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In vitro and in vivo differentiation of boundary cap neural crest stem cells into mature Schwann cells

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Abstract

Boundary cap cells can generate neurons as well as peripheral glia during embryonic development (Maro, G.S., Vermeren, M., Voiculescu, O., Melton, L., Cohen, J., Charnay, P., Topilko, P., 2004. Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci. 7 (9), 930–938), and, recently, the boundary cap was shown to contain multipotent stem cells (Hjerling-Leffler, J., Marmigère, F., Heglind, M., Cederberg, A., Koltzenburg, M., Enerbäck, S., Ernfors, P., 2005. The boundary cap, a source of neural crest stem cells generating multiple sensory neuron subtypes. Development. 132 (11), 2623–2632). The ability of stem cells to generate mature functional glial phenotypes has not been addressed. In this study, we have explored the competence of boundary neural crest stem cells (bNCSCs) to differentiate into mature functional Schwann cells (SCs) in vitro and in vivo. bNCSCs failed to differentiate into SCs in vitro when cultured in a defined media and in vivo when grafted into adult rat sciatic nerves. However, in the presence of neuregulins, during long-term cultures, the majority of bNCSCs differentiated into SCs. After analysis of the in vivo expression of Sox2, Sox10, S100, GFAP, fibronectin and Krox20 in the glial lineages, we used these markers to characterize differentiation of the bNCSCs. Gliogenesis of bNCSCs proceeded similar to that in vivo by sequentially adopting a SC precursor and immature Schwann cell before maturing into myelinating and non-myelinating SCs. In co-culture with explanted dorsal root ganglia (DRG) as well as in vivo in transplants to the axotomized sciatic nerve, these bNCSC-derived SCs myelinated axons as shown by ensheathing of neuronal processes and expression of myelin basic proteins (MBP). These results show that, under appropriate conditions, bNCSCs can generate mature SCs that are functional and can myelinate axons in regenerating nerves.
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Introduction

Neurons regenerate axons if they are surrounded by a permissible environment for growth, and, if provided with myelinating cells, nerve conduction can also be restored. Much attention has been focused on the identification of suitable substrates and interventions to achieve regeneration and remyelination. The neural-crest-derived SCs have attracted particular interest as a source of cells for cell-based therapies because they are myelin forming cells that support axonal regrowth. Consistently, SCs have been successfully used in experimental models for nervous system regeneration and demyelinating diseases (Honmou et al., 1996; Stangel and Hartung, 2002; Takami et al., 2002). However, their clinical use is limited because of the limited availability of cells (Mimura et al., 2004).

Lineage tracing and transplantation experiments have shown that the migrating neural crest is multipotent and can generate both neurons and glia. The commitment to specific fates is a continuous process starting in the migratory cells and continuing at the sites of differentiation (Anderson, 2000; Le

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Douarin, 1986). Both neurons and Schwann cells can be generated from self renewing NCSCs isolated from the migrating neural crest or from tissues derived from it such as the sciatic nerve and the enteric nervous system (Anderson, 1997; Bixby et al., 2002; Morrison et al., 1999; Rao and Anderson, 1997). Both in vivo during neural crest migration and in vitro, multipotent NCSCs as well as glial committed precursors express the high mobility group transcription factor Sox 10. Differentiation into glial cells may be a consequence of a lack of instruction of commitment to a neuronal fate, a hypothesis consistent with the role of Sox10 for multipotency of the migrating neural crest as well as its requirement for glial specification (Britsch et al., 2001; Kim et al., 2003; McKeown et al., 2005; Paratore et al., 2001).

Glial differentiation depends on the intrinsic competence to respond to extrinsic signals favoring glial development including neuregulins (NRGs) and Notch (Shah et al., 1994; Morrison et al., 2000) and the lack of signals such as BMP that inhibits glial maturation and instructs a neuronal fate (Dore et al., 2005; Shah et al., 1996). NRGs play an important role during gliogenesis that extends beyond cell fate commitment. During development, SCs are precisely matched to the axons that they support. This is controlled by the presence of axonal β-neuregulins delivered in an anterograde fashion (Meyer et al., 1997). NRGs are a family of growth factors which include several alternatively spliced forms, arising from a single gene (Marchionni et al., 1993). NRGs suppress neuronal differentiation and promote a glial fate on progenitors at the expense of other fates, that is, it instructs a glial fate (Dong et al., 1995; Shah et al., 1994). NRGs are important for the initial establishment of the SC lineage, and, in neuregulin null mutant embryos, SC precursors fail to develop normally (Meyer and Birchmeier, 1995; Riethmacher et al., 1997). NRGs are also critical for maturation and survival of the SC lineage (Chen et al., 2003; Garratt et al., 2000; Jessen and Mirsky, 1999, 2002; Zorick and Lemke, 1996) and are key factors for a normal myelination process (Garratt et al., 2000; Michailov et al., 2004).

