THESIS ON NATURAL AND EXACT SCIENCES B87

Deciphering Molecular Basis of Schwann Cell Development

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TUT PRESS

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The dissertation was accepted for the defence of the degree of Doctor of Philosophy in Natural and Exact Sciences on November 2, 2009

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Defence of the thesis: December 14, 2009

Declaration
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for a doctoral degree at Tallinn University of Technology, has not been submitted for any academic degree.

This dissertation was supported by European Social Fund.

ISSN 1406-4723
ISBN 978-9985-59-953-2

LOODUS- JA TÄPPISTEADUSED B87

Schwanni rakkude arengu molekulaarsete mehhanismide selgitamine

MARKO PIIRSOO

TTÜ KIRJASTUS

CONTENTS

INTRODUCTION

Nervous system is traditionally considered as one of the most, if not the most, complex organ systems in a multicellular organism. This belief dates back to the ideas of ancient Greek scholar Hippocrates, who stated that brain is the place of sensation and intelligence – two expressions of a living form that are still the most enigmatic ones. This notion of complexity has gathered many bits of evidence at physiological, anatomical, histological, cell and molecular biological levels during the development of scientific knowledge from ancient Greeks to present day. Despite the long history in the studies of the nervous system, we probably are still at the very beginning of understanding how it works.

The vertebrate nervous system can be divided into the central nervous system (CNS) and the peripheral nervous system (PNS). CNS consists of brain and spinal cord, whereas PNS is composed of networks of nerves throughout the body. On a rough cellular level the nervous system consists of two types of cells, namely neurons and glial cells. Glial cells are traditionally considered as "the other" cell type in the nervous system, while outnumbering neurons tenfold. There is a belief that more than 10,000 different types of neurons exist in the nervous system, while only a few have been identified within glial cells. This is a clear example of our ignorance in understanding the functioning of the nervous system, which is challenged in the last 20 years, when glial biology has also come to focus and the functions attributed to glia have widened considerably.

Glial cells are divided into two main cell types, namely macroglia and microglia. The main function of microglia is to act as an executive of the immune system inside the nervous system. Basically, they are residing mesoderm derived monocytes and macrophages in the nervous system. Macroglia, or true glia, consists of astrocytes and oligodendrocytes in CNS, and these cell types have different developmental origins. In PNS cells from single origin are considered to compose all macroglial cells.

The term glia comes from a Greek word for glue, giving an immediate impression of the function of these cells, to insulate and glue together the network of neurons. This single function of glial cells has been challenged by numerous studies during the last 20 years showing that glial cells have also an essential function during the development and in plasticity of the nervous system. Glia is absolutely necessary in keeping neurons alive, in modulating the synaptic transmission and in regulating nerve conductance, to name just a few functions. This has put glia as an equally important cell type of the nervous system next to neurons.

As mentioned earlier, CNS contains two types of macroglial cells – oligodendrocytes and astrocytes, whereas in PNS the function of those two is

combined in Schwann cells. Besides myelinating and non-myelinating Schwann cells, there are a number of specialized glial cell types in PNS, all of which share a common origin with Schwann cells. Among others, these types include satellite cells of sympathetic, parasympathetic and sensory ganglia, perisynaptic Schwann cells in the neuromuscular junctions and enteric glial cells of the in the autonomic ganglia in the gut wall.

One clear difference between the macroglial cells of CNS and PNS is that while Schwann cells are able to support the regeneration of a damaged axon, macroglia of the CNS is inhibitory for this process. This knowledge leads to a fundamental difference in PNS and CNS, namely that PNS injuries are generally curable, while the CNS injuries are not. Curiously enough, Schwann cells can also, under certain conditions, support the regeneration of a damaged axon while transplanted into CNS. This phenomena has greatly speeded up the research in the field of the PNS glia, as this property of Schwann cells could be developed in future for a therapeutic measure curing the CNS injuries.

OUTLINE AND AIMS OF THIS THESIS

The current thesis aims to add some bits to our understanding about the biology of Schwann cells. In the first part of the thesis I will describe our current knowledge about a number of aspects of Schwann cells biology, by starting with the developmental history of Schwann cells. This will be followed by an overview of myelin, a specialized membrane formation around axons unique to oligodendrocytes and Schwann cells. Next I will characterize the transcription factors that are important in Schwann cell differentiation and the functions of neurotrophic factors in Schwann cells (**Chapter 1**). The second part of the thesis will address some questions in air in the development of Schwann cells, namely what is the function of POU domain transcription factor Oct-6 in Schwann cells during development and regeneration (**publication I**), how are the functions of Oct-6 shared by another transcription factor Brn-2 (**publication II**), what are the target genes for Oct-6 during Schwann cells development and differentiation (**manuscript I**) and describe mRNA expression patterns of neurotrophic factors and their receptors in Schwann cells and during peripheral nerve development (**manuscript II**). Finally I will describe mRNA expression pattern of the calcium activated chloride channel gene family, that are the target genes of Oct-6 in the nervous system (**publication III**).

ORIGINAL PUBLICATIONS

I Ghazvini, M., Mandemakers, W., Jaegle, M., **Piirsoo, M**., Driegen, S., Koutsourakis, M., Smit, X., Grosveld, F., Meijer, D. (2002). A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration. EMBO Journal, 21, 4612 – 4620

II Jaegle, M., Ghazvini, M., Mandemakers, W., **Piirsoo, M**., Driegen, S., Levavasseur, F., Raghoenath, S., Grosveld, F., Meijer, D. (2003). The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. Genes and development, 17, 1380 – 1391

III Piirsoo, M., Meijer, D., Timmusk, T. (2009). Expression analysis of the CLCA gene family in mouse and human, with emphasis on the nervous system. BMC Developmental Biology, 9, 10

MANUSCRIPTS

I Piirsoo, M., Küry, P., Müller, HW, van der Spek, P., Grosveld, F., and Meijer, D. Defining the transcriptome of differentiating Schwann cells.

II Piirsoo M., Kaljas A., Tamm K., Timmusk T. Expression of NGF and GDNF family members and their receptors during peripheral nerve development and differentiation of Schwann cells *in vitro.*

ABBREVIATIONS

ARTN	artemin
BDNF	brain derived neurotrophic factor
BMP	bone morphogenetic protein
cDNA	complementary DNA
cAMP	cyclic adenosine monophosphate
cdk	cyclin dependent kinase
CLCA	chloride channel, calcium activated
CMT	Charcot-Marie-Tooth Disease
CNS	central nervous system
Cx	connexin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
E	embryonic day
ECM	extracellular matrix
EMSA	electrophoretic mobility shift assay
GDNF	glial cell-derived neurotrophic factor
MAG	myelin associated glycoprotein
MBP	myelin basic protein
mRNA	messenger RNA
NCSC	neural crest stem cell
NGF	nerve growth factor
NRG-1	neuregulin-1
NT	neurotrophin
NTN	neurturin
P	postnatal day
PCR	polymerase chain reaction
PMP	peripheral myelin protein
PNS	peripheral nervous system
PSPN	persephin
qRT-PCR	quantitative reverse transcriptase PCR
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SCE	Schwann cell enhancer
TK	tyrosine kinase

1. REVIEW OF THE LITERATURE

1.1 Developmental history of the Schwann cell lineage

Most, if not all, Schwann cells are derived from neural crest, a transitory structure in the vertebrate embryo. Neural crest, a violation of the central dogma of the germline theory, appears at the dorsal aspect of the neural tube and its cells migrate along defined routes to various tissues, giving rise to a number of different cell types (Dupin et al., 2006 for a review). Localization of neural crest in the vertebrate embryo is depicted in figure 1. Neural crest cells have rostro-caudal specification along the neuraxis, with specific cell types emerging at different levels. In principle the differentiated cells originating from the neural crest can be divided into ectomesenchymal and non-ectomesenchymal cell types. Ectomesenchymal cell types include connective tissue, odontoblasts, osteocytes and chondrocytes. Non-ectomesenchymal cell types include sensory, enteric, sympathetic and parasympathetic neurons, Schwann cells and melanocytes (Donoghue et al., 2008). Ectomesenchymal cell types arise exclusively from cephalic neural crest in chick *in vivo* (LeDouarin et al., 2008). In mice, it has been shown that one ectomesenchymal cell type, a subset of endoneurial fibroblasts, arise from trunk neural crest (Joseph et al., 2004). Neurons of the PNS arise from strict locations among the rostro-caudal axis of the neural crest. Melanocytes arise from both cephalic and trunk neural crest in chick (Baker et al., 1997), but only from trunk neural crest in mice (Wilkie et al., 2002). Schwann cells are the only type of neural crest derivative, that emerge at all levels of the neural crest in mice. Ventral part of the neural tube has been proposed to be another source of Schwann cells in the avian embryo (Rickmann et al., 1985), but even if true, this probably comprises a minority of the Schwann cells eventually emerging in the nerve.

Numerous *in vivo* and *in vitro* studies have indicated that at least a subset of migrating neural crest cells are multipotential progenitors, that upon environmental signals are able to differentiate into various derivatives of neural crest. Moreover, the differentiation potential of isolated neural crest cells *in vitro* exceeds their potential *in vivo* (Anderson 1997; Delfino-Machin et al., 2007; LeDouarin et al., 2008). For instance, it has been shown that exogenous Sonic hedgehog is able to induce mesectodermal cell fates in isolated quail cephalic neural crest cells *in vitro* (Calloni et al., 2007). These results indicate that the differentiation of neural crest derivatives is rather plastic. This plasticity is also exemplified by the finding that at least *in vitro,* embryonic Schwann cells of chick are able to trans-differentiate to melanocytes (Dupin et al., 2003).

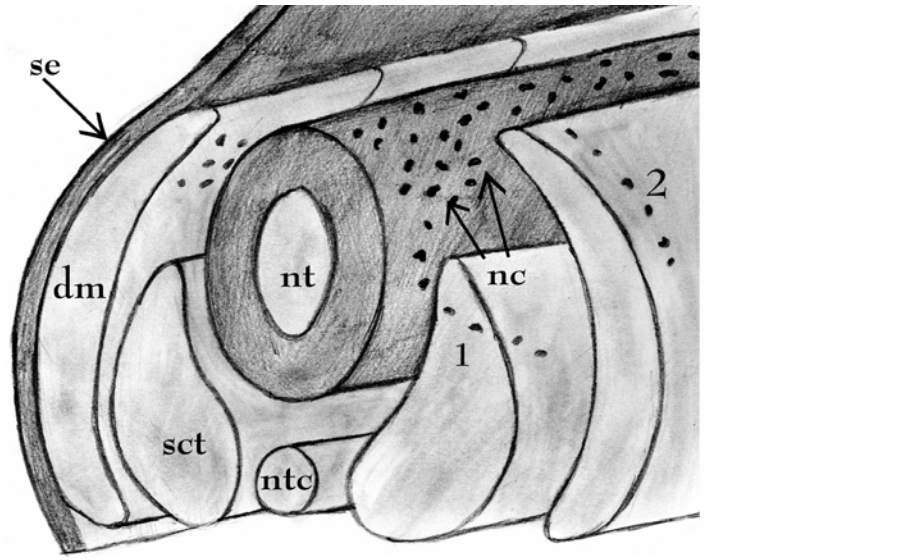

Figure 1. Location of neural crest in the vertebrate embryo. For sake of clarity, surface ectoderm has been removed from the right side of the embryo. Two distinct migratory pathways of trunk neural crest cells are indicated (1 and 2). dm – dermamyotome; nc – neural crest; nt – neural tube; ntc – notochord; sct – sclerotome; se – surface ectoderm. Adapted from Gilbert, 2006

Neural crest cells isolated from E10.5 rat embryo, are able to generate both neurons and Schwann cells *in vitro* (Stemple and Anderson, 1992). These cells have been considered as neural crest stem cells (NCSC) and have also been shown to be able to differentiate towards smooth muscle cell fate (Shah et al., 1996). Cells with such characteristics can also be identified from E14.5 rat sciatic nerve and dorsal root ganglion (DRG) (Morrison et al., 1999; Hagedorn et al., 1999). At E14.5 in rat, migration of neural crest cells is over, peripheral ganglia have been formed and glial cell precursors are already associated with outgrowing peripheral axons. Multipotent NCSC is characterized by the expression of low affinity nerve growth factor receptor p75 and transcription factor Sox-10 (Stemple and Anderson 1992; Paratore et al., 2001). Interestingly, multipotent neural crest cells, isolated after neural crest migration cessation, also express several early glial markers including P0, Pmp22 and Desert hedgehog (Hagedorn et al., 1999; Joseph et al., 2004).

Instructive signals have been shown to promote the generation of cells with differentiated phenotype from NCSC. In case of Schwann cells, the most prominent signalling molecule identified is neuregulin-1 (NRG-1, also known as NDF, GGF, ARIA, SMDF or heregulin) (Shah et al., 1994; Dong et al., 1995). Other fates of NCSC are induced by transforming growth factor beta (TGFβ)

(smooth muscle cells), bone morphogenetic protein (BMP) 2/4 (neurons) and endothelins (melanocytes and enteric nervous system progenitors) (Morrison et al., 1999 and refs therein). When a combination of differentiation inducing factors is applied to NCSC *in vitro*, TGFβ and BMP2/4 act dominantly over NRG-1, indicating that Schwann cell phenotype might be the default fate of NCSC (Shah and Anderson 1997). This notion is also supported by the fact that a co-receptor for NRG-1, ErbB-3 is initially expressed in most, if not all, neural crest cells, and that its expression is subsequently switched off in most of them, except Schwann cells (Meyer and Birchmeier, 1995; Garratt et al., 2000). Mice deficient for NRG-1 signalling have drastically reduced numbers of neural crest derived cells in the peripheral nerves, whereas the number of neural crest derived non-neural cells in the peripheral ganglia (satellite cells) remains normal (Garratt et al., 2000). It is possible that compensatory mechanisms for the lack of NRG-1 signalling occur near neuronal bodies, in the ganglia, but not in the peripheral nerves. Alternative explanation for the lack of requirement of NRG-1 signalling in the developing peripheral ganglia is that glial cells of the ganglia are of distinct lineage compared to Schwann cells in the peripheral nerves. An indication that the latter explanation might hold true, comes from the fact that *in vivo* it is possible to differentiate between satellite cells and Schwann cells by the expression Ets domain transcription factor Erm, which is expressed only in satellite cells (Hagedorn et al., 2000).

An initial intermediate cell type has been proposed in the course of emergance of Schwann cells from the migrating neural crest cells. This cell is called Schwann cell precursor and it is first detected at embryonic day (E) 14.5 in rat and E12.5 in mouse sciatic nerves (Jessen et al., 1994; Dong et al., 1999). Main intermediate cell types emerging during development within Schwann cell lineage is depicted in figure 2. At E14.5 in rat, Schwann cell precursors are associated with multiple axons, which contrasts to the adult nerve, where a myelinating Schwann cell ensheaths a single axon (Figure 2). At this stage the Schwann cells precursors are not associated with the basal lamina. Initially, Schwann cell precursors are found at the edges of nerves, within larger nerve trunks and in the immediate vicinity of growth cones (Jessen et al., 1994; Wanner et al., 2006). These cells express p75 low-affinity neurotrophin receptor, which is a marker of migrating neural crest cells, but do not express Schwann cells markers like Ca++ binding protein S-100 and lipid antigen O4. They can be distinguished from NCSCs by the expression of growth associated protein 43 (Gap-43). Also, they fail to survive without axons and die rapidly within 24 hours, when put into cell culture. Cell death can be prevented by conditioned medium from neuron cultures or NRG-1, showing that NRG-1 acts as a survival factor for Schwann cell precursors. NRG-1 also promotes

proliferation of Schwann cell precursors and stimulates differentiation of precursors into embryonic Schwann cells (Jessen et al., 1994).

NRG-1 belongs to a family of factors, with homology to Epidermal Growth Factor. It signals via heterodimers of ErbB family members of receptor tyrosine kinases (Falls 2003; Birchmaier and Nave 2008 for recent reviews). Many different protein isoforms are encoded by a single *NRG-1* gene. These protein isoforms arise both due to the involvment of different promoters and alternative splicing. Mice deficient for all NRG-1 isoforms show significant reduction in the number of nerve associated crest cells already at E10.5 (Meyer and Birchmeier, 1995). Subsequent analysis of isoform specific *NRG-1* knockouts has revealed that type III NRG-1 isoform, but not type I and II isoforms, is important in Schwann cell generation (Meyer et al., 1997). Type III NRG-1 isoform was initially identified as a soluble molecule, but was later shown to be mainly a transmembrane protein. This isoform is expressed in sensory and motoneurons, axons of which are associated with Schwann cell precursors. It has also been shown that the membrane bound form of type III NRG-1 is more potent inducer of Schwann cell differentiation *in vitro* as compared to a soluble form (Leimeroth et al., 2002).

High-affinity receptor for NRG-1 in Schwann cells is composed of a heterodimer of ErbB2/ErbB3. Mice with targeted inactivation of either of these genes show similar phenotype in Schwann cell progenitors as NRG-1 knockout animals, which is a severly reduced number of Schwann cell precursors (Riethmacher et al., 1997; Lee et al., 1995).

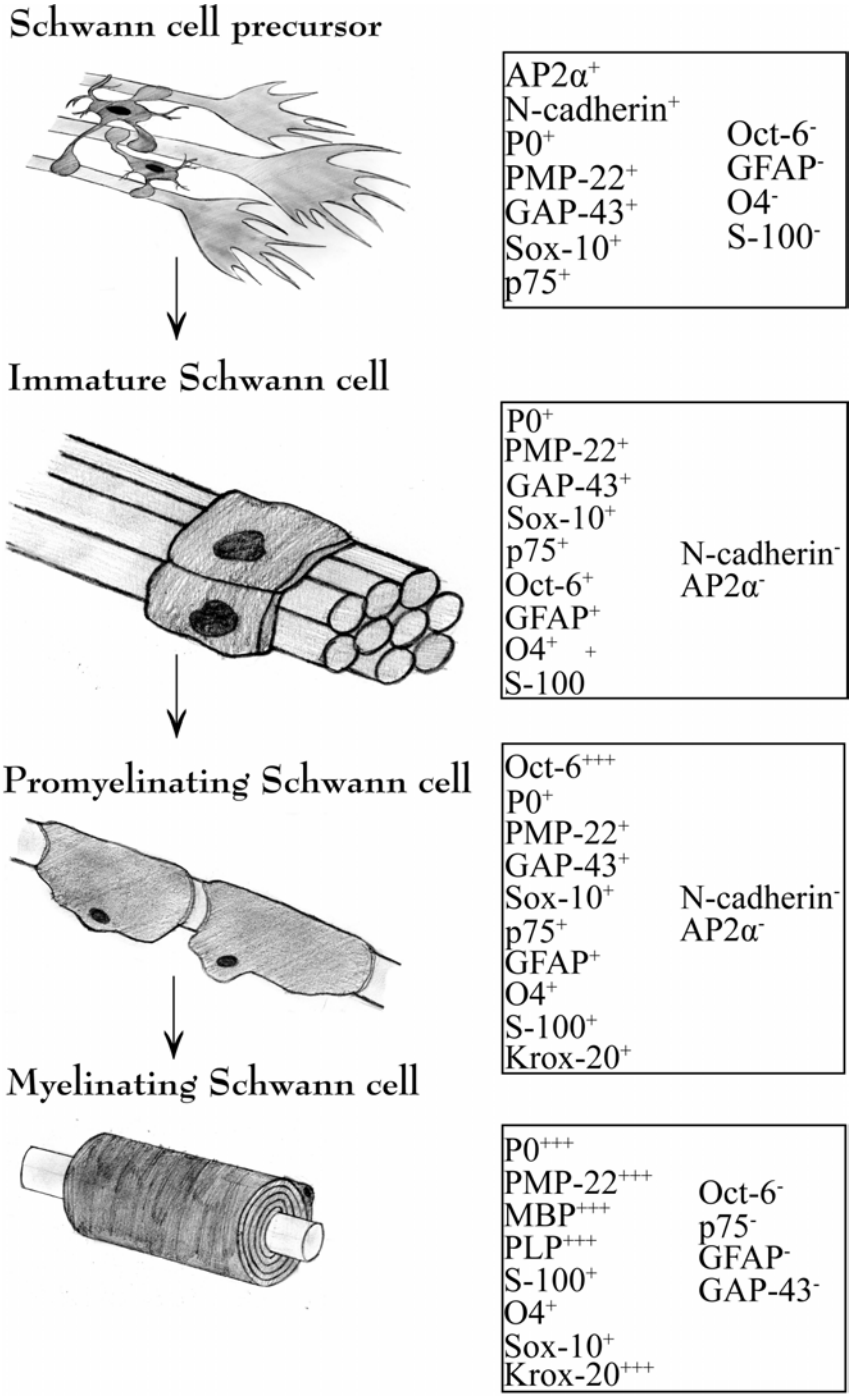

Figure 2. Schematic illustration of main cell types and developmental transitions in Schwann cell lineage. Some important markers for respective cell types are shown on the right side of the figure

Absence of neuregulin signalling in the embryonic peripheral nerve results also in deleterious effects on the neurons. Sensory connections in *ErbB3* knockout mice do form initially, but there is massive death of sensory neurons later in embryonic development. Motoneurons at cervical and lumbar level also start to form in these animals, but eventually degenerate. However, thoracic motoneurons are minimally affected (Riethmacher et al., 1997; Woldeyesus et al., 1999). This shows that Schwann cells express trophic factors for both sensory and motoneurons. These factors include CNTF, GDNF, BDNF, LIF, PDGF, FGF and NT-3 (Davies 1998; Nave and Trapp 2008).

NRG-1 responsivness in Schwann cell precursors is maintained and increased by Notch signalling pathway. It has been shown that Notch signalling induces ErbB2 expression, increases precursor sensitivity to NRG-1 signalling and by doing so, acts as a positive regulator of precursor Schwann cell transition (Woodhoo et al., 2009).

Another factor, FGF2, is also able to promote survival of Schwann cell precursors and can act as a positive regulator of precursor to Schwann cell transition. FGF2, together with IGF-1, is able to promote only short term survival of rat Schwann cell precursors, but act as long term survival factor on mouse Schwann cells. There is another difference between mouse and rat Schwann cell precursors in their response to FGF2, namely that this growth factor stimulates the proliferation of mouse Schwann cells, while fails to do so with rat Schwann cell precursors (Gavrilovic et al., 1995; Dong et al., 1999).

Entothelins have also been implicated in regulating the precursor-Schwann cell transition. Endothelins, in combination with IGF-1, are able to promote long-term survival of Schwann cell precursors. However, in contrast to NRG-1 and FGF2, endothelins are unable to promote precursor-Schwann cell transition. Moreover, they are able to delay the maturation promoting effects of NRG-1 *in vitro*. Endothelins are also functional in Schwann cell differentiation *in vivo*. Sl rats, animals lacking functional endothelin receptorB, have premature transition of precursors to embryonic Schwann cells. These premature Schwann cells express S-100, a Schwann cell marker, but lack autocrine survival support, which is characteristic to embryonic Schwann cells (Brennan et al., 2000).

It has also been reported that high concentrations of PDGF-BB and NT-3 are able to mediate Schwann cell precursor survival (Lobsiger et al., 2000).

Whether the identified Schwann cell precursor represents a commited progenitor or is it an intermediate NSCS-like cell, biased to glial fate is questionable. The identified Schwann cell precursor expresses low levels of peripheral myelin protein P0. P0 was originally identified as a protein expressed only in Schwann cells. However, P0 positive cells from rat E14.5 sciatic nerve are, in principle, competent to differentiate also to neurons and smooth muscle cells *in*

vitro. The same holds true for P0 positive cells from rat E14.5 DRG (Hagedorn et al., 1999; Morrison et al., 1999).

At around E16 in rat and E14 in mouse, Schwann precursors in the embryonic peripheral nerve differentiate to immature Schwann cells (Figure 2) (reviewed in Woodhoo and Sommer 2008). Immature Schwann cells start to express at high levels a number of markers, that are expressed at very low levels in the precursors. These include S-100, O-4 antigen, GFAP and transcription factors Oct-6 and Krox-20 (reviewed in Jessen and Mirsky, 2005). The precursor-Schwann cell transition is mediated by axon-derived NRG-1. When precursors are exposed to NRG-1 *in vitro*, they differentiate to Schwann cells (Dong et al., 1995). Although this differentiation is synchronous and rather rapid *in vivo*, there is a proximal to distal gradient, with first Schwann cells appearing in the nerve roots and later in distal parts of the nerve. Immature Schwann cells do not depend critically on axons for their survival, instead they obtain an autocrine survival loop. This autocrine survival loop involves several growth factors including IGF, NT-3, PDGF (Meier et al., 1999).

