Cell Research (2012) 22:288-304.

www.nature.com/cr

REVIEW

Neural crest stem cells: discovery, properties and potential for therapy

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Neural crest (NC) cells are a migratory cell population synonymous with vertebrate evolution. They generate a wide variety of cell and tissue types during embryonic and adult development including cartilage and bone, connective tissue, pigment and endocrine cells as well as neurons and glia amongst many others. Such incredible lineage potential combined with a limited capacity for self-renewal, which persists even into adult life, demonstrates that NC cells bear the key hallmarks of stem and progenitor cells. In this review, we describe the identification, characterization and isolation of NC stem and progenitor cells from different tissues in both embryo and adult organisms. We discuss their specific properties and their potential application in cell-based tissue and disease-specific repair.

***Keywords*:** neural crest cells; stem and progenitor cells; self renewal; multipotency; ES cells; iPS cells

Cell Research (2012) **22**:288-304. doi:10.1038/cr.2012.11; published online 10 January 2012

Introduction

The neural crest (NC) is a population of cells that was first described by the Swiss embryologist Wilheim His in 1868 as a "Zwischenstrang" (the intermediate strand) due to their location between the dorsal ectoderm and the neural tube in vertebrate embryos. The tissue was later renamed NC by Arthur Milnes Marshall as a more precise description of its anatomical position [1]. NC cells (NCCs) are born during vertebrate embryogenesis within the dorsal margins of the closing neural folds. Initially, they are integrated within the neuroepithelium where they are morphologically indistinguishable from the other neural epithelial cells. Upon induction by signals that come from contact-mediated tissue interactions between the neural plate and the surface ectoderm, NCCs delaminate through an epithelial-to-mesenchymal transition and start migrating extensively to several different locations in the embryo where they contribute to a remarkably diverse array of different tissue types ranging from the peripheral nervous system (PNS) to the craniofacial skeleton (Figure 1) [2].

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NCC derivatives originate from four different segments of the neuraxis: cranial, cardiac, vagal, and trunk. The cranial NC, which probably represents the most astonishing example of the multipotentiality of these cells, gives rise to the majority of the bone and cartilage of the head and face, as well as to nerve ganglia, smooth muscle, connective tissue and pigment cells (Figure 1A, 1B). The cardiac NC contributes to heart development by forming the aorticopulmonary septum and conotruncal cushions, whereas the vagal NC gives rise to enteric ganglia of the gut (Figure 1C, 1D). Finally, the trunk NC will give rise to neurons and glia, and contribute to the PNS, to secretory cells of the endocrine system and to pigment cells of the skin (Figure 1E) [1]. The remarkable developmental capacity of neural ectoderm-derived NCCs to differentiate into both neuronal and mesenchymal derivatives has resulted in the NC being considered a fourth germ layer [3]. A controversial aspect of the nature of NCCs concerns the "stemness" of these cells. Stem cells in the strictest sense are cells that upon division continually give rise to an identical daughter cell (self-renewal) and to a cell with more restricted potentials (differentiation). An explosive amount of work has taken place in the last 15 years testing and demonstrating the multipotentiality and self-renewal capacities of NCCs both *in vitro* and *in vivo*. However, many caveats remain. One such caveat is that NCCs are only generated

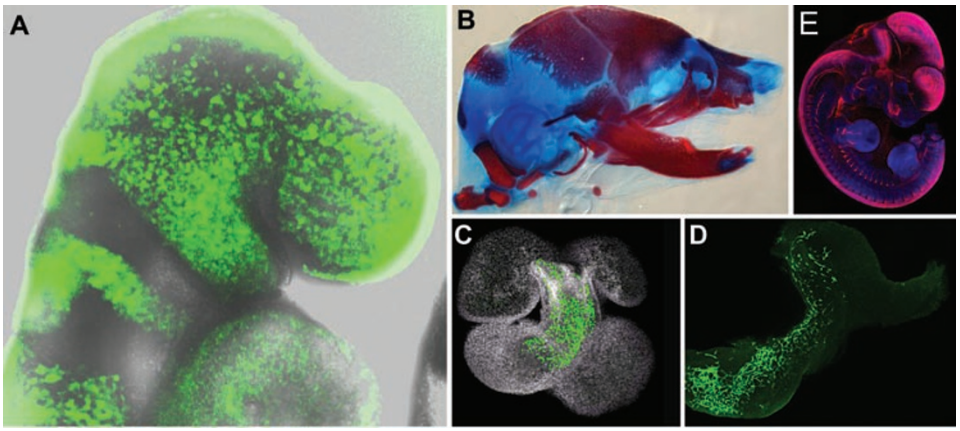

Figure 1 NC cell-derived structures in the mouse. **(A)** NCCs are marked with GFP using the knock-in Pax3-GFP transgenic line at E8.5 [109]. The cranial NC gives rise to the bone (red) and cartilage (blue) of the face as seen by alizarin and alcian staining **(B)**, the cardiac NC contributes to the aorticopulmonary septum and conotruncal cushions of the heart **(C)**, the vagal NC will give rise to the enteric ganglia of the gut (Wnt1CreYFP) **(D)**, and the trunk NC will give rise to neurons, which will contribute to the PNS as seen by TuJ1 immunostaining in an E11.5 embryo **(E)**.

transiently within the embryo. Therefore, it may be more appropriate to describe the majority of NCCs as progenitor cells rather than true stem cells [4]. Progenitor cells like stem cells have the capacity to self-renew and differentiate, but in contrast to stem cells both capacities are more limited. Despite these semantic differences, NCCs continue to fascinate scientists because of their importance in vertebrate development, evolution and disease. In this work, we review the classic studies that pioneered the field of NC stem cells (NCSCs); the efforts to isolate embryonic and adult NCSCs; and the generation of human and mouse embryonic stem cell (ESC)- and induced pluripotent stem (iPS) cell-derived NCSCs. We also evaluate the current therapeutic applications of NCSCs and examine the potentials of these cells in regenerative medicine.

The multipotency of NCCs: the classical work

A central question is whether a single NCC is multipotent or whether the fate of each NCC is determined prior to migration. The development of the quail-chick chimeras system opened the doors for pioneering work, which demonstrated for the first time with certainty the plasticity of the premigratory NCCs [5]. In these experiments, a specific region of trunk neural tube that normally gives rise to cholinergic neurons was ablated before the onset of NCC migration in a chick embryo and was replaced by a different region from a stage-matched quail embryo that would typically produce adrenergic neurons [1]. These heterotopic grafting experiments revealed that presumptive adrenergic NCC precursors could generate cholinergic neurons if placed in the right environment. Thus, the fate of NCCs is not fully determined before these cells migrate. Shortly thereafter, *in vitro* growth of multilayered cultures of avian trunk NCCs demonstrated that differentiation into melanocytes and adrenergic cells can take place simultaneously raising the possibility that NCCs were multipotent [6]. However, this conclusion was limited by the heterogeneity of the cells cultured. Some years later, the first data demonstrating the multipotency of single NCCs were obtained from *in vitro* and *in vivo* experiments. The developmental potential of single quail premigratory trunk NCCs was analyzed *in vitro* and it was revealed that in cell culture these cells can give rise to at least two types of cells: melanocytes and neuronal cells [7]. When these quail cell colonies were reintroduced into a chick embryo host it was discovered that they maintain their ability to migrate along the NCC paths and contribute to tissues and organs similar to their endogenous counterparts [8]. In particular, these single cell-derived colonies gave rise to different populations of neurons and also contributed to the sympathetic ganglia, adrenal gland, and aortic plexus. An important finding of these studies was that a single cell could give rise to two daughter cells of different types such as melanocytes and adrenergic neurons thus establishing the NC as a multipotent cell population.

