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Schwann Cells in Neuroblastoma

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Why should we consider Schwann cells when we are interested in the biology of neuroblastomas (NBs)? Although we are familiar with the term "stroma-rich" NB, we basically think of a favourable prognostic subgroup, histologically distinguished by the development of a prominent Schwann cell-stroma. According to current opinion on the maturation processes in NBs, the NB-associated Schwann cell is believed to represent a differentiation product of the NB cell, and we therefore do not envisage the Schwann cell as having any important role in NBs. However, our interest was raised after having realised that Schwann cells in NBs are normal cells, very likely attracted to the neoplastic neuroblasts. But what role does this cell play in these tumours? Can we still reduce the appearance of Schwann cells in NBs to an epi-phenomenon or is this cell population responsible for the differentiation of certain NBs? If so, will it be possible to use their strategies to induce differentiation of neuroblasts and so render them non-aggressive, mature ganglionic cells? To shed light on the possible interactions between normal Schwann cells and NB cells, the maturation capacity of NBs and the genetic constitution of the two main cell populations in these tumours are briefly reviewed. Some data leading to the current view on the origin of the Schwann cells in NBs, and several physiological aspects of the Schwann cells, including normal neurone–Schwann cell interactions, are detailed.

Key words: neuroblastoma, maturation, regression, Schwann cells, Schwann cell–axon interactions, growth factors, *in situ* hybridisation, 1p deletion, ploidy
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THE NEUROBLASTOMAS AND THEIR CAPACITY TO UNDERGO MATURATION

THE NBs show some peculiar characteristics which are essentially based on their embryonal nature and their capacity for regression and complete organoid maturation. Embryonal tumours, as defined by Willis, are thought to arise during fetal and postnatal development from tissues which are still immature, but in which differentiation is already determined and restricted. Those NBs which become clinically manifest in the first year of life mainly present as undifferentiated or immature tumours with a varying degree of mitotic activity. Differentiating tumours (Figure 1) develop a dense Schwann cell stroma, and are called ganglioneuroma when exclusively consisting of mature ganglionic cells and Schwann cells (and a variable collagen stroma). Ganglioneuromas are usually diagnosed in older children and also rarely in adults. Based on the existence of these mature NBs, the relatively high incidence of NBs *in situ* [1], the increasing incidence when mass screening is performed in the first months of life [2], the IVS tumours [3, 4] and localised not totally resected tumours showing spontaneous regression [5, 6] (Ambros and colleagues, this issue pp. 510–515), we can assume that at least some of the immature NBs occurring in infancy would regress or mature after a period of proliferative activity. Therefore, it is crucial to detect genetic, biological, and also morphological features which allow the distinction of aggressive tumours from those tumours with maturation or regression capacity. Amplification of the *MYCN* gene is one of the best known and characterised indicators of a poor prognosis [7–11]. However, *MYCN* amplification is only present in a subgroup of aggressive NBs, and a significant number of unfavourable stage III and IV tumours lack amplification. Deletions at the short arm of chromosome 1 (1p36.3) are the most common cytogenetic aberrations found in advanced stage NBs [12–15], and in local tumours which showed progression (Ambros and colleagues, this issue pp. 510–515). Differences in ploidy were

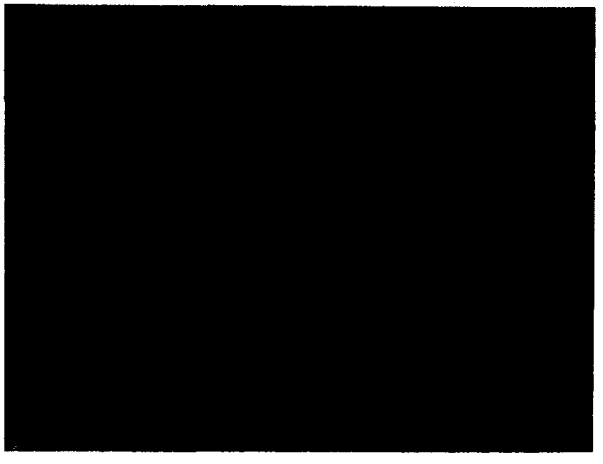

Figure 1. Favourable stroma-rich NB composed of highly differentiated ganglionic cells and Schwann cells (H&E stain). Magnification 300×.