During early postnatal development the number of Schwann cells has to be adjusted with the number of axons, since myelinating Schwann cells adopt 1:1 realtionship with axons. This adjustment is achieved through a balance between cell proliferation and death. Both Schwann cell precursors and immature Schwann cells are mitotically active cells. The proliferation of Schwann cells in the peripheral nerve peaks around birth in rodents, before declining as the cells differentiate terminally during first two weeks of postnatal life (Stewart et al., 1993). Schwann cell apoptosis is observed *in vivo* during early postnatal development in rodents (Grinspan et al., 1996; Syroid et al., 1996). Apoptotic cells in the perinatal nerve have promyelinating phenotype. Apoptosis can be induced by axotomy and inhibited by the adminsitration of exogenous NRG-1 in early postnatal nerve. However, later during development, there is markedly reduced induction of apoptosis following axotomy (Grinspan et al., 1996). Signalling through p75 low-affinity neurotrophin receptor has been proposed to be a mediator of apoptosis in Schwann cells (Soilu-Hanninen et al., 1999). Indeed, Schwann cells express high levels of p75 receptor (Lemke and Chao 1988) and this receptor has been shown to act as a mediator of apoptosis (reviewed in Casaccia-Bonnefil et al., 1999). However, *in vivo* the percentage of apoptotic Schwann cells in the early postnatal nerves of p75 deficient mice is comparable to wild-type (Syroid et al., 2000). This indicates, that there is possibly a second mediator of early postnatal Schwann cell death in rodents. p75 probably still has an important function in mediating Schwann cell apoptosis, since apoptosis is supressed in p75 deficient animals after axotomy, when Schwann cells have lost contact with axons (Syroid et al., 2000). It is possible that the factor mediating Schann cell apoptosis in early

postnatal nerves is TGFβ. TGFβ is produced by Schwann cells themselves and acts through its receptor and c-jun to mediate apoptosis (Parkinson et al., 2001).

The final stage of differentiation in Schwann cells involves the generation of myelinating and non-myelinating Schwann cells in the peripheral nerve. The decision between these fates is made by an axon calibre, with small calibre axons becoming ensheated by non-myelinating Schwann cells and large calibre axons with myelinating Schwann cells (Voyvodic 1989). The molecular nature of this signal is largely unknown. It has recently been shown that part of the signalling complex involved in sorting large axons by Schwann cells involves interaction of laminins in the basal lamina with their receptor β1 integrin in the Schwann cell membrane. The downstream effectors of this signalling cascade include Rac1 GTPase (Benninger et al., 2007; Nodari et al., 2007).

1.2 Structural aspects of a myelinated nerve fiber

Compact myelin is unique to vertebrates. Although many non-vertebrate chordates and non-chordates do have glial ensheathment, that resembles myelin, its molecular structure, as known to date, differs considerably from “true” myelin. The myelin like structures discovered in annelids and crustaceans are probably products of convergent evolutionary processes, rather than been evolved from the same phylogenetic origin.

Myelin sheath around axons reduces the capacitance and increases the resistance, thereby reducing the current flow across axons. Thus, the myelin sheath is responsible for the saltatory nature of the nerve impulses.

Myelin can be viewed as a multilamellar specialized membrane structure of an oligodendrocyte or Schwann cell ensheathing the axon. It has been shown that glial cells, that ensheath larger axons, form myelin, whereas glial cells around smaller axons do not. The threshold size is usually put at 1 μm of axon diameter.

Myelinating Schwann cell is in many aspects analogous to a polarized epithelial cell (reviewed in Arroyo and Scherer, 2000). Similarly to an epithelial cell it synthesises basal lamina and has highly organized network of cytoplasmic channels that divide the cell into apical and basal parts. Since the major function of myelin sheath is to insulate axons electrically, myelin is mainly composed of non-conducting macromolecules and aqueous cytosolic material is excluded from it as much as possible. The lipid-rich plasma membrane of myelinated Schwann cell forms major dense line and the extracellular surface between the membrane wraps forms intraperiod line. The entire myelin structure is accessible to the Schwann cell soma via cytoplasmic channels, called Schmidt-Lanterman incisures, that run spirally through the myelin sheath. The innermost tip of the myelinated Schwann

cell that contacts the axon is called inner mesaxon, and the tip of the cell facing basal lamina is called outer mesaxon.

Myelinated axons are completely covered by myelin sheath except at nodes of Ranvier, that are small gaps between successive Schwann cells along myelinated fiber. One Schwann cell covers a space between two nodes of Ranvier. Myelin sheath is organized into two domains. These domains of myelin include compact and non-compact myelin (reviewed in Salzer et al., 2008). Schematic illustration of different domains of a myelinated nerve figure is shown in figure 3. Compact myelin is found in internodes (space between two node of Raniver) and non-compact myelin in Schmidt-Lanterman incisures, juxtaparanodes, paranodes (the latter two lie in lateral borders of the myelin sheath, adjacent to the nodes of Ranvier). Compact myelin and non-compact myelin differ in their protein composition (reviewed in Garbay et al., 2000). Non-compact myelin can be viewed primarily as giving the regulating properties of the myelin sheath, while the compact myelin gives insulating properties.

Myelin associated glycoprotein (MAG) is generally considered to be a marker for most if not all structures of the non-compact myelin. MAG belongs to the immunoglobulin superfamily of cell adhesion molecules (Salzer et al., 1987). MAG is expressed at high levels already at early stages of myelination and is thought to be involved in regulating the interactions between axon and the myelinating Schwann cell (Martini and Schachner, 1986). MAG is able to bind *in vitro* to specific gangliosides present in the axonal membranes (Collins et al., 1997). Analysis of mice with mutated *MAG* gene show however, that MAG is not essential for myelination (Li et al., 1994). The subtle phenotype of *MAG* mutant animals includes delayed myelination (Montag et al., 1994) and decrease in the number of myelinated axons (Li et al., 1998). Both of these defects were observed in the CNS, however. The fact that the presence of MAG controls the number of myelinated axons in a nerve, together with its role in the axonal regrowth during nerve regeneration, suggests that MAG might act in a cell non-autonomous way (Schafer et al., 1996). This would mean that the defect in the *MAG* mutant mice lies in the axons. Indeed, there is chronic axonal atrophy, together with the decreased neurofilament spacing and reduced neurofilament phosphorylation in the MAG deficient mice (Yin et al., 1998). This influence of MAG onto axonal structure has been proposed to involve a kinase cascade in neurons that involve ERK kinases and cdk5 (Dashiell et al., 2002).

Similarly to epithelial cells, myelinating Schwann cell contains adherens junctions, tight junctions and gap junctions. These structures are usually found between cells, but in myelin sheath they join adjacent layers of myelin. All of these specialized structures are found in the paranodes, Schmidt-Lanterman incisures and

in inner as well as outer mesaxons, which are the regions of non-compact myelin (Spiegel and Peles, 2002).

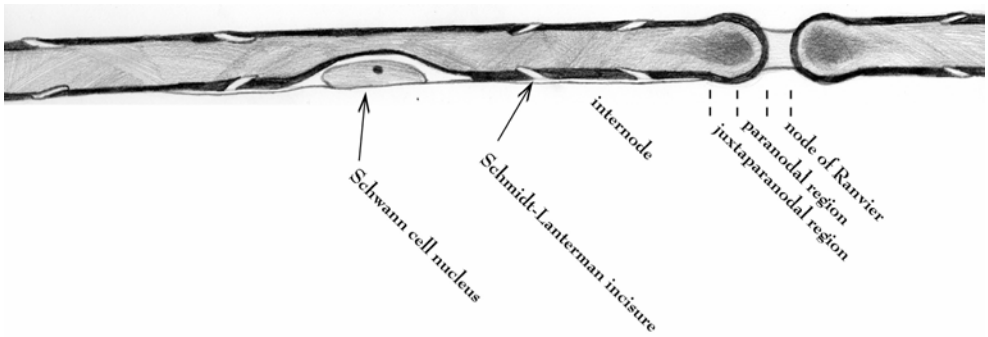

Figure 3. Schematic illustration of a myelinated nerve fiber. Major structural domains of the fiber are shown. Adapted from Cajal, 1999

Adherens junctions contain E-cadherin, an adhesion molecule that forms homophilic dimers (Fannon et al., 1995). The cytoplasmic domain of E-cadherin is able to bind α-catenins, that link E-cadherin to actin cytoskeleton (Shapiro et al., 1995). The formation of adherens junctions is dependent on correct structural formation of myelin sheath, since inactivation of either *P0* or *PMP22* genes in mice, which are components of compact myelin, alters the distribution of E-cadherin in myelin. E-cadherin is diffusely expressed in the nerves of mutant animals, as opposed to being located into the adherens junctions in normal animals (Neuberg et al., 1999; Menichella et al., 2001). On the other hand, E-cadherin mediated formation of adherens junctions is not necessary for the structural integrity of myelin, since peripheral nerves of mice lacking E-cadherin myelinate normally. E-cadherin is necessary only for the proper formation of outer mesaxon (Young et al., 2002). It is possible that other cadherins are able to compensate for the loss of E-cadherin (Wanner and Wood, 2002). It has been postulated that adherens junctions function in stabilisation of the newly formed myelin wraps during development (Fannon et al., 1995).

Tight junctions, functioning as selective cell-cell permeability barriers in epithelial cells, are also found in Schmidt-Lanterman incisures as well as paranodal loops of the myelinating Schwann cells. The molecular composition of tight junctions in different compartments of non-compact myelin appears to be different. Besides creating a diffusion barrier, it has been proposed that tight junctions in myelinating Schwann cells might function as a border between compact and non-compact myelin. A polarized vesicle transport function has also been attributed to tight junction (Zahraoui et al., 2000) and this function could be imagined also to

contribute to creating the border between the two myelin compartments. The vesicle transport function can also deliver components of myelin into required places within myelin sheath.

Tight junction formation is dependent on the proteins of Claudin family *in vitro* (Furuse et al., 1998) and also *in vivo* in CNS myelin (Gow et al., 1999). Claudins are membrane proteins with four transmembrane domains. There are at least five members of the family expressed in myelinating Schwann cells (Poliak et al., 2002). Claudin 5 is expressed in Schmidt-Lanterman incisures, whereas Claudin 1 in paranodal loops and mesaxon. Claudin 2 is expressed at the node of Ranvier and its expression colocalizes with the components of ERM proteins in the microvilli. This raises the possibility of the existence of tight junctions also between two adjacent Schwann cells (Poliak et al., 2002).

Gap junctions are traditionally intercellular channels facilitating the movement of ions and low-molecular weight molecules between the cells (reviewed in Goodenough and Paul, 2003). Gap junctions consist of two hemichannels, called connexons. Each connexon is composed of six connexin molecules. Connexins oligomerize intracellularly and are transported into the plasma membranes as hexamers. There are more than 20 connexin genes in a mammalian genome and the proteins encoded by these genes can hetero-oligomerize, thus giving a repertoire of specificities to the gap junctions.

It has been shown that two connexins – Cx32 and Cx29 are expressed in myelinating Schwann cells (Bergoffen et al., 1993; Altevogt et al., 2002; Li et al., 2002). Cx32 is localized in the outer aspects of paranodes and incisures (Scherer et al., 1995). Ultrastructurally recognizable gap junctions are mostly composed of Cx32 and again, contrary to the intercellular gap junctions in the epithelial cells, the gap junctions in the myelin sheath form between the membrane components of the same cell (Scherer et al., 1995). The notion that these gap junctions represent functional channels come from the dye diffusion studies showing that low molecular-weight molecules can diffuse radially within the myelin sheath through these channels (Balice-Gordon et al., 1998). Mutations in *Cx32* gene cause X-linked Charcot-Marie-Tooth disease, a hereditary demyelinating neuropathy (Bergoffen et al., 1993).

The fact that Cx32 is not the only connexin forming gap junctions in myelinating Schwann cells comes from the studies showing that radial dye diffusion through gap junctions is normal in the myelinated fibers of *Cx32* mutant mice (Balice-Gordon et al., 1998). Cx29 is also expressed in Schwann cells, but its localization is different from that of Cx32. Cx29 is localized into the inner mesaxon and in the inner aspects of paranode (Altevogt et al., 2002). Moreover, Cx29 protein is concentrated into developing nodes well before the final formation of nodal structure, indicating that Cx29 might function during myelination.

Although Cx29 is unable to form gap junctions between two cells *in vitro*, it is able to modulate the Cx32 dependent gap junctions (Altevogt et al., 2002). The fact that Cx29 is located in the inner mesaxon and inner aspect of paranode raises an intriguing possibility, that there are also gap junctions between axonal membrane and Schwann cell membrane. The existence of such channels remains to be investigated, however. Alternatively, since Cx29 is colocalized with the K^+ channel Kv1.2 (this molecule is localized in the axonal membrane) it has been postulated that Cx29 connexon functions in the buffering of K^+ ions (Altevogt et al., 2002).

The saltatory conduction along a myelinated fiber is dependent on the proper establishment of the axoglial junctions at and around the node of Ranvier. Voltage-gated Na^+ channels, that are responsible for the inward current flow, are concentrated into the axonal membranes at the node of Ranvier. The major channel subtype located in the node is Nav1.6 (Caldwell et al., 2002), but other members of the family are also located in axonal membranes. Clustering of Na channels into the future node of Ranvier is determined by the Schwann cell attached to the axon (reviewed in Salzer, 2002; Salzer et al., 2008).

Adjacent to the node of Ranvier lie paranodal regions. Axonal membrane in the paranodes contains a specific set of adhesion molecules that interact with their counterparts in the Schwann cell membrane. Axonal membrane proteins in the paranode include Caspr and Contactin (Einheber et al., 1997). Caspr and Contactin interact in cis i.e. when expressed in the same cell (Peles et al., 1997) and form a heterodimeric receptor for Schwann cell components of the paranode. Paranodes have been disrupted genetically in mice, where either *Caspr* of *Contactin* genes are inactivated (Boyle et al., 2001; Bhat et al., 2001). Both of these mice display reduced conductance velocities. They also have disorganized juxtaparanodal regions, indicating that paranode functions as a barrier between node and juxtaparanode, where different voltage gated channels are clustered. The myelin membrane of the paranode contains a cell adhesion molecule Neurofascin-155 (Tait et al., 2000). These three paranodal proteins form a complex and the formation of it is dependent on each other. The other components of the complex fail to cluster into the paranode in *Caspr* or *Contactin* mutant mice (Boyle et al., 2001; Bhat et al., 2001). Paranodal structure is also disrupted in mice deficient for the synthesis of galactolipids (Dupree et al., 1999).

A region between compact myelin and paranode is called the juxtaparanodal region. Axonal membranes of juxtaparanodal regions contain Kv1.1 and Kv1.2, members of the shaker family of delayed rectifier K+ channels. The β subunit associated with these channels in axonal membranes is Kvβ2 (Wang et al., 1993; Rasband et al., 1998). These molecules form a complex with Caspr2 (Poliak et al., 1999).

The most homogenous part of the myelin sheath is thought to be compact myelin in the internodal compartment. By mass it is the most prominent part of myelin. About 75% of total myelin dry mass is composed of lipids. All of the lipids present in the myelin sheath are not specific to myelinating Schwann cells, it is just their abundance that differentiates myelin membranes from conventional cell membranes (reviewed in Garbay et al., 2000).

50-70% of the total myelin protein in the peripheral nerves consists of Protein zero (P0) (Garbay et al., 2000 and refs therein). P0 is a 28 kDa protein that is glycosylated, phosphorylated, acetylated and sulphated (reviewed in Eichberg, 2002). X-ray analysis of P0 protein suggests that the protein forms tetramers (Shapiro et al., 1996; Inouye et al., 1996). This oligomerization property has led to the view that P0 is functioning in two opposing major dense line membranes and is responsible for maintaining the proper diameter of the intraperiod line. This view is supported by the fact that inactivation of *P0* gene in mice leads to the defects in myelin compaction (Giese et al., 1992). These defects include hypomyelination, and abnormal expression of a number of myelin proteins and incomplete formation of intraperiod line (Giese et al., 1992; Martini et al., 1995; Xu et al., 2000). Given a very high abundance of P0 protein in Schwann cells it is surprising that even minor overexpression of P0 in transgenic animals leads to defects in myelination (Wrabetz et al., 2000; Yin et al., 2000). Peripheral neuropathies in humans have also been associated with the mutations in *P0* gene (reviewed in Warner et al., 1999). Among them are relatively mild forms of Charcot-Marie-Tooth Disease (CMT) and more severe ones, like Dejerine-Sottas Syndrome and congenital hypomyelination.

P0 protein interacts with another peripheral myelin glycoprotein PMP22 in the membrane compartment of compact myelin (D'Urso et al., 1999). There is late onset of myelination in mice, where *PMP22* gene is inactivated. There is also initial hypermyelination and the levels of other compact myelin proteins like P0 and MBP are decreased in these mice (Adlkofer et al., 1995). Analysis of *P0* and *PMP22* double mutant animals has shown that the intraperiod line of these animals is considerably wider than in the animals where only *P0* gene is inactivated (Carenini et al., 1999). This indicates that PMP22, together with P0, acts to form compact myelin structures. Part of the phenotype seen in *PMP22* mutant mice is already evident in animals, where one allele of the gene is still functional (Adlkofer et al., 1997). Humans with mutations in *PMP22* gene develop various peripheral neuropathies including CMT and hereditary neuropathy with liability to pressure palsies (HNPP) (reviewed in Warner et al., 1999). The phenotype and severity of the disease depends on the nature of the mutation (point mutations, duplications, deletions). Analysis of natural mouse mutants *Trembler* and *TremblerJ*, with point mutations in *PMP22* gene, has led to the conclusion that these mutations probably

affect trafficking and oligomerization properties of the mutant proteins, rather than being dominant negative (Tobler et al., 2002). The same mutations have also been found in human patients with Dejerine-Sottas neuropathy and CMT1A (Valentijn et al., 1992; Ionasescu et al., 1997).

Myelin basic protein (MBP) is a protein with unusually high percentage of basic residues, and unlike P0 and PMP22, it is present both in CNS and PNS compact myelin. MBP has different isoforms, that differ by the presence of internal peptides, that are encoded by exons two, five and six of the gene (Roach et al., 1983; Roach et al., 1985). A mouse mutant *shiverer* lacks all the isoforms and thereby can be considered as *MBP* gene knockout. *Shiverer* mice have extensive myelination defects in the CNS, while PNS myelin appears to be rather normal (Rosenbluth, 1980). It is believed that in the PNS, P0 is able to compensate for the loss of MBP expression and analysis of mice where both *P0* and *MBP* genes are inactivated provides evidence for that (Martini et al., 1995).

A number of other proteins are involved in the generation and maintenance of the myelin sheath, that include P2, CNP, PLP and periaxin (reviewed in Garbay et al., 2000).

Taken together, it appears that the formation of myelin around axons is an extremely delicate event, where even subtle alterations lead eventually to axonal degeneration.

1.3 Transcription factors involved in Schwann cell differentiation

The status and differentiation potential of a cell can be characterized by the network of transcription factors it expresses. This is due to the fact that majority of decisions in a cell are made at the level of regulating transcription initiation. The number of transcription factors is limited. Bioinformatic analysis has revealed that there are more than 2800 potential transcription factors in the human genome (Transcription factor prediction database, www.transcriptionfactor.org). Additional complexity is brought in by alternative splicing and post-translational modifications. Paradoxically, the complexity is lowered again by the fact that many different transcription factors bind to the same site on DNA. Conversly, the complexity of transcriptional regulation is again increased by the cooperative interactions between different transcription factors and transcription factors with DNA. To date it is impossible to present mathematical values for the extent of complexity lying under the term transcriptional regulation and the principal aim would be to identify the network of active and meaningful complexes of transcription factors in a given cell.

The generation of terminally differentiated Schwann cell from the migrating neural crest cell can be characterized by the serial and partially overlapping

expression of a set of transcription factors.. Unfortunately, we are far from understanding the regulatory network of them, since only a few have been shown to operate also *in vivo* during this differentiation pathway. Transcription factors that are shown to be important in Schwann cell development *in vivo* are shown in figure 4.

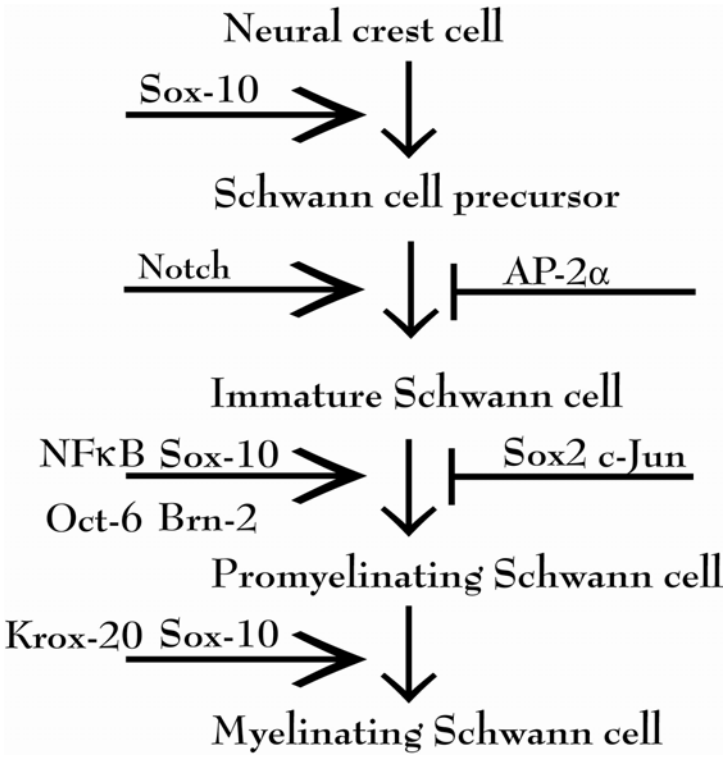

Figure 4. Schematic representation of major transitions taking place in Schwann cell lineage during development. Transcription factors shown to be important *in vivo*, regulating the respective transition are shown. Arrows indicate positive regulation, blunt lines indicate negative regulation

The first step of Schwann cell generation involves the emergance of Schwann cell precursors from the migrating neural crest cells. The only transcription factor shown to be important *in vivo* in this transition is Sox-10. Sox-10 is a protein with high-mobility-group (HMG) domain as its DNA binding domain (Wilson and Koopman, 2002). Sox-10 is initially expressed broadly in the neural crest and only later the expression is confined in cells of glial lineage of the PNS (Kuhlbrodt et al., 1998). The factors that regulate the expression of Sox-10 in the neural crest cells and in the cells of Schwann cell lineage are unknown at present. One candidate transcription factor that could participate in the induction of Sox-10

expression in the neural crest is a zinc-finger containing transcription factor Snail. There is moderate induction of Sox-10 expression in Xenopus embryos injected with Snail RNA and injection of the dominant negative form of Snail leads to inhibition of Sox-10 expression in the early neural crest (Honore et al., 2003). This phenomena has not been addressed in mammalian system. Mice deficient for the *Snail* gene die at E8.5, thus making the direct *in vivo* experiment impossible (Carver et al., 2001). Another study has demonstrated that *in ovo* electroporation of a transcription factor Sox-9 into chick embryos leads to the induction of Sox-10 in early neural crest (Cheung and Briscoe, 2003), however this expression is delayed and might very well account for the ability of Sox-9 to induce proliferation of prospective neural crest cells. Ability of Sox-9 to induce Sox-10 expression has also not been tested in a mammalian system. Mice heterozygous for *Sox-9* gene have severe skeletal defects and die perinatally (Bi et al., 2001). However, an indication that Sox-9 is involved also in the differentiation of other cell types than those of skeletal elements, comes from a study, where *Sox-9* gene was inactivated in CNS (Stolt et al., 2003). It appears that Sox-9 is essential for the generation of myelin-forming oligodendrocytes and astrocytes in the CNS.

Mutations in *Sox-10* gene lead to a combination of defects in the tissues of neural crest origin (Herbarth et al., 1998). In mice there are spontaneous mutations in Sox-10 locus called *dominant megacolon (dom).* In homozygous state these mutations lead to embryonic lethal phenotype with one of the most prominent defects being the lack of prospective Schwann cells. In heterozygous state the *dom* mutations lead to pigmentation defects and dominant megacolon (Herbarth et al., 1998). *Dom* mutations are nonsense or missense mutations, which most likely act in a dominant-negative manner, since complete absence of the Sox-10 results in the perinatal death of animals as compared to the embryonic death of *dom* animals (Britsch et al., 2001).

