CHAPTER 13

Neural Crest Stem Cells

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Abstract

Stem cells are defined by their ability to both self-renew and give rise to multiple lineages in vivo and/or in vitro. As discussed in other chapters in this volume, the embryonic neural crest is a multipotent tissue that gives rise to a plethora of differentiated cell types in the adult organism and is unique to vertebrate embryos. From the point of view of stem cell biology, the neural crest is an ideal source for multipotent adult stem cells. Significant advances have been made in the past few years isolating neural crest stem cell lines that can be maintained in vitro and can give rise to many neural crest derivatives either in vitro or when placed back into the context of an embryo. The initial work identifying these stem cells was carried out with premigratory neural crest from the embryonic neural tube. Later, neural crest stem cells were isolated from postmigratory neural crest, presumably more restricted in developmental potential. More recently it has been demonstrated that neural crest stem cell progenitors persist in the adult in at least two differentiated tissues, the enteric nervous system of the gut and the whisker follicles of the facial skin. In all cases, the properties of the stem cells derived reflect their tissue of origin and the potential of the progenitors becomes more restricted with age. In this chapter we will review this work and speculate on future possibilities with respect to combining our knowledge of neural crest gene function in the embryo and the manipulation of adult neural crest stem cells in vitro and eventually in vivo.

Neural Crest Stem Cells

At the onset of migration, neural crest (NC) cells are a heterogeneous mixture of cell populations with extensive proliferative and developmental potential. Later, the postmigratory crest appears either fate-restricted, highly committed or unipotent. However, there are some limited number of long-term pluripotent progenitor cells derived from the embryonic NC that persist in the adult. These rare cells have the capacity for both self-renewal and generation of multiple differentiated progenies in vitro, and are therefore bona fide stem cells. They exist in several NC-derived tissues in both the embryo and postnatal animal and represent an enticing source of stem cells with potential for therapeutic applications. Figure 1 illustrates the location of these progenitors at different stages and in specific locations of the developing mouse embryo that will be referred to throughout.

Identification of Neural Crest Stem Cells (NCSCs)

Self-renewing multipotent NCSCs were originally isolated by Stemple and Anderson from premigratory neural crest by taking advantage of expression of the low affinity nerve growth

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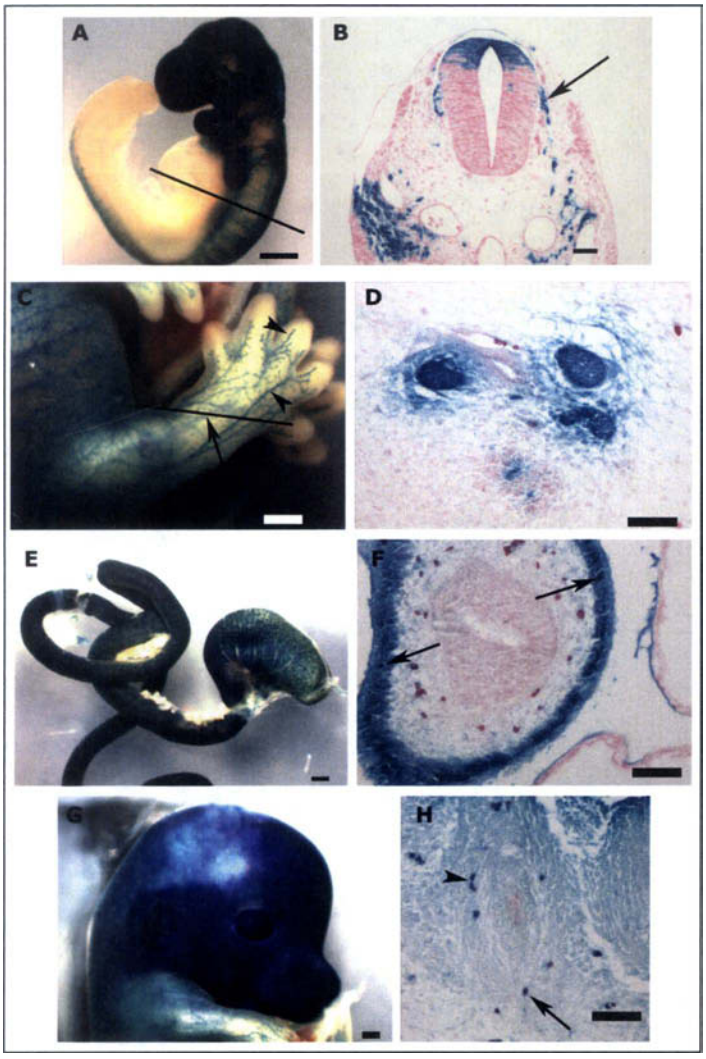