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described for low and high stage NBs [11, 16] (see [17] for a review).

Expression of the nerve growth factor (NGF) receptor by NB cells presumably represents one of the prerequisites for the maturation processes. Both receptor subunits, the high affinity NGF receptor with tyrosine kinase activity ($p140^{trkA}$), and a transmembrane glycoprotein, the low affinity NGF receptor ($p75^{NGFR}$), are thought to be required for high affinity NGF binding, and probably for signal transduction [18, 19]. Nakagawara and associates [20] showed that detectability of the NGF receptor subunit $p140^{trk}$, expressed at different levels by the majority of NBs, was associated with lower stages of disease, lower age of patients, and was inversely related to *MYCN* amplification. Analysing mRNA expression of both receptor subunits in NBs, Kogner and associates identified three prognostic subgroups [21]. The most favourable one, expressing $p140^{trkA}$ and $p75^{NGFR}$, was associated with young age, favourable clinical stages and lack of *MYCN* amplification.

The well known diversity of genotype, phenotype and prognosis of NBs is also reflected in the morphological appearance of these tumours. Interpretation of histopathological features and assessment of reliable prognostic criteria may still present difficulties, especially in the subgroup of undifferentiated NBs. In general, tumour grading is mainly based on the differentiative status and the proliferative activity of the tumour cells, with the most undifferentiated forms thought to represent the most malignant subtypes. However, a grading system exclusively based on these features cannot be applied to NBs for the reasons mentioned above. In order to escape these difficulties, Shimada and colleagues [22] created a classification system in which age at diagnosis and the occurrence of the Schwann cell stroma play an essential role. Maturing, (Schwann cell-) stroma-rich NBs consist of two main cell populations: neuronal cells with all steps of cytodifferentiation, ranging from few undifferentiated neuroblastic cells to mature ganglionic cells, and Schwann cells. Due to limited mitotic activity or even lack of proliferation, typical for favourable stroma-rich NBs, these tumours have escaped conventional cytogenetic analysis, and little is known about their genetic make-up. Investigations concerning the DNA content of this subset of NBs are sparse and controversial, but a relatively large number were shown to be diploid. Taylor and associates [23] analysed ploidy in a series of ganglioneuromas using flow cytometry (FCM), and found DNA aneuploidy in more than 50% of tumours and diploidy in the remaining cases. To determine whether the maturation capacity of NBs depends on genetic features, that is 1p deletions and ploidy, we investigated maturing NBs ([24], Ambros IM, Children's Cancer Research Institute, Austria) which were classified as favourable intermixed or well differentiated stroma-rich NBs following the classification by Shimada and associates [22] (Figure 2).

GENETIC ABERRATIONS OF MATURING STROMA-RICH AND REGRESSING NEUROBLASTOMAS

To overcome the difficulties mentioned above, we performed peroxidase- and fluorescence- based *in situ* hybridisation (ISH) experiments and FCM analysis on more than 20 stroma-rich intermixed and well-differentiated NBs ([24], Ambros IM, Children's Cancer Research Institute, Austria). The probes used were alphoid probes specific for the centromeres of different chromosomes and a VNTR probe identifying the subtelomeric region of the short arm of chromosome 1 (for further details of the method see Ambros and colleagues, this issue pp. 510–515). All tumours analysed so far have shown a near-triploid (or

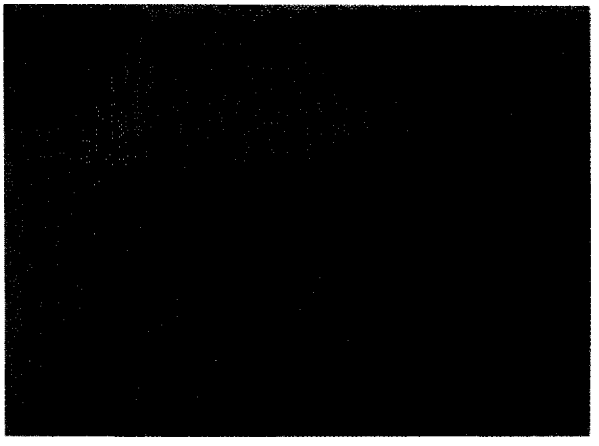

Figure 2. Ganglionic cells derived from an intermixed stroma-rich NB showing 3 signals (brown) with the centromere-specific probe D1Z1, indicating 3 chromosomes 1. Peroxidase *in situ* hybridisation was performed on a paraffin section. Magnification 1000×.