Part of Sox-10 function in prospective cells of Schwann cell lineage is the activation of neuregulin signalling system. There is a severe downregulation of *ErbB3* gene expression, which is a receptor for NRG-1 in Sox-10 *dom* embryos. It is noteworthy, however, that markers of neural crest like p75 and Cadherin-6 are not downregulated. Therefore, expression of Sox-10 is essential for the generation and/or maintenance of Schwann cell precursors, but not for neural crest cells (Britsch et al., 2001).

Forced expression of Sox-10 inhibits generation of neuronal and smooth muscle cells from NCSC *in vitro*. *In vivo*, Sox-10 is initially expressed in all migrating neural crest cells, while later the expression is extinguished in all cell types other than glia (Kim et al., 2003). Instructive clues for neuronal and smooth muscle differentiation act dominantly over neuregulin signalling (Shah et al., 1996) and therefore it is possible that expression of Sox-10 leads the cells to the default

differentiation pathway, followed by the elimination of others. Needless to say that glial fate is the default fate if one follows this scenario.

Another important target gene for Sox-10 in Schwann cell lineage is P0. Sox-10, but not a related Sox factor Sox-11 or Krox-20, is able to activate *P0* promoter *in vitro*. This activation is abolished by *dom* mutated Sox-10 proteins. Moreover, at E12.5, there are decreased levels of P0 in *dom* embryos as compared to wt littermates (Peirano et al., 2000).

Sox-10 is not able to activate transcription by its own *in vitro* (Kuhlbrodt et al., 1998). Instead, it cooperates with various other transcription factors, like Oct-6 and Pax-3, to activate transcription in a cooperative manner, given that binding sites for both proteins are present in a favourable configuration (Kuhlbrodt et al., 1998; Lang and Epstein 2003).

A general feature of HMG-domain proteins is to induce bends in DNA upon binding. In case of Sox-10, this feature is amplified due to the fact that unlike most HMG domain containing proteins, Sox-10 is able to bind DNA as a dimer in a cooperative manner. This dimeric binding increases the DNA bending and stability of the protein on DNA (Peirano and Wegner, 2000). Cooperative binding of Sox-10 is essential for the activation of *P0* promoter (Schlierf et al., 2002).

After the generation Schwann cell precursors from migrating neural crest cells, the next, precursor-embryonic Schwann cell transition, involves the function of transcription factors of AP-2 family. It has been shown that two of the three family members, AP-2α and AP-2γ are expressed in Schwann cell precursors and their expression is downregulated upon differentiation. Forced expression of AP-2α in Schwann cell precursors delays the differentiation of precursors to embryonic Schwann cells. However, there is no delay in embryonic Schwann cell generation in *AP-2α* knockout animals (Stewart et al., 2001). This might be due to the presence of AP-2γ in these cells. The Schwann cell lineage phenotype of *AP-2γ* knockout animals has not been analyzed due to the early embryonic death of these animals (Auman et al., 2002).

Embryonic Schwann cells receive signals from axons to initiate terminal differentiation process of the cells. This differentiation process involves serial activation of a set of transcription factors, timely first of which is NFκB. NFκB is activated by serine/threonine kinase PKA in response to elevated cAMP levels (Zhong et al., 1998). Active NFκB is a heterodimeric nuclear protein. Active heterodimer can be composed of different subunits. Schwann cells express p65 and p50 subunits (Nickols et al., 2003). Part of its activity is regulated by an inhibitor protein IκB, that retains the heterodimer of the transcription factor in the cytoplasm (Ghosh and Karin, 2002). Active NFκB is required for myelination *in vitro* and Schwann cell precursors from *p65* -/- animals are impared in myelination. Activity of NFκB in Schwann cells coincides with the time of myelination in mice (Nickols

et al., 2003). At the same time, the expression of the inhibitor IκBα is readily detectible in the myelinated Schwann cells of human sural nerve (Andorfer et al., 2001). Since the antibodies used in the cited studies recognize preferentially nuclear, activated forms of NFκB, it is possible that in fact the direct target gene, regulated by axons, is not NFκB, but the inhibitor IκB.

NFκB is required for the activation of POU-homeodomain containing transcription factor Oct-6 in cultured Schwann cells. NFκB regulates Oct-6 protein, but not mRNA levels suggesting regulation of Oct-6 activity at post-transcriptional level (Nickols et al., 2003; Yoon et al., 2008). Oct-6 (also known as Pou3f1/Tst-1/SCIP) belongs to class III subfamily of POU domain transcription factors. It shares highest homology with Brn-1 and Brn-2 transcription factors. In mice Oct-6 expression can be detected at E13 in Schwann cell precursors (Blanchard et al., 1996). Higher level of Oct-6 expression in Schwann cell lineage is induced later during embryonic development and induction of expression coincides with the time when large caliber axons acquire one-to-one relationship with Schwann cells. Expression of Oct-6 in Schwann cells is the highest around birth and is gradually decreasing during early postnatal development. Oct-6 expression is downregulated in myelinating Schwann cells, whereas it is retained in many non-myelinating Schwann cells throughout the adult life (Blanchard et al., 1996; Mandemakers et al., 1999; Zorick et al., 1996).

Schwann cell specific over-expression of dominant negative form of Oct-6 (lacking transcriptional activation domain) in transgenic mice leads to developmentally advanced and abnormal myelination in the PNS (Weinstein et al., 1995). This result shows that Oct-6 acts as a negative regulator of myelination. Indeed, it has also been shown that in co-transfection experiments Oct-6 can repress *P0* and *MBP* promoter (Monuki et al., 1993). Further support to the idea of Oct-6 being a negative regulator of myelination comes from the studies where full-legth Oct-6 is over-expressed in transgenic mice. Mice with elevated full-length Oct-6 in Schwann cells exhibit persistent hypomyelination and have decreased levels of P0, MBP and PMP22 mRNA (Ryu et al., 2007).

Inactivation of *Oct-6* in mice leads to a transient arrest of the myelination of PNS at the promyelin stage with a severe delay in myelination suggesting a function for Oct-6 as a positive regulator of myelination (Jaegle et al., 1996; Bermingham et al., 1996). The results from these studies are in conflict with idea that Oct-6 acts as a negative regulator of myelination. To resolve the conflict it has been proposed that Oct-6 activates genes that are important for Schwann cell differentiation but represses expression of myelin structural proteins. This suggestion highlights the importance of *Oct-6* gene downregulation during Schwann cell differentiation *in vivo*. It has been speculated that Oct-6 is responsible for its downregulation itself, since Oct-6 mRNA expression is not

downregulated in *Oct-6* mutant animals (Jaegle and Meijer 1998). Another posssiblity is that transcription factor Krox-20 (also known as Egr2) is involved in the downregulation of Oct-6 in myelinating Schwann cells as Oct-6 expression is not downregulated in Krox-20 deleted Schwann cells (Zorick et al., 1999). Indeed, it has been shown that Krox-20 can interact with Nab corepressor proteins and act as a transcriptional repressor (Russo et al., 1995; Svaren et al., 1996) Another possibility to explain the apparent conflict of explaining Oct-6 function as a positive or negative factor in myelination in the peripheral nervous system is that the expression level of Oct-6 in Schwann cells is critical and ectopic over-expression studies might lead to biologically meaningless conclusions.

The transient nature of the developmental arrest in Oct-6 deficient mice suggests a compensatory transcriptional activity in Schwann cells. It has been found that, beside Oct-6, Schwann cells express Oct-1, Brn-5 and Brn-2 (Blanchard et al., 1996; Wu et al., 2001; Jaegle et al., 2003). Brn-5 is expressed in the Schwann cells of adult animals and not during promyelin stage (Wu et al., 2001). Oct-1 is expressed in Schwann cells at a constant level throughout differentiation (Blanchard et al., 1996). Therefore they are unlikely candidates to compensate for the loss of Oct-6. Brn-2 expression in Schwann cells is regulated in a similar manner as that of Oct-6. Also, it has been shown that the expression of Brn-2 is higher in the nerves of Oct-6 deficient mice. Schwann cell specific ablation of Brn-2 in mice does not result in myelination phenotype. Overexpression of Brn-2 in Oct-6 deficient Schwann cells partially rescues the developmental defect of Oct-6 absence. In addition, it has been shown that Schwann cell specific inactivation of both *Oct-6* and *Brn-2* genes in mice results in more severe delay in myelination than incativation of *Oct-6* gene alone (Jaegle et al., 2003).

Terminal differentiation of myelinating Schwann cell requires zinc finger transcription factor Krox-20. Inactivation of *Krox-20* gene in mice leads to a complete absence of myelin (Topilko et al., 1994). It has also been shown that Krox-20 is required for maintenance of peripheral nerve myelination (Decker et al., 2006). Genetic studies have indicated that Oct-6 is required for the expression of Krox-20 in Schwann cells as Krox-20 expression in Schwann cells is downregulated in *Oct-6* null animals (Jaegle et al., 1996; Bermingham et al., 1996). It has also been shown that Oct-6 and Brn-2 in synergy with Sox-10 directly activate *Krox-20* Schwann cell specific enhancer (Ghislain and Charnay, 2006). The importance of Krox-20 in PNS myelination is further demonstrated by the fact that in human *Krox-20* mutations are associated with several peripheral neuropathies (Warner et al., 1998; Bellone et al., 1999; Mikesova et al., 2005). It has been shown that most major myelin genes are direct or indirect Krox-20 target genes (Topilko et al., 1994; Nagarajan et al., 2001). These include *P0, MBP, PMP22, Cx32* and *MAG*. Besides that, Krox-20 is involved in the activation of

some genes involved in the activation of lipid biosynthesis during myelination, such as *HMG CoA* reductase (Leblanc et al., 2005).

Krox-20 also acts as a transcriptional repressor in Schwann cells (Mager et al., 2008). In cultured Schwann cells, enforced expression of Krox-20 is sufficient to suppress expression of c-jun, a component of AP-1 transcription factor complex. c-Jun is present in embryonic Schwann cells and its expression *in vivo* is downregulated upon myelination. *In vitro* experiments suggest that supression of c-jun expression is prerequisite for myelination (Parkinson et al., 2004, 2008). The *in vivo* function c-jun during myelination remains enigmatic however, because there is no myelination defect in mice, where c-jun expression is conditionally ablated in Schwann cells (Jessen and Mirsky, 2008).

Therefore, on one hand, Krox-20 acts as transcriptional activator for many myelin genes, but on the other hand, together with Nab corepressors it is able to act as a negative regulator of transcription and represses genes like *Oct-6* and *c-jun*.

1.4 Neurotrophic factors in the development of peripheral nervous system

Neurotrophic factors are important regulators of development of the nervous system and maintenance of homeostasis of the neural tissue in the adult animal. Central concept of neurotrophic factor hypothesis postulates that neurotrophic factors are secreted molecules, being released from the targets of neuronal innervation at limited amounts. This limited secretion ensures balance between the size of the target organ and the number of innervating axons. Two main families of neurotrophic factors have been identified in vertebrates. These involve NGF family of neurotrophins and GDNF family of neurotrophic factors.

In mammals NGF family of neurotrophins consists of four related proteins nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) (reviewed in Bibel and Barde, 2000). Avian species lack NT-4 and at least six members of the family have been identified in bony fishes (Hallböök, 1999). Neurotrophins are synthesized as pre-pro-proteins and processed neurotrophins are secreted from the cells. The biological activity of neurotrophins is exerted through binding of neurotrophin dimers to a transmembrane receptor. Two classes of neurotrophin receptors have been identified. Tropomyosin-related tyrosine kinase (Trk) receptors consitute high affinity neurotrophin receptors and it has been shown that neurotrophins are able to directly bind and induce dimerization these receptors. Trk receptors exibit specificity for neurotrophins with Ntrk1/TrkA being receptors for NGF, Ntrk2/TrkB for BDNF and NT-4 and Ntrk3/TrkC for NT-3 (Figure 5A). All Trk receptors have multiple isoforms that are generated through alternative splicing.

Many of these isoforms lack tyrosine kinase domain and are believed to attenuate neurotrophin signalling. Another receptor for neurotrophins is the low-affinity receptor p75NTR, which does not possess any known enzymatic activity and all neurotrophins bind to it with a similar affinity (reviwed in Bibel and Barde, 2000; Huang and Reichardt, 2001).

GDNF family of neurotrophic factors consist of four members, glial derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), also known as neublastin and enovin, and persephin (PSPN). They bind to GPI linked GFRα receptors. The high affinity receptor for GDNF is GFRα1, for NRTN GFRα2, for ARTN GFRα3 and for PSPN GFRα4 (Figure 5A). The signalling cascade for GDNF family of neurotrophic factors is initiated mainly through common Ret coreceptor, which exhibits tyrosine kinase activity (reviewed in Airaksinen and Saarma, 2002).

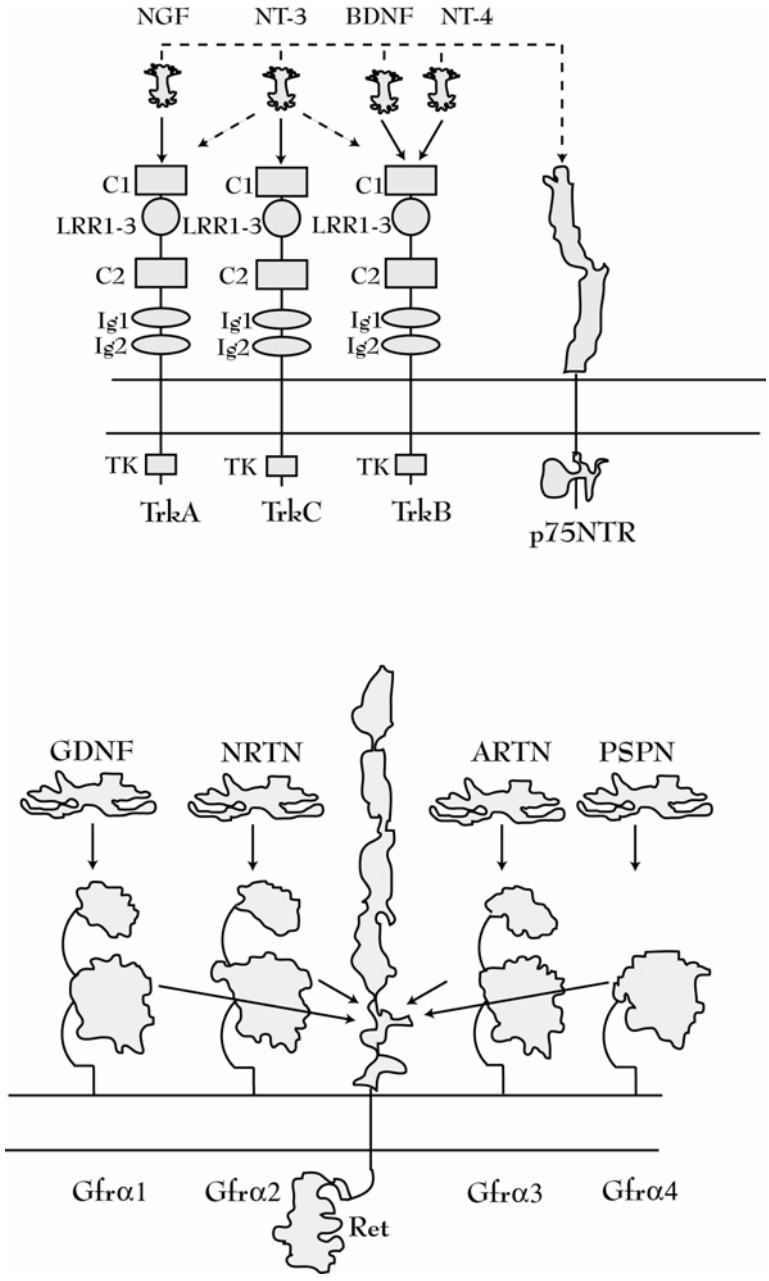

Figure 5. Ligand–receptor interactions of NGF and GDNF family of neurotrophic factors. A) All mature neurotrophin dimers are able to bind p75NTR receptor, but exhibit specific binding to Trk receptors. NGF Binds to TrkA, BDNF and NT-4 to TrkB and NT-3 to TrkC receptor. NT-3 is also able to interact with TrkA and TrkB with smaller efficiency. B) Each mature GDNF family ligand binds as a dimer to its specific alpha receptor, which is followed by activation of Ret transmembrane tyrosine kinase. Adapted from Reichardt, 2006; Bespalov and Saarma, 2007

All neurotrophins are expressed in rodent sciatic nerve and Schwann cells (Heumann et al., 1987; Meyer et al., 1992; Chan et al., 2001) .It has also been shown that Schwann cells express TrkB, TrkC and p75NTR receptors (Chan et al., 2001).

In vivo, BDNF and NT-3 elicit direct effect onto Schwann cell lineage. It has been shown that BDNF is a positive regulator of myelination in the PNS. Addition of exogenous BDNF into DRG neuron/Schwann cell co-culture results in enhanced formation of myelin in this system. Conversly, removal of endogenous BDNF by the addition of TrkB-Fc receptor inhibits myelination in cell culture system. Direct injection of BDNF into the sciatic nerve of newborn mice also results in the enhanced formation of myelin (Chan et al., 2001). Overexpression of BDNF in mice results in the increase in rate of myelination and myelin thickness and to lesser extent axon caliber. BDNF overexpression does not, however, influence the number of myelinated nerve fibers (Tolwani et al., 2004). Endogenous BDNF is also important for the regeneration of the injured peripheral nerve, as intraperitoneal injection of BDNF antibody into the animal that has undergone nerve lesion, results in the decrease in the number of myelinated axons after regeneration (Zhang et al., 2000).

During myelination BDNF signals through two BDNF receptors expressed in Schwann cells. First, p75NTR acts as a positive regulator of myelination. p75NTR is expressed in the Schwann cell lineage early on. Its expression is maximal around birth and is downregulated after that. Administration of blocking antibody to p75NTR inhibits myelination in DRG neuron/Schwann cell co-culture and in mice. Also, peripheral myelin thickness is smaller in *p75NTR* knockout animals (Cosgaya et al., 2002). It has also been shown that exogenous administration of BDNF inhibits Schwann cell migration in p75NTR dependent manner (Yamauchi et al., 2003). Another BDNF receptor expressed in Schwann cells is T1 isoform of TrkB. TrkB-T1 lacks intracellular tyrosine kinase domain and is considered to act as a dominant negative regulator of BDNF signalling. It has been suggested that after the onset of myelination TrkB-T1 expression is induced and binding of BDNF to it titers the neurotrophin off from its binding to p75NTR (Cosgaya et al., 2002). It has been proposed that the major source of BDNF in the peripheral nerve during development are sensory neurons and that BDNF is transported and secreted in anterograde fashion (Ng et al., 2007). Thus BDNF acts as a general positive inducer of myelination onto all myelinating Schwann cells, since every embryonic Schwann cell expresses p75NTR (Shah et al., 1994).

NT-3, on the other hand, is a negative regulator of myelination in PNS. It has been shown that additon of exogenous NT-3 in DRG neuron/Schwann cell co-culture inhibits myelination and removal of endogenous NT-3 from the cell culture system promotes myelination. Also, subcutaneous injection of NT-3 into newborn

mice inhibits myelination in the PNS (Chan et al., 2001). The negative effect of NT-3 on PNS myelination is executed through its high-affinity receptor TrkC (Yamauchi et al., 2003). It has been shown that NT-3 induces RhoGTPases Rac1 and Cdc42, and c-Jun N-terminal kinase dependent Schwann cell migration (Yamauchi et al., 2005). Whether the positive effect of NT-3 on Schwann cell migration is directly connected to its negative effect on myelination remains to be investigated.

NGF has also been shown to be a positive regulator of myelination in PNS. However, the positive effect of NGF on myelination in PNS is mediated through TrkA receptor, not through p75NTR, as it is in the case of BDNF (Chan et al., 2004). To date it has been shown that Schwann cells do not express TrkA and that the positive effect of NGF in myelination is on TrkA expressing neurons (Offenhauser et al., 1995; Chan et al., 2004). It has been proposed that NGF function is more indirect than that of BDNF, and that it regulates neuronal signals to glia. It is interesting to note that NGF acts as a negative regulator of myelination by oligodendrocytes, indicating fundamental differences in the regulation of myelination in the PNS and CNS (Chan et al., 2004).

A separate function for Schwann cell derived neurotrophic factors has been proposed in the development of PNS. BDNF (as well as GDNF) induces rapid release of axonal heparin binding forms of NRG-1 from motoneurons and NGF (and GDNF) induces rapid release of heparin binding forms of NRG-1 from sensory neurons, thus positively regulating myelination (Esper and Loeb, 2004). Whether this function of Schwann cell derived neurotrophic factors in the development of PNS is interconnected with the above-mentioned functions of neurotrophins has remained elusive so far.

A possible role for GDNF in the development of PNS has also been proposed from its source in the developing nerve. Schwann cells expresss GDNF and it has been suggested that Schwann cells are the source of biologically active GDNF for peripheral neurons (Springer et al., 1994; Choi-Lundberg and Bohn, 1995). A direct effect of GDNF onto Schwann cell myelination has been demonstrated in the study showing that exogenous GDNF is able to promote myelination in DRG neuron/Schwann cell co-culture system. It has also been shown that administration of exogenous GDNF to adult rats leads to hypermyelination in the PNS, with many normally unmyelinated axons adopting 1:1 relationship with Schwann cells and becoming myelinated (Hoke et al., 2003).

Taken together, a number of studies have established neurotrophic factors as important modulators of PNS development and myelination.

2. AIM OF THE STUDY

Aim of the study was to gain knowledge about the function of Oct-6 in Schwann cells and analyze changes in gene expression profile during differentiation of Schwann cells.

For this purpose the following tasks were carried out:

1. Generation and analysis of Schwann cell specific *Oct-6* knockout mice.
2. Identification of a novel POU domain transcription factor in Schwann cells.
3. Analysis of Brn-2 function in Schwann cells.
4. Global analysis of gene expression changes during Schwann cell differentiation using microarray and differential display methods.
5. Analysis of changes in mRNA expression levels of neurotrophic factors and their receptors during Schwann cell differentiation *in vitro* and *in vivo*.
6. Expression analysis of calcium activated chloride channel (CLCA) gene family, including Oct-6 target genes, in mouse and human nervous system.

3. MATERIALS AND METHODS

All molecular biology methods were performed according to standard practices (Sambrook and Russell, 2001; Nagy et al 2003) or according to instructions provided by the producer of the consumable or chemical. The following methods were used:

- Immunohistochemistry and western blotting (publications I, II and manuscript I);
- Electrophoretic mobility shift assay (publication II);
- Primary Schwann cell culture and transfections (publication III and manuscript I);
- Microarray analysis (manuscript I);
- Molecular indexing (manuscript I);
- RT-PCR and qRT-PCR analysis (publications II, III and manuscripts I and II);
- In situ hybridization (publication III);
- Analysis of genetically modified mice (publications I, II and manuscript I);
- Bioinformatic analyses of gene and mRNA structures (publication III and manuscripts I and II).

4. RESULTS AND DISCUSSION

4.1 Deciphering the role of Oct-6 in the development of the peripheral nervous system (publications I and II manuscript I)

An important role for the Class III POU domain transcription factor Oct-6 in the developing peripheral nerve has been established. However, studies of its exact role in nerve development and regeneration have been hampered by the high mortality rate of newborn *Oct-6* mutant animals (Bermingham et al., 1996; Jaegle et al., 1996). To circumvent this problem of early postnatal lethality, we generated Schwann cell-specific *Oct-6* null allele in mice by removing previously identified Schwann cell specific enhancer (SCE; Mandemakers et al., 2000) from the locus by homologous recombination. Mice heterozygous for the targeted allele *(ΔSCE*) were intercrossed and all three possible genotypes were represented in the offspring at expected Mendelian ratios. Analysis of the resulting *ΔSCE* homozygous animals revealed that unlike *Oct-6* null animals, these animals did not have the lethal perinatal defect of respiratory distress and they survived.

Perinatal death of *Oct-6* null animals is due to the lack of its expression in the neurons of the brainstem (Bermingham et al., 1996). Beside expression in the brainstem and Schwann cell lineage, Oct-6 is also expressed at high levels in hippocampus and skin (He et al., 1989; Alvarez-Bolado et al., 1995). Analysis of Oct-6 protein expression in homozygous *ΔSCE* animals showed that Oct-6 expression is retained in hippocampus, brainstem and retina, but not in sciatic nerve, showing that deletion of SCE specifically eliminates Schwann cell expression of the protein. These expression data also indicate that SCE is necessary and sufficient for the control of Oct-6 expression in the peripheral nerves *in vivo*.