The subsequent development of vital dyes as cell fate and lineage-tracing markers provided the opportunity to follow single NCCs *in vivo* from the beginning of their journey [9]. This revealed that a single trunk NCC could give rise to neuronal and non-neuronal descendants, demonstrating the multipotency of not only premigratory

but also of migratory trunk NCCs in avian embryos [9, 10]. Similarly, cranial NCCs were observed to give rise to a number of different cell types including neuronal, glia, and melanocyte lineages. In addition, cranial NCCs give rise to mesectodermal precursors that contribute to cartilage, bone and connective tissue, a feature that sets it apart from the other axial populations of NCCs. Furthermore, clonal *in vitro* analysis revealed that single migrating cranial NCCs contain rare multipotent precursors common to neurons, glia, cartilage and pigment cells. However, the majority of cranial NCCs gave rise to clones composed of only one or two distinct cell types [11]. Recent *in vivo* analyses of single premigratory avian trunk NCCs cells revealed a similar restriction in potency [12]. These findings were indicative of the presence of cells with predominantly restricted developmental potentials. Subsequently, the isolation of individual migratory NCCs from the visceral arches of quail embryos demonstrated their ability to differentiate into up to four different cell types [13]. More recently, the multipotent nature of individual cranial and trunk NCCs in avian and mouse systems has been successfully demonstrated [14-16]. In fact, Sonic hedgehog (Shh) promotes the differentiation of individual NCCs into a diverse array of cell types including neurons, glia, melanocytes, myofibroblasts, chondrocytes and osteocytes *in vitro*. Furthermore, it was previously thought that trunk Kit^+ NCCs gave rise only to melanocytes while Kit^- NCCs gave rise only to neurons and glia. However, Motohashi *et al.* [16] have now demonstrated genetically using ES cell-derived and embryo-derived NCCs that both $Sox10^+/Kit^+$ and $Sox10^+/Kit^-$ cells can differentiate into neurons, glia, and melanocytes.

These results are remarkable and indeed demonstrate that NCCs are multipotent. However, in many instances, these properties were revealed in culture and through the addition of exogenous factors such as Shh and to date have not necessarily been recapitulated *in vivo* in an embryonic environment. Taken together with the fact that NCCs are only generated transiently, this is consistent with NCCs being primarily a progenitor cell population as opposed to a true stem cell population in the strictest sense. The true stem cells may well be the neural stem cells (NSCs) that constitute the neural ectoderm from which NCCs are ultimately derived as suggested from single neuroepithelial cell *in vivo* labeling experiments. In further support of this idea, the transient nature of NCC induction and migration from primitive neuroepithelium, led to the assumption that it would be unlikely for NC precursor cells to persist during the major period of neuroepithelial maturation and central nervous system (CNS) development. However, a very recent study demonstrated that NCCs can indeed still be generated from the cortex of E14.5 embryos and that this capacity depends primarily on the inactivation of Sox2 and the activation of Sox9 [17]. Moreover, following transplantation into the hindbrain of chick embryos, cortical neurosphere-derived NCCs recapitulate endogenous NCC migratory pathways colonizing the proximo-distal extent of the pharyngeal arches and differentiating into sensory neurons within the cranial ganglia. This provocatively implies that the developmental segregation of the CNS and NC and thus NSCs and NCCs may be reversible even over extended periods of time [17].

Isolation of NCSCs

The term NC stem cell (NCSC) is thought to have been coined by Stemple and Anderson [18] after their demonstration of the multipotency and self-renewal of mammalian NCCs *in vitro*. Although mimicking studies performed earlier in avians, Stemple and Anderson pioneered the isolation of a pure or enriched NCSC population. This was achieved by fluorescent activated cell sorting of rodent trunk neural tubes using a non-destructive antibody against the low-affinity nerve growth factor receptor (p75), which was known to be expressed by NCCs. Isolated NCCs gave rise to mainly peripheral neurons and a few immature Schwann-like cells. Secondary cloning of the cells yielded both neuronal and unspecified non-neuronal cells with many of them generating multipotent subclones. Amazingly, the same technique (p75 sorting) was subsequently used to isolate NCSCs from postmigratory NCC populations and specifically from the rat fetal sciatic nerve [19]. Since p75 is also expressed in the PNS glia at the stages of isolation, the authors also selected against P0, the peripheral myelin protein expressed by these differentiated cells in order to purify a true NCSC population. The isolated postmigratory NCSC population differentiated into neurons, Schwann cells, and smooth muscle-like myofibroblasts *in vitro*; when transplanted directly in the trunk of chick embryos without any intervening period of growth in culture, they gave rise to neurons and glia in diverse locations of the PNS [19]. The avoidance of *in vitro* culturing by directly transplanting the isolated cells into the host chick embryo reassuringly demonstrated that the observed differentiation was not due to a culture artifact, an issue that continues to require close scrutiny in the NCSC field.

Postmigratory cranial NCCs have also been isolated from the first branchial arch of E10.5 mouse embryos [20] and at this developmental stage, NCCs in the first branchial arch are purportedly undifferentiated based on

the lack of expression of single cellular markers characteristic of neurons, glial cells, and smooth muscle cells. The lack of these markers is a good indication of their undifferentiated state but at the same time it does not exclude the possibility that these markers are simply not expressed early enough in the differentiation process, especially since only one marker per cell type was used. Nevertheless, these postmigratory cranial NCCs were shown to remain undifferentiated under certain conditions and to give rise to cells expressing markers representative of neurons, glia, osteoblasts, and myofibroblasts *in vitro*. It is worth noting, however, that in the same study, it was shown that some of these isolated cranial NCC progenitors when allowed to differentiate expressed SMA, a smooth muscle marker and ALP (alkaline phosphatase), an osteoblast marker at the same time. Since differentiation was driven by specific media conditions, it is possible that growth factors included in the media drive expression of the genetic markers used to determine cell types, and this does not necessarily reflect the actual fate of that cell. Additionally, the expression of only one marker does not mean that an individual cell became a neuron, or a glia or any other cell type and the conclusions drawn must reflect this. Nonetheless, follow-up assays subsequently demonstrated self-renewal and differentiation of postmigratory cranial NCCs into osteoblasts *in vivo* [21]. Amazingly, postmigratory cranial NCCs transplanted into a host with a calvaria defect differentiated into osteocytes and contributed to the repair of the defect.

Recently, a highly multipotent progenitor cell type from early migratory cranial NCCs that gave rise to all the NC-derived cell types: neurons, glia, melanocytes, myofibroblasts, chondrocytes, and osteocytes was identified [15]. However, the long-term self-renewal capacity of these cells has yet to be demonstrated. Additionally, in the presence of Shh these multipotent progenitors appeared more frequently suggesting a role for the Shh pathway in survival and proliferation of multipotent cranial NCSCs [15]. It would be interesting to test the *in vivo* potentials of these highly multipotent progenitor cells when transplanted into cranial, cardiac, trunk or vagal NC migrating pathways to determine if they are indeed multipotent in all four different environments. As mentioned above, however, their self-renewal capacity has yet to be challenged in order to satisfy the strictest definition of a stem cell.

