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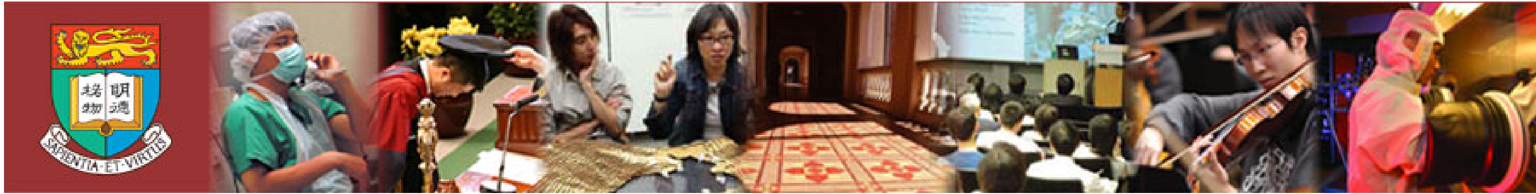

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**Review**

# Neural crest stem cells and their potential therapeutic applications


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**Abstract**

The neural crest (NC) is a remarkable transient structure generated during early vertebrate development. The neural crest progenitors have extensive migratory capacity and multipotency, harboring stem cell-like characteristics such as self-renewal. They can differentiate into a variety of cell types from craniofacial skeletal tissues to the trunk peripheral nervous system (PNS). Multiple regulators such as signaling factors, transcription factors, and migration machinery components are expressed at different stages of NC development. Gain- and loss-of-function studies in various vertebrate species revealed epistatic relationships of these molecules that could be assembled into a gene regulatory network defining the processes of NC induction, specification, migration, and differentiation. These basic developmental studies led to the subsequent establishment and molecular validation of neural crest stem cells (NCSCs) derived by various strategies. We provide here an overview of the isolation and characterization of NCSCs from embryonic, fetal, and adult tissues; the experimental strategies for the derivation of NCSCs from embryonic stem cells, induced pluripotent stem cells, and skin fibroblasts; and recent developments in the use of patient-derived NCSCs for modeling and treating neurocristopathies. We discuss future research on further refinement of the culture conditions required for the differentiation of pluripotent stem cells into axial-specific NC progenitors and their derivatives, developing non-viral approaches for the generation of induced NC cells (NCCs), and using a genomic editing approach to correct genetic mutations in patient-derived NCSCs for transplantation therapy. These future endeavors should facilitate the therapeutic applications of NCSCs in the clinical setting.

**Keywords**: Neural crest stem cells; Induced pluripotent stem cells; Human embryonic stem cells; Melanocytes; Mesenchymal.

## Introduction

The neural crest (NC) represents a transient cell population with stem cell-like properties that emerges from the dorsal neural plate border during gastrulation. Molecular signals derived from the non-neural ectoderm and underlying mesoderm play essential roles in the specification of the border region where multipotent NC stem cells (NCSCs) are formed. These neuroepithelial NCSCs then undergo a process called epithelial-mesenchymal transition (EMT), in which cell-cell adhesion and cytoarchitecture are altered leading to mesenchymal migration as they delaminate from the dorsal neuroepithelium (or premigratory NC domain). Depending on their axial origin and environmental guidance cues, multipotent NCSCs migrate along stereotypical routes to different locations in the embryo, where they can differentiate into various cell types, including ectomesenchymal tissue (cartilage and bones), sensory neurons and enteric ganglia in the PNS, melanocytes in the skin, and smooth muscle cells in the cardiac outflow tract (LeDouarin and Kalcheim, 1999). The NC development involves a cascade of molecules that are functionally linked and can be integrated into a gene regulatory network that define the processes at different developmental stages. Genetic mutations can result in dysregulated NC development leading to many congenital human diseases, such as cardiovascular defects, craniofacial abnormities, and intestinal aganglionosis, collectively known as neurocristopathies (Mayor and Theveneau, 2013). The identification and isolation of multipotent NCSCs derived from adult tissues, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and skin fibroblasts provide promising cellular sources for the treatments of neurocristopathies. In this review, we provide an overview of the molecular regulation involved in different NC developmental stages; strategies for the isolation and characterization of NCSCs from embryonic, fetal, and adult tissues; specific strategies to differentiate pluripotent stem cells or reprogram somatic cells into NCSCs; and their therapeutic potential for treating neurocristopathies.

## Molecular network of neural crest induction and epithelial-mesenchymal transition (EMT)

The process of NC induction starts at the early gastrula stage and continues through to neural tube (NT) closure. Tissue grafting experiments in amphibian and chick embryos showed that NC was formed via interactions between the neural plate and the non-neural ectoderm (Mancilla and Mayor, 1996; Selleck and Bronner-Fraser, 1995). Meanwhile, NCCs could be induced in vitro via interactions between the paraxial mesoderm and neural plate (Bonstein et al., 1998; Selleck and Bronner-Fraser, 1995). Gain- and loss-of-function studies in the mouse,

chick, frog, and zebrafish demonstrated that several signaling molecules including BMP, Wnt, Notch/Delta, and FGF were involved in the induction of NC within the neural plate border region (Chang and Hemmati-Brivanlou, 1998; Endo et al., 2002; Garcia-Castro et al., 2002; Glavic et al., 2004; Ikeya et al., 1997; Kengaku and Okamoto, 1993; LaBonne and Bronner-Fraser, 1998; Lewis et al., 2004; Liem et al., 1997; Liem et al., 1995; Marchant et al., 1998; Mayor et al., 1997; Monsoro-Burq et al., 2003; Nguyen et al., 1998). Although the timing and relative importance of these signaling molecules varies among the species, it is believed the integated action of these signaling molecules is crucial for establishing the neural plate border region distinct from the neural and non-neural ectoderm. The neural plate border cells respond to early inductive signals by expressing a distinct set of transcription factors termed neural plate border specifiers, such as *Tfap2*, *Msx1, Zic1, Pax3*, and *Pax7* (Bang et al., 1997; Garnett et al., 2012; Knight et al., 2003; Mansouri et al., 1996; Marchal et al., 2009; Mitchell et al., 1991; Streit and Stern, 1999). Once these factors are initiated, they maintain their expression via regulatory interactions with each other (Bhat et al., 2013; Monsoro-Burq et al., 2005). They cooperate with the NC signaling pathways to specify NC progenitors within the neural plate border by activating the expression of NC specifier genes, such as those encoding *Snail*, *FoxD3,* and *SoxE* family (mainly *Sox9* and *Sox10*) transcription factors (Cheung and Briscoe, 2003; Plouhinec et al., 2014; Simoes-Costa et al., 2014; Simoes-Costa et al., 2012). Several functional studies in various animal models have demonstrated these molecules are important in defining NC identity (Cheung and Briscoe, 2003; Dottori et al., 2001; Mayor et al., 1995; Mori-Akiyama et al., 2003; Nieto et al., 1994; Sasai et al., 2001). Overexpression or knockdown of one NC specifier generally affected the expression of another, indicating that like neural plate border specifiers, the NC specifier genes form an interconnected regulatory loop, believed to be crucial for maintaining NC progenitors in a multipotent state (Aybar et al., 2003; Cheung et al., 2005; Honore et al., 2003; O'Donnell et al., 2006). Recently, Labonne's group further revealed that some NC regulators (*Id3*, *TFAP2*, *Ets1*, *FoxD3,* and *Snail1*) were co-expressed with the core pluripotency factors in *Xenopus* blastula cells and promoted pluripotency in both NC and blastula cells. These results suggest that NCCs differentiated from a subset of blastula cells still retained pluripotent potential and had greater plasticity for developmental cell fate than previously anticipated (Buitrago-Delgado et al., 2015).

The NC specifiers have an additional function of conferring NC progenitors with the ability to undergo EMT through alteration of cell-cell adhesion and regulation of Rho GTPases,

essential for remodeling actin cytoskeleton dynamics (Cheung et al., 2005; Liu and Jessell, 1998; Sit and Manser, 2011). Previous studies showed that Snail2 directly repressed *cadherin6B*, which is a prerequisite for EMT in cranial NCCs (Coles et al., 2007; Taneyhill et al., 2007). Further studies revealed that Smad-interacting protein 1 (Sip1), a transcriptional repressor, was a critical requirement for cells to become fully mesenchymal via the regulation of the switch from E-cadherin to N-cadherin (N-Cad) during cranial NC EMT (Rogers et al., 2013). In addition, Sip1 regulated *FoxD3* expression, which was shown to inhibit N-Cad expression, while upregulating integrin β1, laminin, and cadherin 7 in trunk NC, indicating that Sip1 indirectly regulated N-Cad via *FoxD3* in cranial NCCs (Cheung et al., 2005; Rogers et al., 2013). Besides its function to specify NC identity, Sox9 also cooperates with Snail2 to promote EMT by inducing morphological change and migratory behavior in trunk NCCs (Cheung et al., 2005; Liu et al., 2013). Therefore, the combined expressions of NC specifier genes can define the characteristics of delaminating NCCs. To determine how Sox9 and Snail2 exert their influence on NC motility, new effectors will need to be identified by transcriptional profiling of sorted $GFP^+$ cells using RNA-seq. Gain- and loss-of function studies can be used to validate the epistatic relationships of these effectors within the transcriptional network, although they cannot distinguish between direct and indirect transcriptional regulation.

Comparative genomic analysis across different species identified conserved and functional enhancers containing sequence-specific binding motifs for direct transcriptional input that triggered the expression of NC specifiers. For example, *Sox10* expression in cranial NCCs was directly regulated by *Sox9*, *c-Myb*, and *Ets-1* (Betancur et al., 2010), while *Ets-1* expression was further subjected to transcriptional regulation by *tfap2, Msx1/2,* and *Pax7* (Barembaum and Bronner, 2013). In addition, dissection of *FoxD3* non-coding genomic elements revealed two separate enhancers NC1 and NC2, which triggered its expression in cranial and trunk NCCs, respectively. Interestingly, *Pax7* and *Msx1/2* functioned as common inputs to both enhancers, where axial specificity was determined by cranial *Ets-1* and trunk *Zic1*, which exhibited high posterior to low anterior gradient expression (Simoes-Costa et al., 2012). Recently, Simoes-Costa and Bronner took advantage of these two enhancers to isolate pure populations of NCCs for comparative transcriptional profiling (Simoes-Costa and Bronner, 2016). RNA-seq analysis comparing the two different cell populations revealed enrichment of genes in the cranial NCCs. Each regulator was individually knocked down to

establish a hierarchical order of the cranial-specific gene regulatory network at early and later time points in the development. Importantly, some of these cranial-specific transcriptional regulator genes, *Sox8, Tfap2b,* and *Ets1*, were able to reprogram trunk NCCs into chondrogenic lineage, which are normally generated only by cranial NCCs. These findings indicate the plasticity of NCC fate, in which the axial identity and lineages can be driven by transcriptional regulators. Together, these functional and enhancer studies establishing direct transcriptional relationships between regulators have been used to assemble the NC gene regulatory network comprising nested sub-modules that define the distinct developmental events of specification, delamination, migration, and differentiation.