pentaploid) DNA content and none of them displayed a deletion at the short arm of chromosome 1. Interestingly, in every case, the numerical chromosome aberrations were restricted only to the neuronal cells (Figure 2), independent of their maturation status. The Schwann cell population consistently showed a disomic hybridisation pattern with all probes used (Figure 3). In the FCM analyses, two DNA peaks were identified, one in the triploid (pentaploid) range and one in the diploid range, the latter obviously indicating the DNA content of the Schwann cells. Since maturation of a triploid neuroblastic cell into a triploid ganglionic and a diploid Schwann cell seems to be unlikely, we concluded that the NB-associated Schwann cell is a normal cell and not a neoplastic cell. Furthermore, it seems evident that the potential for organoid maturation is restricted to non-diploid NBs without deletions at 1p36.3, and that the cell of origin of, at least, the non-diploid NBs is not a pluripotent neural crest cell, due to the exclusively undirectional differentiation of NB cells into ganglionic cells.

CURRENT OPINION ON THE ORIGIN OF THE NEUROBLASTOMA-ASSOCIATED SCHWANN CELL

Based on ontogenetic considerations, (the common ancestry of sympathetic neurones and Schwann cells), the coexistence of

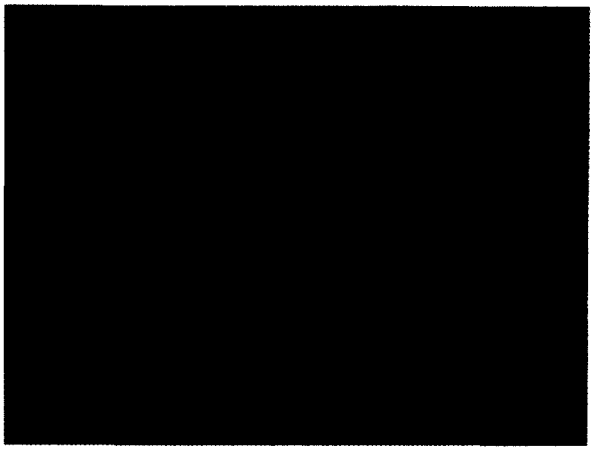

Figure 3. Schwann cells "invading" an intermixed stroma-rich NB showing 2 or less signals (brown) with the centromere-specific probe D1Z1, indicating 2 chromosomes 1. Peroxidase *in situ* hybridisation was performed on a paraffin section. Magnification 1000×.

both cell types in the differentiated types of NBs, and *in vitro* differentiation experiments, it has been claimed that NB cells have the potential to differentiate into ganglionic cells as well as into Schwann cells. Therefore, it is widely accepted that the Schwann cell in NBs represents a neoplastic cell. *In vitro* differentiation experiments, i.e. treatment of NB cell lines with differentiating agents, such as NGF [25], retinoic acid (RA) [26, 27], dibutyryl cyclic AMP (dbcAMP) [27], 5-bromo-2'-deoxyuridine (5-BrdU) [28] and 12-O-tetradecanoylphorbol-13-acetate (TPA) [29] induced, in some NB cell lines, morphological and biochemical differentiation, i.e. the occurrence of neuronal (N-) appearing cells with neurite outgrowth, tyrosine hydroxylase and dopamine-β-hydroxylase activites as well as the occurrence of flat- (F-) or substrate- (S-) adherent, epithelial-like cell forms not expressing enzymes of catecholamine synthesis. The F-cells were shown to differ in ganglioside biosynthesis from N-cells [30], to produce collagen proteins, including basement membrane collagen (type IV), laminin and fibronectin, and to express the enzyme, cyclic nucleotidyl phosphohydrolase (CNP) [27]. An increased expression mainly of the α subunit and to a lesser extent also of the β subunit of the S-100 protein, a widely used Schwann cell marker (see below), was demonstrated by Tsunamoto and associates in two NB cell lines [28]. The non-neuronal appearing cells were interpreted in different ways by different authors, and the F- or S-cells were thought to represent various cell types, including glial or Schwann cells [27, 28, 30], melanocytes [27, 31], fibroblast-like meningeal cells [32], and multipotent embryonal precursor cells of the neural crest [33]. Furthermore, chromaffin-related genes, that are sequentially expressed by adrenal medullary cells during fetal development, were also shown to be spontaneously expressed in a series of NB cell lines [34].