Figure 1. *Wnt1-Cre; Rosa26R lacZ* positive cells during different stages of mouse embryogenesis illustrate the various NC lineages from which NCSCs have been generated. A,B) At 9.5 dpc early migrating NC is exiting the neural tube and populating the branchial arches. Cells such as those indicated with the arrow in panel B are the ones that Stemple and Anderson isolated in vitro to first culture NCSCs.[1] C) At 14.5 dpc the peripheral nervous system has extended well into the developing limbs. The developing sciatic nerve is internal and indicated by the arrow. Arrowheads indicate superficial peripheral nerves. D) A section through the hindlimb of a 14.5 dpc embryo counterstained with eosin shows a developing sciatic nerve similar to that which Morrison et al isolated NCSCs.[5] E,F) At 17.5 dpc the entire digestive tract is populated with NC-derived enteric nervous system. This is the region from which gut derived NCSCs are isolated.[25] A section from a 14.5 dpc gut in F shows the location of the NC in the outer layers of the myenteric plexus and not in the gut epithelium. G,H) The 14.5 dpc facial skin has a very high contribution of NC cells. Some NC derived cells are found near the bulge region of the whisker follicles (arrowhead in H) while some are found near the dermal papilla (arrow in H). There are many other NC-derived cells scattered throughout the skin. These cells are the progenitors for NC-derived facial SKPS and eNCSCs.[26,31] Scale bars are equal to 0.5mm in A, C, E and G and 0.05 mm in panels B, D, F and H.

factor receptor (also known as p75 or p75NTR) and nestin in a limited population of cells.[1] The p75 neurotrophin receptor binds several related growth factors: nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4. This receptor has been demonstrated to play an important role in modulating the susceptibility of specific cell populations to programmed cell death (reviewed in ref. 2). Nestin, an intermediate filament expressed in neuroepithelial stem cells is often expressed in multi-lineage progenitor cells and differentiation of the cells is associated with loss of immunoreactivity to nestin. Embryonic rat neural tubes (E10.5) were placed in culture and cells that migrated away from the neural tube (presumably the premigratory NC initiating migration) were isolated either by fluorescence-activated cell sorting (FACS) for p75 expression or manually. Serial subcloning of cells and extended culture times demonstrated that these cells underwent self-renewal in vitro. Prolonged time in culture revealed that NCSCs were multipotent based on their ability to differentiate into peripheral neurons, Schwann cells and smooth muscle cells as assessed by expression of lineage specific markers.[1,3]

Molecular Control of NCSC Differentiation

Multiple factors affect either the self-renewal and/or the multipotency of NCSCs; addition of fetal bovine serum (FBS), containing a myriad of uncharacterized growth factors, causes NCSCs to differentiate. Alterations in the substrate molecules fibronectin (FN) and poly-D-lysine (pDL) influence cell fate. The addition of bone morphogenic proteins 2 and 4 (Bmp2 and 4) induces neurogenesis in NCSCs, transforming growth factor beta molecules (Tgfβ1, 2 and 3) promote smooth muscle differentiation, and glial fates are observed by adding Neuregulin I (NrgI; also called glial growth factor 2) to the culture medium.[1,3,4]