**Neural crest derivatives and their signaling regulation**

The delaminated NCCs undergo extensive migration following stereotypical routes throughout the embryo to eventually settle and differentiate (LeDouarin and Kalcheim, 1999; Trainor, 2013), which is mediated largely by their axial origin within the NT and by the myriad of complex environmental cues they encounter during migration. Cranial NCCs derived from the mid-diencephalon to somite 5 exhibit a unique differentiation capacity by forming craniofacial structures of the head including cartilage and bone tissues in the skull, facial, and pharyngeal skeleton, but they can also differentiate into cranial neurons, glia, and connective tissues of the face. The trunk NCCs, which arise from the NT caudal to the somite 5, initially migrate through the anterior portion of sclerotome, where they form the dorsal root ganglia (DRG) containing sensory neurons and satellite glia. Some of them further migrate to form Schwann cells along the spinal nerves, sympathetic ganglia, and endocrine cells of the adrenal gland. A subset of trunk NCCs then take a dorsolateral route beneath the skin to form pigment-producing melanocytes. Another population of trunk NCCs lying opposite to somites 1-7 (vagal) and posterior to somite 28 (sacral) contribute to the formation of enteric ganglia throughout the length of the gut. Finally, cardiac NCCs derived from somites 1-3 that overlap with the anterior portion of the vagal region form the muscle and connective tissue in walls of large arteries and the septum in the outflow tract.

Besides the importance of genetic determinants in regulating specific rostral-caudal identities of NCCs, environmental cues along the migratory path play crucial roles in directing the differentiation of NC progenitors into different lineages at their destination. A similar set of signaling molecules to those involved in NC induction also play important roles in NC differentiation. For example, activation and inhibition of canonical Wnt signaling in NC

progenitors resulted in promoting and inhibiting sensory neuron formation, respectively (Hari et al., 2002; Lee et al., 2004). In addition, BMP2 and BMP4 secreted by the dorsal aorta induced NCCs to differentiate into sympathetic neurons (Schneider et al., 1999; Shah et al., 1996; Varley and Maxwell, 1996). Meanwhile, FGF signaling was found to play a crucial role in determining the skeletogenic fate of cranial NCCs. Functional studies in mice and chick demonstrated that FGF secreted by the pharyngeal ectoderm induced cranial NCCs expressing Fgf receptor 1 to differentiate into cartilage tissue in the branchial arches (John et al., 2011; Li et al., 2010; Partanen et al., 1998; Sarkar et al., 2001). Finally, Delta/Notch signaling mediated lateral inhibition resulting in the neuron-glial fate of NC progenitors in the DRG (Wakamatsu et al., 2000).

**Developmental potency of neural crest precursors**

The remarkable differentiation capacity of NCCs underlies their multipotency. The diversity of NC-derived lineages was first demonstrated by Le Douarin et al., who used chick-quail chimeras to establish the sites of origin of NC derivatives generated along the anterior-posterior axis of embryos (LeDouarin and Kalcheim, 1999). Subsequent heterotrophic transplantations before the onset of migration, in which chick neural domain was replaced with quail neural primordium from a different axial level, showed that quail NCCs destined to be adrenergic neurons acquired cholinergic identity. This indicates that premigratory NCCs are not pre-determined to a particular cell fate, rather they appear to be specified by environmental signals and multiple cellular interactions during migration (Le Douarin et al., 1975). However, these transplantation experiments revealed only the plasticity of bulk NCC fate at a particular axial level, but not the developmental potential of a single NCC. To resolve this issue, an in vitro clonogenic assay was established, which demonstrated the existence of multipotent quail NCCs that gave rise to at least two progeny, melanocytes and neuronal cells (Sieber-Blum and Cohen, 1980). Consistently, these cultured single NCCs were able to migrate like their endogenous counterparts, and differentiate into melanocytes and neurons when placed in a chick embryonic environment (Bronner-Fraser et al., 1980), indicating that some premigratory NCCs were multipotent. Recently, culture conditions (bFGF, retinoic acid, and IGF1) were established to maintain chick premigratory NCCs as "crestospheres" that could self-renew and remain in a multipotent state for 7 weeks. Heterogeneous and dynamic expressions of NC markers, *Sox10* and *FoxD3*, in crestospheres indicated a subpopulation of the crestosphere cells (~10%) were multipotent NCSCs with self-renewal capacity, whereas the rest were fate-restricted NC precursors (Kerosuo et al.,

2015). It is possible that culture conditions could alter and probably introduce bias to the developmental fate of NCCs, which may not reflect the in vivo situation. To demonstrate the presence of multipotent NCCs in vivo, cell lineage tracing studies were conducted on premigratory and migratory trunk NCCs in avian embryos using either vital fluorescence dye or retroviral vectors expressing marker proteins. These studies showed the majority of the labeled clones were multipotent before and after delamination, as their descendants could generate more than one differentiated cell type (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). Similarly, in vivo dye injection into single premigratory trunk NCCs in mouse (Serbedzija et al., 1994), *Xenopus* (Collazo et al., 1993), and zebrafish embryos (Raible and Eisen, 1994) revealed multipotent clones, although the proportions varied between species. Both in vitro clonal/crestosphere analysis and in vivo lineage tracing studies demonstrated that many individual premigratory and migratory NCCs were multipotent.

The stochastic labeling of premigratory NCCs by microinjection also resulted in the identification of clones that were fate-restricted to a single cell type (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988). Indeed, previous studies in cultured trunk NCCs suggested that a major portion of the initial NC population contained a heterogeneous mixture of neuronal and glial precursors followed by melanogenic lineages (Erickson and Reedy, 1998; Henion and Weston, 1997). The order of trunk NC derivative generation was consistent with results from a discrete lineage analysis of cells in the dorsal NT, in which early emerging cells migrated ventrally to generate sympathetic ganglia, Schwann cells in the peripheral nerves, neurons, and glial in the DRG, while late emerging cells migrated dorsolaterally to generate melanocytes (Erickson et al., 1992; Serbedzija et al., 1989). This stereotypical ventral to dorsal order of colonization of NC derivatives appeared to be determined by the spatiotemporal location of NC progenitors within the premigratory domain of the NT, in which most dorsal cells that were first to delaminate generated ventral derivatives, while ventral cells emigrating later generated melanocytes (Krispin et al., 2010). However, the cell labeling experiment was performed in an open book culture of NT that may not reflect the cell behavior in vivo. Recent studies using in vivo cell labeling and time-lapse imaging confirmed no correlation between ventrodorsal movement of premigratory NC precursors and their fate restriction, and cells from different subregions of the dorsal NT were equipotent in their ability to differentiate into specific trunk derivatives (McKinney et al., 2013). Although there is no strict correlation between the choice of NC derivatives and the

position of their progenitors in the dorsal NT, both studies demonstrated that the NC was comprised of a heterogeneous population of fate-restricted progenitors before departure from the NT, whereas previous single cell lineage experiments in vivo showed premigratory NCCs were multipotent (Bronner-Fraser and Fraser, 1988). These discrepancies could be due to differences in the timing of cell labeling in the NT. Cell labeling was performed in the chick NT at stage 10-17 in Bronner-Fraser's study, at stage 14 in Krispin's study and at stage 13-17 in Mckinney's study. It is possible that labeling primitive neuroectodermal progenitors (in Bronner-Fraser's study) in early stages prior to segregation between NT and NC lineages resulted in more labeled single cells contributing to multiple cell types, whereas labeling cells in later stages (all three studies) resulted in more labeled fate-restricted premigratory NCCs. In vivo time-laspe imaging of single trunk premigratory NCCs labeled at stage 10-17 may be able to resolve these issues. This approach was supported by a recent in vivo fate mapping study of single trunk NCCs in a R26R-Confetti mouse model that demonstrated most of the premigratory and postmigratory murine NC populations were multipotent (Baggiolini et al., 2015).

**Isolation and characterization of embryonic NCSCs**

The ability of an undifferentiated NC population to undergo self-renewal and acquire progressive lineage restriction for different cell types fulfils the basic criteria for stem cells. Therefore, multipotent NCCs have "stemness" properties, whereas NC progenitors are fate-restricted precursor cells that are either unipotent (form one lineage) or bipotent (form two lineages).

The idea of NC stem cells (NCSCs) was originally proposed by Stemple and Anderson, who used flow cytometry to isolate a rodent NC population that expressed a low affinity nerve growth factor receptor (NTR) $p75^{NTR}$. These $p75^{NTR+}$ cells were able to self-renew to form colonies and could generate neurons and glia in the PNS and myofibroblasts (Stemple and Anderson, 1992). The same research group also fractionated E14.5 rat fetal sciatic nerve into five distinct subpopulations using flow cytometry with antibodies against $p75^{NTR}$ and P0 (marker for peripheral myelin protein). One of the enriched $p75^{NTR+}P0^{-}$ subfractions exhibited self-renewal capacity, and formed multipotent colonies that gave rise to neurons, Schwann cells, and smooth muscle-like myofibroblasts in vitro. Upon transplantation, these post-migratory NCSCs generated neurons and glia in diverse regions of the PNS in chick embryo, demonstrating the persistence of multipotency of NSCSs in postmigratory targets of

embryonic NC (Morrison et al., 1999; White et al., 2001). The DRG is another target site containing substantial amounts of $p75^{NTR+}P0^{+}$ or $p75^{NTR+}PMP22^{+}$ NCSCs that could generate multilineage colonies of glia, neurons, and smooth muscle-like cells, each of which increased in response to NRG1 (neuregulin1), BMP2, and TGF-β, respectively. Whether they undergo self-renewal has not been assessed (Hagedorn et al., 1999). In contrast, $p75^{NTR-/low}P0^{+}$ populations isolated from sciatic nerve generated smooth muscle cells, indicating that cells expressing P0 exhibited differentiation potential other than glial lineage (Morrison et al., 1999). Similarly, P0 and PMP22, both major components of peripheral myelin, were expressed in the glial lineage long before myelination, and were markers for the differentiation of multipotent NCSCs into neurons, glia, and smooth muscle-like cells in the embryonic DRG in response to instructive extracellular cues (Hagedorn et al., 1999). Another postmigratory NCSC population was derived from E14.5 embryonic rat gut by flow cytometry enrichment using antibodies against cell surface proteins $p75^{NTR}$ and α4 integrin. Upon plating 1% to 2% of the sorted cells for culture, only 60% survived to form colonies, and 80% of these differentiated into neurons, glia and myofibroblasts, indicating the colonies derived from embryonic gut were multipotent NCSCs (Iwashita et al., 2003). It is important to note that the NCSCs isolated from different postmigratory target sites were intrinsically different, underlying their differentiation potentials. In normal development, gut-derived NCSCs are biased toward a neuronal fate, whereas sciatic nerve-derived NCSCs are biased toward a glial fate (Kubu et al., 2002); however, both cell types remained multipotent in vitro and were able to generate neurons and glia upon transplantation in diverse locations in chick embryos (Bixby et al., 2002; Mosher et al., 2007). Although neurogenic and gliogenic factors were expressed in these two tissue-specific NCSCs, gut-derived NCSCs were more sensitive to the neurogenic effects of BMP4 than sciatic nerve derived NCSCs. In contrast, sciatic nerve-derived NCSCs exhibited a stronger response to the gliogenic effects of soluble Delta than gut-derived NCSCs. These results demonstrated that cell-intrinsic differences encoded in NCSCs and regional environmental influences determine the generated cell types in different embryonic locations (Bixby et al., 2002). In addition to the trunk NCSCs, an undifferentiated progenitor population derived from early migratory cranial NCCs in mouse and chick embryos also exhibited self-renewal capacity and differentiated into diverse cell types such as neurons, melanocytes, osteocytes, and chondrocytes (Calloni et al., 2009; Chung et al., 2009; Zhao et al., 2006).