Since their initial isolation, multipotent NC progenitor cells have been identified not only in other late gestation embryonic tissues but also in adults (Table 1), a discovery that has opened the door to the use of NCC as a source for therapeutic applications such as tissue engineering and repair. For example, multipotent enteric progenitor cells were discovered both in the fetal and the adult gut (Figure 1D and Table 1) [22-24]. Both populations exhibit self-renewal capacity and differentiation into neurons, glia, and myofibroblasts in culture, although the adult gut progenitor cells display less-efficient capacity in both characteristics. Interestingly, transplantation of uncultured NC progenitor cells freshly isolated from rat fetal gut into a chick embryo host, gave rise primarily to neurons, whereas the adult equivalent progenitor cells gave rise predominantly to glia. Additionally, whereas the fetal progenitor cells migrated away from the transplantation site and differentiated into neurons in more distal places like Remak's ganglion and the gut, the adult progenitor cells engrafted only in structures proximal to the hindlimb bud somites (sympathetic chain, peripheral nerves) into which they were transplanted [22-24]. Therefore, it seems that the developmental potential of the gut progenitor cells decreases with age, a concept that is at odds with the properties of true stem cells. In addition, a comparison of differentiation potentials between fetal sciatic nerve and fetal gut NCSCs revealed intrinsic differences between these two populations, with the sciatic nerve NCSCs undergoing mostly gliogenesis whereas the gut NCSCs undergoing mostly neurogenesis [25]. This implies that there are intrinsic differences

Table 1 Sources of NCSCs in the embryo and adult mouse/rat

Tissue	Embryo	Postnatal/Adult
Bone marrow	-----	✓
Carotid body	-----	✓
Cornea	-----	✓
Dental		
DFPCs	-----	✓
DPSCs	-----	✓
PDLSCs	-----	✓
SCAP	-----	✓
SHED	-----	✓
Dorsal Root Ganglion	✓	✓
boundary caps (BC)	✓	-----
Gut	✓	✓
Heart	✓	✓
cardiac side population (SP)	-----	✓
Palatum	-----	✓
Sciatic nerve	✓	-----
Skin		
whisker pad- EPI-NCSCs	-----	✓
facial dermis-SKPs	-----	✓
trunk dermis-SKPs	-----	✓

among the postmigratory NCC progenitors that are isolated from different regions of the animal.

Interestingly, transplantation of postmigratory enteric progenitor cells in the aganglionic distal gut of a rat model of the Hirschsprung's disease resulted in their engraftment and differentiation into cells expressing neuronal markers [26, 27]. Hirschsprung's disease is a congenital human condition characterized by absence of enteric ganglia in the distal part of the colon, which results in the inability to coordinate peristaltic movements of the bowel and is fatal if not treated [28]. Several genes have been implicated in Hirschsprung's disease and one of them is endothelin receptor B (EDNRB) [28]. Remarkably, when fetal enteric NCC progenitors were taken from *Ednrb*-deficient rats, cultured, and then transplanted into the aganglionic region of the gut of an *Ednrb*-deficient host, they were able to engraft and undergo neurogenesis [25]. Therefore, isolation of multipotent enteric NCCs from the ganglionic region of the gut of a Hirschsprung's disease patient and their subsequent transplantation into the aganglionic regions could provide a successful treatment of the disease, while at the same time overcoming issues of histocompatibility and immunosuppression typical of transplantation surgery. Multipotent postmigratory NCC progenitors have also been isolated from the dorsal root ganglia (DRG) of both embryos and adults (Figure 1E) [29-32]. Embryonic-derived rat progenitor cells exhibit a very restricted capacity and generate mostly neurons and glia when grown in standard medium, whereas addition of instructive cues promotes their differentiation into smooth muscle actin (SMA)-positive non-neuronal cells as well [29]. Self-renewal capacity, however, was not examined in this study. Additional studies isolated clones of cells that formed small clusters, which were derived from cultured embryonic boundary caps (BC), a group of cells located at the dorsal root entry zone [30]. BC cells derive from late emigrating trunk NCCs and were shown to differentiate into sensory neurons and glia *in vivo* [33]. BC cells can be propagated for up to 6 months after cloning and express previously characterized NCSC markers including nestin and p75. Additionally, the gene expression profile of these cells is very similar to that of stem cells. Interestingly, when compared to the cells isolated from the central part of the ganglion, the BC cells displayed a significantly higher number of clone-forming stem cell-like cells [30]. BC-NCSCs were also shown to self-renew and differentiate into neurons, glia, and smooth muscle-like cells *in vitro*. An important difference between the BC-NCSCs and premigratory NCSCs is the inability of the former to differentiate into sensory neurons in the absence of cellular cues. This indicates that their differentiation potential is more restricted and furthermore that differentiation of these cells can be influenced by environmental cues [30]. Very similar results were shown for progenitor cells derived from adult DRG [32]. However, not surprisingly, their self-renewal capacity was more restricted than that of the embryonically derived cells. These adult DRG progenitors are hypothesized to be derived from satellite glial cells, which originate from embryonic BC cells, that are derived from NCCs [32]. However, substantial *in vivo* analysis is needed before these progenitor cells can be defined as true stem cells.

A more precise way to isolate progenitor cells of NCC origin in a mammalian model is through the use of P0 and Wnt1 promoter-Cre/Floxed-EGFP reporter mice [31]. Although P0 is a Schwann cell protein, it has been shown to be transiently activated in embryonic NCCs. Therefore, using these mice, presumably only the NC-derived cells will express EGFP and this has facilitated the identification of NC-derived cells in the adult DRG, whisker pad (WP) and bone marrow (BM). $EGFP^+$ cells in the BM were also positive for p75 and Sox10, two markers typically expressed by NCCs. Isolation of $EGFP^+$ cells by flow cytometry from the BM, DRG, and WP of adult mice revealed their neurosphere-forming capacity *in vitro*, a characteristic of proliferative NC-derived cells. These spheres when placed on a serum-containing differentiation medium exhibited trilineage differentiation potential by giving rise to neurons, glial cells, and myofibroblasts. Interestingly, the frequency of trilineage potential although being high in the DRG-derived cells (74.6%) was very low in the WP-derived (7.3%) and the BM-derived cells (3.3%). Cells derived from these tissues were mostly bipotent or unipotent with WP-derived cells giving rise mostly to neurons and myofibroblasts and BM-derived cells giving rise to mostly myofibroblasts. These conclusions, however, were based solely on the expression of a single marker for each lineage and have not been confirmed *in vivo*. The self-renewal capacity of the cells derived from the three distinct tissues was reflective of their differentiation capacity with the frequency of secondary sphere formation being highest in cells derived from the DRG [31].

The discovery of NC-derived progenitors in the BM is a significant finding since it reinforces the link between mesenchymal stem cells (MSCs) and NCCs. Although MSCs were known to derive from bone and BM, their developmental origin had remained elusive. However, by isolating NC-derived cells using P0- and Wnt1-Cre/Floxed-EGFP mice it was demonstrated that these cells exhibit MSC characteristics of self-renewal and differentiation into mesenchymal cell lineages including osteocytes, chondrocytes, and adipocytes [34, 35]. Importantly, appropriate care was taken to use only the trunk,

thus avoiding the head, which contains cranial NC that endogenously gives rise to skeletogenic cells and connectve tissue. This helps to explain the capacity of BM-derived MSCs to differentiate into neuronal derivatives [36, 37]. Although it was postulated that BM-derived cells could transdifferentiate into neurons, the discovery of NC-derived progenitors in the BM, however, indicates that the differentiation of BM-derived cells into neurons is very likely due to the presence of NC-derived MSCs in the BM. Nevertheless, it should be pointed out that the above studies concluded that MSCs do not derive solely from the NC and that other currently unknown sources probably exist [34, 35]. Indeed, it was recently shown by fate mapping that a small but significant number of Mesp1+ cells, a basic HLH protein, which is expressed in the paraxial mesoderm, reside in the BM and exhibit MSC characteristics [38]. It remains to be determined if additional source(s) for MSCs besides the NC and the paraxial mesoderm also exist.

It is worth mentioning here a discrepancy between the two transgenic mice used in the aforementioned studies [31]. The $EGFP^+$ cells in the BM of the P0-Cre/Floxed-EGFP mice were positive for both PECAM-1 and SMA-1, markers for endothelial and smooth muscle cells, respectively, whereas the equivalent group of cells in the Wnt1-Cre/Floxed-EGFP mice did not express either of the two markers. Wnt1, however, is expressed in the BM of adult rodents hence there is a possibility that the EGFP expression was due to the ongoing activity of Wnt1. Additionally, with respect to the use of the P0 promoter, it should be remembered that P0 is a marker for differentiated Schwann cells. Discrepancies and issues like these must be taken into consideration by researchers in the field with respect to the conclusions one could draw.