The developmental defect of delay in myelination in *ΔSCE* animals was found to be slightly milder than in *Oct-6* null animals. Analysis of Oct-6 expression in postnatal day eight sciatic nerves by western blotting showed minute amounts of Oct-6 protein expression. Therefore it is likely that homozygous *ΔSCE* animals are strong Oct-6 hypomorphs.

We also showed that SCE drives Oct-6 expression in reactive Schwann cells during regeneration, because we detected high level of Oct-6 expression in distal nerve stumps eight days after axotomy. Oct-6 protein expression was not detected in the regenerating nerves of homozygous *ΔSCE* animals. These observations indicate that, as in development, re-myelination is delayed in the absence of *Oct-6* gene function.

In conclusion, we generated a novel Schwann cell-specific allele of *Oct-6* through deletion of the major Schwann cell-specific regulatory element, the SCE. Analysis of these mice reveals a Schwann cell autonomous function for Oct-6 in nerve development and regeneration.

The transient nature of the developmental arrest in Oct-6 deficient mice suggests a compensatory activity of one or more transcription factors in Schwann cells. It is prudent to speculate that additional transcription factor(s) has similar DNA binding activity as Oct-6. We analyzed expression of POU domain transcription factors in rat Schwann cells by EMSA analysis. We showed that Schwann cells express three POU domain proteins. In addition to Oct-6, we could detect the expression of Class I POU protein Oct-1 and Class III POU protein Brn-2. Oct-6-DNA and Oct-1-DNA complex gave stronger signals in our assay than Brn-2-DNA complex. Given that all three POU proteins bind to consensus octamer binding site with similar affinity, it could be concluded that expression of Oct1 and Oct6 is higher than expression of Brn-2 in rat Schwann cells.

Expression of Oct-1 in the peripheral nerve has been shown previously. It has been shown that the dynamics of Oct-1 mRNA expression partly antagonizes that of Oct-6 during Schwann cell development. During embryonic development the level of Oct-1 mRNA expression is the lowest at E17, whereas Oct-6 mRNA expression level is gradually increasing in the developing peripheral nerve (Blanchard et al., 1996). This suggests that Oct-1 and Oct-6 have separate functions during Schwann cell development.

Dynamics of Brn-2 expression in the peripheral nerves during development is similar to that of Oct-6. Also, the two the dipartite DNA binding domains, consisting of POU specific and homeodomain, are virtually identical between the two proteins. There are only 6 differences in the 144 amino acid long DNA binding domains of Oct-6 and Brn-2.

Our analysis showed that Brn-2 was expressed in peripheral nerves *in vivo*. Also, we could readily detect Brn-2 expression in Oct-6 deficient peripheral nerves. We also showed that both mRNA and protein levels of Brn-2 were elevated in developing peripheral nerves in the absence of Oct-6.

These data indicate that Brn-2 is a good candidate for being the compensatory factor for Oct-6 in regulating myelination in PNS. In order to test this hypothesis we generated transgenic mice that express Brn-2 and another POU protein Brn-5 in Schwann cells. It has been suggested by others that Brn-5 might also activate myelination program in the absence of Oct-6 (Wu et al., 2001). Expression of transgenes was under the control of Oct-6 Schwann cell enhancer (SCE; Mandemakers et al., 2000). Analysis of these transgenic mice in Oct-6 deficient genetic background revealed that Brn-2 and not Brn-5 is able to partially rescue the developmental myelination defect of Oct-6.

To elucidate the role of Brn-2 in myelination further, we generated Schwann cell specific knockout of *Brn-2* gene. The full knockout of *Brn-2* has been described previously and showed vital requirement of Brn-2 in the development of endocrine neurons of the hypothalamus (Nakai et al., 1995; Schonemann et al.,

1995). Homozygous Brn-2 deficient animal die before postnatal day 10 and therefore are unsuitable for studying the function of the protein in myelination. Schwann cell specific targeted deletion of *Brn-2* gene in mice did not affect peripheral nerve development and myelination. Schwann cell specific deletion of both *Brn-2* and *Oct-6* resulted in a hypomyelinating phenotype that was more severe than deletion of *Oct-6* alone.

Functional overlap between related transcription factors has been described in other tissues. For instance, closely related transcription factors Brn-1 and Brn-2 have overlapping roles in cortical neuron development (McEvilly et al., 2002; Sugitani et al., 2002). Also, it has been shown that Oct-6 and Skn-1 function synergistically in keratinocyte differentiation program (Andersen et al., 1997). Our data shows that Brn-2, but not Brn-5, can substitute for the requirement of Oct-6 during peripheral nerve development.

Brn-2 is expressed during peripheral nerve development similarly to Oct-6, albeit at somewhat lower levels. However, endogenous expression of Brn-2 is not sufficient for supporting proper time course of peripheral nerve development in the absence of Oct-6. One possibility to explain this phenomenon is that a specific interaction between POU protein and another transcription factor is needed, in order to activate necessary target genes for Schwann cell differentiation. It has been shown that N-terminal activation domain of Oct-6 functions synergistically with Sry box transcription factor Sox-10 (Kuhlbrodt et al., 1998). Sox-10 acts synergistically with POU proteins in the activation of *Krox-20*, an Oct-6 target gene, in Schwann cells (Ghislain and Charnay, 2006). Brn-2, on the other hand, does not interact functionally with Sox-10, but acts synergistically with another Sry box transcription factor, Sox-11 (Kuhlbrodt et al., 1998). Sox-11 is not expressed in Schwann cells. Therefore, it is possible that higher than endogenous levels of Brn-2 are required during peripheral nerve development in the absence of Oct-6, to overcome the lack of synergistic partnering of POU/Sox factors.

It has also been shown that Brn-1, another Class III POU protein, can fully replace Oct-6 during Schwann cell development (Friedrich et al., 2005). In their studies, mice with Oct-6 coding sequence replaced with that of Brn-1, had normal development of peripheral nervous system. These results argue against hypothesis about the existence of a specific Sox/POU partnering during Schwann cell development since Brn-1 does not partner with Sox-10, but with Sox-11 (Kuhlbrodt et al., 1998). However, these results can also be interpreted in the view of Sox/POU partnering hypothesis. For instance, it is possible that the level of Brn-1, when expressed under control of Oct-6 regulatory elements, is sufficient to overcome the requirement for Sox/POU partnering. Alternatively it is possible that Brn-1 is able to partner with a different Sox protein in Schwann cells, such as Sox-4, which is expressed in Schwann cells (Verheijen et al., 2003).

Taken together these data show that Brn-2 function largely overlaps with that of Oct-6 in peripheral nervous system development.

One of the important target genes of Oct-6 in Schwann cells is Krox-20 (Ghislain and Charnay, 2006). We were interested, what other genes might Oct-6 regulate in the Schwann cell lineage. In order to identify genes that have dynamic expression during Schwann cell development, we used modified *in vitro* culture model of rat Schwann cell differentiation (Brockes et al., 1980).

To analyze the gene expression changes upon Schwann cell differentiation *in vitro*, we used rat GEM-4 microarray chip of Incyte Pharmaceuticals, Palo Alto, CA. containing 8144 brain-enriched cDNAs. Quantification of the results of microarray experiment, showed the following. Out of 8144 datapoints, 315 were differentially regulated upon differentiation by at least 2-fold. Out of those 315 differentially expressed datapoints, 61 different annotated cDNAs were upregulated and 62 different annotated cDNAs downregulated upon Schwann cell differentiation. Broad functional classification of the differentially expressed genes showed that, as expected, myelin genes were upregulated during Schwann cell differentiation. Also, we found that more ECM protein genes were upregulated than suppressed during Schwann cell differentiation, indicating synthesis of basal lamina in differentiated Schwann cells. Same number of genes coding for cytoskeletal proteins and ion transporters were upregulated and suppressed showing broad rearrangements of cell shape and homeostatic balance taking place during Schwann cell differentiation. Interestingly, we found that more cell surface receptors were downregulated than upregulated during Schwann cell differentiation. Broad classification genes that were regulated during Schwann cell differentiation *in vitro* is shown in figure 6.

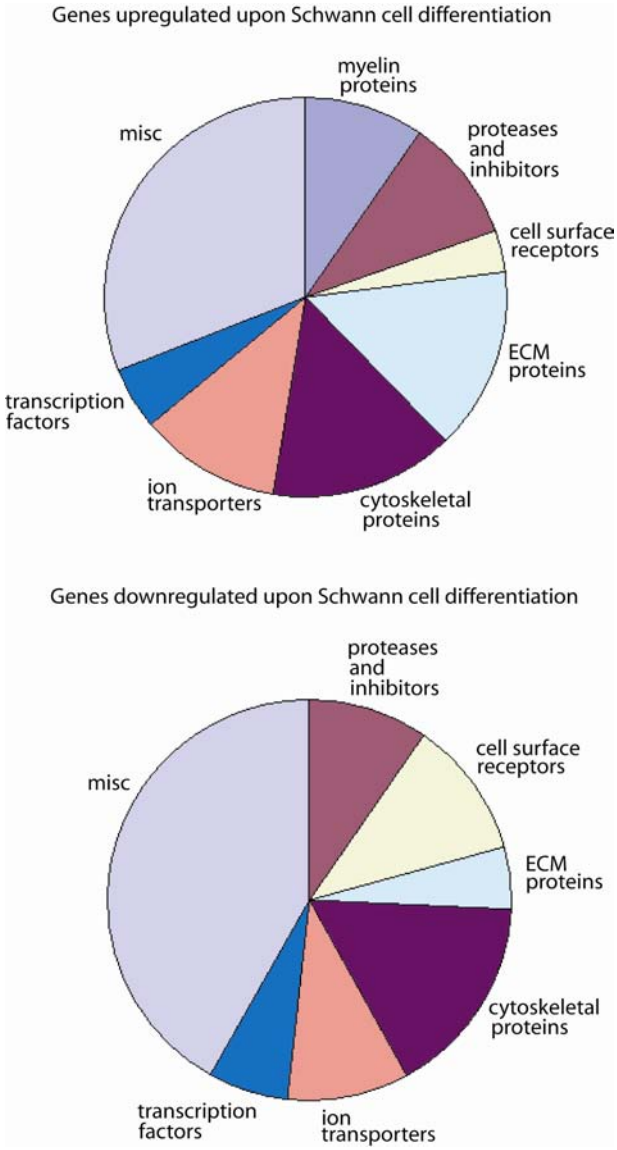

Figure 6. Broad classification of genes that are differentially regulated during Schwann cell differentiation *in vitro*

As a complement and alternative to microarray analysis we used modification of a differential display method called molecular indexing (Mahadeva et al., 1998) to compare cDNA populations derived of non-differentiated and differentiated Schwann cells. Using molecular indexing we identified a total of 50 cDNAs differentially expressed in non-differentiated versus differentiated Schwann cells.

We also asked whether the genes for which differential expression was established *in vitro* were also differently regulated in the perinatal peripheral nerves of Oct-6 deficient mice as compared to nerves of wt animals. We performed semi-quantitative RT-PCR analysis of 14 mRNAs with altered expression patterns during Schwann cell differentiation *in vitro*. The levels of transcription factor Krox-20 and Rho GTP exchange factor Rgnef were induced during Schwann cell differentiation *in vitro* and severely reduced in the nerves of the mutant animal as compared to the wt. The levels of two transcription factors – Zfand5 and Mitr – were suppressed during Schwann cell differentiation *in vitro* and the respective mRNA levels in mutant nerves were significantly higher as compared to wt nerves.

Taken together, our results give a glimpse into the transcriptional hierarchies operating during Schwann cell differentiation. We have shown that the terminal myelin gene expression is activated by Krox-20, which in turn is activated by Oct-6. Beside activating Krox-20, there is a separate function for Oct-6, it activates genes like Rgnef and Fibulin5.

4.2 Analysis of changes in mRNA expression levels of neurotrophic factors and their receptors during Schwann cell differentiation *in vitro* and *in vivo* (manuscript III)

Members of both NGF and GDNF families of neurotrophic factors are important in the development of the vertebrate PNS (Bibel and Barde, 2000; Airaksinen and Saarma, 2002). Despite their importance, expression patterns of the neurotrophic factors during nerve development have not been studied thoroughly. We have analyzed the expression of NGF and GDNF family members and their receptors during rat sciatic nerve development and in three differentiation stages of cultured rat Schwann cells.

Our analysis of mRNA expression of NGF family of neurotrophic factors showed that NGF and BDNF were expressed in cultured Schwann cells, whereas NGF, BDNF and NT-3 were expressed in the developing peripheral nerve *in vivo*. Expression of neurotrophins was higher in embryonic peripheral nerves. We also showed that all Trk receptors and p75NTR were expressed in the developing peripheral nerve *in vivo*. Cultured Schwann cells did not express full length TrkB receptor, but expressed all other receptors for NGF family ligands. Expression of full length TrkA and TrkB receptors was higher in the embryonic nerves as compared to later developmental timepoints and expression of truncated TrkB T1 isoform did not change significantly during development. Expression of both the full length and truncated TrkC as well as p75NTR receptors had a peak of expression perinatally and mRNA levels decreased thereafter.

Analysis of mRNAs for GDNF family of neurotrophic factors revealed that GDNF and Pspn, but not Artn and Nrtn, were expressed in the developing peripheral nerve and in cultured Schwann cells. Similarly to NGF family ligands, expression of GDNF and Pspn mRNA was higher in embryonic, as compared to postnatal nerves. We also found that Gfr alpha 1-3 receptors were expressed in the developing peripheral nerves and in Schwann cells. Surprisingly, we also detected Ret expression in the developing peripheral nerves, but not in Schwann cells. Schwann cells were previously considered as a Ret negative cell type, where signalling of GDNF family ligands is executed through NCAM co-receptor (Paratcha et al., 2003). Our analysis indicates that *in vivo*, there still might be active GDNF signalling through Ret co-receptor.

Our results demonstrating that neurotrophin expression is high in embryonic sciatic nerves and the levels are decreasing during development suggest two possible scenarios. First, it is possible that Schwann cells in the embryonic nerves are not a homogenous population of glial cells, but rather the phenotype of a Schwann cell depends on the type of axon it is contacting with. This means that during late embryogenesis different Schwann cell populations express different set of neurotrophins. Alternatively it is possible that embryonic Schwann cells exhibit homogenous phenotype, but express higher level of neurotrophins to provide maximal support for growing axons.

4.3 Expression analysis of the CLCA gene family in mouse and human with emphasis on the nervous system (publication III)

In a previous study (manuscript I) a member of Clca family was identified as a target gene of Oct-6. Members of the calcium-activated chloride channel (Clca) gene family have been suggested to possess a variety of functions including cell adhesion and tumor suppression (Hartzell et al., 2005). Expression of Clca family members has mostly been analyzed in non-neural tissues. We described the expression of mouse and human Clca genes in the nervous system.

RT-PCR analysis with cDNAs from adult mouse brain showed that mClca1, mClca2 and 4 are expressed at low levels in mouse brain. In accordance with previously published data we could not detect mClca3, 5 and 6 expression in adult mouse brain. Quantitative real-time PCR analysis of mClca1, 2 and 4 expression during mouse brain development showed that expression of mClca1 was increasing during postnatal brain development and reached maximum levels in the adult mouse brain. mClca2 expression did not change during mouse brain development and mClca4 expression was low during embryonic development, highest around birth of the animal and the level of respective mRNA was gradually decreasing during postnatal development.

To further analyze the cellular distribution of mClca1, 2 and 4 expression in brain, we performed in situ hybridization on adult brain sections. Since mClca1 and 2 genes share 95% identity, we were unable to design probes that distinguish between these genes. Therefore, we consider the expression pattern of mClca1 and 2 together. It should be noted however, that mClca2 was expressed at very low levels in the adult mouse brain and therefore most of the signal likely corresponds to mClca1 expression. Our analysis showed that mClca1/2 and 4 genes are expressed in the olfactory nerve layer of the adult brain. High magnification imaging of mClca1/2 expression in the adult mouse brain showed expression in cells next to the glomerular layer of the olfactory bulb, namely layer of entering olfactory nerve fibers, which is populated by the olfactory ensheathing cells.

Since three mouse Clca gene family members were expressed in brain, we were interested if any of the four human CLCA genes are expressed in the nervous system. RT-PCR analysis revealed that hCLCA2 and hCLCA4 were expressed in the adult human brain. The highest level of hCLCA2 and hCLCA4 expression was found in optic nerve. The expression level of hCLCA2 and hCLCA4 in optic nerve exceeded 58 and 35 times the expression level of the corresponding gene in cerebral cortex. The expression of hCLCA2 and hCLCA4 was also significantly higher in medulla and olfactory tract as compared to the expression level in cerebral cortex. Very low levels of hCLCA2 and hCLCA4 expression were found in cerebral cortex, cerebellum and spinal cord.

Taken together our analysis of mouse and human Clca gene expression indicates that the gene family is expressed at higher levels in the cells that ensheath cranial nerves.

CONCLUSIONS

1. Class III POU domain transcription factor Oct-6 has a cell autonomous function in the developing and regenerating peripheral nerve.

2. Oct-6 and a related transcription factor Brn-2 have partially overlapping functions and regulate positively transition from promyelinating to myelinating Schwann cells. This positive regulatory function cannot be substituted by class VI POU domain transcription factor Brn-5.

3. Oct-6 has several target genes other than Krox-20 in Schwann cells.

4. NGF and GDNF family of neurotrophic factors and their receptors have dynamic and specific expression pattern in Schwann cells.

5. Members of the Clca family of potential calcium activated chloride channels are expressed at high level in glial cells ensheathing nerves.

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ACKNOWLEDGEMENTS

This work has been carried out in Department of Cell Biology and Genetics at Erasmus University Rotterdam, The Netherlands and in Department of Gene Technology at Tallinn University of Technology, Estonia.

I am deeply thankful to Dies Meijer and Tõnis Timmusk, my supervisors, for their guidance in science, their enthusiasm, patience and belief in me. Working (and sometimes partying) together with Dies and Tõnis has taught me many things in science and also outside of it.

I am grateful to professors Frank Grosveld and Erkki Truve for giving me the opportunity to work at Erasmus University and Tallinn University of Technology.

I would like to thank all co-authors of my publications and manuscripts for sharing their results with me.

Many thanks go to Wim Mandemakers, Martine Jaegle, Mehrnaz Ghazvini and Jaan-Olle Andressoo from Rotterdam and Mart Speek, Richard Tamme, Andres Veske, Epp Väli, Priit Pruunsild, Indrek Koppel and Mari Sepp from Tallinn for being such nice and helpful labmates.

I am also thankful to Maria Maloverjan for her excellent drawing skills and Kaur Jaanson for his help in computer stuff.

Olen tänulik Annele ja Kenile sõpruse ja igakülgse abi eest.

Lõpetuseks sooviksin südamest tänada Allat, Evat, oma vanemaid ja Mihklit toetuse, julgustamise ja kõige muu eest.

PUBLICATION I

A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration

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EMBO Journal 21 (2002) 4612–4620

The EMBO Journal Vol. 21 No. 17 pp. 4612–4620, 2002

A cell type-specific allele of the POU gene *Oct-6* reveals Schwann cell autonomous function in nerve development and regeneration

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While an important role for the POU domain transcription factor Oct-6 in the developing peripheral nerve has been well established, studies into its exact role in nerve development and regeneration have been hampered by the high mortality rate of newborn *Oct-6* mutant animals. In this study we have generated a Schwann cell-specific *Oct-6* allele through deletion of the Schwann cell-specific enhancer element (SCE) in the *Oct-6* locus. Analysis of mice homozygous for this allele (Δ*SCE* allele) reveals that rate-limiting levels of Oct-6 in Schwann cells are dependent on the SCE and that this element does not contribute to Oct-6 regulation in other cell types. We demonstrate a Schwann cell autonomous function for Oct-6 during nerve development as well as in regenerating nerve. Additionally, we show that Krox-20, an important regulatory target of Oct-6 in Schwann cells, is activated, with delayed kinetics, through an Oct-6-independent mechanism in these mice.

Keywords: enhancer/glia/myelin/Oct-6/POU domain

Introduction

Over the years, a considerable research effort has focused on how the myelination programme in Schwann cells is regulated. This cellular differentiation programme is characterized by dramatic metabolic and morphological changes, including polarization of the cell by deposition of a basal lamina, the production of massive amounts of cell membrane, incorporating myelin-specific lipids and proteins, and the spiralling of these lamellae around the axon (reviewed in Mezei, 1993; Garbay *et al*., 2000). The myelin organelle further matures into structurally and functionally distinct domains of compact and non-compact myelin such as the Schmidt–Lantermann incisures and paranodal loops (Arroyo and Scherer, 2000; Peles and Salzer, 2000; Pedraza *et al*., 2001). The synthesis and maintenance of myelin is an exquisitely sensitive process, as demonstrated by the many, inherited or acquired, demyelinating and dysmyelinating diseases such as Guillain-Barré Syndrome and the hereditary motor and sensory neuropathies. If we are to understand the interactions between glial cells and neurones that shape and maintain the functionally mature histo-architecture of the nerve or lead to pathogenesis, it is important to elucidate the molecular basis of the myelination programme.

Myelination involves the coordinate and sequential activation of sets of genes whose expression is controlled by transcription factors that are modulated during Schwann cell differentiation. While many transcription factors are known to be present in premyelinating and myelinating Schwann cells, two transcription factors have gained prominence in recent years for their important role in regulation of the myelination programme (Wegner, 2000; Topilko and Meijer, 2001). These transcription factors are the zinc finger protein Krox20 (Egr-2) and the POU-homeodomain protein Oct-6/SCIP/Tst-1 (referred to as Oct-6 in this paper) (Monuki *et al*., 1989; Meijer *et al*., 1990; Suzuki *et al*., 1990; Topilko *et al*., 1994). Both genes are dynamically expressed within the Schwann cell lineage, during development as well as during nerve regeneration, and their regulated expression depends on continued axonal contact (Scherer *et al*., 1994; Zorick *et al*., 1996). Genetic and cell biological studies have revealed that these transcription factors act in a genetic cascade (Topilko and Meijer, 2001). In promyelin Schwann cells, *Oct-6* expression is strongly increased in response to an unknown axonal contact-related signal and subsequently activates a set of genes that includes *Krox-20*. Induction of high-level Krox-20 expression leads to the activation of an additional set of genes including the major myelin genes and those involved in lipid metabolism (Nagarajan *et al*., 2001). Oct-6 is strongly down-regulated after the peak of myelination. A third transcription factor, Sox-10, is expressed throughout the development of the Schwann cell lineage and possibly interacts with both Oct-6 and Krox-20 in regulating their target genes (Kuhlbrodt *et al*., 1998).

Further study into the role of Oct-6 in nerve development and regeneration is hampered by the fact that *Oct-6* knock-out mice die shortly after birth because of breathing insufficiency, most likely caused by a defect in migration and differentiation of neurones in the brainstem (Bermingham *et al*., 1996). To circumvent this problem of early postnatal lethality, one would have to generate a viable Schwann cell-specific allele for *Oct-6*. Such a mouse would be of great value, allowing studies into the role of Oct-6 in nerve regeneration and allowing study of

Oct-6 protein domains, target genes and potential Oct-6 redundant proteins.

Recently, we have identified putative regulatory elements within the *Oct-6* locus using DNase I hypersensitivity mapping. Eight hypersensitive sites were mapped within a region of 35 kb. Using a deletion mapping approach in transgenic mice, we characterized a major *cis*-acting element within the *Oct-6* locus on which intracellular signalling pathways converge to activate *Oct-6* gene expression (Mandemakers *et al*., 2000). This element, the *Oct-6* Schwann cell enhancer or SCE, is characterized by two DNase I hypersensitive sites. The SCE was shown to be sufficient to drive regulated expression within the Schwann cell lineage of transgenic mice. However, endogenous *Oct-6* gene expression is not restricted to the Schwann cell lineage but is also expressed in the developing nervous system and skin. Expression is particularly high in the hippocampus, cortex, superior colliculus and brainstem nuclei, such as those of the hypoglossus and facial nerves (He *et al*., 1989; Alvarez-Bolado *et al*., 1995). No consistent transgene expression was observed in any of these brain regions in mice carrying a β-*galactosidase* reporter gene under the control of the SCE.