**Characterization of adult tissue-derived NCSCs and their regenerative potential**

Multipotent NCSCs have been identified not only in the early embryonic stage but also in adulthood. The discovery of NCSCs in multiple rodent and human adult tissues including DRG (Li et al., 2007), bone marrow (BM) (Shi et al., 2016), whisker pad (WP) (Nagoshi et al., 2008), skin (Toma et al., 2001; Wong et al., 2006), gut (Kruger et al., 2002), carotid body (CB) (Pardal et al., 2007), heart (Tomita et al., 2005), and several cranial tissues such as the cornea (Brandl et al., 2009), iris (Kikuchi et al., 2011), dental pulp (Janebodin et al., 2011), hard palate (Widera et al., 2009), and oral mucosa (Davies et al., 2010) provide potential attractive sources of cells for replacement therapy (Fig. 1).

***BM-, DRG-, and WP-derived NCSCs***

In addition to the surface markers for the prospective isolation of NCSCs from various embryonic and adult tissues (Morrison et al., 1999; Nagoshi et al., 2008; Stemple and Anderson, 1992), transgenic mice carrying NC-specific expression of Cre recombinase with continuously activated floxed-EGFP reporter driven by P0 and Wnt1 promoter-sequences were used to derive NCSCs from the BM, DRG, and WP by flow cytometry (Nagoshi et al., 2008). Besides being a marker for Schwann cell lineage (Lemke et al., 1988), P0 is also expressed in migrating NCCs like Wnt1 at E10.5 of mouse embryos (Jiang et al., 2000; Nagoshi et al., 2008). When cultured at the same density, DRG-derived EGFP$^{+}$ NCSCs generated the highest number of primary and secondary spheres consistent with high expression levels of NCSC marker genes (*p75$^{NTR}$*, *Sox10*, *Nestin,* and *Musashi1*) compared with BM and WP. In addition, most of the DRG-derived spheres (74.6%) showed trilineage differentiation potential (neurons, glia, and myofibroblasts) compared to WP-derived spheres (7.3%) and BM-derived spheres (3.3%), indicating that DRG contained the highest proportion of NCSCs. These results support the previous view that tissue-dependent signals also conferred intrinsic differences in the adult NCSCs in terms of their differentiation potency and self-renewal capacity.

***CB-derived NCSCs***

The carotid body is another tissue with therapeutic value as a potential source of cells. It is an oxygen-sensing organ located at the bifurcation of carotid artery, which contains clusters of $O_2$-sensitive, neuron-like glomus cells enveloped by glial cells. They support organ growth during acclimatization to sustained hypoxia (Pardal et al., 2007). Genetic lineage tracing studies using a *Wnt1-Cre* reporter line demonstrated the glial cells were multipotent stem

cells of NC origin. These CB stem cells were able to form neurospheres, self-renew, and differentiate into dopaminergic neurons and smooth muscle cells both in vitro and in vivo. Interestingly, activation of CB stem cells in hypoxia by adjacent glomus cells induced switching of phenotypes from glial (GFAP$^+$) to Nestin$^+$ proliferative intermediate progenitors, which in turn differentiated into glomus cells and other cell types (Pardal et al., 2007). Because of their highly dopaminergic nature, glomus cells have been successfully used in transplantation studies in animal models of Parkinson's disease (PD) with promising results (Munoz-Manchado et al., 2013; Villadiego et al., 2005). However, the small amount of available tissue in the CB limits its use in transplantation. Therefore, expansion of CB progenitors in vitro from resident adult stem cells could provide sufficient quantities of cells to differentiate into glomus cells for the treatment of PD. These studies demonstrate for the first time a potential physiological application of adult NCSCs in vivo.

***Cardiac-derived NCSCs***

Cardiac NCCs participate in the septation of the cardiac outflow tract into the aorta and pulmonary artery (Kirby et al., 1983). In vitro clonal analysis of the cardiac NC population emigrated from NT explants revealed only a small fraction of them were NCSCs capable of self-renewal and generating various NC derivatives (Ito and Sieber-Blum, 1991; Youn et al., 2003), but whether these cells exhibit long-term self-renewal capacity remains to be determined. Cardiac NCSCs were identified in fetal and adult heart within the cardiac side population (SP). SP cells are found in various tissue types and are considered to be tissue specific progenitors that are mostly dormant. Stem cell fractions from rodent neonatal heart, which were enriched and expanded in culture as proliferating cardiospheres, were found to express undifferentiated markers, *Nestin* and *Musashi-1*. In addition, cardiosphere-initiating cells were found within neonatal and adult cardiac non-myocyte cell populations. Cells dissociated from cardiospheres could differentiate into NC-derived cell types such as PNS neurons, glia and smooth muscle cells, and cardiomyocytes. When transplanted into the migration staging area between the neural tube and somite of chick embryos, many cardiosphere-derived cells behaved like NCCs by following normal migratory routes and contributing to trunk derivatives. Lineage analysis using *P0-Cre/Floxed EGFP* double transgenic mice revealed that the NC contributed to some of the dormant cardiosphere-initiating cells, which remained as resident stem cells in the adult heart (Tomita et al., 2005). This finding was supported in a recent study using high-resolution genetic fate-mapping approaches with *cKit*$^{CreERT2/+}$ and *Wnt1:Flpe* mouse lines to show that *cKit* delineates cardiac

NC progenitors, which possess the full capacity to generate cardiomyocytes and other cardiac NC derivatives (Hatzistergos et al., 2015). In another study, a subpopulation of resident cardiac progenitors expressing Nestin migrated to the infarct region following ischemic damage and contributed to reparative vascularization (El-Helou et al., 2008; El-Helou et al., 2013). Therefore, understanding the molecular regulation involved in the establishment and maintenance of these stem/progenitor populations, as well as how they differentiate into target tissues could be of therapeutic value for the treatment of various heart diseases.

***Gut-derived NCSCs***

Fetal gut-derived NCSCs can be enriched by antibodies against $p75^{NTR}$ and $\alpha 4$ integrin (Nagoshi et al., 2008). These cells persisted in the gut of adult rodents (Kruger et al., 2002) and could be isolated to a high purity by positive selection using CD49b (integrin $\alpha 2$) (Joseph et al., 2011). The sorted $CD49b^+$ cells expressed markers characteristics of NCSCs (p75, Sox10, and Nestin) and enteric glia (S100B and GFAP). Immunofluorescence staining revealed that 60% of enteric glia were positive for CD49b, but only 44% of cells formed multipotent primary neurospheres in culture, indicating heterogeneity of adult enteric glia. Upon dissociation into single cells, primary neurospheres could generate secondary neurospheres, indicating their ability to self-renew. However, multipotent enteric progenitors derived from the fetal gut exhibited a greater degree of self-renewal and differentiation capacity compared to adult gut progenitor cells (Kruger et al., 2002; Mosher et al., 2007). After transplantation into chick embryos, the former cells gave rise to mainly neurons, whereas the latter differentiated into predominantly glia (White and Anderson, 1999). Further probing of the physiological role of $Cd49b^+$ NCSCs in the adult gut revealed they were fated to form mainly glia instead of neurons under steady-state conditions and after injury (Joseph et al., 2011). In vivo grafting of fetal or postnatal intestinal NC-derived neurospheres into the colon of postnatal mice showed the cells derived from these neurospheres could migrate, proliferate, and generate ganglion-like clusters with neurochemical, morphological, and electrophysiological properties of enteric neurons, and were able to receive synaptic input (Dettmann et al., 2014; Hotta et al., 2013). These studies indicated that progenitor cells isolated from the postnatal gut might have therapeutic potential for the treatment of enteric nervous system (ENS) disorders. However, further proof of concept studies (e.g., transplanting cells isolated from adult mouse gut into adult mice) are needed to demonstrate that gut-derived NCSCs can be used to treat adult enteric neuropathies.

***Skin-derived NCSCs***

The identification and isolation of multipotent progenitors from the skin of adult rodents and humans is an important discovery in the field of adult stem cells because skin tissue is readily available (Fernandes et al., 2004; Sieber-Blum et al., 2004; Toma et al., 2001; Toma et al., 2005). Using *Wnt1-Cre* reporter mice, genetic lineage tracing of skin-derived precursors (SKPs), which reside in the base of facial hair follicles and dermal papillae, revealed they were of NC origin (Fernandes et al., 2004; Sieber-Blum et al., 2004). They expressed genes (*Snail1/2*, *Pax3*, *Twist,* and *Sox9,* but not *Sox10* or $p75^{NTR}$) characteristics of embryonic NC precursors with self-renewal capacity and multipotency to differentiate into neurons, smooth muscle cells, Schwann cells, and melanocytes in vitro. Furthermore, transplantation of SKP-derived neurospheres into chick embryos showed they dispersed along NC migratory routes and colonized NC-derived structures such as the DRG and the peripheral nerve (Fernandes et al., 2004). Whereas facial SKPs are derived from the NC, trunk SKPs originate from somite/mesenchymal tissues as shown in somite-specific *Myf5-Cre/Floxed-YFP* (Jinno et al., 2010) and dermal-specific *Dermo1-Cre/Floxed-YFP* reporter mice (Jinno et al., 2010; Krause et al., 2014). Despite the distinct tissue origins, both trunk and facial SKPs are very similar at the transcriptome level, and have similar differentiation potentials and functional properties. Another multipotent cell population of NC origin, termed epidermal NCSCs, was identified in the adult mouse bulge region of whisker follicle (Nagoshi et al., 2008; Sieber-Blum et al., 2004). Although found in different locations, these cells behaved similarly to SKPs with the ability to self-renew and differentiate into various NC derivatives. Gene expression profiling identified a panel of 19 signature genes (*Pcbp4, Msx2, H1fx, Thop1, Vars2, Myo10, 2700094K13Rik, Ets1, Pygo2, Adam12, 5730449L18Rik, Rex3, Bdac1, Cair, Cryab, Peg10, AU041707, Crmp1,* and *Ube4b*) common between epidermal NCSCs and embryonic NCSCs, but distinct from epidermal stem cells that generate keratinocytes, despite sharing the bulge as their niche. Some of these genes are related to NC development. For example, *Msx2* regulates cranial NCC differentiation into skeletal lineages (Han et al., 2007; Takahashi et al., 2001); *Ets1* regulates NC formation through the recruitment of an epigenetic regulator to repress BMP signaling (Wang et al., 2015) and cardiac NC migration (Gao et al., 2010); and *Myo10* is critical for cranial NC migration (Nie et al., 2009). Other genes are involved in neural/neural-crest related diseases (*Thop1, Adam12, Ube4b, and Vars2*) (Baertling et al., 2016; Caren et al., 2006; Pollio et al., 2008; Shao et al., 2014), regulation of Wnt signaling pathways (*Pygo2*) (Belenkaya et al., 2002), and invasiveness (*Crmp1, Adam12*) (Cai et al., 2016; Shao et al., 2014). Interestingly, a detailed comparison of gene expression revealed

epidermal NCSCs did not express any NC marker genes (*Snail1/2, Twist, Pax3, Sox9, nexin, Nestin, fibronectin, Wnt5a, Sca-1, Shox2,* and *Dermo-1*) in SKPs (Fernandes et al., 2004; Hu et al., 2006), suggesting at least two molecularly distinct populations of NCSCs are located in the hair follicle.

In mouse models of spinal cord injury, grafted murine epidermal NCSCs were found to integrate with the surrounding host spinal tissue leading to significant improvements in sensory connectivity and touch perception (Sieber-Blum et al., 2006). Importantly, even though epidermal NCSCs share some stem cell genes with iPSCs, they do not form tumors at the grafting site, which is an essential prerequisite for stem cell-based therapies. NC-derived Schwann cells are glial cells of the PNS responsible for axonal myelination and ensheathing as well as tissue repair following severe nerve damage in spinal cord injury. These glial type cells promise to be useful for nerve regeneration by contributing to axon regeneration and remyelination. A previous study developed an efficient protocol for generating SKP-derived Schwann cells (Biernaskie et al., 2006). Studies showed that transplantation of SKP-derived Schwann cells were more effective than SKPs in contributing to the recovery from spinal injury, including lesion site bridging effects, increasing the size of spared tissue, reducing reactive gliosis, and most importantly, enhancing locomotor recovery (Biernaskie et al., 2007).