While these initial experiments demonstrated that premigratory NC contained multipotent progenitors, later work by Sean Morrison in Anderson's laboratory showed that the postmigratory embryonic NC also contained progenitors with similar potential.[5] Sciatic nerves from late gestation rat embryos (up to E17.5) contained NCSCs that gave rise to neurons, glia (Schwann cells) and myofibroblasts, but the potential of the progenitor cells decreased with increasing embryonic age (E14.5 compared with E17.5). Expression of p75 and the lack of expression of P_0, a peripheral myelin protein normally expressed by Schwann cells, was used to enrich the NCSC progenitors, although the populations are still not pure and the precise in vivo location of the progenitors is still not identified.

Strikingly, however, NCSCs isolated from sciatic nerve respond differently to Bmp2 than do premigratory NCSCs from the neural tube, presumable reflecting potency changes in the cells.[6] Other non-NC stem cell populations require Tgfβ related molecules to remain undifferentiated and self-renewing: Bmp4 is sufficient to derive embryonic stem cells (ES cells) and Nodal and/or Activin is required to derive trophoblast stem cells (TS cells),[7-10] but in the case of NCSCs it appears that Bmp2 and/or Bmp4 promote neuronal differentiation of progenitors. *Bmp2-/-* embryos contain fewer NC cells in vivo than wild type,[11] however, NCSCs have not yet been isolated from *Bmp2-/-* embryos to determine if they have defects in neurogenesis. Genetic evidence from mouse embryos carrying a mutation in *Bmpr1a*, a Bmp2/4 type I receptor normally expressed in the dorsal neural tube, showed a requirement for Bmp signaling in the NC cells that form the outflow tract of the heart,[12] but again, the role of this receptor has not been examined specifically in NCSCs.

This observation that NCSCs from different sources respond differently to growth factors was reinforced by Morrison's observations that NCSCs isolated from embryonic gut (the enteric nervous system) versus sciatic nerve respond differently to Bmp4; both types of NCSCs form neurons when exposed to Bmp, but gut NCSCs were 5 to 10 fold more responsive to Bmp4 than NCSCs from the sciatic nerve.[13] However, gut NCSCs are not simply more sensitive to all growth factors. Gut NCSCs were less likely than sciatic nerve NCSCs to form glia at lower concentrations of the Notch ligand Delta. A similar response was seen with Neuregulin, which activates members of the Epidermal Growth Factor Receptor/ErbB family of

proto-oncogenes. This is perhaps expected, since progenitor cells in the sciatic nerve would be primarily involved in forming glial cells while NC progenitors of the enteric nervous system are more likely to form neurons, but it is important to note this bias of NCSCs from different regions of the embryo to differentiate into terminal fates. In fact, it is known that between 10.5dpc and 14.5dpc, neural crest stem cells (NCSCs) become increasingly sensitive to Delta.[14] In vivo evidence also supports the observation that not all NCSCs are equal; when NCSCs from either gut or sciatic nerve were transplanted into the neural crest migratory stream of the chick embryo, gut NCSCs gave rise primarily to neurons while sciatic nerve NCSCs gave rise exclusively to glial cells.[13] These data support the hypothesis that postmigratory NCSCs are not equivalent and that both the stem cell niche and age of the cells influence the multipotency of these progenitor cells. However, these data do not distinguish whether these effects are cell autonomous and/or if the qualities of the NCSC niche are changing over time.