Based on these results, we hypothesized that deletion of the SCE from its normal chromosomal context would result in a Schwann cell-specific *Oct-6* null allele. To test this hypothesis, we have generated mice homozygous for this deletion allele, the Δ*SCE* allele, and found that *Oct-6* gene expression is affected in the Schwann cell lineage but not in any other cell type examined. These results demonstrate that the SCE is the decisive *cis*-regulating element governing Schwann cell-specific expression of the gene and that the SCE does not contribute to other aspects of the *Oct-6* expression pattern. Consequently, these mice, which are viable, have allowed us to study, for the first time, the role of Oct-6 in regeneration. Our results demonstrate that activation of *Oct-6* gene expression in reactive Schwann cells in regenerating nerves depends on the SCE and that the temporally correct activation of the myelination programme requires Oct-6. Also, our results demonstrate that the peripheral nerve phenotype observed in *Oct-6* mutant animals results from a loss of function of Oct-6 in Schwann cells and not in neurones. Additionally, we provide evidence that Oct-6 protein levels are rate limiting in the differentiation of promyelin Schwann cells into myelinating cells, demonstrating the importance of precise quantitative expression during development and regenerative processes. Furthermore, we show that *Krox-20* gene expression is activated in these mice with delayed kinetics, involving an Oct-6-independent mechanism.

Results

Deletion of the Oct-6 SCE through gene targeting

To delete the 4.3 kb SCE, a gene targeting vector was constructed in which the SCE was replaced with a *puromycin* selection cassette (Figure 1A). A negative selection cassette was introduced (*Py-TK*) flanking the 5′ homologous region, allowing counterselection of randomly integrated targeting constructs. The *puromycin* selection cassette was flanked by LoxP sites, which allowed deletion of the *puromycin* gene and its regulatory sequences from the targeted allele using Cre recombinase (Le and Sauer, 2001). Of the 141 embryonic stem (ES) cell clones that were puromycin resistant and ganciclovir insensitive, four were found to contain a homologous recombination event, as judged by Southern blot analysis (Figure 1B). Of those four, three had additional random integrations of the targeting cassette and were discarded. The one correctly targeted ES cell clone had a correct number of chromosomes and was used to generate chimeric mice. Chimeric animals were mated to *Zp3-Cre* transgenic female animals (D.Drabek). *Zp3-Cre* transgenic animals express high levels of the Cre recombinase in the oocyte, resulting in the removal of the puromycin cassette on the paternal chromosome in the zygote. Offspring in which the puromycin cassette was removed and the *Zp3-Cre* transgene was absent were identified using Southern blot analysis. These mice were used for further analysis.

Deletion of the SCE results in loss of Oct-6 expression in the Schwann cell lineage but not in other lineages

Adult mice heterozygous for the targeted allele, $Oct\text{-}6^{\Delta SCE/+}$, were intercrossed and offspring were genotyped. All three genotypes were represented in the offspring of these intercrosses at the expected Mendelian frequencies (out of 122 pups, 38 were $Oct\text{-}6^{\Delta SCE/\Delta SCE}$, 54 were $Oct\text{-}6^{\Delta SCE/+}$ and 30 were wild type). To examine whether Oct-6 expression in Schwann cells of the developing nerve was affected by the homozygous deletion of the SCE, we collected sciatic nerves from $Oct\text{-}6^{\Delta SCE/+}$ and $Oct\text{-}6^{\Delta SCE/\Delta SCE}$ pups at day 8 after birth and processed them for immunohistochemistry. While large numbers of Oct-6-positive Schwann cell nuclei were observed in nerves of $Oct\text{-}6^{\Delta SCE/+}$ animals, no Oct-6-positive nuclei were observed in the nerve of $Oct\text{-}6^{\Delta SCE/\Delta SCE}$ animals (Figure 2A and B). Thus, homozygous deletion of the SCE results in a strong reduction of Oct-6 expression to levels beyond detection in our immunohistochemistry experiment.

As mentioned above, $Oct\text{-}6^{\Delta SCE/\Delta SCE}$ and $Oct\text{-}6^{\Delta SCE/+}$ genotypes were found among the offspring of heterozygote crosses at the expected Mendelian ratios. In contrast, heterozygous crosses between mice carrying an insertional null allele for *Oct-6* (the *βgeo* allele) produced only a few offspring alive at 10 days post-partum, and homozygous for the null allele (Bermingham *et al*., 1996; Jaegle *et al*., 1996). It was found that most $Oct\text{-}6^{\beta geo/\beta geo}$ animals die of respiratory distress shortly after birth (Bermingham *et al*., 1996). This high incidence of lethality in newborn *Oct-6* null mice was attributed to a disorganization or reduction in cell number of cervical motor neurone groups of the phrenic nucleus and possibly medullar nuclei involved in breathing regulation, such as the nucleus tractus solitarius. Thus, the apparent lack of respiratory distress in neonatal $Oct\text{-}6^{\Delta SCE/\Delta SCE}$ mice suggests that the function of these nuclei is not affected by the deletion of the SCE. We therefore examined whether Oct-6 expression in a number of brain regions was affected by deletion of the SCE. We first examined Oct-6 expression in the medulla of the same animals as presented in Figure 2A and B. As can be seen in Figure 2C and D, Oct-6 is highly expressed in a subset of neurones in the nuclei of the hypoglossal nerve (XII) and the

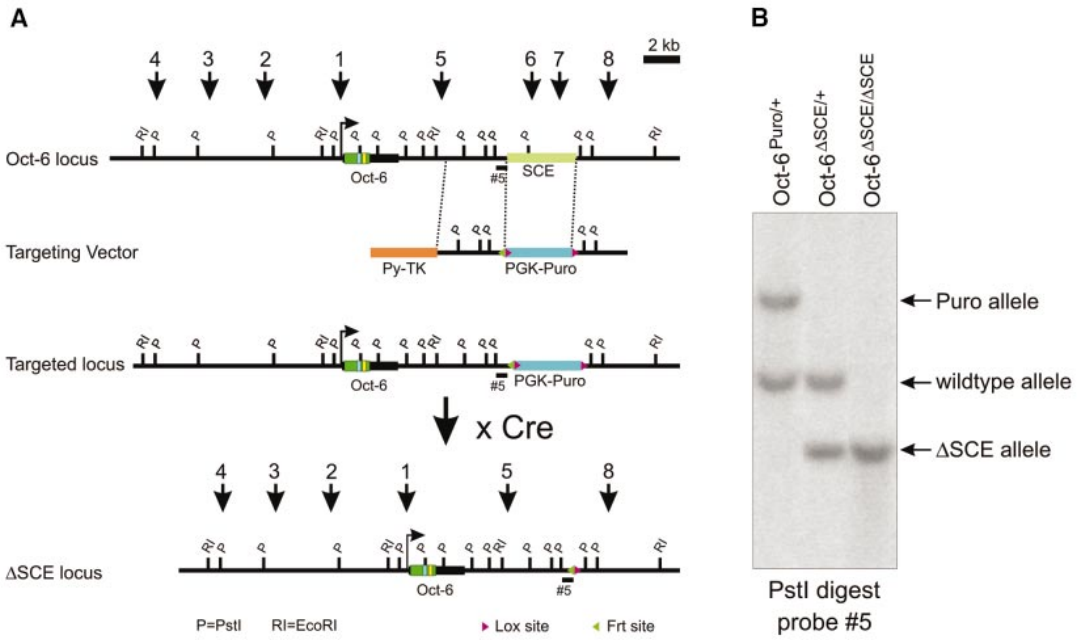

Fig. 1. Deletion of the 4.3 kb SCE from the *Oct-6* locus. (**A**) Gene targeting scheme for the *Oct-6* SCE. The SCE is indicated with a light green bar and is located ~12 kb downstream of the *Oct-6* gene CAP site. The intronless *Oct-6* transcription unit is indicated with a thick black line and the open reading frame is dark green with the POU-specific domain and POU homeodomain highlighted in blue and yellow, respectively. The relative positions of the mapped DNase I HSSs are indicated by numbered arrows. The SCE contains HSS6 and HSS7. In the targeting vector, a *puromycin* selection gene driven by the *pgk-1* promoter replaces the 4.3 kb *Hpa*I–*Msc*I fragment containing the SCE. LoxP sites (red triangles) flank the selection cassette, while an FRT site (green triangle) is present directly 5′ of the 5′ LoxP site. The orange box represents the counterselection cassette containing the HSV *TK* gene plus promoter linked to a polyoma virus enhancer. The probe used to identify the correctly targeted allele at the 5′ end is indicated (5). The locus after the predicted homologous recombination event and removal of the *puromycin* cassette by Cre recombinase is shown. (**B**) Southern analysis of mice carrying the targeted allele before and after removing the selection cassette. Using probe 5, the targeted allele is identified by a 3.5 kb *Pst*I fragment, while a 1.8 kb band identifies the wild-type allele. Removal of the selection cassette is demonstrated by the appearance of a 1.1 kb *Pst*I fragment with probe 5.

solitary tract. The identity of these Oct-6-positive neurones was confirmed by, in addition to anatomical criteria, immunostaining with antibodies directed against choline acetyltransferase, a general marker for cholinergic neurones (data not shown). Oct-6 expression in these neurones was not affected by the deletion of the SCE. In addition, we found that Oct-6 is normally expressed in neurones of the CA1 field of the hippocampus, putative amacrine neurones in the inner nuclear layer of the retina, superior colliculus and the skin (Figure 2E–H; data not shown). In fact we have not observed a tissue or cell type other than Schwann cells in which deletion of the SCE affects Oct-6 expression. These results demonstrate that deletion of the SCE from its normal genomic context leads to a severe reduction of *Oct-6* gene expression in Schwann cells, while expression in other tissues is not affected, thus providing a plausible explanation for the viability of Δ*SCE* homozygous animals. Thus, the SCE is required for Schwann cell-specific expression of the *Oct-6* gene, but does not contribute to regulation of the gene in other cell types.

Developmental delay in peripheral nerve development

Mice homozygous for complete loss-of-function alleles show delayed peripheral nerve development with Schwann cells transiently arrested at the promyelin stage of differentiation. It has been assumed that this developmental delay results from loss of Oct-6 function in the Schwann cell lineage. However, as Oct-6 is widely expressed during embryonic development throughout the neurectoderm, it is possible that part of the phenotype results from loss of Oct-6 function in neurones or their precursors (Alvarez-Bolado *et al*., 1995; Zwart *et al*., 1996). Analysis of peripheral nerve development in Δ*SCE* homozygous animals should resolve this issue, as in these animals Oct-6 expression is selectively lost in the Schwann cell lineage only. Therefore, we examined electron microscopically the developmental maturation of the sciatic nerve in *Oct-6*$^{\Delta SCE/+}$ and *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals at different postnatal stages (Figure 3). In the sciatic nerve of heterozygous animals at postnatal day 4 (P4), many Schwann cells are actively myelinating, with significant numbers of cells still at the promyelin stage (Figure 3A). Four days later, at P8, most, if not all, prospective myelinating cells have progressed beyond the promyelin stage and are actively engaged in elaborating myelin around their associated axon (Figure 3C). In contrast, in nerves of animals homozygous for the SCE deletion, a majority of Schwann cells are found in a promyelin configuration during the first week of postnatal

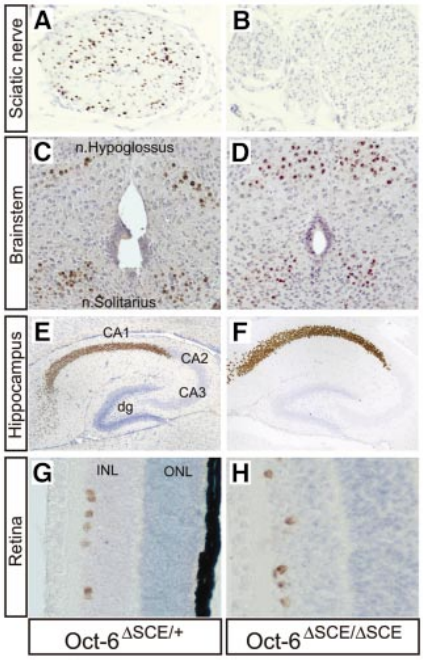

Fig. 2. The Δ*SCE* allele affects Oct-6 expression in the Schwann cell lineage only. (**A**) A brown precipitate identifies Oct-6 protein-expressing Schwann cell nuclei in transverse sections of sciatic nerve of *Oct-6*$^{\Delta SCE/+}$ mice at P8. (**B**) None of the Schwann cells in the sciatic nerve of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice expresses high levels of Oct-6 at this stage. In contrast, Oct-6 expression is not affected in the brainstem of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice, in particular the nucleus hypoglossus or nucleus solitarius [compare (**C**) and (**D**); P8]. Also, neurones in the hippocampal CA1 field express high levels of Oct-6 and this expression is not affected by the deletion of the SCE [compare (**E**) and (**F**); P8]. Oct-6 is expressed in a subset of neurones in the inner (INL) but not the outer (ONL) nuclear layer of the developing retina (P8). Although we made no further attempt to identify these neurones, their position within the nuclear layer corresponds to amacrine neurones. Again, expression of Oct-6 in these neurones is not affected by deletion of the SCE [compare (**G**) and (**H**)]. All paraffin sections are counterstained with haematoxylin.

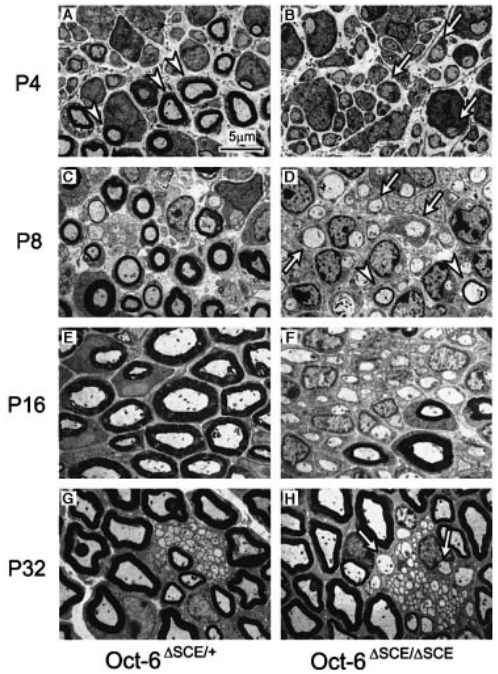

Fig. 3. Schwann cell differentiation is delayed at the promyelin stage in developing nerves of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice relative to heterozygous mice. (**A**–**H**) Representative electron micrographs of transverse sections through the sciatic nerve of homozygous and heterozygous animals at different postnatal stages of development. While condensed myelin figures are abundantly present at P4 in heterozygous animals [arrowheads in (A)], such myelin figures are only appearing at P8 in homozygous animals [arrowheads in (D)]. Most Schwann cells at P4 and P8 in homozygous animals are morphologically (and molecularly; see Figure 2) at the promyelin stage [arrows in (B) and (D)]. Promyelin figures are still found at P32 in mutant animals [arrows in (H)], while in heterozygous animals all myelin-competent Schwann cells are at later stages of myelination.

life (Figure 3B and D). Only in the second week are increasing numbers of myelinating cells observed (Figure 3F). By 4.5 weeks of postnatal development, most myelinating Schwann cells have elaborated myelin, although few promyelin figures are still observed at this time, especially around groups of non-myelinated low-calibre fibres (arrows in Figure 3H). Thus, while most prospective myelinating Schwann cells in heterozygous nerves have initiated myelination by P8, the vast majority of such cells in homozygous animals only do so between P16 and P32. These results suggest that the delay in nerve development, as observed in *Oct-6*$^{\beta geo/\beta geo}$ and *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice, results primarily from loss of Oct-6 function in Schwann cells and not in neurones.

The SCE deletion is a Schwann cell-specific Oct-6 hypomorphic mutation

The developmental delay in peripheral nerves of mice homozygous for the Δ*SCE* allele appears slightly milder than that observed in mice homozygous for the *βgeo* allele (full knock out). This is particularly evident at P8 (Figure 4A). At this stage, no myelin figures are observed in the nerves of *Oct-6*$^{\beta geo/\beta geo}$ mice, while few myelin figures are present in the nerves of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice. This difference in severity of peripheral nerve phenotype could be due to non-Schwann cell autonomous or systemic effects of the *Oct-6 βgeo* allele that add to the Schwann cell autonomous effect. Alternatively, it is possible that the Δ*SCE* allele is a hypomorphic *Oct-6* allele characterized by low-level residual expression of Oct-6 protein not detected in our immunohistochemistry experiments (Figure 2A). We therefore examined Oct-6 expression at P8 in nerves of animals heterozygous or homozygous for the Δ*SCE* allele using the more sensitive western blotting technique (Figure 4B; see also Figure 6). Low amounts of Oct-6 protein are observed in P8 nerve extracts of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice, while *Oct-6*$^{\beta geo/\beta geo}$ mice do not express Oct-6 (data not shown). It is, therefore, likely that the Δ*SCE* allele is a strong hypomorphic allele.

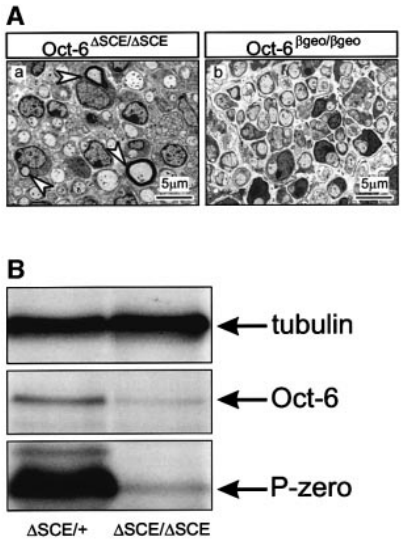

Fig. 4. The Δ*SCE* allele is a hypomorphic allele of *Oct-6*. (**A**) Comparison of sciatic nerve morphology in *Oct-6*$^{\Delta SCE/\Delta SCE}$ (a) and *Oct-6*$^{\beta geo/\beta geo}$ (b) mice at P8 reveals that a minority of Schwann cells in *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice have progressed to form compact myelin (arrowheads). In contrast, all Schwann cells are still at the promyelin stage of differentiation in sciatic nerve of *Oct-6*$^{\beta geo/\beta geo}$ mice. (**B**) Mice homozygous for the Δ*SCE* allele express strongly reduced levels of Oct-6 in Schwann cells of the developing nerve at P8. Western blot experiments showing low levels of Oct-6 protein in nerve extracts from *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals. The amounts of protein loaded per lane were similar, as demonstrated by the similar intensities of the α-tubulin immunoreactive band. In accordance with the delayed myelination status of sciatic nerve in *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals, low levels of P-zero protein are detected at this stage. Nerve development in heterozygous mice is normal and myelinating Schwann cells express high levels of P-zero.

Krox-20 activation is delayed in Schwann cells of Oct-6$^{\Delta SCE/\Delta SCE}$ *mice*

Schwann cell differentiation is arrested at the promyelin stage in *Oct-6* and *Krox-20* null mice. However, this differentiation arrest is transient in *Oct-6* mutant mice, while the arrest is permanent in *Krox-20* null mice, although these mice die before 3 weeks of age. Previously, we have shown that one important target of Oct-6 regulation in myelinating Schwann cells is the zinc-finger transcription factor Krox-20 (Ghislain *et al*., 2002). In particular, we have shown that Krox-20 is not expressed in Schwann cells during the first week of postnatal development in *Oct-6* null mice. One could speculate that the failure to initiate myelination on schedule in *Oct-6* mutant animals results from a failure to activate *Krox-20* gene expression. The transient nature of the differentiation block in *Oct-6* mutant animals then suggests that *Krox-20* is activated at a later stage in an Oct-6-independent manner.

To address this question, we collected sciatic nerves of *Oct-6*$^{\Delta SCE/+}$ and *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice at different postnatal stages, and examined the temporal expression of Oct-6 and Krox-20 and myelin protein P-zero by immunohistochemistry (Figure 5). P-zero is the major myelin protein in peripheral myelin and its accumulation in the compacting myelin sheath provides a convenient measure for the

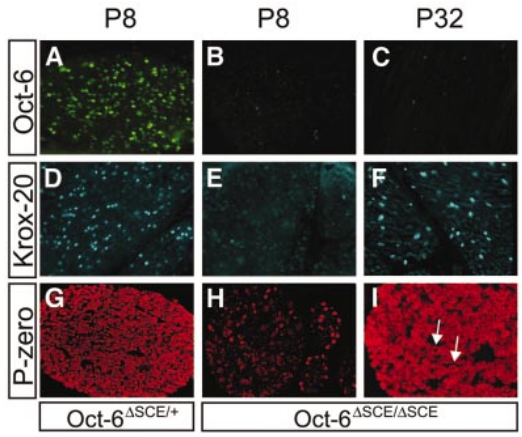

Fig. 5. Oct-6 expression is lost in Schwann cells of mice homozygous for the Δ*SCE* allele. (**A**) Homozygous deletion of the SCE results in loss of Oct-6 expression in Schwann cells of the developing nerve and delayed appearance of Krox-20 and the major myelin protein P-zero. Transverse sections of paraffin-embedded sciatic nerves at P8 or P32 from *Oct-6*$^{\Delta SCE/+}$ and *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice were incubated with antibodies against Oct-6, Krox-20 or P-zero. P-zero immunoreactivity reveals the typical ring-like myelin structures indicated by arrows in (**I**).

progression of myelin formation in the developing nerve (Greenfield *et al*., 1973). We first confirmed that Schwann cells in P8 *Oct-6*$^{\Delta SCE/+}$ nerves express high levels of Oct-6, while no Oct-6 expression was observed in Schwann cells of *Oct-6*$^{\Delta SCE/\Delta SCE}$ nerves (Figure 5A). Also, in agreement with our previous observations, Krox-20 expression is undetectable in P8 *Oct-6*$^{\Delta SCE/\Delta SCE}$ nerves, while Schwann cells in *Oct-6*$^{\Delta SCE/+}$ nerves do express Krox-20 at this stage (Ghislain *et al*., 2002). In addition, P-zero protein expression is severely reduced in Schwann cells of P8 nerves of *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals. However, at P32, Krox-20 is expressed in Schwann cells in nerves of *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals and extensive myelination is evident by the high level of P-zero immunoreactivity, showing characteristic ring structures in transverse sections (arrows in Figure 5I). Thus, in the absence of Oct-6 function, Krox-20 expression is eventually activated at the time extensive myelination is observed.

Nerve regeneration

Oct-6 gene expression is strongly increased in reactive Schwann cells during nerve regeneration (Scherer *et al*., 1994; Zorick *et al*., 1996). Previous work has suggested that the SCE is sufficient to mediate this reactivation of *Oct-6* gene expression during regeneration (Mandemakers *et al*., 2000). However, in these experiments, the SCE was coupled to the *Oct-6* promoter and upstream region. It is, therefore, possible that activation of *Oct-6* gene expression in reactive Schwann cells is mediated through elements outside the SCE, such as the promoter. To assess whether the SCE is also necessary for reactivation of *Oct-6* gene expression and, if so, whether Oct-6 function is required in reactive Schwann cells in regenerating nerves, we first comparatively examined Oct-6 expression in regenerating nerves of *Oct-6*$^{\Delta SCE/+}$ and *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals. Oct-6 is highly expressed in Schwann cells of the regenerating

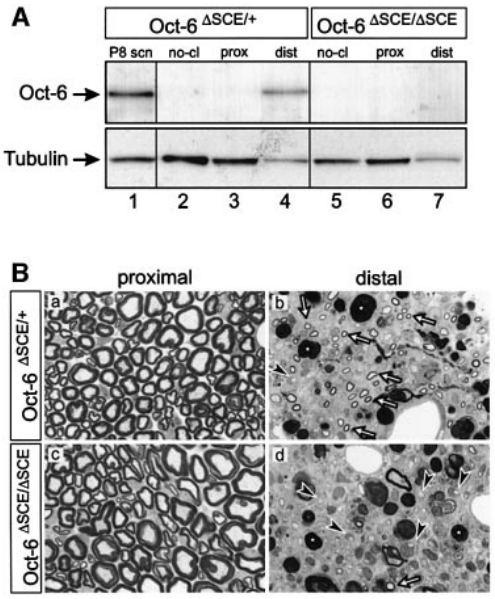

Fig. 6. Oct-6 regulation and function in the regenerating nerve. (**A**) Reactivation of Oct-6 expression is mediated through the SCE. Western blot analysis of sciatic nerves 12 days after crush lesion from adult *Oct-6*$^{\Delta SCE/+}$ (lanes 2–4) and *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice (lanes 5–7). Nerves were divided in a proximal part (prox, lanes 3 and 6) and a distal part (dist, lanes 4 and 7) of equal length. Controls included are the undamaged nerves, contralateral to those operated on (no-cl, lanes 2 and 5), and developing sciatic nerve at P8 (P8 scn). Oct-6 is not expressed in Schwann cells of the adult nerve, while at P8, Schwann cells express high levels of Oct-6. Tubulin served as loading control. However, tubulin expression is reduced in the distal part of the regenerating nerve because of incomplete regeneration. (**B**) Schwann cell myelination is delayed in regenerating nerves of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice. Regenerating nerves, 12 days post-operation, were examined by light microscopy. Nerves were embedded in plastic and semi-thin sections were cut at 5 mm proximal (a and c) and 3 mm distal (b and d) to the lesion site. Sections were stained with ppd. In both genotypes, much myelin debris is still present (asterisk in b and d). Many regenerating fibres in the *Oct-6*$^{\Delta SCE/\Delta SCE}$ mouse are ensheathed by Schwann cells that have not elaborated compact myelin yet (arrowheads in b and d). In contrast, many compact myelin figures are found surrounding the regenerating fibres in *Oct-6*$^{\Delta SCE/+}$ mice (arrows in b and d).

distal nerve stump 8 days after axotomy (Scherer *et al*., 1994; Zorick *et al*., 1996; Mandemakers *et al*., 2000; Figure 6A). In contrast, Oct-6 expression is not detectable at this stage in the regenerating nerve of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice (Figure 6A). Thus, the SCE is also required for reactivation of *Oct-6* gene expression during regeneration.