A subset of neural crest cells contributes to a neural-crest-derived structure called the boundary cap (Altman and Bayer, 1984). These cells migrate specifically to the axonal entry and exit points of the spinal cord and provide an interphase between the central nervous system and the periphery (Niederlander and Lumsden, 1996) and participate in the entry of sensory and exit of motor axons (Golding and Cohen, 1997; Vermeren et al., 2003). Boundary cap cells express Sox10, as other neural crest cells do, but they have the unique property of expressing Krox20 before starting migration within the dorsal or ventral roots that they themselves contribute to. In a recently study by Maro et al. (2004), using gene targeting to trace the lineage of the Krox20 expressing cells, it was shown that approximately 5% of the dorsal root ganglion neurons are derived from the boundary cap. The boundary cap contains multipotent stem cells (Hjerling-Leffler et al., 2005) that in vivo differentiate into both kinds of peripheral glia: Schwann cells and satellite cells (Maro et al., 2004). While Schwann cells are abundant in peripheral nerves, satellite glial cells are exclusively found in sensory ganglia, interacting with neuronal somata. The bNCSCs express neural crest stem cells markers, self-renew and can generate glia, sensory neurons and smooth-muscle-like cells in vitro (Hjerling-Leffler et al., 2005). Since cells of the boundary cap proliferate throughout embryogenesis (Altman and Bayer, 1984) and there is only a defined number that differentiate into neurons, the majority presumably acquire glial fates.

In previous studies of stem cell differentiation into a peripheral glial fate, it has not been addressed whether these can generate mature myelinating Schwann cells in vitro or in vivo (Morrison et al., 2000; Shah et al., 1994). In the present study, we have examined the potential of in vitro propagated bNCSCs to differentiate into SCs and studied the ability of the bNCSC-derived SCs to myelinate peripheral axons in vitro and in vivo.

Materials and methods

Isolation and propagation of bNCSCs

The boundary cap NCSCs were isolated as previously described (Hjerling-Leffler et al., 2005). In brief: E11 DRGs from the Rosa26 mouse (ubiquitously expressing lac-Z) and their roots and boundary caps were microdissected out by using sharpened tungsten needles. For dissociation, tissue was incubated 20 min in collagenase/dispase (1 mg/ml, Roche) and DNAse (0.5 mg/ml, Sigma) in N2 medium (DMEM/F12 medium with N2 supplement) with B27 supplement (Gibco). Cells were mechanically dissociated and plated in the above medium supplemented with EGF and bFGF (both 20 ng/ml, R&D Systems; propagation medium).

Differentiation cultures

bNCSC clones were dissociated using collagenase/dispase plus DNAse for 15 min, and the single cells were plated (3×10^3 cells/cm^2) on cover slips (Menzel, Germany) coated with 1/20 of growth factor reduced Matrigel (BD Biosciences). For 5-h cultures, cells were kept in propagation medium. In the 5.5-day condition, N2 medium was supplemented with B27, heparin (5 μg/ml, Sigma), laminin (1 μg/ml, R&D Systems), forskolin (5 μM, Sigma), NGF (10 ng/ml, Promega), BDNF (10 ng/ml, Promega), NT3 (10 ng/ml, Promega) and GDNF (10 ng/ml, Promega). When indicated, medium was supplemented with the EGF domain of human-recombinant β1-heregulin (125 ng/ml, Sigma). The differentiation medium of 30-day cultures contained: N2, B27, heparin (5 μg/ml), laminin (1 μg/ml) and forskolin (5 μM). On day 8 of incubation, NGF (30 ng/ml) was also added and, 3 days later, ascorbic acid (50 μg/ml, Sigma). When indicated, medium was supplemented with β1-heregulin (125 ng/ml from day 0 to 8 and 50 ng/ml from day 8).

Schwann cell morphology in vitro was defined as thin and very elongated cells, with 2 or 3 main processes, which present oval blunt-ended nuclei oriented longitudinally relative to its main long axis and are more frequently grouped in clusters of cells parallel to each other, in pavement-like arrangements.

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