Multipotent SKPs have also been isolated from human neonatal foreskin and adult trunk skin (Toma et al., 2005; Wong et al., 2006). These multipotent cells are likely to be of NC origin based on the expression of *Sox10* and $p75^{NTR}$ (Wong et al., 2006), although they are limited in their use in human research. Human epidermal NCSCs located in the bulge of hair follicle, similar to their mouse counterparts, are multipotent cells that express markers characteristics of NCSCs and iPSCs, and can undergo self-renewal and give rise to all major NC derivatives including neurons, osteoblasts/chondrocytes, Schwann cells, and smooth muscle cells (Clewes et al., 2011). Importantly, they do not form tumors in vivo even though they express stem cell genes, making them attractive candidates for cell-based therapies. Indeed, human epidermal NCSCs can be differentiated into clinically relevant cell types including bone cells, midbrain dopaminergic neurons (Narytnyk et al., 2014), and Schwann cells that could be used in nerve repair (Sakaue and Sieber-Blum, 2015). Whether these differentiated cell types can be used for tissue regeneration needs to be evaluated in animal disease models before conducting clinical trials.

***Craniofacial tissue-derived NCSCs***

- *Cornea*

In addition to trunk tissue-derived NCSCs, several aforementioned cranial NC-derived tissues also contain multipotent NCSCs. One such tissue is the cornea, which is a transparent avascular structure that functions to cover the front of the eye and together with the lens helps to focus light on the retina. The cornea is composed of three major cellular components: a stratified epithelium, a thick collagenous stroma containing keratocytes, and a single layered endothelium. The homeostasis and integrity of these components is vital in preserving transparency and optical precision. Chick-quail chimeras revealed that quail-derived cranial NCCs contributed to the corneal endothelium and keratocytes by producing keratan sulfate proteoglycans (KSPGs) that interact with the stromal collagen regulating the collagen fibril diameter and spacing required for transparency (Hassell and Birk, 2010). Keratocytes undergo division in the fetal stage, withdraw from the cell cycle in newborns, and become quiescent throughout the adult life. However, in response to the injury, these non-proliferative keratocytes can resume migration, mitosis, wound healing, and repair via re-expression and secretion of extracellular matrix, indicating cellular plasticity (Fini, 1999). This was demonstrated by transplantation of quail NC-derived keratocytes from late embryos into the early chick embryo, which resulted in the cells following normal NC migratory routes and differentiating into NC derivatives, such as smooth muscle, myofibrils, keratocytes, and endothelial cells, but not neurons of cranial ganglia and branchial arch cartilage, suggesting stromal keratocytes have partially restricted NCSC-derived progenitors (Lwigale et al., 2005). This study demonstrated that keratocytes were not terminally differentiated, but maintained a degree of plasticity and multipotency. Consistently, putative multipotent keratocyte precursor cells (also called cornea-derived precursors, COPs) were identified in the corneal stroma of adult mice (Yoshida et al., 2006). Genetic lineage tracing using *Wnt1-Cre*/*Floxed-EGFP* and *P0-Cre*/*Floxed-EGFP* reporter mice showed that COPs were of NC origin, but not derived from the BM because they expressed embryonic NC marker genes (*Twist, Snail1/2,* and *Sox9*). Clonal sphere-forming assay showed that single COPs formed spheres, which could be further subcultured for more than 18 passages, indicating self-renewal potential. Moreover, COPs demonstrated multipotency and had the ability to differentiate into keratocytes, fibroblasts, and myofibroblasts as well as adipocytes, chondrocytes and neural cells when cultured in differentiation-inducing media. Human corneal stromal stem cells (CSSCs) present in the limbus also exhibited self-renewal capacity and broad differentiation potential, as well as appeared to be of NC origin (Du et al., 2005).

Consistently, gene array analysis revealed a panel of multipotent mesenchymal stem cell (MSC) genes (*ABCG2, BMi1, CD166, cKIT, Notch1, Pax6,* and *Six2*) were highly expressed in CSSCs, but were weakly expressed in keratocytes. Nonetheless, when cultured in low-mitogen, ascorbate-containing media, CSSCs differentiated and expressed genes (*keratocan*, *ALDH3A1*, *CXADR*, *PTDGS*, and *PDK4*) characteristics of keratocytes. Human CSSCs injected into corneal stroma of lumican-null mutants ($Lum^{-/-}$), which exhibit reduced corneal transparency due to defects in collagen fibril assembly (Chakravarti et al., 1998), adopted keratocytic functions by depositing human extracellular matrix components in the stroma (Du et al., 2009). Electron microscopy analysis 3 months post-injection revealed improved collagen fibril organization, resulting in restoration of both transparency and thickness of $Lum^{-/-}$ corneas. Most importantly, transplanted human CSSCs did not trigger T-cell-mediated immune rejection, making them excellent candidates for allogeneic transplantation for the treatment of scarred stroma.

- *Iris*

Fate mapping analysis using transgenic mice and chick embryos revealed that cranial NCCs also contributed to the development of the iris (Gage et al., 2005) (Kikuchi et al., 2011). The isolated $EGFP^+$ iris cells from *P0-Cre/floxed-EGFP* reporter mice formed primary and secondary spheres on non-adherent plates, indicating self-renewal capacity. These iris-derived sphere-forming cells expressed marker genes for neural stem cells (*Sox2*, *Nestin,* and *Musashi*) and NCCs ($p75^{NTR}$, *Sox10, Sox9, Snail2,* and *AP-2β*), and were able to differentiate into neurons, glia, smooth muscle, and chondrocytes (Kikuchi et al., 2011). These NC-derived multipotent stem cells, which can be isolated from murine iris and expanded by sphere culture, have properties that make them potential sources of cells for regenerative therapy for eye diseases.

- *Teeth*

Another accessible source of NCSCs can be derived from the dental pulp and periodontal ligament in both mouse and human. Dental pulp is located at the center of each tooth encased in mineralized dentin and is comprised of connective tissue, odontoblasts, mesenchymal cells, nerve fibers, lymphatic, and blood vessels. The primary function of the dental pulp is to produce dentin and maintain its biological and physiological vitality. In response to teeth eruption, a population of cranial NC-derived dental pulp stem cells (DPSCs)/progenitor cells

in the pulp tissue of teeth is induced to differentiate into odontoblasts to form reparative dentin, which protects the dental pulp from degradation (Huang et al., 2006; Janebodin et al., 2011). Consistent with their NC origin, DPSCs expressed NCSC marker genes (e.g., *GFAP, HNK-1, Nestin,* $p75^{NTR}$, and *S-100*) (Almushayt et al., 2006). These DPSCs are multipotent with the capacity to differentiate into chondrocytes, adipocytes, odontoblasts, and neural-like cells in vitro as well as form ectopic dentin and associated pulp tissue in vivo (Gronthos et al., 2002). In addition, they possess biological properties similar to BM-derived MSC, such as fibroblast-like morphology, clonogenicity, and ability to adhere to plastic tissue culture dishes, and they expressed MSC surface markers (CD29, CD44, CD59, CD73, CD90, CD105, and CD146), but not hematopoietic markers (CD34, CD45, or CD11b) (Liu et al., 2015). However, detailed quantitative comparison studies revealed that DPSCs had faster population doubling time, higher numbers of stem/progenitor cells, and increased mineralization potential compared to BM-MSCs (Alge et al., 2010). Consistently, DPSCs exhibited a higher osteogenic potency than BM-MSCs both in vitro and in vivo (Jensen et al., 2016). Transplantation of a biocomplex comprised of human DPSCs and a hydroxyapatite tri-calcium-phosphate paste in parietal defects of immunocompetent rats produced significant improvements in the calcification rate and bone mineral density at 8 weeks post-surgery compared to the untreated control group (Asutay et al., 2015). Moreover, DPSCs could be cryopreserved for 2 years without altering their proliferation rate, viability, morphology, surface antigens, level of gene expressions, and osteogenic differentiation capacity, making them an attractive cell source for long-term cryopreservation and bone regeneration (Papaccio et al., 2006). The DPSCs could also differentiate into other clinically relevant cell types in vitro, such as neurons (Gervois et al., 2015) and islet cell aggregates (Govindasamy et al., 2011), which could have potential for regenerative therapies in neurological disorders and diabetes, respectively.

The periodontal ligament (PDL) is a soft connective tissue embedded between the tooth root and the alveolar bone socket, which is also derived from cranial NCCs (Chai et al., 2000), Like the dental pulp, PDL contains multipotent NCSCs with characteristics similar to MSCs as they are able to self-renew and differentiate into neural and mesenchymal lineages (Seo et al., 2004). Consistently, PDL-derived stem cells expressed marker genes of NCCs (*Notch1, BMP2*, *Snail1/2, Nestin,* and *TuJ1*), MSC (*CD44*, *CD90,* and *Vimentin*), and ESCs (*c-Myc, Klf4, Nanog*, and *Oct4*), suggesting that PDL may contain a pluripotent stem cell population. Under appropriate culture conditions, PDL could undergo pancreatic differentiation into

insulin-producing cells with endodermal lineage (Huang et al., 2009). In addition, PDL-derived stem cells could be directed to generate retinal progenitors with competence for photoreceptor differentiation, underlying their therapeutic potential in retinal cell regeneration (Huang et al., 2013). Importantly, both DPSCs and PDL-derived stem cells exhibited low immunogenicity as demonstrated in rodent (de Mendonca Costa et al., 2008), sheep (Mrozik et al., 2013), and swine models (Ding et al., 2010), which would make them promising candidates for allogeneic transplantation. Together dental pulp- and PDL-derived stem cells obtained from the readily accessible human teeth material possess powerful ex vivo expansion, versatile differentiation potential, and immunomodulatory ability, providing a rich source of cells for various tissues regeneration therapies.

- *Palatum*

The palate of adult rats was shown to have NC-related stem cells, which expressed markers characteristic of both NCSCs and pluripotent ESCs, and they propagated into neurosphere-like clusters that differentiated efficiently into neurons and glial cells. In addition, mRNAs of the four transcription factors (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) involved in iPS induction were detected in the human palate (Widera et al., 2009). These findings suggest dormant NC-related stem cells in the palate might be a possible donor source for iPS derivation, and also contribute to its highly regenerative capacity following wound injury (Kahnberg and Thilander, 1982). However, we still do not know if palate-derived stem cells originated from embryonic NCCs, if they can differentiate into other lineages besides neurons and glial cells, or if they can undergo self-renewal through multiple passages of neurospheres.