We next examined how nerve regeneration at the morphological level was affected in the absence of *Oct-6* reactivation. The sciatic nerves of *Oct-6*$^{\Delta SCE/+}$ or *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals were crush lesioned at mid-femoral level. The extent of regeneration was assessed by serial sectioning and microscopic analysis of the regenerating nerves. At 12 days post-transection, many regenerating axon fibres were seen at 3 mm distal of the lesion. In addition, we observed many myelin ovoids that had not been cleared yet by macrophages or had not been autophagocytosed (arrowheads in Figure 6B, b and d). The degree of degeneration and axonal ingrowth of the distal nerve stumps was similar in *Oct-6*$^{\Delta SCE/+}$ and *Oct-6*$^{\Delta SCE/\Delta SCE}$ nerves. Many regenerating nerve fibres are being actively myelinated in the *Oct-6*$^{\Delta SCE/+}$ nerves, as demonstrated by the many thin compact myelin figures (arrow in Figure 6B, b). In contrast, myelination of regenerating fibres in *Oct-6*$^{\Delta SCE/\Delta SCE}$ nerves was much less advanced at this stage. Many fibres that had not yet progressed beyond the promyelin stage of ensheathment and those that were myelinated had thinner myelin sheaths. These observations indicate that, as in development, myelination is delayed in the absence of *Oct-6* gene function.

Discussion

In the work described here, we have generated a viable and Schwann cell-specific *Oct-6* knock-out mouse through deletion of the SCE. Analysis of this mouse allowed us to address questions related to the regulation and function of Oct-6 during peripheral nerve development and regeneration.

Deletion of the SCE results in a Schwann cell-specific hypomorphic Oct-6 allele

An *Oct-6* allele in which the SCE is deleted was created through homologous recombination in ES cells and removal of the LoxP-flanked *puromycin* cassette by Cre recombinase. The selection cassette was removed as such cassettes have frequently been found to interfere with expression from the targeted locus (see for example McDevitt *et al*., 1997). Oct-6 expression was found to be severely reduced in the Schwann cell lineage of mice homozygous for the Δ*SCE* allele. These low residual levels of Oct-6 expression could be visualized only by western blotting (Figure 4) and electrophoretic mobility shift assays (not shown), and not by immunohistochemistry (Figures 2 and 3). We estimated that the residual level of Oct-6 expressed in the sciatic nerve at P8 is 5–10% of wild-type Oct-6 levels. These low levels of Oct-6 are not sufficient to sustain normal differentiation of Schwann cells, as *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice exhibit a peripheral nerve phenotype that is only slightly less severe than that observed in *Oct-6*$^{\beta geo/\beta geo}$ mice.

Expression of Oct-6 in neurones in the hippocampus, brainstem and retina was not affected by the deletion of the SCE. These neurones express high levels of Oct-6 at the correct developmental time point. In fact, we have not found any cell lineage that normally expresses Oct-6 (apart from the Schwann cell lineage) in which Oct-6 expression was affected by deletion of the SCE. Thus, *Oct-6* expression in these cell types is under the control of additional elements within the *Oct-6* locus not requiring interaction with hypersensitive site (HSS) 6 and/or HSS7 within the SCE. Other regulatory elements may include some of the HSSs that we have mapped previously (see Figure 1; Mandemakers *et al*., 2000).

The fact that Oct-6 expression is selectively lost in Schwann cells while expression is not affected in neurones of homozygous Δ*SCE* mice helps to resolve the long-standing question of cell autonomy of the peripheral nerve phenotype in *Oct-6* mutant animals. Our results now unequivocally demonstrate that this phenotype results

from a loss of *Oct-6* function in Schwann cells and not in neurones.

Furthermore, it has been suggested previously that the high incidence of neonatal death of mice carrying a null allele (βgeo allele) of the *Oct-6* gene is caused primarily by migration or differentiation defects in neurones involved in breathing regulation, and not by defects in peripheral nerves as a consequence of delayed Schwann cell differentiation. The fact that the Δ*SCE* allele does not affect Oct-6 expression in these neurones and that *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals are viable with no evidence of breathing problems, but with the same Schwann cell differentiation defect as in *Oct-6*$^{\beta geo/\beta geo}$ mice, strongly supports the notion that neonatal death in *Oct-6*$^{\beta geo/\beta geo}$ mice does indeed result from a neuronal defect, as originally suggested by Bermingham *et al*. (1996).

Function of the Oct-6 Schwann cell enhancer

Why is expression of *Oct-6* from the Δ*SCE* allele not completely lost in Schwann cells? Traditionally, enhancers have been thought to function by increasing the rate of transcription initiation from a linked promoter. In recent years, it has been shown that in some cases enhancers not so much influence the rate of transcription initiation, but instead increase the chance that a linked promoter is activated. In this probabilistic model, enhancers are thought to function through a mechanism that involves modifications to the local chromatin configuration or relocation to an active centre within the nucleus (Fiering *et al*., 2000; Hume, 2000). This model predicts that an enhancer increases the percentage of cells in a population expressing the gene. In transgenic mice experiments, such a mechanism might explain the often observed variegated expression of the transgene (Elliott *et al*., 1995; Milot *et al*., 1996). The low level of Oct-6 expression we observed in the developing nerve of *Oct-6*$^{\Delta SCE/\Delta SCE}$ animals could thus result from either a small number of Schwann cells expressing the gene at normal levels, or a very low expression in most Schwann cells. We did not observe individual Schwann cells expressing normal levels of Oct-6 in P8 *Oct-6*$^{\Delta SCE/\Delta SCE}$ nerves (see Figure 2B: a field containing >100 nuclei). Therefore, it appears that the SCE functions as a classical enhancer in Schwann cells by modulating the rate or the frequency of transcription of the linked gene.

The reduced levels of Oct-6 expressed in Schwann cells of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice result in a slightly less severe peripheral nerve phenotype than that observed in *Oct-6*$^{\beta geo/\beta geo}$ mice, which do not express Oct-6 at all. In particular, we found that the number of Schwann cells that have entered the myelinating phase of differentiation at P8 is lower in *Oct-6*$^{\beta geo/\beta geo}$ mice than in *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice. These observations suggest that the level of Oct-6 determines the rate at which a Schwann cell progresses through the promyelin stage of differentiation. This suggests that increased levels of Oct-6 might result in an increased rate of differentiation of Schwann cells, potentially resulting in early onset of myelination and hypermyelination. Weinstein *et al*. (1995) have previously shown that expression of a mutant form of the Oct-6 protein (ΔSCIP) under the control of the P-zero promoter in Schwann cells of transgenic animals results in early onset of myelination and hypermyelination. Although these results were initially interpreted differently, involving a dominant-negative action of the ΔSCIP protein, more recent interpretation suggests that the protein acts as a dominant positive (Wu *et al*., 2001). This reinterpretation strongly suggests that the levels of Oct-6 are rate limiting in Schwann cell differentiation. Such transcription factor dose-dependent differentiation has also been demonstrated for a number of other systems, including the haematopoietic system (McDevitt *et al*., 1997; Vivian *et al*., 1999). For example, it has been demonstrated that 80% reduction in Gata-1 expression levels results in a decreased rate or efficiency of red blood cell maturation.

How would the rate of Schwann cell differentiation depend on the level of Oct-6 protein? It is possible that high levels of Oct-6 are needed to saturate all potential binding sites in the *cis*-acting elements of target genes. Lower levels of Oct-6 would then result in lower transcription rates of these targets and a longer time for the differentiation programme to complete. One potential target of Oct-6 is the *Krox-20* gene. The relevant *Krox-20* myelination-associated enhancer (MSE; myelinating Schwann cell element) contains at least one high-affinity Oct-6 binding site, and several lower-affinity binding sites (Ghislain *et al*., 2002). Although the relevance of these binding sites for *Krox-20* enhancer function has not been assessed genetically, it is possible that full *Krox-20* enhancer activation depends on maximum occupancy of the Oct-6 binding sites. In addition, high levels of Oct-6 protein might be required for efficient interaction with other proteins, such as Sox-10 (Kuhlbrodt *et al*., 1998). As these types of interaction are often of low affinity, high protein concentrations are needed. Following activation, Krox-20 expression is maintained through a mechanism that does not involve Oct-6.

We found that Krox-20 expression is activated through an Oct-6-independent mechanism in Schwann cells of Δ*SCE* homozygous animals, albeit with a delay of 10–14 days (Figure 5). Although it is not known whether this delayed activation is mediated through the *Krox-20* MSE, it is possible that an 'Oct-6-like' function, activated after the first week of postnatal development, is involved in Krox-20 activation. Recently, a potential candidate for this function has been postulated (Wu *et al*., 2001). *Brn-5*, a class VI POU domain gene, is expressed at higher levels in advanced stages of nerve development and expression is not dependent on Oct-6. Although the optimal DNA binding sequence for Brn-5 differs from that of Oct-6, both factors can bind to the octamer and octamer-related sequences present in the MSE (Rhee *et al*., 1998). If Brn-5 does indeed serve an Oct-6-redundant function in Schwann cell differentiation, it is expected that expression of Brn-5, from a transgenic construct controlled by the *Oct-6* SCE, will result in a substantial alleviation of the delayed myelination phenotype in an *Oct-6* mutant background. These experiments are currently under way.

Myelination is delayed in regenerating nerves in the absence of Oct-6

Using a nerve lesion paradigm that allows regeneration, we have shown that reactivation of *Oct-6* gene expression in reactive Schwann cells requires the SCE and that Oct-6 is important, as it is in development, for the progression of

Schwann cell differentiation. Both in developing and regenerating nerves, myelination is delayed in the absence of Oct-6. It is, therefore, most likely that the transcriptional programme regulated by Oct-6 is the same in Schwann cells during development as well as during regeneration. Furthermore, we did not observe differences between the two genotypes in the extent and numbers at which regenerating axons enter the distal nerve stump. Also, the extent of clearance of myelin debris did not differ between the two genotypes. Therefore, the Δ*SCE* allele has no obvious effect on Wallerian degeneration. Whether the delayed myelination in *Oct-6* mutant nerves results in reduced functional recovery of the regenerated nerve is not known.

Results presented here and elsewhere could be helpful in the development of strategies to improve peripheral nerve regeneration in several ways (Gondre *et al*., 1998; Mandemakers *et al*., 2000). First, the SCE would be an excellent choice for inclusion in gene therapy vehicles to express neurotrophic factors such as BDNF and GDNF in Schwann cells during a tight window of nerve regeneration. These factors have proven beneficial for regeneration and functional recovery (Xu *et al*., 1995; Menei *et al*., 1998; Terenghi, 1999; Ramer *et al*., 2000). The inclusion of the SCE in such vectors will alleviate complications that arise from continued administration of these factors to the lesioned nerve. Secondly, regenerated axons generally have a lower calibre, thinner myelin sheath and shorter internodes (Beuche and Friede, 1985). Based on the observation that Oct-6 protein levels are limiting in Schwann cell differentiation, we hypothesize that increased Oct-6 levels will increase the rate and extent of myelination of Schwann cells, resulting in restoration of myelin thickness and axonal diameter to near normal. We are currently testing this hypothesis.

In conclusion, we have generated a novel Schwann cell-specific allele of *Oct-6* through deletion of the major Schwann cell-specific regulatory element, the SCE. Analysis of these mice reveals a Schwann cell autonomous function for Oct-6 in nerve development and regeneration. We have further shown that Krox-20 is activated in Schwann cells of these mice through a mechanism that does not involve Oct-6. This new mouse mutant, together with the possibility to generate transgenic mice expressing genes selectively in the Schwannn cell lineage, provides a unique and excellent genetic system to address future questions related to the transcriptional targets of Oct-6, potential Oct-6 redundant functions in Schwann cell development, the study of functional domains of the Oct-6 protein and the role of Oct-6 in nerve regeneration.

Materials and methods

Targeting of the Oct-6 SCE

A genomic clone encompassing the 4.3 kb *Hpa*I–*Msc*I Schwann cell enhancer fragment was subcloned from cosmid clone pTBE 6Cos. From this clone, a 3.2 kb *Msc*I–*Hpa*I fragment, containing homologous genomic sequences 5′ of the SCE, was cloned behind the negative selection cassette py-TK. This selection cassette consists of the herpes simplex virus (HSV) *thymidine kinase* gene including its own promoter and a variant polyoma virus enhancer. A second clone was generated containing the *puromycin* resistance gene driven by the *phosphoglycerate kinase-1* (PGK) promoter. This clone was flanked on both sites by LoxP sites that have the same orientation. An FRT site was introduced immediately 5′ of the 5′ LoxP site. This also introduces a unique *Swa*I site at the 5′ end of this clone. Downstream of the 3′ LoxP site, a unique *Sna*BI site was used to introduce a 2.8 kb *Msc*I fragment containing the 3′ genomic homologous region. The entire fragment, encompassing the FRT–LoxP–PGK–puromycin–LoxP–3′ 2.8 kb *Msc*I, was excised as a *Swa*I–*Not*I fragment and cloned in the *Hpa*I–*Not*I-linearized Py-TK plasmid. This resulted in the targeting vector, as depicted in Figure 1A. The targeting vector was first linearized using *Not*I before electroporation into E14 ES cells. Electroporation and selection of cells in which homologous recombination had occurred were carried out as described. G418-resistant and ganciclovir-insensitive ES cell clones were screened for homologous recombination by Southern blotting of genomic DNA digested with *Pst*I using a ^{32}P-labelled DNA probe derived from the 5′ homologous region (see Figure 1A). Chimeric mice were generated by injection of ES cells from the correctly targeted clone into C57BL/6 blastocyst embryos. Chimeric males were crossed with FVB females and offspring were genotyped by Southern blotting of tail DNA digested with *Pst*I using the probe described above. Offspring carrying the chromosome with the targeted SCE allele were identified (see Figure 1B) and subsequently crossed to mice carrying the *Zp3-Cre* transgene to obtain offspring in which the *puromycin* cassette was removed. These mice were then inter-crossed to obtain mice homozygous for the deleted SCE allele (see Figure 1B).

Immunohistochemistry and western blotting

For western analysis, nerves were isolated and directly lysed in loading buffer, followed by sonication and heating in a boiling water bath. Equal amounts of nerve extracts were resolved on a 12.5% SDS–PAGE gel and transferred to a PVDF membrane (Millipore) by electroblotting. Membranes were blocked with 3% bovine serum albumin (BSA), 0.05% Tween-20 in phosphate-buffered saline (PBS) for 1 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at room temperature. Primary antibodies used were an Oct-6 rabbit polyclonal antiserum used at 1:300 dilution (Jaegle *et al*., 1996), a P-zero mouse monoclonal (clone P07; Archelos *et al*., 1993) used at 1:1000 dilution, a tubulin antibody (Sigma T-6793) and a rabbit Krox-20 antibody. Filters were subsequently washed five times in 0.5% Tween-20 in PBS and incubated with secondary antibodies conjugated with horseradish peroxidase or alkaline phosphatase (Dako) for 1 h in blocking buffer. Following five washes in 0.5% Tween-20 in PBS, the antigens were visualized by luminol (in the case of horseradish peroxidase) or NBT/BCIP (in the case of alkaline phosphatase) detection methods.

For immunohistochemistry, paraffin sections were dewaxed in xylene and rehydrated in a descending series of alcohol. Sections were blocked in 1% BSA, 0.05% Tween-20/PBS for 2 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at room temperature. Secondary antibodies used were Oregon Green-conjugated goat anti-rabbit IgGs (Molecular Probes) and Texas Red-conjugated goat anti-mouse IgGs (Molecular Probes). Cell nuclei were visualized by DAPI staining.

Electron microscope analysis

Wild-type and mutant littermates (P4–P32) were anaesthetized with pentobarbital and perfused transcardially, first with PBS followed by 3% paraformaldehyde/1% glutaraldehyde in 100 mM cacodylate buffer pH 7.3. Sciatic nerves were dissected out and immersion fixed overnight in the same fixative or formalin at 4°C. Nerves were then rinsed in 100 mM cacodylate and postfixed in 1% osmium tetroxide/ferricyanide in 100 mM cacodylate overnight at 4°C. Following dehydration through an ascending alcohol series, nerves were embedded in Epon resin as described. One-micrometre sections were cut, mounted on a microscope slide and stained with paraphenylenediamine (ppd; Estable-Puig *et al*., 1965). Sections were examined under an Olympus BX40 light microscope and photographed using an Olympus DP50 digital camera. For electron microscopy, sections were cut at 50–60 nm and mounted on grids. Sections were contrasted with lead acetate and uranyl citrate, and examined using a Philips CM100 transmission electron microscope. Photographs were taken using a Megaview II digital camera.

Animal surgery

Young adult mice were anaesthetized by inhalation of halothane and placed on a heating pad. The sciatic nerve in the left leg was exposed and crushed for two times 15 s at the midfemoral level using No. 5 biology forceps. Animals were killed 12 days after the operation and the lesioned and contralateral nerves were isolated for western analysis. For light and

electron microscope analysis, animals were perfused as described in the previous section.

Acknowledgements

The authors wish to thank Sjaak Philipsen, David Whyat, Elaine Dzierzak and Ben Barres for their critical comments on the manuscript. Juan José Archelos (University of Graz) is thanked for the monoclonal antibody against P-zero protein (clone P07). Hans van den Berg's assistance in animal surgery procedures is greatly acknowledged. Dubi Drabek generated the *Zp3-Cre* transgenic mouse. This work was funded by grants from the Dutch research council (ALW 805-17.281 and MW 903-42-195) and the European Community (Biomed 2 PLp62069).

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Received May 22, 2002; revised July 15, 2002; accepted July 17, 2002

PUBLICATION II

The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development

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Genes and Development 17 (2003) 380-91

The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development

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The genetic hierarchy that controls myelination of peripheral nerves by Schwann cells includes the POU domain Oct-6/Scip/Tst-1 and the zinc-finger Krox-20/Egr2 transcription factors. These pivotal transcription factors act to control the onset of myelination during development and tissue regeneration in adults following damage. In this report we demonstrate the involvement of a third transcription factor, the POU domain factor Brn-2. We show that Schwann cells express Brn-2 in a developmental profile similar to that of Oct-6 and that *Brn-2* gene activation does not depend on Oct-6. Overexpression of Brn-2 in Oct-6-deficient Schwann cells, under control of the *Oct-6* Schwann cell enhancer (SCE), results in partial rescue of the developmental delay phenotype, whereas compound disruption of both *Brn-2* and *Oct-6* results in a much more severe phenotype. Together these data strongly indicate that Brn-2 function largely overlaps with that of Oct-6 in driving the transition from promyelinating to myelinating Schwann cells.

[*Keywords*: POU domain; myelin; Schwann cell; nerve development]

Received December 20, 2002; revised version accepted April 10, 2003.

The high conduction velocity of nerve fibers is a hallmark of the nervous system of higher vertebrates and depends on structural and molecular specializations that are elaborated during development. These specializations occur through intimate and continued interactions between the neuron and its associated glial cells and result in the elaboration by glial cells of myelin, the important membranous structure that ensheaths and insulates axons (Arroyo and Scherer 2000; Fields and Stevens-Graham 2002; Mirsky et al. 2002). Two glial cell types produce myelin: the oligodendrocyte in the central nervous system (CNS) and the Schwann cell in the peripheral nervous system (PNS). Although very similarly organized, the molecular composition of CNS and PNS myelin differs significantly, and oligodendrocytes and Schwann cells have adopted different, but overlapping, sets of transcriptional regulators to coordinate myelogenesis (Hudson 2001; Topilko and Meijer 2001). These differences reflect their distinct embryonic origins. Whereas oligodendrocytes originate from the neuroepithelial precursors that line the lumen of the spinal cord and ventricles of the brain, Schwann cells derive mainly from the neural crest, a transient embryonic stem (ES) cell population that generates a wide variety of cell types including sensory and autonomic neurons and melanocytes (Le Douarin and Kalcheim 1999; Richardson 2001). Schwann cell precursors populate the early outgrowing nerve bundles, where they proliferate and segregate individual and groups of fibers until the number of Schwann cells and fibers is eventually matched. During the first few days of postnatal development, many Schwann cells establish a 1:1 relationship with axons, cease to proliferate, and initiate myelin formation such that by the end of the first postnatal week of development, all myelin-competent axons are actively being myelinated. Schwann cells that remain associated with groups of lower-caliber fibers will segregate these fibers in cytoplasmic cuffs without myelinating them (Webster 1993). Although virtually nothing is known about the molecular identity of the axon-associated signal(s) that divert Schwann cells along either a myelinating or a nonmyelinating differentiation pathway, significant information has accumulated in recent years about the transcription factors involved in Schwann cell differentiation and myelination.

To date, several transcription factors have been found to be involved in the differentiation of Schwann cells and include the zinc-finger protein Krox20 (Egr-2), the Sry box protein Sox10, and the POU domain protein Pou3f1/

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.258203.

Oct-6/Scip/Tst-1 (referred to here as Oct-6; Topilko and Meijer 2001). Gene targeting experiments in mice have revealed insight into the functional roles of each of these factors and their possible order within a genetic hierarchy. Sox10 is required early in development for the establishment and/or maintenance of Schwann cell precursors from the neural crest (Britsch et al. 2001). At later stages of development, Oct-6 and Krox20 are both required for the differentiation of myelinating Schwann cells at two respective progressive steps in the genetic hierarchy (Topilko et al. 1994; Bermingham et al. 1996; Jaegle et al. 1996; Ghislain et al. 2002). During fetal development, Oct-6 gene expression is induced in immature Schwann cells and peaks in promyelinating and early myelinating cells during the first week of postnatal life (Scherer et al. 1994; Blanchard et al. 1996; Arroyo et al. 1998). Consequently, Oct-6 regulates a set of downstream genes that includes Krox-20 (Blanchard et al. 1996; Ghislain et al. 2002). Krox-20 regulates an additional set of target genes including the major myelin genes and those involved in lipid metabolism (Nagarajan et al. 2001). In addition, Sox10 may continue to participate in these transcription programs, as it interacts with both Oct-6 and Krox20 when bound to adjacent DNA-binding sites (Kuhlbrodt et al. 1998b).

Schwann cell differentiation in nerves of *Krox20* or *Oct-6*-deficient mice is arrested at the promyelin stage (Topilko et al. 1994; Bermingham et al. 1996; Jaegle et al. 1996). However, this differentiation block in *Krox20*$^{-/-}$ mice is permanent, and is transient in *Oct-6* mutant animals (*Oct-6*$^{\beta geo/\beta geo}$). Thus, *Oct-6*$^{\beta geo/\beta geo}$ Schwann cells do eventually activate Krox20 expression and commence myelination, albeit with a delay of 7–10 d, thus suggesting some functional redundancy in the genetic program (Ghazvini et al. 2002). We previously proposed that the transient nature of the differentiation block is the result of an unknown Oct-6-like activity in Schwann cells, with this putative factor acting at a later developmental time than Oct-6 in the Schwann cell lineage (Jaegle and Meijer 1998). Alternatively, the transient block could be the result of a factor that regulates part or all of the transcriptional targets of Oct-6, but does so less efficiently. The most likely candidates for this Oct-6-like activity are the other members of the POU domain transcription factor family, of which there are 15 members in mammals (Ryan and Rosenfeld 1997). Interestingly, two POU domain transcription factors, Brn-1 and Brn-2, have virtually identical DNA-binding characteristics compared to Oct-6. Hence, to provide insight into the genetic program acting alongside Oct-6 in promyelinating Schwann cells, we examined the expression and function of the candidate POU domain transcription factors during development and in Oct-6-deficient mice.