There is considerable interest in the molecules that regulate stem cell maintenance and inhibit differentiation. Microarray analysis of gut-derived NCSCs has generated a list of many transcription factors, secreted ligands and cell surface receptors that are certainly involved in these processes.[15] One of these is the HMG transcription factor Sox10. *Sox10* mRNA was almost 13 times more abundant in gut NCSCs than in the fetus as a whole. Sox10 is expressed in migrating NC cells, then maintained in the glial lineage while downregulated in NC-derived neurons and required for the differentiation of peripheral glial cells.[16] Sox10 plays multiple roles in NC development and it is suggested that it may be especially important for NCSC maintenance. Anderson's lab has shown that Sox10 can override the effect of soluble lineage restricting growth factors on NCSC; both neurogenic and gliogenic differentiation capacity is maintained despite exposure to Bmp2 and/or Tgfβ. At the same time, higher levels of Sox10 inhibit both neuronal and smooth muscle differentiation, thereby maintaining multipotency of the cells.[17] Another transcription factor, Erm, a member of the Pea3 subfamily of Ets domain proteins, is expressed in multipotent neural crest progenitor cells and glial cells. Blocking Erm function in NCSCs did not disrupt differentiation or survival but it severely impeded proliferation of the cells.[18] The basic helix-loop-helix protein Twist is required within the first branchial arch for the proper migration of NC cells into the arch. Later, Twist is also required for normal differentiation of first arch derivatives into bone, muscle, and teeth, but it has not been investigated if this protein is similarly required for differentiation of NCSCs.[19] Mouse mutants in both Snail and Foxd3, two proteins expressed in early NC cells that have been shown to play a role in NC formation in Xenopus and chick, die before the NC can be specified so tissue-specific deletions will be required to address the role of these transcription factors in the NC and in NCSCs.[20,21] Curiously, a mutation in the Snail-related transcription factor Slug has no phenotype in the NC and it is likely that the expression of Snail may compensate for the mutated *Slug* gene.[22]

As discussed above, NCSCs have different responses to growth factors based on the location and age of the progenitor cells. However, like central nervous system neural progenitors, NCSCs require bFGF to remain undifferentiated. Although the stem cell culture medium is fairly well defined (no serum is present) there is a high percentage (15%) of chicken embryo extract added that provides many undefined factors. Therefore, the minimally required factors to maintain the stem cell characteristics of NCSCs are still unknown. Many other stem cell populations utilize Wnt signaling to control proliferation, thereby expanding the stem cell pool and maintaining the transit amplifying pool of progenitors. However, NCSCs are unlike these stem cell populations in their requirement for the Wnt signaling pathway. Wnt signaling in migrating NC cells does not affect the population size; instead, β-catenin activation by Wnt signaling promotes differentiation of sensory neurons at the expense of other neural crest derivatives.[23] However, Wnt1, in combination with Bmp2, maintains p75 and Sox10 expression in NCSC cultures and inhibits differentiation of the cells.[24] Therefore, much is left to explain in terms of the minimal requirements for secreted signals, and it is likely to be a combination of factors that will change with respect to the location of the progenitor cells.

Postnatal NCSCs, Disease and Cell Therapy

Further work from Morrison's group showed that postnatally, sciatic nerve derived progenitors are either not present or at least not receptive to the same culture conditions. However, gut-derived NCSCs progenitors exist in the gut as late as postnatal day 110 in the rat.[25] These gut NCSC progenitor cells change their cell surface markers over time; E14.5 cells are p75+, α4integrin+, while the postnatal gut progenitors are largely p75+, α4integrin-. But more importantly, their ability to self-renew and differentiate into tyrosinase hydroxylase (TH) or dopamine-β-hyroxylase (DβH) expressing neurons decreases substantially with age.[25]

The link between stem cell function in vivo and stem cell dysfunction in disease is well illustrated in the case of NCSC defects in Hirschsprung's disease which is caused by a failure of the enteric nervous system to innervate the gut properly. Microarray analysis of gut NCSCs revealed elevated expression of several of the genes linked to Hirschsprung's disease including *Ednrb*, *Gfra-1*, *Ret*, and *Sox10*. NCSCs from *Ret-/-* mouse embryos were examined and it was confirmed that the anterior-most NCSCs in the esophagus were normal while the more posterior NCSC progenitors in the stomach and intestine were reduced in number.[15] The ability to isolate and characterize NCSCs from other mouse models of NC disorders may reveal more about the genes required to maintain this progenitor pool in the embryo and control their differentiation in the adult. So far it appears that the stem cell potential for postnatal NC-derived tissues is far less than that of embryonic tissue, but this raises the question of why these progenitors exist at all in the postnatal setting. Perhaps they are an accessible source of progenitor cells to repair tissues subject to either injury or degeneration, or perhaps they are simply remnants of embryogenesis. It is also possible that these progenitors are the stem cells of origin for neuroblastomas, one of the most common pediatric tumors that originates from the neural crest.