NCSCs in other embryonic and adult tissues

NC-derived progenitor cells have been identified in and isolated from rodent embryonic and adult hearts (Figure 1C) [39-41]. Cardiac NCCs contribute to the aorticopulmonary septum of the outflow tract and ablation of the cardiac NC results in cardiac outflow tract defects such as truncus arteriosis [42]. Presumptive cardiac NCCs isolated from primary neural tube explants and grown as single-cell suspensions were shown to be multipotent by giving rise to smooth muscle cells, neurons, Schwann cells, pigment cells, and chondrocytes [39]. Although short-term survival was also demonstrated, the number of passages was not described leaving open the question of long-term self-renewal. In this same study, two additional types of cardiac NCCs were also evident; a group of fate-restricted cells that could give rise to SMA^+ cells, as well as to SMA^- cells, and another group that was committed to the smooth muscle lineage [39]. These *in vitro* data, however, still remain to be replicated *in vivo*.

More recently, a multipotent NCC population was identified in the neonatal and adult mouse heart within the cardiac side population (SP) [40]. SP cells are found in several tissue types and are considered to be tissue-specific progenitor cells, which are mostly dormant. Isolated cardiac SP cells formed spheres of proliferating cells in culture termed cardiospheres, which are similar to neurospheres. These cardiospheres express nestin and musashi-1, markers of stem/progenitor cells. Dissociated cardiospheres differentiate into neurons, glia, melanocytes, chondrocytes, and myofibroblasts. Most importantly, when cardiosphere-derived DiI-labeled cells are transplanted into chick embryos, they are capable of migrating along endogenous NCC migrating pathways and contributing to the PNS in the form of DRG, sympathetic ganglia and the ventral spine nerve, as well as to the heart where they contribute to the outflow tract and the controncus. Significantly, these cardiosphere-derived cells differentiated accordingly in each tissue they colonised [40]. Interestingly, a population of nestin-expressing cells that exist in the adult rat and in both normal and infarct human hearts is believed to be related to the SP cells [41]. When these sphere-forming, nestin-expressing cells isolated from infarct regions of an adult rat heart are transplanted into the infarct region of another adult rat, they contribute to newly formed small blood vessels by presumably differentiating into vascular smooth muscle cells based on SMA expression [41]. The identification of a cardiac progenitor cell population with the ability to contribute to heart injury repair offers considerable therapeutic promise.

Multipotent NC progenitors have also been found in the postnatal and adult mouse cornea [43, 44]. The cornea is the transparent tissue in the front part of the eye that transmits and refracts light to the retina. The corneal stroma represents the majority of the corneal thickness and is comprised of extracellular matrix produced by cranial NC-derived keratocytes, which also have the ability to heal the cornea throughout life [45]. It is therefore possible that the healing ability of keratocytes is related to the stem cell-like properties of NCCs from which they are originated. The multipotency of keratocytes was first demonstrated using the chick/quail chimera [46]. Transplantation of late quail keratocytes into early chick embryos resulted in their migration to many sites normally populated by cranial NCCs and their contribution to the cornea, smooth muscle, and myofibrils [46]. The link between healing ability and multipotency of the NC-

derived keratocytes was confirmed by isolating multipotent keratocyte precursor cells from adult mouse corneas [43]. These cells were called COPs for cornea-derived precursors and the NC origin of COPs was confirmed using the P0- and Wnt1-Cre/Floxed-EGFP mouse lines. Like other multipotent NC progenitor cells described so far, single COPs can form spheres in culture that can be repeatedly passaged (> 18) suggesting their self-renewal capacity. Furthermore, COPs have the ability to differentiate into keratocytes, fibroblasts, and myofibroblasts and when induced they can also differentiate into adipocytes, chondrocytes, and neural cells thus demonstrating their multipotency [43]. A similar cell population was isolated from the juvenile mouse cornea showing analogous characteristics of NC-derived progenitors such as self-renewal and multipotency [44]. One discrepancy, however, was the inability of the latter study to isolate NC-derived progenitor cells from adult corneas as opposed to the former study. A possible explanation is that different progenitor populations may exist in the cornea before (juvenile) and after (adult) the eyelid opens; however, it could also be due just to technical differences in the isolation processes [44].

Interestingly, NC-derived stem cell-like cells with neurogenic abilities have also been identified in the adult murine carotid body (CB) [47]. The CB is an oxygen-sensing neural organ located at the bifurcation of the carotid artery and is formed from the sympathoadrenal lineage, which originates from the NC. The CB exhibits a high degree of plasticity such that under hypoxic conditions it increases in size and yet, it recovers to its original size upon returning to normal conditions [47]. Pardal *et al*., identified a group of cells within the CB that can form neurospheres in culture, exhibit self-renewal capacity and differentiate into neurons including dopaminergic neurons, and SMA^+ cells both *in vitro* and *in vivo*. By fate-mapping analysis using Wnt1-Cre-driven recombination in mice, they were able to show that this group of cells is indeed derived from the NC. The two major cell classes in the CB are mature glomus cells (TH^+) and sustentacular type II cells ($GFAP^+$, $nestin^-$). Sustentacular type II cells rapidly convert into intermediate progenitors, which give rise to mature TH^+ glomus cells [47]. Although CB progenitors and NCSCs are claimed to have the same multipotency capabilities, in reality the differentiation capability of the CB-derived cells is more restricted. They can give rise to neurons and to SMA^+ cells, but this does not necessarily mean these SMA^+ cells are smooth muscle cells. NCSCs on the other hand give rise to neurons, glia, and myofibroblasts. Nonetheless, an important implication of the results in this study is the potential of CB progenitor cells for therapeutic application in tissue engineering and repair. Glomus cells are highly dopaminergic and have been used for transplantation studies in Parkinson's disease with promising results. One drawback, however, is the limited tissue quantity available for their isolation. Therefore, the ability of the CB progenitor cells to differentiate into glomus cells *in vitro* may offer a solution to this problem of tissue quantity.

Lastly, NCCs with stem cell characteristics were also isolated from the palatum of adult rats and were called palatal neural crest-related stem cells (pNC-SCs) [48]. Isolated pNC-SCs expressed NSC markers like Nestin and Sox2, as well as NCC markers such as p75, Slug, Twist, and Sox9. Upon neural induction by retinoic acid, pNC-SCs differentiated into $TuJ1^+$ neuronal cells, whereas upon glial induction by fetal calf serum, they differentiated into $GFAP^+$ cells with a typical glial morphology. The authors do not mention whether differentiation towards other types of cells was observed or even attempted. Furthermore, in the same study, cells expressing stem cell markers like Nestin and Oct3/4 were also isolated from human palate. The self-renewal capacity or multipotency of these cells, however, was not demonstrated. Although the discovery that a stem cell-like population may exist in the human palate is very exciting, further work is needed to demonstrate their NC-origin, and any potential stem cell or progenitor cell characteristics like self-renewal and multipotency.

