primordium (including the premigratory trunk NC) and arise from determined progenitors that do not respond to BMP2 autonomic cue [35]. Signals that instruct multipotent NC cells to form sensory neurones have been shown recently to involve activation of Wnt/β-catenin signalling, which is not only required [36], but also sufficient to trigger sensory neurogenesis [37].

These studies performed with mammalian NC cells did not elucidate whether pigment cells also arise from the more multipotent NC cells. In vitro cultures of individual quail NC cells grown on fibroblast feeder-layers and in the presence of endothelin3 demonstrated the

presence of pluripotent cells that could generate glial, neuronal, myofibroblast, and melanocyte cells [19]. The vasoactive peptide endothelin3 that acts in a paracrine way on migrating NC cells through endothelin receptors of the B and B2 types in the avian embryo [38,39] is a strong mitogen for melanocyte precursors, as well as for their ancestors endowed with both glial and melanogenic potentials [40]. Moreover, we showed that the self-renewal ability of these bipotent progenitors is dependent upon endothelin3 signalling, which allowed the colonies derived from NC cell founders to be propagated along several rounds of subcloning [19]. These experiments also revealed that a distinct category of bipotent cells, those yielding myofibroblasts and glia, are able to self-renew without requiring the presence of endothelin3.

Therefore distinct types of multipotent progenitors in the trunk and cephalic NC display the self-renewal property characteristic of stem cells, and their fate decisions are regulated by cell-type specific environmental factors.

4. Reprogramming capacity of glial cells and melanocytes

The plasticity of NC cell differentiation raises the issue as to whether their differentiation state is fixed during embryogenesis. The traditional concept that development is unidirectional and that cells isolated from differentiated tissues are fully restricted in the types of cells they can generate has been challenged by several lines of evidence (reviewed by Blau et al. [41] and Zipori [42]). For example, in the CNS a combination of extracellular signals was reported to induce oligodendrocyte precursors to revert to multipotent neural stem cells in culture [43]. It was later suggested that this process results from epigenetic alteration in the regulatory sequences of the Sox2 transcription factor [44].

In the NC lineages, differentiated glial cells of the PNS nerves and pigment cells exhibit phenotype plasticity in culture and convert into each other in vitro upon mitogenic stimulation by endothelin3 [45–48]. Schwann cells isolated from quail embryonic sciatic nerves and cultured as single cells yielded a mixed progeny of melanocytes and myofibroblasts, in addition to parental-like glial cells. Moreover, when transplanted to the branchial arch of chick host embryos, the Schwann cell progeny contributed to the vascular smooth muscle layer of cranial blood vessels [45,46]. In vitro experiments showed that single pigment cells purified from the quail epidermis are able to yield glial, melanocytic, and myofibroblastic cells in clonal cultures [47,48] (Fig. 4). Such phenotypic reprogramming occurred in the majority of clonogenic pigment cells, even when they had been taken from epidermis isolated until the hatching stage. More importantly, serial subcloning experiments have revealed that the pigment cells undergo dedifferentiation into multipotent progenitors, which are able to self-renew and recapitulate expression of early NC markers such as HNK1 and *Sox10*, *FoxD3*, *Pax3*, and *Slug* transcription factors [48].

Therefore, when removed from their differentiated tissues and subjected to new environmental conditions, embryonic pigment cells and Schwann cells can reverse their differentiation programme and recover properties of their multipotent NC ancestors. These findings indicate that differentiated cell types derived from the NC are phenotypically unstable and capable of broad differentiation plasticity, which suggests they could be mobilized for potential tissue repair in disease or after injury.

5. Persistence of multipotent NC progenitors in late embryonic and adult tissues

At post-migratory stages, restrictions of NC cell developmental potentials clearly occur. However, the search for pluripotent NC cells that had persisted in NC derivatives led to first identify multipotent progenitors in the rat PNS nerves, where they derive from stem cell division of common progenitors for neurones, glial cells, and myofibroblasts [49]. Similar progenitors were also found in dorsal root ganglia [50] and in the foetal gut [51]. In the gut, these progenitors are still present at postnatal stages, opening new possibilities in the understanding and treatment of enteric neural defects in children, like Hirschsprung's disease [52]. In the mammalian adult skin, progenitors have been recently isolated either from the dermal papillae or from the epidermal bulge area of hair follicles [53–57]. These cells were identified as NC-derived by the use of *Wnt1*-genetic fate mapping in the mouse and further characterized in the human skin [56]. Skin-derived NC cells behave in vitro as pluripotent stem cells endowed with both neural and mesenchymal lineage potentials. Although their response to growth factors might slightly differ from that of early NC stem cells [58], these cells revealed new therapeutic promise in regenerative medicine. Indeed, the transplantation of follicle stem cells in injured adult mouse sciatic nerves promoted regenerative axonal growth, resulting in the recovery of peripheral nerve function [59,60]. Epidermal NC stem cells derived from the hair follicles have been also recently instrumental in spinal cord repair [61].