Taken together, the results of the *in vitro* differentiation experiments of NB cells are controversial and heterogeneous, and even the common neural crest origin of Schwann cells and neuroblasts does not necessarily support the hypothesis that the NB-associated Schwann cell is derived from a NB cell, since the commitment of neural crest cells takes place at a very early developmental stage.

SOME PHYSIOLOGICAL AND DEVELOPMENTAL FEATURES OF THE SCHWANN CELL

Schwann cells, initially described as a population of cells which become mitotically active after peripheral nerve damage, have an established crucial role as axon ensheathing and myelin forming cells in the peripheral nervous system. The sheath cells cover virtually the entire surface of the neuronal cells, thus preventing contact with the connective tissue. Schwann cells and the other glial cell types of the peripheral nervous system (PNS) are derived from the neural crest, which also gives rise to the neurones of the PNS, adrenal medullary cells, certain other endocrine cells, the melanocytes and the facial mesenchyme which is a derivative of the rostral segments of the neural crest. The neural crest origin of Schwann cells was first demonstrated by Harrison in 1924 [35], who found that motor axons innervating axial musculature were not accompanied by Schwann cells after ablation of the neural crest in amphibian embryos. Experiments in the past decades using radiolabelling of neural crest cells [36] or marking techniques carried out with interspecific chimeras [37] confirmed the neuroectodermal derivation of Schwann cells. More recent experimental work has endeavoured to elucidate the time of embryonic development at which commitment to glial or neuronal cell differentiation is specified (for reviews see [38, 39]). These data suggest that the commitment of neural crest cells takes place during the migratory period of neural crest cells and early ganglion formation.

Besides the function of axon ensheathment and myelin formation and maintenance, Schwann cells have the capacity to perform several other activities, including a marked potential for proliferation after nerve fibre injury, macrophagic and secretory properties. The sheath cells produce a variety of precursor molecules for extracellular collagens and other extracellular matrix glycoproteins, and they are responsible for the formation of the basal lamina and express laminin and collagen type IV as their most important constituents [40]. When the decision is made whether a Schwann cell becomes a myelin-forming or a non-myelin-forming cell, the expression pattern changes and each expresses a characteristic set of proteins [41]. The Schwann cell precursors and the non-myelin-forming Schwann cells express the low affinity NGF receptor and the cell adhesion molecule L-1 [42], and share some other antigens. The precursor cells lack expression of S-100 protein and glial fibrillary acidic protein (GFAP), both present on non-myelinating Schwann cells, together with the neural cell adhesion molecule (N-CAM). The myelin-forming cells lose NGF receptor and N-CAM positivity, and start to express a set of myelin and myelin-associated proteins and S-100, but not GFAP [41].

INTERACTIONS BETWEEN NON-NEOPLASTIC NEURONAL CELLS AND SCHWANN CELLS

The close and very complex interactions between these two highly specialised cell types of the PNS have been well documented in recent decades. In contrast to fibroblasts, purified Schwann cells proliferate only very slowly in conventional serum-containing culture medium. In 1973, Wood and Bunge [43] demonstrated that sensory neurones derived from dorsal root ganglia are able to provoke Schwann cell proliferation *in vitro*. The authors showed that Schwann cell division is only induced by direct contact with axons, and concluded that proliferation of Schwann cells during development is dependent on a mitogenic signal provided by the growing axon. In the following years, surface components of neuronal cells, including sympathetic neurones and PC12 cells, carrying mitogenic signals which stimulate Schwann cell division by direct contact were also described by other authors [44–49], and a limited number of soluble mitogens acting on Schwann cells were reported. Raff and associates [50] showed that agents raising the intracellular level of cyclic AMP (cAMP), such as cholera toxin or dbcAMP, stimulated Schwann cell proliferation. Crude extracts of bovine pituitary and brain also exert a pronounced proliferation stimulus on cultured Schwann cells, but without increasing the intracellular cAMP level [51]. Platelet-derived growth factor B (PDGFB), expressed by neurones of the central nervous system [52], has been identified as a chemo-attractant for glial cells [53]. Furthermore, PDGFB and fibroblast growth factor (FGF) act as mitogenic factors on Schwann cells in the presence of physiological agents capable of raising intracellular cAMP [54], which is known to mediate induction of growth factor receptor expression [55]. Glial growth factor (GGF) and transforming growth factors-beta (TGFβ) exert their mitogenic stimulation even without agents raising the intracellular cAMP level, and TGFβ can replace cAMP elevation and act synergistically with PDGFB and FGF [56, 57] (for overview see [58–60]). GGF was partially purified from bovine pituitary glands in 1984 [61]. In 1993, three major isoforms of this protein were purified, GGF I, II and III [62]. It turned out that the GGFs are alternatively spliced