The most potent NCSCs discussed so far originate from internal tissue sources and embryonic and/or early postnatal ages, making them relatively impractical for cell therapeutic approaches. Embryonically derived stem cells are difficult to obtain and are fraught with ethical and political controversies and restrictions, so the development of adult sources for stem cells is ideal for therapy. The most accessible tissue of the body and one in which most patients easily tolerate biopsy is the skin. Freda Miller's group demonstrated that it was possible to culture self-renewing, multipotent cells from the dermis of rats, mice and human.[26-28] These SKP cells (for **sk**in derived **p**recursor cells) are different from previously described NCSCs; they are p75$^-$, they require Fgf2 and Egf in the culture medium, and they grow as nonadherent spheres similar to central nervous system-derived neural stem cells. However, many of their characteristics are similar to both NC and NCSCs; they can differentiate into neurons, glia and smooth muscle, are more easily generated from embryonic versus adult skin and express a number of NC specific molecular markers: *Slug*, *Snail*, *Twist*, *Pax3* and *Sox9*. The SKP progenitors were not present in embryonic skin before embryonic day 14, coincident with the time that the NC derived peripheral nerves populate the skin. Although all regions of the skin have progenitors that generate SKPs, Miller's group showed conclusively that SKPs isolated from facial dermis are derived from the NC.[26] This was accomplished by indelibly marking the NC with *lacZ* using a *Wnt1-Cre* transgene in combination with the *R26R* reporter allele activated by Cre expression.[29,30] SKPs derived from facial dermis expressed beta-galactosidase and are therefore NC-derived. Miller's group went on to show that cells in or near the dermal papilla of both hair and whisker follicles in the face contain the NC-derived cells that are presumably the SKP progenitors.[26] However, Sieber-Blum and colleagues demonstrated that similar NC-derived progenitor cells give rise to what they termed eNCSC (for epidermal NCSC) and reside in the bulge area of the hair and whisker follicles.[31] It is not apparent what the differences are between these two populations, but it is clear that both groups are studying NC derived adult stem cells. SKPs have been derived from both adult human scalp samples and juvenile foreskin samples,[27,28] and the human SKPs can generate neurons, glia and smooth muscle, and maintain a normal karyotype for over a year in culture. It is intriguing to imagine the possibility of using these multipotent human NCSCs to generate differentiated cells for therapeutic uses.

The discovery of NCSCs in postnatal skin raises the possibility that other NC niches might contain progenitors that could be readily available for isolation, manipulation and potential therapeutic uses. One tantalizing and very accessible postnatal niche is the tooth. Progenitor cells have been isolated from human teeth; these so-called SHED cells (for stem cells from human exfoliated deciduous teeth) grow as multipotent spheres in vitro. SHED cells express markers common to mesenchymal stem cells (Stro-1 and CD146) and can differentiate not only into odontoblasts in vitro and in tumor models, but also into neurons and glia in vitro and when transplanted into the dentate gyrus of a mouse hippocampus.[32] Lineage labeling using the *Wnt1-Cre*; *Rosa26R* mice, as described above, revealed that the dental pulp originates from the NC, so it is possible that SHED cells also derive from NC.[29] This remains to be tested, but it is possible that such an accessible source of postnatal stem cells would be readily adaptable for cell therapies. Our knowledge of the factors that control the specification, migration, patterning and multipotency of the embryonic NC, when combined with the experience of stem cell biologists should lead to promising avenues of exploration and hopefully, to the development of effective stem cell therapies in the future.

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