NCSCs in the skin

A very important and exciting finding was the discovery of NC-derived multipotent progenitors in the skin of adult mice [49, 50]. Toma *et al*. [51] had initially identified and isolated a group of mutlipotent cells from juvenile and adult rodent dermis (skin) that were able to proliferate and differentiate in culture and which they called skin precursor cells (SKPs). At the time, the origin of SKPs was unknown and in fact the authors excluded an NC origin based on the absence of PSA-NCAM and p75 expression, two common markers of NCSCs. Later, however, the same group was able to demonstrate the NC origin of SKPs residing in the facial dermis using Wnt1-Cre recombination reporter mice [49, 50]. SKPs were shown to reside in the base of the facial hair follicle and the dermal papilla (DP) and to exhibit self-renewal capacity and multipotential differentiation into neurons, smooth muscle cells, Schwann cells, and melanocytes *in vitro*. Furthermore, transplantation of SKP-derived neurospheres from the back skin of a mouse into a chick embryo, resulted in their migration and occupancy of NC-derived structures such as the DRG and the spinal

nerve [50]. In the trunk, skin derives from the mesodermal somites and therefore it was assumed that trunk SKPs are not of an NC origin. Indeed, first Wong *et al*. [52] were able to demonstrate by fate mapping that the DP and the dermal sheath (DS) of the mouse trunk skin are not of NC-origin unlike in the facial skin, but they argued that the spherogenic cells derived from the back skin are associated with the glial and the melanocyte lineages, which are of NC origin. Recently, Biernaskie *et al*. [53] demonstrated that mouse trunk SKPs derive from $Sox2^+$ cells that originate from the DP and DS of the hair follicle. Remarkably, they were able to show that this population is capable of dermal maintenance, wound-healing, and hair follicle morphogenesis, properties attributed to a dermal stem cell [53]. In a follow-up study, the same group convincingly showed that SKPs from the trunk skin are not of NC-origin but instead of a somite origin [54]. Fate mapping using the NC-specific Wnt1-Cre/Floxed-EGFP and the somite-specific Myf5-Cre/Floxed-EYFP was employed to confirm the origin of these precursors. Interestingly, however, both facial and trunk SKPs have very similar transcriptional profiles, differentiation potentials, and functional properties. Most surprisingly, trunk SKPs can differentiate into functional Schwann cells, a cell type that is believed to be derived only from the NC [55]. This means that either another type of precursor cell can give rise to Schwann cells other than the NC, or that the cells isolated from the Myf5-Cre/Floxed-EYFP mouse included cells of the melanocyte lineage, which are known to reside in the hair follicle bulge region. Therefore, based on the limitations that transgenes can have, fate-mapping results need to be very carefully interpreted. Multipotent progenitor cells have also been isolated from human neonatal foreskin and adult trunk skin [52, 56]. Their NC origin, however, has only been assumed by the expression of p75 and Sox10, due to experimental limitations in humans [52].

A different kind of multipotent cell of NC origin distinct from SKPs has also been identified in the adult bulge region of whisker follicles [31]. Termed epidermal NC stem cells (EPI-NCSCs), they are similar to SKPs, and when cultured can give rise to neurons, glia, smooth muscle cells, and melanocytes. Amazingly, preliminary results highlight the potential for using both SKPs and EPI-NCSCs in the treatment of injured spinal cord, although the two populations exhibit some major differences. When both murine and human neonatal SKPs were isolated and exposed to cues known to promote Schwann cell differentiation, they gave rise to myelinating Schwann cells both *in vitro* and *in vivo* [55]. Moreover, when transplanted into *shiverer* mice, which are deficient in myelin basic protein, both differentiated and non-differentiated SKPs associated with both PNS and CNS axons and adopted a myelinating phenotype. It remains to be determined however whether this association and myelinating phenotype is actually functional. Additionally, it is also not known what fate the rest of the non-differentiated SKPs assume when transplanted in the *shiverer* mice.

EPI-NCSCs derived from mouse whisker follicles and expanded in culture in a serum-free medium (i.e., with no differentiation inducers) were transplanted into injured spinal cords of mice [57]. In contrast to the SKPs in the aforementioned study, grafted EPI-NCSCs did not differentiate into Schwann cells but instead formed CNS cells expressing markers for GABAergic neurons and oligodendrocytes. This is also different from the results of Fernandes *et al*. [58], which showed that transplantated undifferentiated SKPs in a CNS environment (rat hippocampal slice cultures) failed to migrate and induce CNS or PNS neurons, whereas transplantation of differentiated SKPs gave rise to migratory cells and PNS neuronal phenotypes. These differences may be due to the distinct sources of SKPs and EPI-NCSCs or simply due to the absence of Schwann cell differentiation cues in the culture. The latter, however, seems less likely since Schwann cell differentiation has been observed after transplantation of non-differentiated SKPs [55]. Nevertheless, when whisker follicle-derived EPI-NCSCs were transplanted into spinal cord lesions, they caused improvement in sensory connectivity and touch perception [59]. Although transplantation took place only on one side of the spinal cord and migration was not evident, a bilateral improvement was observed. An explanation for this is that the grafted EPI-NCSCs express genes that encode neurotrophic factors, angiogenic factors, and metalloproteases and that bilateral improvement is due to the secretion and diffusion of these factors capable of modifying scar formation [59]. In agreement with this, SKP-derived Schwann cells promote recruitment of endogenous myelinating Schwann cells when transplanted in the rat-injured spinal cord [60]. More studies, however, need to be done to prove the incorporation of SKPs and EPI-NCSCs or their derivatives in the injured spinal cord and their functionality *in vivo*. In fact, when SKP-derived neuron-like cells were subjected to electrophysiological analysis, they failed to exhibit any electrophysiological function [58]. A promising finding, however, is that transplantation of EPI-NCSCs in the spinal cord did not result in uncontrolled proliferation and tumorigenesis [57].

Recently, Li *et al*. [61] have isolated a distinct group of dermal stem cells (DSC) in the human foreskin that could give rise to melanocytes, among other NC fates

such as cells expressing neuronal, chondrocytic, adipocytic, and smooth muscle markers. When pre-differentiated DSCs were introduced into a human 3D skin reconstruct, they migrated towards the epidermis-dermis surface, differentiated into melanocytes and localized in the epidermis where melanocytes normally reside. This is in contrast to SKPs isolated from human foreskin in which no melanocytic lineage differentiation was observed [56]. The two progenitor populations are probably distinct, although they were both isolated from dissociated dermis. It could, however, be the same population, and just that the culture conditions that were used were either favorable or unfavorable for melanocytic differentiation. Indeed, Li *et al*. used culture conditions known to favor melanocyte formation of human ES cells, whereas the original study did not.

Melanoblasts (NC-derived precursors of melanocytes) isolated from mouse skin exhibit limited capacity for self-renewal *in vitro* [62]. They are, however, multipotent as evident by their capacity to generate $TuJ1^+$, SMA^+, and $GFAP^+$ cells in culture. A number of experiments have been performed to confirm the melanoblast identity of these cells. First, these cells were isolated based on the presence of Kit expression, which is a melanoblast marker and the absence of CD45 which is a hematopoietic marker. RT-PCR confirmed the presence of melanoblast transcripts and absence of neuronal transcripts and lineage-trace analysis confirmed their melanoblast identity. However, ~26% of these isolated cells did not express the melanoblast marker Mitf. Therefore, it is possible that this 26% fraction contaminated the melanoblast pool and is responsible for the presence of different types of differentiated cells. Indeed, in a recent study, $Mitf^+/Sox10^+$ cells were shown to associate with nerves that innervate the skin in chick and mouse embryos. Ablation and Schwann cell precursor (SCP) lineage-tracing experiments demonstrated that a large number of SCPs acquire a melanoblast fate, which gives rise to melanocytes in the skin [63]. Furthermore, the decision of an SCP to become a mature Schwann cell or a melanoblast, depends on its association with a nerve and is probably mediated by Neuregulin signaling. When an SCP retains its association, it matures into a myelinating Schwann cell, but if this contact is lost, it becomes a melanoblast. This was nicely demonstrated by cutting part of the sciatic nerve and observing a large increase in melanocytes in the injured region [63]. The reason for the capacity of SCPs to give rise to melanoblasts is still unknown, but it may help explain the findings of the aforementioned study where foreskin-derived melanoblasts were found to be multipotent. It remains possible that these melanoblasts were isolated together in a mixture with SCPs that were either associated or not with cutaneous nerves, and retained the ability to differentiate into both neuronal and non-neuronal cell types. The potential of SCPs to give rise to a non-neuronal lineage is fascinating since it could mean that potentially more cell types could originate from these precursors. Additionally, it points towards a link between neurological disorders and changes in skin pigmentation that may help in the diagnosis and understanding of these disorders [63]. Additionally, the latter study which revealed the derivation of melanoblasts from SCPs highlights several stages of the NCC differentiation process that have not been previously appreciated. However, the plasticity of these stages might have led researchers to prematurely proclaim the "stemness" of NCCs, especially of those isolated in the adults.