variants of a single gene which also encodes the heregulins, the neu differentiation factor (NDF) and the ARIA protein (acetylcholine receptor inducing activity) [62]. These gene products are ligands of the $p185^{erbB2/neu}$, a member of the epidermal growth factor receptor family with receptor tyrosine kinase activity, which is also expressed on Schwann cells in developing nerves and following nerve transection [63]. It is possible that a membrane-bound form of the GGFs is identical to the axon-associated Schwann cell mitogen described by Wood and Bunge, and also to the neuronal cell surface mitogen reported by Ratner and associates in 1988 [64], possessing a heparin-binding activity.

In addition, it is well known that the phenotype of a fully differentiated mature Schwann cell is essentially determined by the presence of axons. The expression pattern of various proteins and lipids of isolated Schwann cells and Schwann cells co-cultivated with neurones was the object of many investigations of the past decades. Bunge and co-workers reported that the presence of nerve cells (axons) is required for the generation of basal lamina on the Schwann cell surface [65, 66]. Mirsky and colleagues [67] observed that rat Schwann cells in cell culture require a continuing signal from appropriate axons to make detectable amounts of myelin-specific glycolipids and proteins, and the axon itself determines whether a Schwann cell differentiates into a myelin-forming or non-myelin-forming sheath cell (for overview see [59, 60]). Further specific neuronal–glial cell interactions also concern the synthesis of S-100 protein by glial precursors, which produce high levels only when co-cultivated with neurones [68, 69].

Schwann cells, however, play a crucial role in the survival, growth inhibition and differentiation of neuronal cells by expressing neurotrophins, such as NGF, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor- (CNTF) and leukaemia inhibitory factor (LIF), and glia maturation factor β (GMFβ) (for overview see [58]). During embryonic development and in response to peripheral nerve injury, the sheath cells express NGF and the low affinity NGF receptor ($p75^{NGFR}$). Axonal contact suppresses receptor expression (for review see [70]). Experiments carried out by Anton and associates [71] led to the hypothesis that one function by elevated levels of the low affinity receptors and NGF may be the promotion of Schwann cell migration, which plays an essential role in development and regeneration. BDNF, known to be produced by cultured fibroblasts and Schwann cells [72], belongs to the neurotrophin family and acts on neurones that are not responsive to NGF. After nerve injury, BDNF prevents death of axotomised motor neurones [73]; CNTF was initially described as a neurotrophic factor for cholinergic ciliary neurones. Subsequently, it turned out that CNTF also affects the *in vitro* survival of various other neuronal cell types including neurones of sympathetic ganglia (for review see [58]). CNTF is expressed in the PNS by Schwann cells, and, unlike NGF and BDNF, is down-regulated in the distal part of a lesioned peripheral nerve, and re-expression occurs with regeneration [74]. The expression of GMFβ, a 17-kDa acidic protein isolated from bovine brain [75] by Schwann cells is assumed to be induced by loss of the normal Schwann cell–axon contact [76], and to play a role in peripheral nerve regeneration [77]. In addition, GMFβ was shown to have an antiproliferative effect on cultured NB cells [78] and on other types of neoplastic cells [79].

Altogether, the investigations concerning axon–Schwann cell and Schwann cell–axon interactions indicate that Schwann cells require neuronal stimuli for survival, proliferation, differentiation and expression of their highly specialised functions, and neuronal cells are dependent on Schwann cells in terms of survival and differentiation. Importantly, the Schwann cells do not promote proliferation of neurones, but, in contrast, exert a growth inhibiting influence. This point is of great interest especially in the context of NB.