- *Oral mucosa*

Although it has been demonstrated that cranial NCCs contribute to the formation of mesenchymal tissues of the head and other craniofacial structures (Cordero et al., 2011), it was only recently confirmed that $Lgr5^+$ cranial NCCs contribute to the stromal cell population in oral mucosa lamina propria (OMLP) using *Lgr5-EGFP-ires-CreERT/Rosa26-Tomato* reporter mice. These $Lgr5^+$ cells expressed NCSC markers with the capacity for clonal growth and multipotent differentiation (Boddupally et al., 2016). Similar to the palate, the rapid healing capacity of oral mucosa and lack of scar formation could be due to the existence of a primitive NCSC-derived population in the OMLP. On the other hand, human (h) OMLP-derived cell populations exhibited rapid proliferative capacity, expressed ESC and

NCSC markers, and had pluripotent potential to differentiate into three germ layers in vitro. hOMLP-derived cells treated with dexamethasone (osteogenic inducer) formed tumors comprising of both mesodermal and ectodermal tissues when transplanted in severe combined immune deficiency mice, demonstrating their capacity to form mesodermal tissues in vivo. Some of the tissues formed in tumors were cranial NC derivatives including cartilage and bone (Marynka-Kalmani et al., 2010). These findings indicate that hOMLP harbors primitive stem cells and NCSC population. Another study demonstrated similar mesodermal and ectodermal lineages could be obtained from in vitro differentiation of hOMLP-derived pluripotent cells, although an in vivo differentiation assay was not performed (Davies et al., 2010). A recent study showed that cells isolated from excised human oral mucosa could form into spheres in neurosphere culture conditions, and were capable of self-renewal and expressed NC-related genes (Abe et al., 2016). These human oral mucosa-derived cells were multipotent and capable of differentiating into NC lineages in vitro and generated ectopic bone tissues in vivo, indicating they could be a potential source of cells for bone tissue regeneration.

**Development of experimental approaches to derive NCSCs from pluripotent stem cells and somatic cells**

***Human NC markers***

Although characterizations of NCSCs from embryonic and adult tissues in animal models have provided insights into their molecular and functional properties, little is known about the mechanisms of human NC development and the pathology of neurocristopathies. In humans, the NC starts to migrate before NT closure as early as embryonic stage 9, around the third to fourth week of gestation (O'Rahilly and Muller, 2007). Because of the ethical and scientific challenges involved in isolating human fetal NCCs for experimental manipulation, these transient structures have remained largely unexplored. Increasing efforts have focused on the derivation of NCCs from human (h) ESCs and iPSCs for use in defining the cellular and molecular mechanisms governing their specification, migration and differentiation, and as a cellular platform for disease modeling and drug screening. The expression of NC genes in different stages of human embryo development and in human NCC lines derived from pharyngulas have already been documented (Betters et al., 2010; Thomas et al., 2008). These studies revealed several commonly expressed NC genes among human and other animal models, such as neural plate border specifiers (*Pax7, Pax3, and Msx1*) and NC progenitors (*Sox9, Sox10, Snail2, and FoxD3*). These genes have been used as markers to identify both

mouse (m) ESC- and hESC/iPSC-derived NCCs (Milet and Monsoro-Burq, 2012). Consistently, cross-species comparison also showed extensive overlap among human, mouse, and avian NCC transcriptomes, though some molecular cascades appeared to be unique to humans or mice, which was linked to the plasticity of the NC lineages that engendered differences in the derivatives generated between species. In addition, the transcriptional profile of hNCCs possessed molecular signatures of hESCs, including but not restricted to the expression of transcription factors *SOX2, NANOG,* and *OCT4*, indicating hNCCs exhibited unique molecular and phenotypic features of pluripotent stem cells (Thomas et al., 2008). In agreement with this notion, a recent study in *Xenopus* demonstrated key NC regulatory factors were expressed in blastula animal pole cells and promoted pluripotency in both NC and blastula cells (Buitrago-Delgado et al., 2015), suggesting that NCCs may have evolved from a subset of blastula animal pole cells retaining activity of the regulatory network underlying pluripotency.

In chick embryos, HNK1 was found to be a marker for migrating NCCs (Tucker et al., 1984), but could only label a small population of $SOX10^{+}$ hNCCs. In contrast, $p75^{NTR}$ could broadly mark hNCCs and other non-NC cell types (Betters et al., 2010). Fluorescence-activated cell sorting (FACS) using antibodies against both surface markers has been used to enrich hNCCs differentiated from a mixed population of cells. In the past decade or so, many approaches have been established for the efficient generation of hNCCs and its derivatives from mESCs, hESCs, and hiPSCs (Kunisada et al., 2014; Milet and Monsoro-Burq, 2012). Here, we highlight key developments in the derivation protocols involving the use of stromal feeder-layers, an intermediate stage in the formation of neural rosettes or embryoid bodies (Bajpai et al., 2009; Elkabetz et al., 2008), and the manipulation of signaling pathways (Table 2, Fig. 2).

***Stromal cell-derived inducing activity (SDIA)***

Mizuseki et al. were first to establish the conditions to induce differentiation of mouse and primate ESCs into NC-like cells by co-culture with the mouse stromal cell line PA6 and exposure to BMP4 after 4 days of co-culture. These ESC-derived NC-like cells could be further differentiated into sensory and autonomic lineages with low (0.5 nM) and high (5 nM) concentrations of BMP4, respectively (Mizuseki et al., 2003). Using similar PA6 co-culture, hESCs were differentiated into hNCCs expressing characteristic marker genes (*SNAIL2* and *SOX9*) or into neural progenitors using noggin to inhibit BMP signaling, which could then be further differentiated into PNS derivatives such as sensory and sympathetic neurons (Brokhman et al., 2008; Pomp et al., 2005). The yield of peripheral sensory neurons was

further improved via the formation of hESC-derived neurospheres under non-adhesive conditions (Pomp et al., 2008). Combining the PA6 co-culture with FACS allowed enrichment of $p75^{NTR+}$ NCCs within 1 week. These $p75^{NTR}$-enriched cells formed neurospheres with self-renewal capacity and could be differentiated into multiple NC lineages including peripheral nerves, glia, and myofibroblastic cells. Importantly, these sorted cells migrated and differentiated into NC derivatives when transplanted into chick embryos (Jiang et al., 2009). Another co-culture study used the BM-derived stromal cell line ST2 to induce differentiation of mESCs into NC-like cells with melanocyte identity as an intermediate cell type after sorting against its cell-surface protein, c-Kit. A single c-$Kit^{+}$ cell was able to form colonies containing neurons, glial cells, and melanocytes, demonstrating their multipotency (Motohashi et al., 2007; Yamane et al., 1999).

***Neural rosettes***

Co-culture of hESCs with MS5 stromal cells resulted in the efficient differentiation of hESCs into neuroepithelial-like structures, termed neural rosettes, which expressed markers compatible with neural plate identity and showed extensive self-renewal capacity (Perrier et al., 2004). Neural rosettes replated on polyornithine/laminin-precoated dishes formed $p75^{NTR+}HNK1^{+}$ hNCSCs, which could be further expanded in the presence of FGF2/EGF and directed toward PNS lineages and mesenchymal lineages in serum free and serum conditions, respectively. Transplantation of enriched hNCSCs into developing chick and adult mouse demonstrated survival, migration, and differentiation compatible with NC identity (Hotta et al., 2009; Lee et al., 2007). However, some major drawbacks of these approaches were the use of stromal cell lines, in which the secreted factors in the conditioned medium were undefined, and the time-consuming nature of obtaining hNCSCs and their derivatives (~21-40 days). To resolve these issues, microarray studies were conducted that identified several growth factors including SHH, WNT5A, TGFβ, and IGF expressed by PA6 (Swistowska et al., 2010). The PA6-derived factors together with NC induction medium could efficiently induce differentiation of hESCs into neural stem cells via embryoid body formation, which subsequently formed $p75^{NTR+}$ hNCSCs in the periphery of the rosettes (Liu et al., 2012).

***Dual-SMAD inhibition***

The efficiency of the generation of hESC-derived neural progenitor cells (NPCs) was further improved without the need of a feeder layer, which was made possible by using specific SMAD signaling inhibitors (dual-SMAD inhibition) such as noggin and SB 431542 that

blocked BMP and Activin A/Nodal signaling, respectively. FACS analysis of the NPC population revealed the $PAX6^+$ cells exhibited anterior neural identity, whereas $PAX6^-$ cells expressed NC markers (Chambers et al., 2009; Curchoe et al., 2010). Consistent with a strong requirement for Wnt signaling in early NC induction (Garcia-Castro et al., 2002), concomitant activation of WNT signaling using the GSK3β inhibitor BIO combined with dual-SMAD inhibition resulted in diverting neural progenitor fate toward $p75^{NTR+}HNK1^+AP2^+$ NC-like cells. These cells could be clonally propagated and maintained for 25 passages, while retaining multipotency to differentiate into peripheral neurons and mesenchymal cell types (Avery and Dalton, 2016; Menendez et al., 2011). Subsequent optimization of the NC induction conditions revealed a narrow time window (starting at day 2 of the differentiation protocol) for adding CHIR99021 (Chir), a Wnt signaling agonist that selectively inhibits glycogen synthase kinase 3β (GSKβ) (Meijer et al., 2004), which was required for the induction of *SOX10::eGFP* reporter expression in hNCSCs (Lee et al., 2010). Delaying Chir treatment until day 4 of differentiation did not support NC specification, indicating loss of cell competency to NC-inductive WNT signals. Similar temporal suppression of BMP and TGFβ/Activin signaling by treatment with small molecule inhibitors at day 2 to 3 was sufficient to specify NC fate. Optimizing NC conditions by timed exposure to SMAD inhibitors and the Wnt agonist resulted in the induction of *SOX10::eGFP* expression detectable by flow cytometry on day 6 of differentiation and peaking by day 11. In addition, the yield of *SOX10::eGFP* positive cells was more than 20-fold (~53%) compared with the standard dual-SMAD inhibition protocol that only led to low levels of spontaneous NC induction (Chambers et al., 2009; Mica et al., 2013). This NC differentiation protocol also generated a small amount of melanoblasts (or melanocyte precursors, 9%) within the 59% $SOX10^+$ NC precursor population on day 11 of the differentiation. Treatment with BMP4 and endothelin 3 (EDN3) on day 6 of the differentiation not only enhanced melanoblast yield but also further directed their maturation (Callahan et al., 2016; Fang et al., 2006; Mica et al., 2013). A recent study has also established a specific melanocytic medium to directly differentiation of mouse NCSCs into melanocytes (Shakhova and Sommer, 2015). Moreover, hESC-derived $SOX10^+$ NCCs did not exhibit high levels of HOX gene expression, indicating their cranial NC identity (Mica et al., 2013). Addition of retinoic acid (RA) into the differentiation medium on day 6 shifted the regional NCC identity from anterior to posterior vagal fates as demonstrated by the expression of HOXB3 and HOXB5 characteristic of vagal identity (Chan et al., 2005; Fattahi et al., 2016; Fu et al., 2003). Under

these NC optimized conditions, hNCSCs were obtained in 11 days. Further refinement of this method using chemically defined medium (CDM) containing minimal growth factor (insulin) with inhibitors for TGFβ signaling and GSK3β, but not for BMP signaling, efficiently induced multipotent hNCSCs (70%-80%) from hESCs or hiPSCs within ~9 days. The induced hNCSCs expressed cranial NC marker genes and could differentiate into corneal endothelial cells. In addition, induced hNCSCs could be stably expanded in CDM supplemented with EGF and FGF2 for at least 10 passages without significant alteration of gene expression profiles (Fukuta et al., 2014). Further refinement simplified the approach by eliminating the use of dual-SMAD inhibitors, instead employing low-density cultures of dissociated hESCs in defined serum free-media under WNT activation, allowing robust induction of cranial NCCs expressing *SOX10* (~63%), *PAX7* (~78%), *TFAP2A* (~84%) and a panel of NC markers in just 5 days. In contrast to previous protocols, hNCCs generated by this method appeared to arise independently not from neural and mesodermal tissues but from precursors with an early pre-border state (Leung et al., 2016), suggesting these hNCCs were not derived from a neural origin. These findings were further supported by previous embryological evidence that NC formed from non-neural ectoderm did not acquire neural characteristics (Yardley and Garcia-Castro, 2012).