Mouse and human ESC-derived neural crest stem cells

Recently, considerable effort has focused on the derivation of NCSCs from ESCs. Indeed, NC-like progenitors from both mouse and human ESCs that give rise to NC-derived cell types have been produced. This approach could facilitate the study of human NCSCs since the isolation of the endogenous embryonic population is not feasible. Although human NC-derived multipotent progenitors have been isolated from adults, they are very rare and their self-renewal capacity and multipotency have been found to be more restricted than their embryonic counterparts. Additionally, although not much can be done *in vivo* when studying human NC-derived progenitors, studies performed in model organisms and *in vitro* studies using human (h)NCSCs can complement each other.

The first demonstration that NC-derivatives can be induced from mouse and primate ESCs came from studies of ESCs growing on a stromal cell layer in culture, in which their induction into neuroectoderm gave rise to sensory neurons, autonomic neurons, smooth muscle cells, and glial cells [64, 65]. Mouse (m)ESCs differentiate into NC-like cells that express NC markers such as Snail, dHand, and Slug and then differentiate into PNS neurons [64]. Subsequent analysis revealed the derivation of an NC-like population from hESCs, which could differentiate into PNS neurons [66]. These two studies therefore were the first to demonstrate that multipotent NCC progenitors can be derived from both mouse and human ESCs. More recently, ESCs have been shown to give rise to NC-like cells that can differentiate into melanocytes, neurons, and glial cells [67]. However, the markers used to identify neuronal fates were either paneuronal (TuJ1) or CNS-specific (GFAP); therefore, there is a possibil-

ity that the differentiated cells are not NC-derived but NSC-derived. Nevertheless, when c-kit$^+$ (melanoblast marker) cells derived from hESCs were transplanted into the chick NC migratory stream, they migrated towards peripheral NC target sites [67]. However, since very few of these transplanted cells exhibited TuJ1 staining, it was concluded that proper differentiation into NC derivatives did not take place *in vivo*.

There are currently two key issues associated with the properties of NCCs derived from ESCs. Firstly, possible contamination from stromal cells that are used as supporting cells in culture and secondly, the proliferation and differentiation of ESCs in undefined serum. Unanticipated interactions between ESCs and stromal cells may exist but have not been explored. The proliferation and differentiation of ESCs are achieved in serum media containing several unidentified growth factors, which can inadvertently affect conclusions. Efforts have been made to utilize methods that eliminate contamination and the use of media with unknown factors. For example, GFP-ESCs can be purified by flow cytometry thus excluding the stromal cells [67]. However, this does not exclude any prior interactions between ESCs and stromal cells that could potentially affect gene expression in ESCs over the long term. More recently, p75 alone or p75/HNK-1 combination has been used as a read-out for NC-like cells derived from hESC rosettes [68, 69]. Purified p75$^+$/HNK-1$^+$ cells derived from hESCs can form neurospheres and differentiate into NC derivatives. When neuronal or mesenchymal differentiation factors were added in the p75$^+$/HNK-1$^+$ neurospheres, cells expressing neuronal and mesenchymal markers, respectively, emerged [69]. However, it has not yet been demonstrated that a single NC progenitor cell can give rise to both mesenchymal and neural progeny, which may indicate that the hESC-derived NCC population is heterogeneous with no true stem cell characteristics. Additionally, a population of p75$^-$ cells expressing TH but not peripherin, two widely used PNS markers has been observed. This is an example of why multiple markers should be used to characterize cell types, and the danger of drawing conclusions about the identity of a cell based on a single marker, a practice, which should be avoided.

NCCs have also been derived from iPS cells using similar protocols [70]. The use of iPS cells to derive NCSCs is very promising in regenerative medicine since iPS cells and subsequently NCSCs can be derived from the patient therefore overcoming potential histocompatibility problems. When hESC-derived NCCs were transplanted into the trunk of a chick embryo, migration was observed along characteristic NCC migration routes followed by differentiation towards peripheral neurons and smooth muscle cells. The transplantation experiments were only performed in the trunk, hence it will be very important in the future to examine the differentiation potential of these cells in the cranial region where NC gives rise to more diverse cell types, such as bone and cartilage in addition to the neuronal lineages. Interestingly, the generation of melanocytes from human iPS cells was recently achieved and furthermore, it was demonstrated that these melanocytes were derived from NCCs [71]. One of the caveats of using iPS cells, however, is their current derivation through genomic integration of retroviral-transduced genes, which is not ideal for therapeutic applications. Nevertheless, the potential that they have for future therapeutic applications is very exciting.

More recently, p75$^+$ cells isolated from hESC cultures grown on stromal cells were shown to be able to form neurospheres, which express known markers of NC progenitors such as HNK-1, Snail, and Sox9/10 [68]. Furthermore, upon addition of defined medium, these neurospheres gave rise to cells expressing markers for neurons, glial cells, and myofibroblasts. However, individual p75$^+$ cells were not able to proliferate. This indicates that the starting populations were heterogeneous. Moreover, the ability to differentiate into cells of different lineages does not say much about the "stemness" of the original cells. Nonetheless, hESC-derived p75$^+$ cells transplanted into the trunk of chick embryos migrated appropriately along the NCC pathways and SMA and TuJ1 expression was observed in a few cells.

In another twist, multipotent progenitor cells were isolated from embryoid bodies (EB) formed from hESCs that could differentiate into cranial NC derivatives [72]. In this case, Frizzled-3$^+$/Cadherin-11$^+$ cells were selected from the EB in order to ensure a population potentially enriched in migrating cranial NCCs. Both Frizzled-3 and Cadherin-11 are associated with migrating cranial NC *in vivo*. The enriched population was shown to spontaneously give rise to cells expressing markers associated with chondrocytes, glia, neurons, osteoblasts, and smooth muscle cells. However, Frizzled-3$^+$/Cadherin-11$^+$ cells exhibited a very limited self-renewal capacity and spontaneous differentiation occurred at the third passage. Differentiation of these cells into different lineages was based on single-marker analysis, which in the absence of *in vivo* data is not convincing. Moreover, in *Xenopus* Cadherin-11 expression persists during differentiation, whereas in mouse it is found in multiple mesenchymal derivatives [73]. Additionally, Frizzled-3 is expressed throughout the CNS in mice [74]. It is highly possible therefore that the Frizzled-3$^+$/Cadherin-11$^+$-enriched population constituted a heterogeneous population of progenitors and differentiated cells.

Recently, a technique for promoting mESC differentiation into NCCs in a serum-free monolayer culture has been developed. This avoids the presence of undefined growth and differentiation factors as well as the presence of feeder cells that can cause contamination [75]. ESCs grown on laminin and in the presence of BMP-4 and FGF-2 were induced into NCCs based on their expression of NC markers such as Snail, Slug, Twist, Sox9/10, and Pax3. Moreover, in the presence of differentiation media, cells expressing markers for neurons, Schwann cells, smooth muscle cells, chondrocytes, osteoblasts, and adipocytes were present. However, again the conclusions were based on single markers for each lineage and caution should be used in their interpretation. One of the reasons to remain cautious is that the differentiation-inducing media may contain factors that induce expression of specific genes in any kind of cell, and not just in an ES or NC cell. Therefore, multiple markers should be used and if feasible, functional tests should be performed before any conclusions are drawn about the fate of these cells.

One of the main challenges in the studies mentioned in this section is the propagation of undifferentiated ESC-derived NCSCs in culture. Recently, a combination of factors that promote self-renewal of these progenitors resulting in their significant expansion as undifferentiated NCCs was defined [76]. A Sox10-GFP transgene was used to isolate mESCs positive for GFP and these cells expressed NCC markers when cultured. After screening several factors, the authors were able to extend their undifferentiated state by adding Noggin, Wnt3a, Lif, and Endothelin-3 to the culture medium. Amazingly, when this population was grafted into fetal gut cultures, these cells were able to migrate and differentiate into neurons. This study is remarkable as it provides a way for expanding NCCs in an undifferentiated state, which overcomes the limitation of cell number and allows for more extensive studies. However, since Sox10 was used as a marker for cell isolation, this population is probably not an NCSC population. Moreover, the authors only explored the neuronal capacities of these cells, and differentiation into other NC-derived lineages was not examined. Nevertheless, this is an example of the rapid and exciting progress occuring in the field of ESC-derived NCCs and NCSCs.