SOME POSSIBLE INTERACTIONS BETWEEN NEUROBLASTOMA CELLS AND SCHWANN CELLS

Differentiating NBs are very likely to reproduce physiological processes occurring in early developmental stages and during peripheral nerve regeneration. The tumours try to imitate their parent organs, which, at least for the non-diploid NBs, seem to be the autonomic sympathetic ganglia and not the adrenal medulla or sympathetic paraganglia. Based on the fact that the NB-associated Schwann cell is a normal cell which "infiltrates" the tumour, we suspect that the growing axons from NB cells come into direct contact with Schwann cells and promote Schwann cell proliferation, while expressing axon-associated mitogens as is described for non-neoplastic neuronal cells. Another or an additional possibility would be that differentiating NB cells produce soluble chemotactic and/or mitogenic factors acting on Schwann cells. Subsequently, the normal Schwann cells ensheath and follow the neoplastic axonal processes of NB cells, and further differentiating factors acting on Schwann cells may be provided by the NB cells. The Schwann cells, in turn, produce antiproliferative and differentiation promoting factors acting on the neoplastic neuronal cells, and, therefore, most likely play a substantial role in the self-limited process of some NBs. NGF, BDNF and GMFβ are possible Schwann cell-derived candidates responsible for growth inhibition and maturation. This model is in accordance with the present view about Schwann cell–neuronal cell interactions, and is also suppored by the distribution of Schwann cells in undifferentiated stroma-poor NBs. Some of these tumours show a considerable number of S-100 positive cells in the septal portion of the supportive stroma. The DNA content of these NBs are in the triploid range, and no deletion at 1p36.3 or *MYCN* amplification has been observed in the cases analysed so far (Dr IM Ambros, Children's Cancer Research Institute, Austria). Interestingly, Shimada and associates [80] separated prognostic sub-groups in undifferentiated NBs based on the extent of S-100 protein positive cells (in the septae), mitotic karyorrhectic index (MKI) and age. The S-100 positive cells are most numerous at the tumour periphery, where the maturation is usually more advanced. In more central parts of the tumour, they are located at the periphery of the tumour cell nests and in the fibrovascular stroma. Schwann cells increase in number when the maturation of the tumour progresses and the fibrovascular septae with S-100 positive cells become more prominent. Finally, the most differentiated variant of the NBs consists of highly differentiated ganglionic cells, arranged singly or in groups, often surrounded by sustentacular cells, numerous Schwann cells ensheathing neurites and a variable number of fibroblastic cells. These tumours, the ganglioneuromas, often found incidentally, usually show a benign clinical behaviour, and represent one end of the wide biological spectrum of NBs. The other end of this spectrum, the undifferentiated stroma-poor NBs, are characterised by considerable heterogeneity, including highly aggressive tumours and tumours with maturation and/or regression capacity.

CONCLUDING REMARKS

In mammalian PNS, physiological proliferation of normal Schwann cells is observed during embryonic and fetal development and during Wallerian degeneration induced by peripheral nerve trauma. Now, another situation in which proliferation of normal Schwann cells can occur, is evident. Although it was known that extensive growth of Schwann cells in NBs is associated with a good prognosis, the impact of the occurrence of this cell type in NBs was not recognised, since the Schwann cell was regarded as representing the differentiation product of the NB cell. This view was disproved by the detection of lack of numeric chromosome aberrations in Schwann cells, always present in neuroblastic/ganglionic cells in all cases analysed so far. We suppose that, in the maturation processes of NBs, the sheath cells fulfil the same role and functions which they physiologically perform during development and after peripheral nerve injury. Therefore, we assume that the processes leading to complete organoid maturation of NBs are based on the presence of a normal cell, i.e. the Schwann cell. However, the occurrence of this cell type in NBs depends on the potential of the tumour cells to induce Schwann cell proliferation and differentiation. The disclosure of these relations not only necessitates revisions in our thinking about differentiation processes in NBs, but also has implications for future work. It will be of great value to detect the factors acting on, as well as expressed by, Schwann cells. The characterisation of the involved membrane-bound and soluble factors might represent prognostic parameters and possibly are even of therapeutical interest.

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