***SOX10-mediated reprogramming***

In addition to generating hNCSCs and their derivatives from hESCs and hiPSCs using various culture conditions, a recent major advancement using a single transcription factor, SOX10, allowed multipotent induced NCCs (iNCCs) to be generated from human postnatal fibroblasts or hESC-derived fibroblasts harboring the *SOX10::eGFP* reporter (Kim et al., 2014; Lee et al., 2010). Sox10 broadly marks all NCCs during development, and is essential for the maintenance of their multipotency, self-renew, survival, and lineage-specification (Britsch et al., 2001; Kim et al., 2003). Using lentiviral-mediated overexpression of SOX10 combined with extracellular matrix components (laminin/fibronectin) and epigenetic modifiers (Aza/VPA), human fibroblasts could be reprogrammed into iNCCs without going through the pluripotent intermediate state. Importantly, addition of Chir and BMP into the culture medium further increased the yield. These iNCCs had morphological and cellular features and expressed NC gene profiles comparable to hESC-derived NCCs. Using in ovo and ex vivo transplantation assays further demonstrated the ability of iNCCs to migrate and integrate into NC-derivatives, such as the DRG, sympathetic ganglion, and ENS (Kim et al., 2014). It should be noted, one major drawback of iNCC generation is that lentiviral

integration into the host genome may result in tumor formation, which would not be ideal for therapeutic applications. Nevertheless, the lineage conversion strategy generating multipotent iNCCs provides an accessible platform for studying human NC biology and the pathogenesis of neurocristopathies.

**Neural crest-associated diseases and stem cell therapy**

Genetic mutations causing defective migration, proliferation and differentiation of embryonic NCSCs are associated with several congenital disorders or neurocristopathies, many of which primarily affect pediatric patients. Because it is not possible to isolate fetal NCSCs in humans, hNCSCs harboring mutations associated with these diseases have been induced from patient-specific hiPSCs. This serves as a powerful cellular platform for disease modeling that can contribute to the understanding of the pathogenesis of these disorders and can serve as a viable cell source for transplantation therapy and drug screening. More importantly, since hNCSCs are derived from the patients, they can be used for autologous transplantation without immuno-rejection.

Previous studies in modeling melanocytic-specific disorders used patient-specific derived hiPSCs from three distinct genetic syndromes with defects in melanosome vesicle formation or trafficking (Callahan et al., 2016; Mica et al., 2013). By using the NC optimized conditions with timed exposure to dual-SMAD inhibitors and WNT agonist, patient-iPSCs could be directed into NCC fate and then into melanocyte precursors in the presence of BMP and END3 signaling. Although mature melanocytes were derived at comparable efficiencies from patients and control iPSCs, each of the independently derived iPS clones exhibited different degrees of pigmentation defects that faithfully recapitulated the disease-related phenotypes from the respective patients.

Familial dysautonomia (FD) is a neurodegenerative autosomal recessive disorder of the PNS and is characterized by autonomic dysfunction, including progressive loss of sympathetic and sensory neurons leading to gradually diminished pain and temperature sensations. The disorder is mainly caused by a germline point mutation in *ELP1/IKBKAP* (a subunit of the Elongator complex), leading to *IKBKAP* mis-splicing and marked reduction of IKBKAP protein expression (Axelrod, 2004). Conditional knockout of *Ikbkap* in mice NCCs caused aberrant neuronal differentiation and early neuronal death (George et al., 2013). To

investigate the underlying causes of FD, Lee et al. generated iNCCs from FD skin fibroblasts. They showed that FD-derived iNCCs expressed the surface marker CD34 that allows segregation of iNCCs from non-reprogrammed cells, but expressed reduced levels of wild-type IKBKAP and exhibited migration defects compared to the control iNCCs (Kim et al., 2014). Gene expression profiling studies also revealed common downregulated genes in both FD iNCCs and hiPSC-derived hNCCs that were involved in alternative splicing and cell migration. Consistently, aberrantly spliced transcripts of *MEF2C* and *PAX3*, genes involved in determining NC lineages (Bachinski et al., 2010; Wang et al., 2006), were detected in FD iNCCs (Lee et al., 2009). The plant hormone kinetin and the small molecule SKF-86466 were found to restore the expression of wild-type IKBKAP proteins and peripheral neuron markers lost in FD (Lee et al., 2009; Lee et al., 2012).

Another well-studied neurocristopathy, Hirschsprung's disease (HSCR), also called aganglionic megacolon, affects 1 in 5000 newborns (Amiel et al., 2008). Failure of enteric NCCs to migrate to the distal intestine results in the absence of NC-derived enteric ganglia along the variable length of the intestine, leading to intestinal obstruction with massive dilation of the proximal bowel. Depending on the variable length of the intestine affected, HSCR can be classified into Short-segment HSCR (S-HSCR) and Long-segment HSCR (L-HSCR) (Amiel et al., 2008; Brooks et al., 2005). In humans, ten HSCR susceptibility genes (*RET, SOX10, PHOX2B, EDBRB, END3, ECE1, ZFHX1B, GDNF, NRTN* and *KIAA1279*) have been identified (Heanue and Pachnis, 2007). Recent studies on zebrafish showed that embryos with transcription factor Meis3 knockdown exhibited colonic aganglionosis (Uribe and Bronner, 2015), suggesting that *Meis3* may contribute to the pathogenesis of HSCR, but this remains to be determined. The standard treatment for HSCR is surgical removal of the defective bowel, but for L-HSCR patients, the remaining length of bowel may not be sufficient for normal nutrient absorption. A recent alternative treatment for HSCR involves the generation of enteric progenitors from hESCs by adding RA into the NC differentiation medium (Fattahi et al., 2016) as described in (Mica et al., 2013). Enriched CD49D$^+$ (α4-integrin) ENS precursors were maintained in 3D spheroids before differentiating into enteric neurons in the presence of ascorbic acid and glial cell line-derived neurotrophic factor (GDNF) (Fattahi et al., 2016). Mature enteric neurons exhibited a broad range of neurotransmitter phenotypes including serotonin-positive, γ-aminobutyric-acid-positive, and nitric oxide synthase-positive neurons. In vivo transplantation of hESC-derived enteric

progenitors into chick embryos and adult mouse colon showed they could invade into the gut region, which confirmed their enteric NC identity. Importantly, engraftment of hESC-derived ENS progenitors increased survival rate of HSCR mice bearing mutations in *Ednrb,* which develop megacolon due to aberrant peristalsis (Gariepy et al., 1996). Pepstatin A, identified from a small-molecule screen, was shown to restore aberrant migration of *EDNRB*$^{-/-}$ hESC-derived enteric NC precursors both in the scratch assay and after transplantation into adult mouse colon (Fattahi et al., 2016), demonstrating the ability of this candidate therapeutic drug to rescue HSCR-related migration defects.

Bicuspid aortic valve (BAV) is a congenital heart disease where two of the aortic valvular leaflets fuse to form a bicuspid valve instead of a normal tricuspid valve. Patients with BAV are at a higher risk of developing thoracic aortic aneurysms (TAA) (Fedak et al., 2005; Michelena et al., 2011). The aneurysms associated with BAV commonly involve the ascending aorta but not the descending aorta. Fate-mappin g studies have demonstrated that cardiac NCCs contribute to the smooth muscle cells (SMCs) formation in the ascending aorta, while the descending aorta is populated by SMCs from the paraxial mesoderm (Majesky, 2007). A recent study demonstrated that iPSCs from BAV/TAA patients could be differentiated into NCCs with high efficiency, but they exhibited defective SMCs formation with impaired contraction, reduced TGF-β signaling and increased mTOR signaling. Inhibition of mTOR signaling pathway using rapamycin could restore the aberrant SMC differentiation (Jiao et al., 2016). These studies demonstrated the utility of patient-specific iPSC-derived NCSCs in defining the molecular basis of a disease process without knowing the underlying genetic defect responsible for the observed abnormality.

**Concluding remarks and future perspectives**

In the past two decades, advances in molecular biology and functional genomics have allowed us to gain a better understanding of how various molecules expressed in different NC developmental stages are functionally connected together to form a gene regulatory network that defines the formation, migration, and differentiation of NC progenitors. Such basic developmental studies are essential for several reasons. First, they provide insights into how genes evolved in the emergence of NCCs and their derivatives essential for studying the evolution of vertebrates, for example, through modeling the function of the head in terms of the shift from filter-feeders to active predators (Kerosuo et al., 2015). Second, increasing evidence suggests that genes involved in NC development are often dysregulated in NC-

derived tumors such as melanoma and neuroblastoma. Understanding the molecular mechanisms underlying NC formation, migration, and differentiation could shed light on the initiation and progression of NC-related tumors (Maguire et al., 2015). Third, they informs us about the identity of extrinsic and intrinsic molecules involved in expanding and maintaining hESC/iPSC-derived NCCs/iNCCs and adult tissue-derived NCSCs, and in directing their differentiation into specific lineages for use in cell and tissue replacement strategies. For example, a recent study demonstrated that chick crestosphere culture conditions could direct hESCs to form human crestospheres expressing NC markers that were able to differentiate into neural, glial, melanocytic, and mesenchymal tissues (Kerosuo et al., 2015). Fourth, they provide gene signatures that allow accurate assessment of the identity, regulatory, and developmental state of in vitro NCSCs/progenitors derived by direct differentiation from PSCs or by lineage reprogramming from somatic cells.

Although animal models have provided promising results for the development and potential use of NCSCs/progenitors for the treatment of many neurocristopathies, some key issues need to be addressed. The current differentiation protocols using *SOX10::eGFP* reporter or $p75^{NTR+}$/$HNK1^{+}$ enrichment tend to generate hNC precursors with cranial/anterior identity. Recent studies demonstrated that addition of RA into NC differentiation medium promoted posterior vagal NC identity (Fattahi et al., 2016). Similarly, addition of RA promoted specification of trunk NC progenitors, which could be further directed to sympathoadrenal lineage in the presence of BMPs (Huang et al., 2016). However, based on the marker expressions and the differentiation protocol used in this study, it was not clear if the generation of trunk NC progenitors and their derivatives was efficient and whether other lineages such as melanocytes and neurons were present in the culture. Further optimization of the differentiation protocol and identification of unique surface proteins are needed for the enrichment and purification of each NC-derived lineage, respectively. Recently, Simoes-Costa and Bronner used transcriptomics to reveal unique gene signatures between cranial and trunk NC in chick embryos that defined their axial identity. Such information would be important for further refinement of the culture conditions required for the differentiation of hESCs/iPSCs into axial-specific NC progenitors and their derivatives, particularly if they are used in cell therapies for various neurocristopathies.

Although the ability to generate patient-specific hiPSC-derived NCCs is a powerful tool for modeling the pathogenesis of neurocristopathies, these NCCs and their derivatives carry gene

mutations that are not ideal for therapeutic application. However, we can now genetically correct mutation in patient-derived iPSCs using CRISPR/Cas9, TALENs and ZFNs to restore the normal function of specific cell types derived from hNCCs. Even though we can obtain highly enriched and genetically corrected NC progenitors for a specific lineage, we still need to use animal disease models to assess the extent of their integration after transplantation to ensure the NC-derived cells are functionally stable in the long term. On the other hand, non-viral approaches such as plasmid-driven strategies and small molecule compounds can mediate lineage reprogramming of somatic cells into iNCCs that can prevent tumorigenesis after transplantation in the host. Overall, these endeavors will ensure hNCCs and their derivatives are clinically safe and can be functionally integrated into the host tissue for disease treatment.