In this report, we show that Schwann cells express Brn-2 in a developmental profile similar to that of Oct-6. We demonstrate that *Brn-2* gene activation is independent of Oct-6, but that Oct-6 negatively regulates Brn-2 expression levels. Higher expression levels of Brn-2 in *Oct-6* mutant Schwann cells result in a partial rescue of the developmental delay phenotype, whereas homozygous deletion of *Brn-2* in *Oct-6* mutant Schwann cells results in a more severe phenotype. Together these data strongly indicate that Brn-2 function largely overlaps with that of Oct-6 in driving the transition from promyelinating to myelinating Schwann cells.

Results

Brn-2 is expressed and regulated in Schwann cells in a manner similar to Oct-6

The transient nature of the Schwann cell defect in Oct-6-deficient mice suggested some redundancy in transcription factor function. Interestingly, previous work on the expression of octamer binding factors Oct-6 and Oct-1 in the developing chick sciatic nerve suggested the presence of a novel octamer-binding activity and possibly a homologous candidate factor involved in developing mouse sciatic nerves (Levavasseur et al. 1998). Hence, to examine the developmental expression profile of this novel binding activity, we performed electrophoretic mobility shift assays (EMSAs) using whole sciatic nerve extracts derived from chick embryos and young chicks. Three complexes can be distinguished (Fig. 1A). The largest complex contains Oct-1, a ubiquitous POU factor, the levels of which are relatively constant at all stages of nerve development (Blanchard et al. 1996). In contrast, the smallest and fastest migrating Oct-6-containing complex is strongly regulated during development. Like in rodents, Oct-6 expression peaks in promyelinating and early myelinating cells (embryonic day 17 and 20; E17 and E20 in Fig. 1A) and is sharply downregulated at later stages of myelination (postnatal day 3; P3). The third, intermediate complex (X) is regulated similarly to Oct-6, but its expression is maintained at reduced levels at later stages (P20 in Fig. 1A). Using different octamer-related DNA-binding sites, such as the HSV1 TAATGARAT motif, and a mutated octamer motif, we found that this novel activity demonstrated binding site preferences very similar to those of Oct-6 (data not shown).

To identify the protein in this complex, we performed EMSA experiments in the presence of antisera against several POU proteins closely related to Oct-6. Although the mobility or intensity of the intermediate complex X was not affected by antibodies against the chicken Oct-6 protein (Fig. 1B, middle lane), a Brn-2-specific goat antiserum strongly reduced the intensity of this complex, thus identifying the protein in the intermediate complex as Brn-2 (Fig. 1B, right lane).

Until recently, expression of Brn-2 in mammalian peripheral nerves had not been described and, paradoxically, only one octamer complex, in addition to Oct-1, had been observed in cultured rat Schwann cells (Kuhn et al. 1991; Sim et al. 2002). Therefore, we next examined the presence of Brn-2-binding activity in the developing sciatic nerve of the mouse at P4 by EMSA. In accordance with previous data, only one prominent protein/DNA complex, in addition to the Oct-1/DNA complex, was observed (Fig. 1C, lane 1). However, in supershift assays

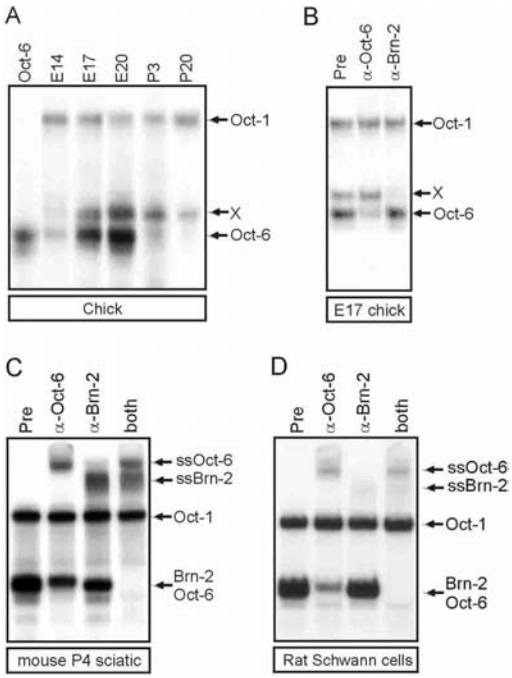

Figure 1. Brn-2 is expressed in chick and mouse sciatic nerves. (*A*) Developmental expression of a novel octamer-binding complex was examined by EMSA using freeze-thaw extracts from chicken nerves at different stages of development [embryonal stages E14, E17, and E20 and postnatal days 3 (P3) and 20 (P20)]. Whole-cell extracts of COS cells expressing chicken Oct-6 (Oct-6) served as a control (Meijer et al. 1990). Free probe is not shown, but all experiments were performed in probe excess. (*B*) Using E17 chick embryonic nerve, extracts identify Brn-2 in complex X, as antibodies directed against the C-terminal part of mouse Brn-2 specifically affect complex X, whereas chicken Oct-6 antibodies (α-Oct-6) affect the Oct-6 complex but not complex X. Preimmune serum (Pre) does not affect either complex. (*C*) Brn-2 is expressed in mouse nerves. Incubation of Oct-6-specific antibodies with P4 mouse whole-nerve extracts and an octamer probe results in the formation of a ternary complex (supershift Oct-6 complex; ssOct-6) and unmasks another complex that is specifically supershifted (ssBrn-2) with mouse Brn-2 antibodies (α-Brn-2). When both antibodies are added all complexes are shifted, demonstrating that no other complexes comigrate with the Oct-6/DNA and Brn-2/DNA complexes. (*D*) Brn-2 is expressed in the Schwann cell lineage. Whole-cell extracts from cultured rat Schwann cells grown in the presence of 20 μM forskolin for 36 h were incubated with the octamer probe in presence of the indicated sera. As in *C*, Oct-6- and Brn-2-specific antibodies identify two comigrating Oct-6 and Brn-2 protein DNA complexes.

using antibodies specific for Oct-6 and Brn-2, this band was found to be composed of two complexes, a Brn-2/DNA complex and an Oct-6/DNA complex (Fig. 1D, lanes 2–4). Thus, Brn-2 protein is expressed in both the developing chick and murine nerve.

Because sciatic nerve extracts are heterogeneous, it is possible that Brn-2 is expressed exclusively in cell types other than Schwann cells, such as endothelial or perineural cells. We therefore examined expression of Brn-2 in pure differentiating primary rat Schwann cell cultures using EMSA (Fig. 1D). These cells express high levels of Oct-6 and moderate levels of Brn-2 (Fig. 1D, cf. lanes 2 and 3). Thus, Brn-2 is expressed in the Schwann cell lineage and is therefore an attractive candidate for the putative Oct-6 like function.

Brn-2 is expressed in Oct-6-deficient Schwann cells

We next examined whether Brn-2 expression is affected by loss of Oct-6 function in Schwann cells. As the vast majority of full *Oct-6* knockout animals ($Oct\text{-}6^{\beta geo/\beta geo}$) are not viable and die shortly after birth, thus precluding studies at postnatal stages of development, we used in this study animals that carry on one chromosome a full knockout allele (βgeo) and on the other chromosome a Schwann cell-specific Oct-6 strong hypomorphic allele (ΔSCE; Ghazvini et al. 2002). The $Oct\text{-}6^{\Delta SCE}$ allele was generated through deletion of the Oct-6 Schwann cell enhancer (SCE; Mandemakers et al. 2000). Both $Oct\text{-}6^{\Delta SCE/\Delta SCE}$ and $Oct\text{-}6^{\beta geo/\Delta SCE}$ mice are viable and exhibit a peripheral nerve phenotype that is indistinguishable from that observed in nerves of $Oct\text{-}6^{\beta geo/\beta geo}$ animals (Ghazvini et al. 2002).

Using EMSA and Western blotting, we analyzed Brn-2 expression in the developing nerve of $Oct\text{-}6^{\beta geo/\Delta SCE}$ mice (Fig. 2). In addition to the expected Oct-1 complex, one abundant octamer complex is observed. Supershift experiments using Oct-6- and Brn-2-specific antibodies identify Brn-2 as the major protein component in this complex (Fig. 2A, cf. lanes 2 and 3), with only very low levels of Oct-6 protein detected, in agreement with previous data (Ghazvini et al. 2002). Both EMSA and Western blot analyses demonstrate that Brn-2 protein levels are increased in $Oct\text{-}6^{\beta geo/\Delta SCE}$ nerves at P4 compared to heterozygous or wild-type nerves (Fig. 2A,B). The higher Brn-2 protein levels in Oct-6-deficient nerves result from increased transcription of the *Brn-2* gene (or increased *Brn-2* mRNA stability), as reverse transcriptase PCR (RT–PCR) data for Brn-2 expression in P1 mice show that steady-state levels of *Brn-2* mRNA are increased in $Oct\text{-}6^{\beta geo/\Delta SCE}$ nerves compared to $Oct\text{-}6^{\Delta SCE/+}$ nerves (Fig. 2C). Immunostaining of single nerve fibers shows that within the nerve, Brn-2 is highly expressed in Schwann cell nuclei (Fig. 2D).

Next we examined developmental regulation of Brn-2 protein expression in nerves of $Oct\text{-}6^{\Delta SCE/+}$ and $Oct\text{-}6^{\beta geo/\Delta SCE}$ mice, using Western blotting (Fig. 2E). In heterozygous animals, Brn-2 expression is up-regulated in the late embryonic nerve and peaks during the first postnatal week of development. At later stages (P32), Brn-2 expression is extinguished. Thus, in both chick and mouse, Oct-6 and Brn-2 are similarly regulated, although Brn-2 down-regulation appears to be faster in mice. In Oct-6 mutant nerves, Brn-2 expression appears to be affected in two ways. First, overall Brn-2 expression levels

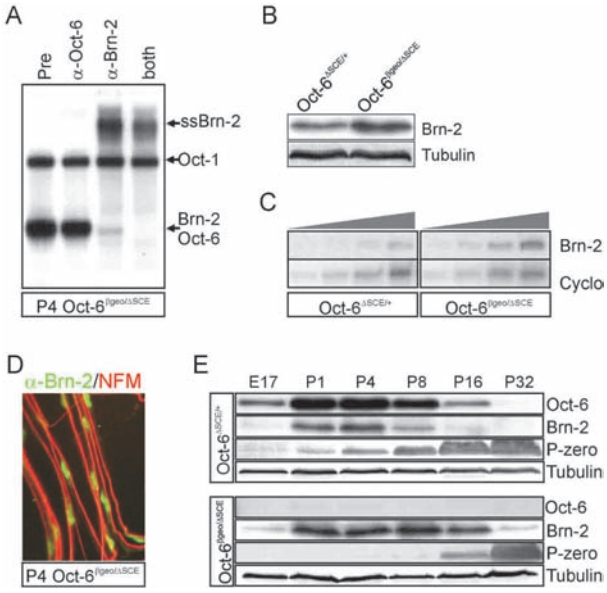

Figure 2. Elevated and protracted expression of Brn-2 in nerves of *Oct-6*$^{\beta geo/\Delta SCE}$ mice. (*A*) EMSA with whole-cell extracts from sciatic nerves of P4 *Oct-6*$^{\beta geo/\Delta SCE}$ mice and a radiolabeled octamer probe reveal a strong complex that migrates at the same position as Brn-2 and Oct-6 DNA complexes. This complex is supershifted with antibodies against Brn-2, unmasking the low residual expression of Oct-6 from the ΔSCE allele. Preimmune serum affects none of these complexes. (*B*) Western blot analysis confirms that Brn-2 protein levels are elevated in the nerves of *Oct-6*$^{\beta geo/\Delta SCE}$ vs. *Oct-6*$^{\Delta SCE/+}$ nerves at P4. Tubulin served as a loading control. (*C*) Semiquantitative RT–PCR using RNA extracted from newborn sciatic nerves shows the higher Brn-2 mRNA steady-state levels in *Oct-6*$^{\beta geo/\Delta SCE}$ mice than in *Oct-6*$^{\Delta SCE/+}$ mice. Cyclophilin mRNA served as a control. (*D*) Brn-2 is expressed in the nucleus of Schwann cells in the nerves of P4 *Oct-6*$^{\beta geo/\Delta SCE}$ mice. Nerves were dissected and teased into single fibers and incubated with antibodies against mouse Brn-2 (α-Brn-2) in green and Neurofilament medium chain (NFM) in red. (*E*) Expression of Brn-2 and Oct-6 was examined in developing nerves of *Oct-6*$^{\Delta SCE/+}$ and *Oct-6*$^{\beta geo/\Delta SCE}$ animals at E17 and P1–P32. Amounts of nerve extract loaded were normalized for acetylated α-tubulin. The build-up of P-zero immunoreactivity over time illustrates the progression of myelination in both genotypes.

are increased (see also Fig. 2A,B). Second, whereas up-regulation of Brn-2 expression follows its normal course, down-regulation of Brn-2 expression is protracted, with significant levels of Brn-2 still present at P32. In both *Oct-6*$^{\Delta SCE/+}$ and *Oct-6*$^{\beta geo/\Delta SCE}$ genetic backgrounds, down-regulation of Brn-2 correlates with the build up of P-zero immunoreactivity, indicating that a myelination-associated signal, which is independent of Oct-6, extinguishes Brn-2 expression.

Together, these data demonstrate that the developmental profile of Brn-2 expression in the nerve is regulated through mechanisms independent of Oct-6, but that Brn-2 expression levels are negatively attenuated by Oct-6.

Role of Brn-2 in Schwann cell development and myelination

Considering the findings that Brn-2 is expressed at relatively high levels in Oct-6-deficient Schwann cells that are transiently blocked in their differentiation, and that Brn-2 DNA-binding characteristics are virtually identical to those of Oct-6, one can suggest several possible roles for Brn-2 in Schwann cells. First, it is possible that Brn-2 regulates the same repertoire of genes as Oct-6 but does so less efficiently, possibly because of different affinities for Oct-6 interacting factors important in target gene regulation. Second, it is possible that Brn-2 function only partially overlaps with Oct-6 and/or that yet another factor, which might be activated at a later developmental time, is responsible for the delayed activation of the myelination program in the absence of Oct-6. It was recently proposed that the POU homeodomain protein Brn-5, a class VI POU protein, might fulfill such a role (Wu et al. 2001). This factor is activated at a later developmental time than Oct-6 and Brn-2, and its activation does not depend on Oct-6. Third, it is possible that Brn-2 negatively regulates Oct-6 targets, possibly through its interaction with the homopolymeric glutamine tract binding protein PQBP-1 (Waragai et al. 1999), and that the balance between Oct-6 and Brn-2 governs the progression of cells into the myelinating phase of differentiation. In the absence of Oct-6 this balance shifts to Brn-2, and cells are inhibited in their differentiation. Thus, to begin to explore the possible roles of Brn-2 and Brn-5 in Schwann cell differentiation, we performed transgenic mouse experiments in which Brn-2 or Brn-5 is overexpressed in the Schwann cell lineage.

To direct expression of Brn-2 or Brn-5 in Schwann cells, we made use of the *Oct-6 SCE* (Fig. 3A). This genetic element drives transgene expression from a generic promoter in Schwann cells with a profile that is identical to that of Oct-6 and very similar to that of Brn-2 but not Brn-5 (Mandemakers et al. 2000). We previously showed that a transgenic construct carrying the *SCE* and an HA-tagged version of Oct-6 rescues the delay in development of the peripheral nerve in Oct-6-deficient mice (Mandemakers et al. 2000). To test whether Brn-2 or Brn-5 overexpression can replace Oct-6 in this rescue, we generated transgenic lines for the three constructs depicted in Figure 3A. These transgenes were subsequently crossed into an *Oct-6*$^{\beta geo/\Delta SCE}$ background, and nerve maturation at P4 was studied by Western blotting and light microscopy (Fig. 3B,C). Additionally, we counted the number of promyelin and myelinating configurations in these nerves and expressed the fraction of myelinating figures divided

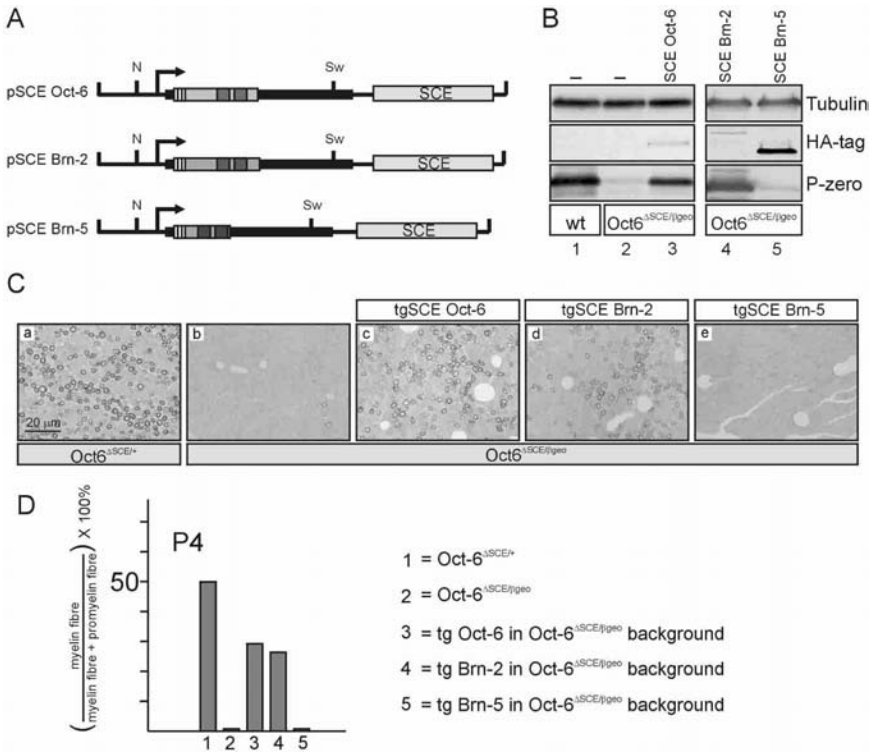

Figure 3. HA-Oct-6 and HA-Brn-2, but not HA-Brn-5, can rescue the developmental delay phenotype of *Oct-6*$^{\beta geo/\Delta SCE}$ mutant mice. (*A*) Schematic representation of the constructs used to generate mice transgenic for HA-Oct-6 (SCE Oct-6), HA-Brn-2 (SCE Brn-2), and HA-Brn-5 (SCE Brn-5). Two restriction sites used in the generation of these constructs are indicated [NotI (N) and SwaI (Sw)]. The SCE is indicated as a gray box and the triple HA-tag as a yellow box. The intron-less Oct-6 gene is shown as a thick black line. The ORF of Oct-6 is in light green, that of Brn-2 is in pink, and that of Brn-5 is in orange, with their POU-specific domain in dark blue and their POU-homeodomain in light blue. (*B*) *Oct-6*$^{\beta geo/\Delta SCE}$ mutant mice that express HA-Oct-6 or HA-Brn-2 show high levels of P-zero protein expression in sciatic nerve. Western blot analysis of P4 sciatic nerve extracts from wild-type (wt; lane *1*), *Oct-6*$^{\beta geo/\Delta SCE}$ (lane *2*), *Oct-6*$^{\beta geo/\Delta SCE}$*/SCE Oct-6* (lane *3*), *Oct-6*$^{\beta geo/\Delta SCE}$*/SCE Brn-2* (lane *4*), and *Oct-6*$^{\beta geo/\Delta SCE}$*/SCE Brn-5* (lane *5*) animals. Levels of HA-tagged proteins are assessed with α-HA antibodies. The amount of protein loaded per lane is estimated by the intensities of the α-tubulin immunoreactive band. (*C*) Comparison of the morphology of cross sections through P4 sciatic nerves of *Oct-6*$^{\Delta SCE/+}$ (panel *a*), *Oct-6*$^{\beta geo/\Delta SCE}$ (panel *b*), *Oct-6*$^{\beta geo/\Delta SCE}$*/SCE Oct-6* (panel *c*), *Oct-6*$^{\beta geo/\Delta SCE}$*/SCE Brn-2* (panel *d*), and *Oct-6*$^{\beta geo/\Delta SCE}$*/SCE Brn-5* (panel *e*) animals. Plastic-embedded osmicated nerves were sectioned at 1 µm and stained with ppd. Myelin is strongly stained by this compound and appears as dark rings in cross sections. Bar, 20 µm. (*D*) Quantification of the promyelin–myelinating transition in nerves of animals in *C*.

by the total number of myelinating and promyelin configurations to obtain a quantitative measure of nerve maturation (Fig. 3D). In the absence of Oct-6, all but a few myelinating Schwann cells are stalled at the promyelin stage of differentiation [Fig. 3C (cf. panels a and b), 3D (50% and 0.5%)], and P-zero protein levels are very low (Fig. 3B, cf. lanes 1 and 2) compared to heterozygous or wild-type animals. Expression of the HA-Oct-6 transgene results in a significant restoration of P-zero protein levels in nerves of *Oct-6*$^{\beta geo/\Delta SCE}$ mice (Fig. 3B, lane 3). At the morphological level, this finding correlates with increased numbers of myelinating Schwann cells, up to 60% of wild-type (Fig. 3C, panel c, 3D). Expression of the HA-Brn-2 transgene also results in significant restoration of P-zero protein levels (Fig. 3B, lane 4) and a strong increase in the numbers of actively myelinating Schwann cells (Fig. 3C, panel d, 3D). In contrast, the HA-Brn-5 transgene is not capable of restoring P-zero protein levels (Fig. 3B, lane 5) and accordingly, no increase in the number of myelinating cells is observed (Fig. 3C, panel e) despite relatively high levels of HA-Brn-5 expression (Fig. 3B, lane 5). Thus, increased Brn-2 expression in early postnatal development results in a significant increase in the number of actively myelinating Schwann cells in transgenic *SCE Brn-2/Oct-6*$^{\beta geo/\Delta SCE}$ nerves. This number approaches that observed in nerves of transgenic *SCE Oct-6/Oct-6*$^{\beta geo/\Delta SCE}$ animals (Fig. 3D). These results demonstrate that Brn-2 does not an-

tagonize Oct-6 function, but instead functionally substitutes for Oct-6 in promoting the transition from promyelinating to myelinating Schwann cells. Furthermore, our results suggest that Brn-2 levels are rate-limiting in Schwann cells of *Oct-6*$^{\beta geo/\Delta SCE}$ mice.

Schwann cell-specific deletion of Brn-2 does not affect peripheral nerve development

The experimental results described above suggest that Brn-2 and Oct-6 share many transcriptional targets, but do not reveal whether Brn-2 has distinct (non-Oct-6) regulatory targets. In addition, the above experiments do not exclude that other, as yet unidentified transcription factors, contribute to the delayed entry of *Oct-6*$^{\beta geo/\Delta SCE}$ Schwann cells into the myelinating phase of differentiation. To address these questions we examined the effect of loss of Brn-2 function in the Schwann cell lineage. A full null allele of *Brn-2* had been generated previously and revealed a vital function for Brn-2 in the development and survival of endocrine neurons in the hypothalamus (Nakai et al. 1995; Schonemann et al. 1995). As a consequence, the hypothalamic–pituitary axis is disturbed and homozygous animals die before postnatal day 10. Therefore, we generated mice carrying a conditional null allele of *Brn-2* in which deletion of the LoxP-flanked Brn-2 open reading frame (ORF; Fig. 4A) depends on activity of the Cre recombinase in Schwann cells. The introduction of LoxP sites at the *Brn-2* locus, together with a neomycin selection cassette and an eGFP reporter, did not compromise *Brn-2* gene function, as *Brn-2*$^{flox/flox}$ animals are healthy and breed normally.