Therapeutic applications of NCSCs: MSCs

As expected, the discovery of adult stem cells has prompted researchers to examine the potential of these cells in regenerative medicine. MSCs have been the focus of most of these efforts as they exhibit self-renewal and differentiation into multiple mesenchymal cell lineages including osteocytes, chondrocytes, and adipocytes *in vitro* [77]. MSCs were first isolated from the BM, but have since been isolated from other tissues including adipose tissues, umbilical cord blood, and, more recently, dental tissues [78]. MSCs from these alternative sources may be advantageous to the BM-derived MSCs due to their non-invasive isolation. Five different dental MSC-like cells have been isolated from humans thus far with potentials of self-renewal and multilinage differentiation with a preference, however, for the odontogenic lineages (Figure 2). These five types are: dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), and dental follicle progenitor cells (DFPCs) [79-83]. The dental mesenchyme, which is the origin of all the five aforementioned cell types, is known to derive from the cranial NC. Although the proper fate-mapping experiments have not been completed to date, it is known that the dental mesenchyme, and interactions between the ectodermal oral epithelium and the cranial NC-derived mesenchymal cells ensure proper tooth development [84]. Therefore, dental MSCs could possess characteristics similar to those of NCC progenitors.

Although the exact relationship between the five types of dental MSCs is not clear, similarities and differences

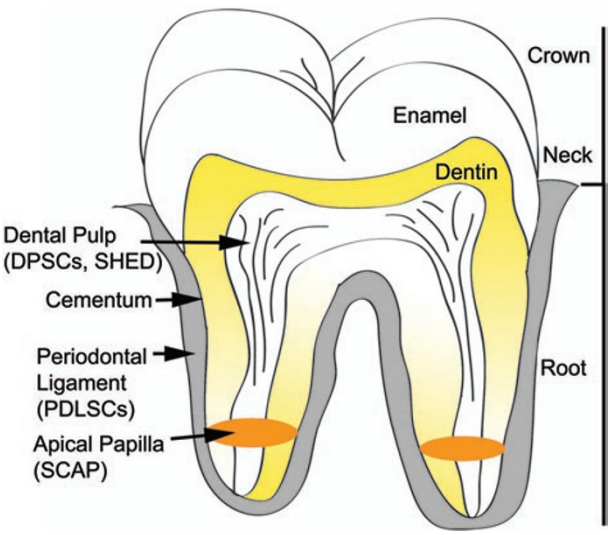

Figure 2 Sources and types of human molar dental stem cells. Five different dental mesenchyme stem cell-like cells have been isolated from humans. These include DPSCs, SHED, PDLSCs, SCAP, and DFPCs [79-83]. The dental mesenchyme, which is the origin of all the five aforementioned cell types, is known to derive from the cranial NC.

in their multipotency and self-renewal capacity have been observed. Both DPSCs and SHED are isolated from postnatal dental pulp, but the former are found in the permanent teeth whereas the latter are found in the deciduous teeth (reflecting immature DPSCs). The higher proliferation rate of SHED and their capacity to form neurospheres *in vitro* possibly reflect the difference in the developmental stages between the two cell populations. Furthermore, when grown on dentin, hDPSCs were shown to give rise to odontoblast-like cells with cell processes extending into the existing dentinal tubules [85]. In addition, when hDPSCs or SHED were transplanted in mice, they gave rise to functional odontoblasts that generated dentin [80, 86, 87]. Unlike DPSCs, however, SHED were unable to regenerate a complete dentin-pulp-like complex. Nonetheless, when induced with the appropriate media, both types of cells expressed markers of adipocytes and neuronal cells, as well as markers for osteoblasts, chondrocytes, and myoblasts [80, 86, 88-90]. Transplantation of SHED in mouse hosts resulted in their engraftment in organs such as the liver, brain, and kidney; however, no follow-up functional analysis was performed [90]. DPSC-derived osteoblasts were able to produce living autologous fibrous bone tissue *in vitro,* which when transplanted into rats formed a lamellar bone containing osteocytes demonstrating, therefore, the functionality of these differentiated cells [89]. SHED on the other hand, were unable to differentiate into osteoblasts when transplanted in mice, but they instead induced recipient mouse cells to differentiate into bone-forming cells [80]. Interestingly, DPSCs can differentiate into both osteoblasts and endothelial cells *in vitro*, which when transplanted into host rats gave rise to functional bone that becomes vascularized by these DPSC-derived endothelial cells [91]. More recently, DPSCs were also shown to spontaneously differentiate into melanocytes whose functionality was demonstrated *in vitro* by exhibiting activity of tyrosinase, the main enzyme involved in melanin biosynthesis [92].

DPSCs were also successfully used to repair a human mandible bone defect [93]. In this study, researchers isolated DPSCs from patients that needed oro-maxillofacial bone tissue repair after extraction of their third molar. The researchers made a biocomplex using DPSCs and a collagen sponge scaffold, which they autologously engrafted at the injury site after the extraction of the mandibular third molars. Three months later, the patients exhibited a complete restoration of periodontal tissue of the alveolar bone and a year later, optimal bone regeneration was evident. This clinical study demonstrated that DPSCs can indeed be used in humans for bone restoration, thus opening the doors to regenerative medicine.

SCAP, similar to DPSCs and SHED, exhibit a self-renewal and multilineage differentiation capacity [82, 94]. When transplanted into mouse hosts, SCAP are able to form a dentin-pulp-like structure and when transplanted simultaneously with PDLSCs in swine, they can regenerate the root/periodontal tissue [82]. PDLSCs constitute another type of dental MSCs, which are isolated from the PDL [81]. These MSCs can give rise to cells expressing osteoblast and cementoblast markers, two types of cells found in the PDL, and when transplanted into rats, they have been shown to integrate into the PDL, although functionality has yet to be tested [81]. More recently, both allogeneic and autologous transplanation of PDLSCs in a swine model for periodontitis resulted in the regeneration of the periodontal tissue [82, 95]. Similar experiments in a dog model of periodontitis have compared the regenerative capacity of three different types of dental MSCs in autologous transplantation and demonstrated that PDLSCs have the best regeneration capacity over DPSCs and DFPCs [96]. DFPCs comprise another type of dental MSCs that were identified in the dental follicle [83]. Upon induction with proper media, DFPCs can differentiate into osteoblasts, adipocytes, or chondrocytes, but upon their transplantation into mice, although they appeared to engraft, they failed to differentiate into any type of functional dental cells as shown by the absence of dentin, cementum, or bone [83, 84].

More recently, *de novo* regeneration of dental pulp in emptied root canal space was demonstrated [97]. In this study, human SCAP and DPSCs were isolated, seeded onto synthetic scaffolds and transplanted into an empty root canal space of a mouse. The transplantation resulted in the formation of vascularized pulp-like tissue and the deposition of a layer of dentin-like tissue. Although the dentin layer was not well organized and no long-term functionality of the regenerated tissues was tested, this study provided the first example of *de novo* dental pulp regeneration.

The challenge in regenerating dental tissue is the complexity of the tooth structure [98]. Several different tissues, such as bone and ligament, have to integrate in order to form a functional tooth. Moreover, whole tooth development and, therefore, regeneration require interactions between two types of cells: epithelial and mesenchymal. Therefore, there has been a focus on identifying epithelial cells in adults capable of inducing odontogenesis after associating with MSCs. One promising type of such cells are the epithelial rests of Malassez (ERM). ERM are found in the PDL near the developed tooth-root and persist throughout adulthood in a quiescent state. When isolated ERM were combined with freshly isolated primary dental pulp cells and then transplanted into a rat

host, they were able to form enamel-like tissues [99]. This is suggestive of the formation of functional ameloblasts.