Multipotent NSCSs have attracted increasing attention in the field of developmental and stem cell biology, because they can be easily obtained from various animal and human adult tissue sources for use in research and treatments. The advent of omics technologies and availability of high throughput drug screening platforms will be instrumental in expanding our understanding of the genetic regulation of hNCCs and their derivatives and will expedite the development of patient-specific therapies to treat neurocristopathies.

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**Table1**
List of abbreviations

| Abbrevations | | Abbrevations | |
|---|---|---|---|
| **(h/m)ESCs** | (Human/mouse) Embryonic stem cells | **iNCCs** | induced neural crest cells |
| **(h)iPSCs** | (Human) induced pluripotent stem cells | **KSPGs** | keratan sulfate proteoglycans |
| **(h)NCCs** | (Human) Neural crest cells | **MSC** | Mesenchymal stem cell |
| **(h)NCSCs** | (Human) Neural crest stem cells | **NA** | Not applicable |
| **(S/L-)HSCR** | (Short-segment/Long-segment-) Hirschsprung's disease | **N-Cad** | N-cadherin |
| **AA** | Ascorbic acid | **NPCs** | Neural progenitor cells |
| **BM** | Bone marrow | **NRG1** | Neuregulin1 |
| **BAV** | Bicuspid aortic valve | **NSPs** | Neurospheres |
| **CB** | Carotid body | **NT** | Neural tube |
| **CDM** | Chemically defined medium | **NTR** | Nerve growth factor receptor |
| **Chir** | Chir CHIR99021 | **OMLP** | Oral mucosa lamina propria |
| **CNS** | Central nervous system | **PD** | Parkinson's disease |
| **COPs** | Cornea-derived precursors | **PDL** | Periodontal ligament |
| **CSSCs** | Corneal stromal stem cells | **PNS** | Peripheral nervous system |
| **DPSCs** | Dental pulp stem cells | **RA** | Retinoic acid |
| **DRG** | Dorsal root ganglia | **SDIA** | Stromal-derived inducing activity |
| **EBs** | Embryoid bodies | **Sip1** | Smad-interacting protein 1 |
| **ECM** | Extracellular matrix | **SKPs** | Skin-derived precursors |
| **EDN3** | Endothelin 3 | **SMCs** | Smooth muscle cells |
| **EMT** | Epithelial mesenchymal transition | **SP** | Side population |
| **ENS** | Enteric nervous system | **TAA** | Thoracic aortic aneurysms |
| **FACS** | Fluorescence-activated cell sorting | **WP** | Whisker pad |
| **FD** | Familial dysautonomia | | |
| **GDNF** | Glial-cell-line-derived neurotrophic factor | | |
| **GSKβ** | Glycogen synthase kinase 3β | | |

Table 2
Derivation and characterization of NCSCs from pluripotent ESCs/iPSCs and skin fibroblasts.

| Pluripotent cell types | Protocols for NCSCs derivation | Brief description of methods | Markers for cell sorting | NC markers analyzed | NC-derived cell types generated | Remarks | References |
|---|---|---|---|---|---|---|---|
| mESCs (EB5) | SDIA | PA6 as feeder layer. Treatment with BMP4 from day 4 increased NCC population | No | Ap2, NCAM | Sensory and autonomic neurons (Brn3a$^+$/Peri$^+$; TH$^+$/Phox2b$^+$/Peri$^+$ ) | | (Mizuseki et al., 2003) |
| hESCs (HES-1, HUES, HUES 1) | SDIA | hESCs co-cultured with PA6 induced peripheral neuronal formation. | No | SOX9, dHAND, MSX1, AP2, SNAIL2 | Sensory and sympathetic neurons (Brn3a$^+$/Peri$^+$; TH$^+$/Peri$^+$ ) | PA6 cells induced NC-like cells before neuronal differentiation. | (Pomp et al., 2005) |
| hESCs (H1, H9) | SDIA | Mitotically inactivated MEF as feeder layer. Treatment with Wnt3a, SCF, and EDN3 induced melanocyte differentiation from hESCs-derived EBs on fibronectin | No | SOX10 | Melanocytes (PAX3$^+$, TYRP1$^+$, DCT$^+$, MITF$^+$) | Differentiated melanocyte showed proper localization and maker expression after grafting into reconstructed human skin. | (Fang et al., 2006) |
| mESCs (D3) | SDIA | ST2 as feeder layer. Treatment with RA promoted NCCs formation | c-Kit+/CD45- | Sox10, Pax3,Snail1/2 | Neuron (Tuj1$^+$), Glial Cells (GFAP$^+$), Melanocytes (MITF$^+$) | C-Kit+ cells showed cell renew capacity and were able to follow the NCCs migratory pathway after in vivo transplantation. | (Motohashi et al., 2007) |
| hESCs (HES1, HUES9) | SDIA | PA6 as feeder layer. | No | AP2/NCAM1 | Sensory neurons (Brn3a$^+$, Peri$^+$) Glial cells (PMP-22$^+$, Sox10$^+$) | Peripheral neurons and NC were observed after 1 week of co-culture. | (Brokhman et al., 2008) |
| hESCs (HUES7, HUES9) | SDIA | hESC co-cultured with PA6 generated NSPs expressing NCCs marker under non-adhesive conditions. | No | SNAIL1, SOX9, MSX1, PAX3 ,FOXD3,NCX/HOX 11L1 | Sensory neurons (Brn3a$^+$, Peri$^+$), Glial cells (GFAP$^+$) | PA6-induced hESC-derived NSPs promoted peripheral neuronal differentiation. | (Pomp et al., 2008) |
| hESCs (H9, H1) | SDIA | PA6 as feeder layer. FACS-based isolation was applied for NCCs enrichment. | p75$^{NTR+}$ | SNAIL1/2, HNK1, SOX10, AP2 and NCAM | Sensory neurons (Peri$^+$), Glial cells( GFAP$^+$), Myofibroblasts (SMA$^+$) | Transplantation of isolated NCCs in chicken and mouse showed proper migration, differentiation and proliferation. | (Jiang et al., 2009) |
| hESCs (HES3, HES4 and ENVY) | SDIA | PA6 as feeder layer. ROCK inhibitor treatment promoted NCCs formation. | No | HNK1, SOX8, SOX9,SOX10 | Enteric neurons (Hu$^+$), Glial cells (S100B$^+$) | Transplantation of isolated NCCs in chicken and mouse showed proper migration, differentiation and proliferation. | (Hotta et al., 2009) |
| hESCs (H9 (WA-09), RUES1-eGFP48, I-8) | SDIA Neural Rosette | MS5 as feeder layer. Treatment with BMP2/FGF2/EGF promoted NCC populations. Different growth factors and culture conditions were developed for NCC and its derivatives. | p75$^{NTR+}$/ HNK1$^+$ | SOX10, PAX3, AP2,ERBB3,SNAIL 2 | Neurons (Peri$^+$,Tuj1$^+$), Schwann cells (GFAP$^+$,MBP$^+$), Smooth muscles (SM22α$^+$,calponin$^+$), Chondrogenic cell (Collagen II$^+$, Aggrecan$^+$), Osteogenic cell (ALP$^+$,BSP$^+$) | Transplantation of hNCSCs into chick embryo and adult mouse hosts showed survival, migration and differentiation compatible with neural crest identity. | (Lee et al., 2007) |
| hESCs (H9, H1, and RU-01eGFP | SDIA Neural Rosette | MS5 as feeder layer. | FORSE-/ N-CAD+ | SOX10,PAX3,AP2, TWIST1,SNAIL2 | NA | Forse1$^-$ neural rosette cells expressed NC markers | (Elkabetz et al., 2008) |
| hESCs and iPS cells | SDIA Neural Rosette | MS5 as feeder layer. | HNK1+ | AP2 | Autonomic and sensory neurons (ASCL1$^+$,Tuj1$^+$, Brn3a$^+$,Peri$^+$) | Generation of NCCs and Peripheral neurons using FD patient-specific iPS cell. | (Lee et al., 2009) |
| hESCs (H9 and H14) | Neural Rosette | No feeder layer. Rapid neuralization of ESCs without Noggin and limited capacity for expansion (up to 5 passages). | No | SOX9 | NA | Committed neural progenitor cells are capable of differentiation into various CNS cell types and NC fates. | (Bajpai et al., 2009) |