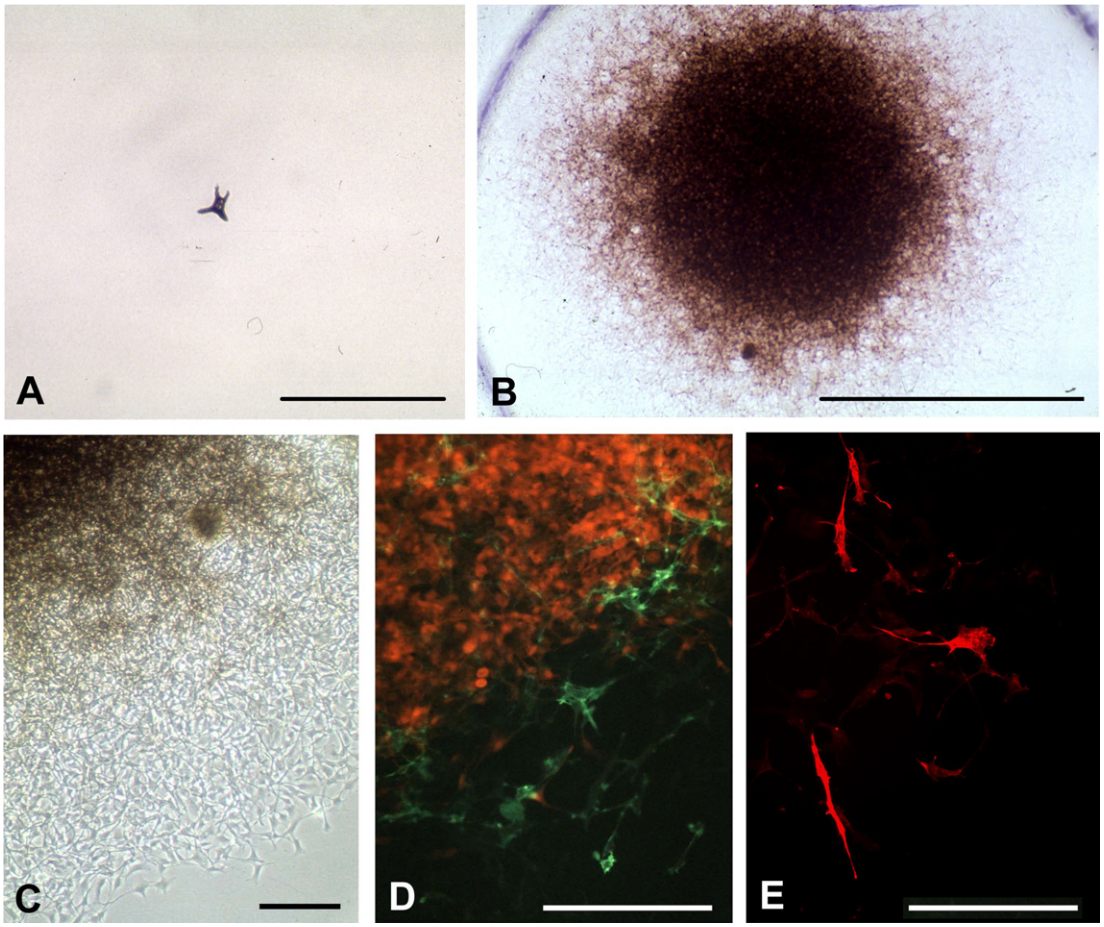

Fig. 4. Pigment cells generate multiphenotypic clonal progeny in vitro. (**A**) Culture of a single melanocyte from quail embryonic epidermis, 4 h after clonal seeding. (**B**) After 13 days in the presence of endothelin3, the melanocyte has generated a large colony, with a highly pigmented core. (**C**) Detailed view shows the periphery of the colony, which comprises unpigmented and pigmented cells. Colony contains melanoblasts and glial cells immunoreactive to MelEM (red) and SMP (green) markers, respectively (**D**) as well as myofibroblasts (positive for α-smooth muscle actin αSMA) (**E**). Scale bars: 100 μm in (**A**) and (**E**), 200 μm in (**C**) and (**D**), 1 mm in (**B**).

Therefore, certain degree of differentiation plasticity and regeneration capacities characterizes particular subsets of NC-derived cells long after organogenesis is completed. In mammals, the opportunity to prospectively isolate NC stem cell populations using cell surface markers, combined with gene targeting mutations, allows further analysis of the stem cell function in normal and pathological conditions.

6. Concluding remarks

Although debate still exists as to whether most individual NC cells are multipotent or whether their fate become restricted shortly after migration, several lines of evidence convincingly demonstrated multipotency and self-renewal capacity of at least various subsets of NC cells in avian and mammalian species. In contrast, early NC cells from Xenopus and zebrafish embryos seem to acquire restrictions of their developmental options as soon as early migratory stages. The presence of multipotent NC cells, not only in the early NC, but also in many of the target tissues of post-migratory NC cells such as the nerves, gut and skin, has underlined the developmental plasticity of developing NC cells. Much remains however to be known with respect to the molecular regulation of stem cell properties and differentiation of the various NC progenitors. These issues are of clinical importance to understand the alterations in NC cell number and function that occur in numerous human pathologies. The recent discovery of resident NC stem cells in adult tissues such as the skin may offer a source for isolating and manipulating autologous NC-derived cells with great potential for regenerative medicine.

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