It is well known that non-dental embryonic epithelium and mesenchyme can differentiate into dental epithelium and mesenchyme upon interactions with odontogenic mesenchyme and epithelium, respectively [100]. Therefore, there has been a concentrated effort in finding alternative sources of non-dental epithelial cells. One possible source may be the oral mucosa epithelium [101, 102]. Transplantation of a mouse line of oral epithelial cells derived from *p53*-deficient embryos combined with embryonically derived dental mesenchymal cells under kidney capsule, exhibited some evidence of tooth formation [101]. Although these results are encouraging, absence of a tumor suppressor gene is not ideal and ultimately isolation of epithelial cells with such capacities needs to become feasible in the adult. In another study, the association of postnatal non-dental epithelium with embryonic dental mesenchyme resulted in tooth-like structures after transplantation [102]. In this study, however, it was not tested whether association of postnatal non-dental epithelium with adult dental MSCs would give similar results, since as mentioned above, the ultimate goal is the autologous regeneration of teeth in adults.

In addition to their ability to regenerate dental tissues, human dental MSCs have been shown to have some neurological regeneration capacities as well. Upon induction with appropriate media, DPSCs were able to differentiate into functional neurons *in vitro*, whereas when transplanted into a chick embryo, host DPSCs gave rise to neuron-like cells [103]. In another study, transplantation of human DPSCs in a chick host, induced chemoattraction of the trigeminal ganglion axons [104]. However, the authors did not mention whether the donor DPSCs gave rise to neuron-like cells. Transplantation of SHED in a rat model of Parkinson's disease partially ameliorated the behavioral deficits, which the authors attributed to the differentiation of SHED into dopaminergic neurons based on the increase of dopamine levels after transplantation [105]. Although very preliminary, these results indicate that dental MSCs could potentially be used in stem cell therapy treatments of neurological disorders. The advantage of these cells is their non-invasive isolation and their availability throughout adulthood. However, SHED are found in the exfoliated teeth and therefore, autologous transplantations would require the isolation and storage of these cells during childhood. One caveat of this strategy is that the effects of long-term storage of these cells have not been studied yet. Nevertheless, the advantage of easy isolation and multilineage differentiation potential of dental MSCs has attracted a lot of attention, which is very likely to yield promising results in the near future.

Concluding remarks

The discovery that NCCs can be isolated at embryonic stages where they exhibit multipotency and self-renewal capacity was pioneering for the field of stem cell biology. These NC progenitor cells can also be isolated from adult tissues that are easily accessible, which overcomes the ethical issues governing ESCs, as well as the rejection problem of foreign transplants, and meets the need for non-invasive isolation procedures. Of great importance was the discovery that NC progenitors with a high degree of multipotency and self-renewal potential exist in the adult skin, a tissue that is highly accessible. More research, however, is needed to further our knowledge about the capacity of the NC progenitors to differentiate into cells of several different lineages. In particular, more studies need to be done *in vivo*, in order to dissect out the interactions the transplanted cells make with different tissue environments, since apart from epidermal NC progenitors, niches for the other tissues have not yet been described. The derivation of NC progenitor cells from hESCs can also be considered a breakthrough in the field since it overcomes the limitation of the quantity of cells that can be harvested from a single individual. However, the use of hESCs still remains controversial. Interestingly, SHED, SCAP, and DPSCs were recently successfully reprogrammed into iPS cells providing the community with an alternative source for generating iPS cells [106]. Additionally, iPS cells have been successfully and efficiently generated from a purified MSC population [107]. When these iPS cells were used to produce chimeras, they showed successful integration and contributed to chimeric mice that were able to reach adulthood indicating the quality of the MSC-derived iPS cells.

Although a great deal of progress has been made in isolating NC progenitors and studying their capacity for differentiation into cell types of different lineages, caution is needed when it comes to the interpretation of the results and the conclusions that are drawn. One major concern is the purity of NC progenitors. The original method for isolating NC progenitors was to culture neural tubes, wait for NCCs to start migrating and then select for $p75^+$ cells [18]. However, using Wnt1-Cre/R26R transgenic mouse, Zhao *et al*. [20], have demonstrated that some non-crest cells also migrate. Additionally, p75 expression is not restricted to progenitor cells but is also observed in differentiated cells. Although the use of lineage-tracing transgenic mice provided researchers with a more accurate way of isolating progenitor cells of NC or-

igin, earlier markers in the development of NCSCs need to be identified and used as a more accurate read-out of their developmental stage. However, very little is known about the key factors or signaling cascades that are essential for mammalian NCC induction. The Wnt, BMP, and FGF signaling pathways, which are well known for playing central roles in NCC formation in avians, fish, and amphibians, appear to be required primarily for later lineage specification of NCCs in mammalian embryos. What is clear, however, is that as NSCs differentiate into NCCs, there is a clear switch in Sox expression state with Sox2 being inactivated in concert with Sox9 and then Sox10 activation in progenitors and migrating NCCs, respectively. These parameters may provide avenues for ultimately identifying the key signals required for mammalian NCC formation.

ESC-derived NC progenitors need to be investigated further for their capacity to give rise to fully differentiated and functional cell types, especially *in vivo*. Caution, however, is still needed when using limited markers to demonstrate differentiation into specific types of cells. Furthermore, although there are several studies where transplantations of NC progenitors took place in live animals, all of them seem to have taken place in the trunk. It will be very important to demonstrate that transplantation of these cells in other regions, like the cranial region also leads to differentiation into the diverse array of cell types that arise from the cranial NC.

Caution also has to be taken when growing stem/progenitor cells *in vitro* for cell-based therapy. Chromosomal aberrations and downregulation of tumor-suppressor genes such as *p16* and *p21* have been reported after several passages of NC-derived mouse corneal progenitor cells [108]. Surprisingly, there are few reports that address the immortalization of NCSCs. Since the growth of NCSCs is a critical step for any potential cell-based therapy, it will be very critical to rigorously determine the long-term safety and stability of these cells. Clearly, analyses of long-term contribution, differentiation and functionality are of paramount importance to satisfactorily demonstrate the utility of NCSCs in regenerative medicine.

In conclusion, on the balance of evidence available, the majority of NCCs appear to be progenitor cells rather than true stem cells in the strictest sense of the term. However, it is clear that some NCCs exhibit the classic stem cell characteristics of self-renewal and multipotency. Even though NC progenitor cells are generated transiently in the embryo, many NCCs appear to retain their capacity throughout embryogenesis and even into the adulthood. Ignoring the semantic issue of whether NCCs are stem versus progenitor cells, what is important is that NCCs and their derivatives have very significant clinical potential in regenerative medicine. Future studies therefore need to thoroughly explore definitive functional differentiation and avoid premature assumptions about fate based on limited marker analysis. Furthermore, every effort should be made to validate the results obtained through *in vitro* cell culture in an *in vivo* animal model.

Lastly, NCCs are synonymous with vertebrate evolution and there is tremendous interest in identifying their specific origin and studying the acquisition of their incredible properties. There is no doubt that these properties were acquired gradually over time. Thus, the studies described above, which investigate the cell renewal capacity and multipotency of NCCs, are not only important for therapeutic applications such as tissue bioengineering and repair, but may also eventually help reveal how the NCCs and their distinctive properties came to be.

Acknowledgments

The authors sincerely thank Drs Kimberly Inman, Lisa Sandell and Amanda Barlow of the Stowers Institute for Medical Research for providing pictures in Figure 1. Research in the Trainor laboratory is supported by the Stowers Institute for Medical Research, the National Institute for Dental and Craniofacial Research (DE 016082) and the March of Dimes FY08-265.

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