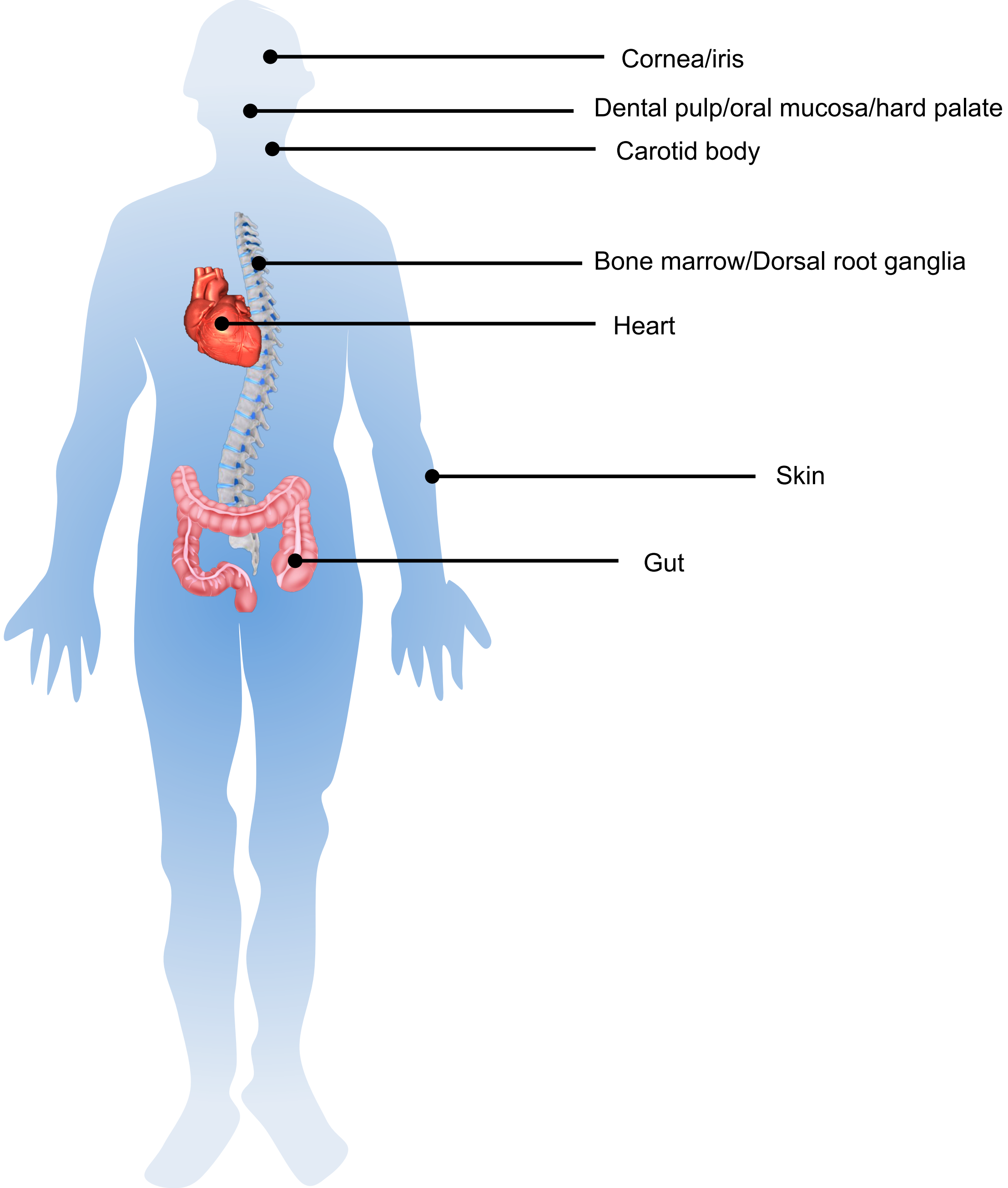
| Pluripotent cell types | Protocols for NCSCs derivation | Brief description of methods | Markers for cell sorting | NC markers analyzed | NC-derived cell types generated | Rearks | References |
|---|---|---|---|---|---|---|---|
| hESCs (H9) | Neural Rosette | No feeder layer. Emigrating hNCCs were obtained by plating of hESCs-derived neurospheres containing rosette structure on fibronectin. | No | PAX3/6,SOX9/10,p $75^{NTR}$, HNK1 | Autonomic neurons ($TH^{+}$/$Peri^{+}$), Sensory neurons ($BRN3a^{+}$/$Peri^{+}$), Smooth muscle ($SMA^{+}$), Melanocyte ($MelanA^{+}$), Glial cells ($PLP^{+}$/$GFAP^{+}$) | Inhibition of BMP and Wnt signaling re-specified NCSCs into CNS cells | (Curchoe et al., 2010) |
| hESCs (H9/14) and iPSCs | SDIA Neural Rosette | PA6 as feeder layer. Schwann cell differentiation was initiated by culturing hNCSCs in MesenPRO medium with 20ng/ml heregulin-β1 for 40 days. | $p75^{NTR+}$ | SOX9/10, $p75^{NTR}$, HNK1,CD44 | Neurons ($Hu^{+}$/$HNK1^{+}$), Schwann cells ($S100^{+}$/$P75^{NTR+}$) | Pure population of Schwann cells were generated and myelinated rat dorsal root ganglia neurons in vitro. | (Liu et al., 2012) |
| hESCs (WA-09) and iPSC lines (iPS-14/27) | Dual-SMAD Inhibition | Feeder layer and EBs formation were not required. Both SB431542 and Noggin induced rapid and complete neural differentiation. | $PAX6^{-}$ | AP2, HNK1,PAX7 ,$p75^{NTR}$ | Melanocytes ($HMB45^{+}$) | Manipulation of the initial hES-cell plating density skewed the ratio of $PAX6^{+}$ CNS and $PAX6^{-}$ NC-like cells. | (Chambers et al., 2009) |
| hESCs (WA-09) and iPSC lines (iPS-14/27) | Dual-SMAD Inhibition | Feeder layer was not required. NC differentiation from hESCs using defined NSB (KSR medium +SMAD inhibitors followed by N2) culture system | $p75^{NTR+}$/ HNK1+ | AP2, HNK1, PAX7,$p75^{NTR}$ | Autonomic Neuron ($MASH1^{+}$/$Peri^{+}$), Sensory neurons ($Brn3a^{+}$/Peri+), Schwann cells (GFAP+,Sox10+), Smooth muscle cells ($SMA^{+}$), Chondrogenic cell (Collagen $II^{+}$) | The yield of $p75^{NTR+}$/$HNK1^{+}$ precursors using the NSB protocol is comparable with the yield using the MS5 co-culture protocol. | (Lee et al., 2010) |
| hESCs and iPSCs | Dual-SMAD Inhibition | Exposure of hPSCs to WNT-activator (CHIR 99021) promoted $Sox10^{+}$ NC population formation by day 6. Subsequent treatment of BMP4 and EDN3 specified melanocytic lineage. | c-Kit+ /SOX10+ | SOX10,HNK1 ,$p75^{NTR}$ | Melanocyte ($SOX10^{+}$/ $MITF^{+}$) ,Melanosomal ($TYRP1^{+}$ /$PMEL^{+}$) | Melanocytes derived from patient-specific iPSCs reproduced pigmentation defects. | (Mica et al., 2013) |
| hESCs (H9) | Dual-SMAD Inhibition | Addition of RA promoted vagal NCCs formation. | $CD49D^{+}$ /$SOX10^{+}$ | HOXB2-9, FOXD3,TBX2,PAX 3,SOX10,TFAT2A/ B,PHOX2A/B | Enteric neurons ($NOS^{+}$,5-$HT^{+}$,$GABA^{+}$,$SSST^{+}$,$TH^{+}$,$CHAT^{+}$) | *In vivo* engraftment of hESC-derived ENC progenitors rescued HSCR mice. | (Fattahi et al., 2016) |
| hESCs (WA01, WA09) iPSCs (Y6 iPSC, RIV9 iPSCs) | Dual-SMAD Inhibition | Low-density of hESCs/iPSCs were cultured in defined serum free media under WNT activation. | No | SOX10, Pax7, TFAP2A | Chondrocytes, osteoblasts, peripheral neurons ($HuC/D^{+}$ (ELAVL3/4), $TUJ1^{+}$ (TUBB3) $PRPH^{+}$, $ISL1^{+}$), glial precursors ($S100\beta^{+}$ $GFAP^{+}$) and melanoblasts ($MITF^{+}$,$SOX10^{+}$) | hNCCs differentiated via an early pre-border state | (Leung et al., 2016) |
| *Sox10::eGFP* reporter hESCs | SOX10-mediated reprogramming | *SOX10::eGFP* fibroblasts were treated with dox-inducible lentiviruses containing SOX10, ECM, epiM and Chir 99021 or BMP4 | $Sox10::eGFP^{+}$ $CD34^{+}$ | HNK1, $p75^{NTR}$ FOXD3, AP2 | Neurons ($Tuj1^{+}$, $Peri^{+}$, $BRN3A^{+}$, $MAP2^{+}$, $Syn^{+}$), Schwann cells ($GFAP^{+}$/$S100B^{+}$/$PRX^{+}$, $MPZ^{+}$), Smooth muscle ($SMA^{+}$), Melanocytes ($MITF^{+}$,$TYRS^{+}$), chondrocytes (Collagen $II^{+}$, $Aggrecan^{+}$), adipocytes ($PPAR^{+}$), osteocytes ($ALP^{+}$, $BSP^{+}$ | FD patient fibroblasts-derived iNC cells displayed defects in cellular migration and alternative mRNA splicing. | (Kim et al., 2014) |

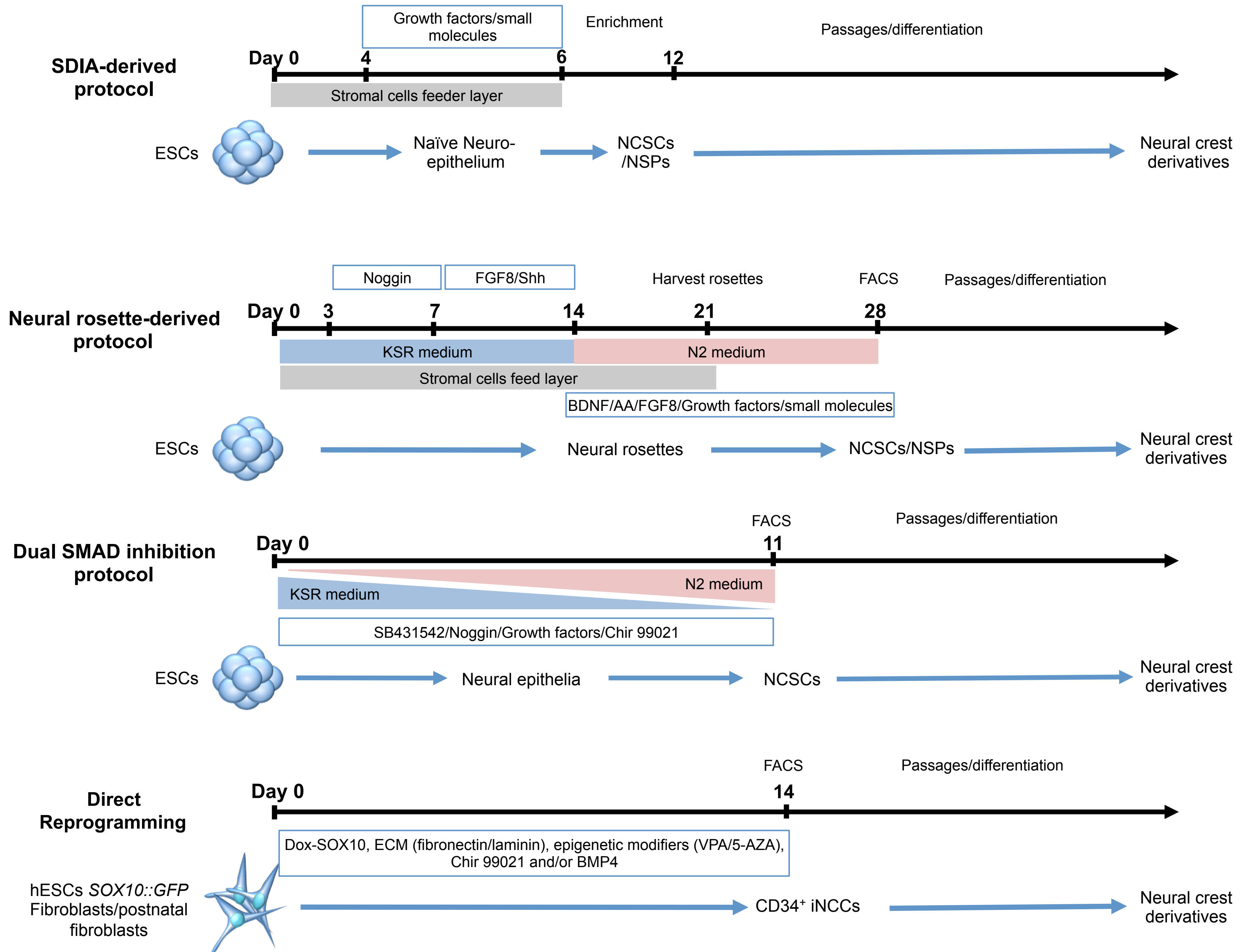
NA: Not applicable. Differentiation of NCCs into different lineages was not demonstrated.

## Figure Legends

**Figure 1**. Schematic diagram showing different adult tissue sources from which NCSCs are derived.

**Figure 2**. Schematic illustration of four different protocols for the derivation of NCSCs from pluripotent stem cells and skin fibroblasts.


Cornea/iris
Dental pulp/oral mucosa/hard palate
Carotid body
Bone marrow/Dorsal root ganglia
Heart
Skin
Gut



SDIA-derived protocol
Growth factors/small molecules
Enrichment
Passages/differentiation
Day 0
4
6
12
Stromal cells feeder layer
ESCs
Naïve Neuro-epithelium
NCSCs /NSPs
Neural crest derivatives
Neural rosette-derived protocol
Noggin
FGF8/Shh
Harvest rosettes
FACS
Passages/differentiation
Day 0
3
7
14
21
28
KSR medium
N2 medium
Stromal cells feed layer
BDNF/AA/FGF8/Growth factors/small molecules
ESCs
Neural rosettes
NCSCs/NSPs
Neural crest derivatives
Dual SMAD inhibition protocol
FACS
Passages/differentiation
Day 0
11
N2 medium
KSR medium
SB431542/Noggin/Growth factors/Chir 99021
ESCs
Neural epithelia
NCSCs
Neural crest derivatives
Direct Reprogramming
FACS
Passages/differentiation
Day 0
14
Dox-SOX10, ECM (fibronectin/laminin), epigenetic modifiers (VPA/5-AZA), Chir 99021 and/or BMP4
hESCs SOX10::GFP
Fibroblasts/postnatal fibroblasts
CD34+ iNCCs
Neural crest derivatives