To achieve Schwann cell-specific deletion of the *Brn-2* gene, we generated transgenic mouse lines in which the Cre recombinase is expressed from *Desert hedgehog* (*Dhh*) regulatory sequences (Fig. 4B). The *Dhh* gene is prominently expressed in Schwann cell precursors of the developing nerves and in Sertoli cells of the testis (Bitgood and McMahon 1995). The cell-type specificity of the recombination event was monitored in offspring of crosses between the *DhhCre* transgenic mice and ROSA26 LacZ reporter (Soriano 1999). Embryos were isolated from these crosses at different developmental stages and stained with Bluo-Gal for β-galactosidase activity. DhhCre activity was observed in the Schwann cell lineage from E12, as evidenced by blue staining of the peripheral nerves (Fig. 4B, panels a,b). DhhCre activity is also observed in the testis, another well documented site of *Dhh* gene expression. Outside the Schwann cell lineage and testis, expression of Cre is ob-

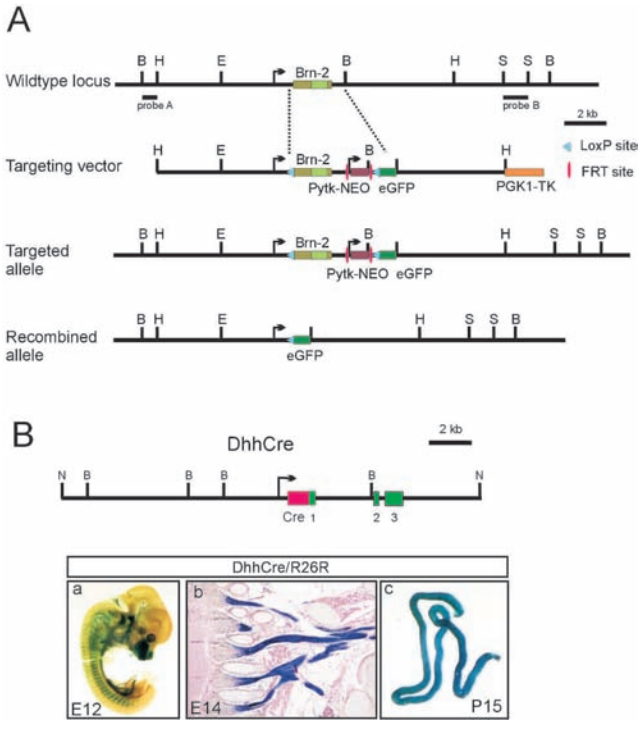

Figure 4. (*A*) The targeting strategy to generate an inducible deletion allele of *Brn-2*. The structure of the wild-type *Brn-2* locus is shown at the *top*. The *Brn-2* single exon gene is represented in olive green with the POU domain in light green. The Southern blot probes A and B are indicated as horizontal black bars. The positions of restriction enzyme cutting sites are shown for BamHI (B), HindIII (H), EcoRI (E) and ScaI (S). In the targeting construct (targeting vector), the neomycin (NEO) expression cassette (purple box) is flanked by FRT sites (vertical red bars) followed by a 3′ LoxP site (blue triangle) and an eGFP reporter gene (dark green box). The orange box represents the counter-selection cassette, containing the thymidine kinase gene (TK). The targeted allele (the floxed allele) obtained after homologous recombination is shown below the targeting construct. Cre-mediated recombination removes *Brn-2* sequences and generates the recombined allele (*bottom*). (*B*) Schwann cell-specific recombination is achieved through expression of the Cre recombinase under control of Dhh gene regulatory sequences. The 19-kb construct containing the entire *Dhh* gene with its three exons (in green) is shown. The Cre recombinase ORF (indicated in red) is preceded by a nuclear localization peptide sequence and cloned in-frame with the *Dhh* ORF. Restrictions sites for NotI (N) and BamHI (B) are indicated. Expression of the Cre recombinase was examined in crossing with the ROSA26lacz (R26R) reporter mouse. (Panel *a*) Whole-mount staining of E12.5 embryo reveals expression in the PNS, the snout, and part of the vasculature. (Panel *b*) Paraffin sections of a stained E14.5 embryo demonstrate the strong expression in immature Schwann cells that populate the peripheral nerves. (Panel *c*) Strong expression is also observed in the seminiferous tubules of the testis, where Dhh is expressed in the Sertoli cells.

served in the skin of the snout, but not in the brain and more specifically the hypothalamic region.

To explore the effect of loss of Brn-2 expression in the Schwann cell lineage, we crossed the *DhhCre* transgene into the *Brn-2*$^{flox/flox}$ background. Offspring from these crosses were healthy and bred normally. Southern blot analysis of DNA derived from sciatic nerves of adult *DhhCre/Brn-2*$^{flox/flox}$ animals revealed that the majority of cells had undergone recombination [cf. the ratio of the 14-kb band (recombined) and the 8.3-kb (not recombined) band in Fig. 5A, lane 4]. The nonrecombined band derives from non-Schwann cells in the nerve that do not express the DhhCre transgene. Recombination within the Schwann cell compartment of the nerve was complete, as judged by the complete loss of Brn-2 expression in nerves of transgenic *DhhCre/Brn-2*$^{flox/flox}$ pups using EMSA (Fig. 5B, cf. lanes 1 and 2). Additionally, loss of Brn-2 did not affect Oct-6 expression levels in these nerves compared with age-matched *Oct-6*$^{\Delta SCE/+}$ nerves (Fig. 5B, cf. lanes 3 and 4). We next examined the effect of complete loss of Brn-2 expression in Schwann cells on nerve development. Light microscopic examination of sciatic nerves of P8 transgenic *DhhCre/Brn-2*$^{flox/flox}$ animals did not reveal clear morphological abnormalities (Fig. 5C). In both *Brn-2*$^{flox/flox}$ and *DhhCre/Brn-2*$^{flox/flox}$ nerves, the majority of Schwann cells associated with larger axons are actively myelinating. Thus, loss of Brn-2 expression in the Schwann cell lineage does not affect the timing or progression of postnatal peripheral nerve development.

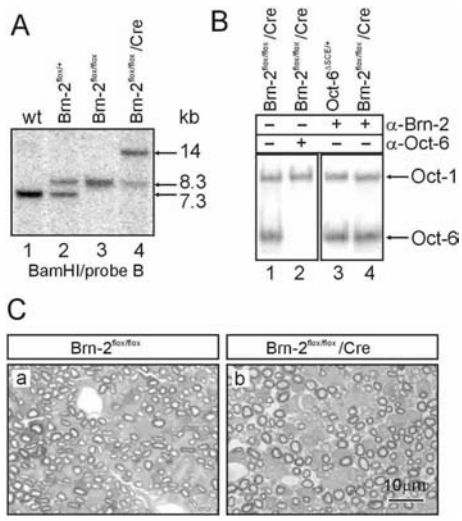

Figure 5. (*A*) The floxed *Brn-2* locus is effectively recombined by the Cre recombinase in peripheral nerve. DNA was extracted from adult sciatic nerve of wild-type (wt), *Brn-2*$^{wt/flox}$, *Brn-2*$^{flox/flox}$, and *Brn-2*$^{flox/flox}$*Cre* (*Brn-2*$^{flox/flox}$ mice transgenic for the *DhhCre* transgene) animals, digested with BamHI, and subjected to Southern blot analysis with probe B. Probe B detects fragments of 7.3 kb (wild-type allele), 8.3 kb (targeted allele), and 14 kb (recombined allele). The nonrecombined band present in lane *4* results from DNA derived from cells that do not express the DhhCre transgene, such as nerve sheath cells. (*B*) The recombination of the *Brn-2* allele in Schwann cells is complete, as *Brn-2*$^{flox/flox}$*Cre* nerves do not express Brn-2 atP4 (lanes *1*,*2*). Complete deletion of *Brn-2* does not affect Oct-6 expression, as Oct-6 expression levels are the same in nerves of *Oct-6*$^{\Delta SCE/+}$ and *Brn-2*$^{flox/flox}$*Cre* animals (cf. lanes *3* and *4*). (*C*) Deletion of *Brn-2* in Schwann cells does not affect the morphological maturation of the nerve. The overall morphology appears very similar in cross sections of nerves from both genotypes. Resin sections (1 μm) of P4 sciatic nerves of *Brn-2*$^{flox/flox}$ (panel *a*) and *Brn-2*$^{flox/flox}$*/Cre* (panel *b*) animals were stained with ppd. Bar, 10 μm (applies to both micrographs).

Schwann cell-specific deletion of Brn-2 and Oct-6 results in a severe hypomyelinating phenotype

To study the role of Brn-2 in Schwann cells deficient for Oct-6, we generated double homozygous mutants by intercrossing transgenic *DhhCre/Brn-2*$^{flox/flox}$*/Oct-6*$^{\Delta SCE/\Delta SCE}$ and transgenic *DhhCre/Brn-2*$^{flox/flox}$*/Oct-6*$^{\beta geo/+}$ mice. The development of peripheral nerves was examined by electron microscopy and quantified as described above. In the absence of Oct-6 function, Schwann cell development is transiently blocked at the promyelin stage (Bermingham et al. 1996; Jaegle et al. 1996; Ghazvini et al. 2002). Whereas >90% of large-caliber axons in nerves of wild-type (data not shown) and heterozygous *Oct-6*$^{\Delta CSE/+}$ animals are myelinated by the third postnatal week (at P16), nerves of *Oct-6*$^{\beta geo/\Delta SCE}$ mice still contain many promyelin configurations (Fig. 6A, panels a,b, 6B, ~60%). A dramatic increase in the severity of this phenotype is observed with the Schwann cell-specific deletion of Brn-2 on the *Oct-6*$^{\beta geo/\Delta SCE}$ genetic background. As shown in Figure 6A, panel c, none of the ensheathing Schwann cells has progressed beyond the promyelin stage of differentiation. To determine whether these cells are permanently blocked in their differentiation, nerve morphology in young adult animals was examined at P56 and P120. At these stages most, if not all, *Oct-6*$^{\beta geo/\Delta SCE}$ Schwann cells have gone on to myelinate their associated axon [Fig. 6A (cf. panels d and e), 6B]. In contrast, nerves of double homozygous animals are abnormal, with many promyelin configurations (~55% at P56 to ~25% at P120) and thinly myelinated axons (Fig. 6A, panels f,i). This morphology resembles that of wild-type nerves during the first week of postnatal development. Therefore, deletion of both *Oct-6* and *Brn-2* results in further delay in the promyelinating–myelinating transition relative to that observed in single *Oct-6* mutants, indicating that, in addition to Oct-6, Brn-2 plays a role in the transition of promyelinating to myelinating Schwann cells.

Discussion

In the present study we have shown that the class III POU domain protein Brn-2 is regulated in parallel with Oct-6 during Schwann cell differentiation. As the previ-

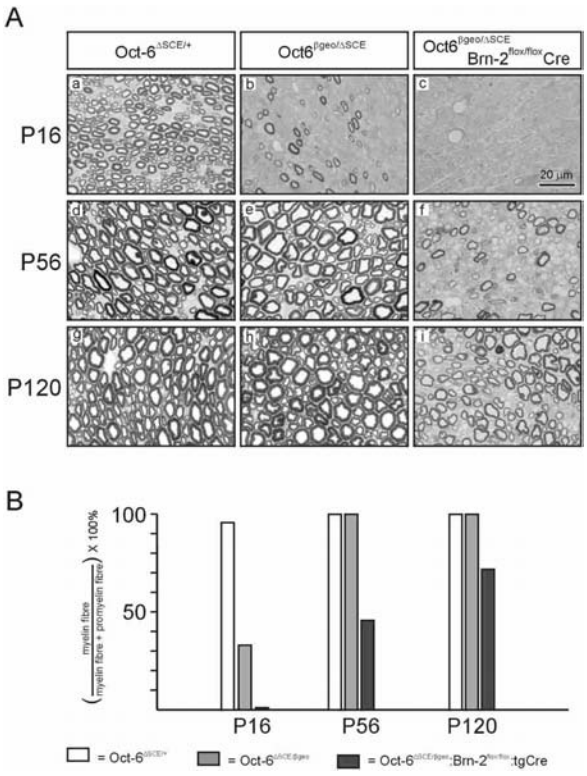

Figure 6. Schwann cell-specific deletion of *Brn-2* in an *Oct-6*$^{\beta geo/\Delta SCE}$ background results in a severe hypomyelination phenotype. (*A*) Representative sections (1 µm; ppd-stained) are shown from sciatic nerves at three developmental time points (P16, P56, and P120). In *Oct-6*$^{\Delta SCE/+}$ mice, most myelin-competent axons are actively being myelinated by P16 (panel *a*). At P56 and P120, these nerves are fully matured (panel *d*). This pattern contrasts with that observed in *Oct-6*$^{\beta geo/\Delta SCE}$ animals, which exhibit a strong delay in the differentiation of Schwann cells (Bermingham et al. 1996; Jaegle et al. 1996; Ghazvini et al. 2002). At P16, a majority of Schwann cells is arrested at the promyelin stage, with only the largest axons myelinating (panel *b*). By P56, most if not all myelinating Schwann cells have elaborated a myelin sheath (panels *e*,*h*; P120). Deletion of *Brn-2* in this genetic background results in a dramatic increase in the severity of the phenotype. At P16, essentially all myelinating Schwann cells are arrested at the promyelin stage of differentiation (panel *c*). Even at P56 and P120, myelination is largely abnormal, with large numbers of cells at the promyelin stage (panel *f*). Those axons that are myelinated have very thin myelin sheaths. Bar: panels *a–f*, 20 µm. (*B*) Quantification of delayed myelination in genotypes presented in *A*.

ous data on the delayed myelination phenotype of Oct-6-deficient mice suggested a redundant activity, we show here that Brn-2 acts as that novel factor. Our genetic evidence, arising from the overexpression of Brn-2 in Oct-6-deficient mice and moreover, the double deficiency of Brn-2 and Oct-6, demonstrates that these POU domain transcription factors share roles as positive regulators of the promyelinating–myelinating transition in Schwann cell development. Considering the importance of peripheral nerve myelination, the activity of both these transcription factors most likely assures the progression of this process.

Regulation of Brn-2 expression in the Schwann cell lineage

Our studies on chick and mouse sciatic nerve at different embryonic and post-hatching or postnatal stages showing that Oct-6 and Brn-2 are regulated similarly during nerve development provided a strong suggestion that Brn-2 plays a role in the onset of myelination. Both genes are activated around the time that most Schwann cells have adopted an immature promyelinating phenotype (E14 in the chick and E17 in the mouse). Expression of both genes peaks in promyelinating Schwann cells and is down-regulated in actively myelinating cells. Using cultured rat Schwann cells as a convenient in vitro system, we found that both Brn-2 and Oct-6 expression is increased upon addition of forskolin (an agent that elevates intracellular cAMP concentration through the reversible activation of adenylyl cyclase; Fig. 1D). Similarly, during nerve regeneration, Sim and colleagues (2002) showed the parallel expression of Brn-2 and Oct-6. Our present studies of Oct-6- and Brn-2-deficient mice demonstrate that the activation of these genes is not interdependent, thus providing a safeguard for myelination in the developing organism.

Intracellular signaling pathways that regulate Oct-6 expression in Schwann cells converge on the SCE, an enhancer element that has been identified in both the mouse and human Oct-6 locus. A first attempt to identify such an enhancer within the Brn-2 locus on the basis of sequence homology has failed thus far. DNaseI hypersensitivity mapping of the Brn-2 locus in Schwann cells should suggest the position of relevant enhancer sequences and facilitate the identification of relevant binding sites within the enhancers of Oct-6 and Brn-2, so as to determine whether the activation of these genes is by means of a similar signaling pathway. One signaling

pathway that is of particular interest in this respect is the NFκB pathway, as Nickols and colleagues (2003) recently provided evidence of its involvement in peripheral nerve myelination and Oct-6 regulation in Schwann cells.

Although Oct-6 and Brn-2 are activated independently, Brn-2 expression levels are attenuated by Oct-6. Brn-2 expression levels are increased (two- to threefold) in Oct-6-deficient Schwann cells compared to wild-type (Figs. 1C, 2A,B). It is at present not known by what mechanism Brn-2 levels are regulated by Oct-6, although our semiquantitative RT–PCR data suggest involvement of transcriptional or posttranscriptional mechanisms rather than translational or posttranslational mechanisms. Brn-2 expression is extinguished through an Oct-6-independent pathway, as Brn-2 expression is down-regulated at later stages of postnatal development, even in the absence of Oct-6 (Fig. 2E).

Role of Brn-2 in Schwann cell development

The coordinated expression of Brn-2 and Oct-6 prompted our in vivo studies of Brn-2 as the additional overlapping activating factor in the Schwann cell developmental program. We found that overexpression of Brn-2 under control of the Oct-6 SCE in Oct-6-deficient Schwann cells results in an increase in the number of Schwann cells that enter the myelinating phase on schedule. Schwann cell-specific deletion of Brn-2 in an Oct-6-deficient background results in a phenotype that is much more severe than that observed in Oct-6 single mutant animals, with Schwann cells arrested at the promyelin stage up to 120 d after birth. These results strongly suggest that Brn-2 shares a role with Oct-6 in Schwann cell development as a positive regulator of the promyelinating–myelinating transition, and identify Brn-2 as the proposed Oct-6 like function in Oct-6 mutant animals (Jaegle and Meijer 1998).

Functional overlap, as demonstrated here for Brn-2 and Oct-6, is a common phenomenon among members of the same transcription factor family and has been described for, among others, members of the Gata and Sp1 family. Within the POU family of transcription factors, overlapping roles have been described for the closely related *Brn-1* and *Brn-2* genes in cortical neuron development (McEvilly et al. 2002; Sugitani et al. 2002). Functional overlap has also been described between *Oct-6* and the more distantly related *Skn-1a/i* POU gene, which are both required for proper differentiation of epidermal keratinocytes (Andersen et al. 1997). In our system, we tested whether the more divergent POU domain gene *Brn-5* could functionally complement *Oct-6*. The choice of *Brn-5* was motivated by the work of Wu and colleagues (2001), who demonstrated that Brn-5 is expressed in myelinating Schwann cells and is regulated independently of Oct-6. High levels of Brn-5 expression did not ameliorate the developmental delay phenotype of nerves in Oct-6-deficient animals. This is not due to a possible inhibitory function of Brn-5, as *SCE-Brn-5/Oct-6*$^{\Delta SCE/+}$ nerves were normally myelinated (data not shown).

Analysis of Brn-2 expression in Oct-6 mutant animals and Brn-2 overexpression studies led us to the conclusion that quantitatively higher levels of Brn-2 protein are required to initiate myelination on schedule. These data, together with the fact that Brn-2 and Oct-6 have very similar DNA-binding preferences, suggest that the differences in biological function between Brn-2 and Oct-6 result mainly from differences in the affinities for interacting factors and/or the repertoire of interacting partners.

POU domain proteins are known to interact with members of the Sry box (Sox) transcription factor family. For example, the activation of the Fgf4 enhancer in ES cells depends on the synergistic interaction between Sox2 and Oct4 proteins (Ambrosetti et al. 1997). In glial cells it has been shown that Sox10 interacts synergistically with Oct-6, but not Brn-1 or Brn-2, to activate transcription when both proteins are bound to adjacent binding sites in an artificial enhancer (Kuhlbrodt et al. 1998a). Synergistic activation required the N-terminal region of Oct-6 and Sox10 (Kuhlbrodt et al. 1998b). Similarly, the oligodendrocyte-enriched Sox11 protein synergizes with Brn2 and Brn-1, but not with Oct-6. Taken together, these data suggest that a specific POU/Sox code exists and postulates that specific POU proteins require specific Sox proteins to exhibit cooperative effects (Kuhlbrodt et al. 1998a,b). If indeed important target genes of Oct-6 in glial cells are regulated through interaction with Sox10, and the pair Brn-2/Sox11 is equivalent to Oct-6/Sox10, the developmental defect in Oct-6-deficient Schwann cells but not in oligodendrocytes (Bermingham et al. 1996) could be explained as follows: Oligodendrocytes express Brn-2/Sox11 in addition to Oct-6/Sox10, whereas Schwann cells express Oct-6/Sox10 and Brn-2 but not Sox11. Further experiments are required to test this hypothesis.

Our data demonstrate that the promyelin-to-myelinating transition is regulated by Oct-6/Brn-2 function, but even in the absence of these POU factors, Schwann cells eventually do enter the myelinating phase of cell differentiation. This is not due to accumulation of Oct-6 protein expressed from the hypomorphic ΔSCE allele (Fig. 2E). Also, as Brn-2 deletion in the Schwann cell lineage is complete, mosaicism of Brn-2 expression cannot explain this delayed myelination (Fig. 5). Two observations are particularly important in understanding the roles of Oct-6 and Brn-2 in Schwann cell differentiation and why even in their absence myelination occurs. First, in the absence of Oct-6, entry into the myelinating phase is delayed *and* the kinetics of this transition are changed (Fig. 6B). Whereas >80% of wild-type Schwann cells have entered the myelinating phase by P8, in the absence of Oct-6 it takes several weeks for the same number of Schwann cells to make this transition into the myelinating phase. In the absence of both Oct-6 and Brn-2, this transition is even further protracted. Second, kinetics of this transition are Oct-6/Brn-2 dosage-dependent. In terms of transcriptional regulation, this suggests that Oct-6 greatly increases the chance that its target genes become activated. The finding that in the absence of

Oct-6 and Brn-2 this chance is not zero indicates that these target genes are activated, albeit less effectively, through other transcription factors, possibly including the ubiquitous POU factor Oct-1.

Notwithstanding, the identification and characterization of Brn-2 and its in vivo role in Schwann cells have provided insight into the transcriptional network of myelination. These results will ultimately lead to a biochemical explanation of the different, yet overlapping roles of Oct-6 and Brn-2 in Schwann cell development and possible intervention strategies to promote myelination in clinical therapeutic settings.

Materials and methods

Gene targeting

A 10.5-kb HindIII fragment of *Brn-2* genomic sequences was isolated from a 129ola ES cell-genomic phage library. Two restrictions sites (HindIII and AgeI) were generated by PCR at the ATG and used to insert a 5′ loxP site. A second clone was generated containing the neomycin gene driven by a thymidine kinase (Pytk) promoter flanked by FRT sites. Next to the 3′FRT site we inserted a 3′ loxP sequence and an eGFP reporter gene. A 1.9-kb fragment containing FRT-Pytk-neomycin-FRT-loxP-eGFP was isolated as a BglII fragment and cloned in a unique BamHI site in the *Brn-2* 3′ untranslated region. We positioned a negative-selection marker gene, a thymidine kinase gene driven by the phosphoglycerate kinase-1 promoter (PGK-tk), downstream from the 3′ homology region.

SpeI-linearized DNA (15 µg) was electroporated into E14 ES cells followed by selection with G418 (200 µg/mL) and 2 µM gancyclovir. Individual clones were screened for homologous recombination by Southern-blot analysis of BamHI and HindIII-digested genomic DNA with 5′ and 3′ external probes (probes A and B). One correctly targeted ES cell clone with a correct karyotype was isolated and injected into C57Bl/6 blastocysts. Male chimeric mice were mated with FVB/N females to transmit the modified *Brn-2* allele to the germ cells (*Brn-2*$^{wt/flox}$). Heterozygous offspring were back-crossed to generate homozygous mutant *Brn-2*$^{flox/flox}$ mice. The different *Brn-2* alleles were genotyped either by Southern blotting or by PCR with the following primers: 5′-GCGCGGCTCCTTTAACCAGAGCGCC-3′ and 5′-CTGGTGAGCGTGGCTGAGCGGGTGC-3′. The wild-type allele will yield a 210-bp PCR product, and *Brn-2*$^{flox/flox}$ will yield a 250-bp PCR product.

Transgenic mice

For the generation of Cre recombinase-expressing mice, we used an 18-kb NotI *Dhh* genomic clone encompassing the *Dhh* promoter, exons 1, 2, and 3 (gift from Dr. Andy MacMahon, Harvard). The *Cre* gene was extended at its N terminus with a nuclear localization peptide sequence and cloned in exon 1 on the start codon of *Dhh*. The 19-kb transgene construct was separated from vector sequences and microinjected into fertilized FVB/N oocytes. Founder lines were crossed back to FVB/N mice. The genotype of the mice was determined by PCR analysis of genomic DNA isolated from mouse tails. Cre-specific primers 5′-ACCCTGTTACGTATAGCCGA-3′ and 5′-CTCC GGTATTGAAACTCCAG-3′ were used to amplify a 300-bp fragment from the DhhCre construct.

The HA-tag expression cassette was generated by subcloning a 2.3-kb NotI/HindIII fragment containing the HA-tagged *Oct-6* fusion gene described before (Mandemakers et al. 2000) into pBluescript (pHA-Oct-6). A BglII/SpeI/NcoI synthetic polylinker was inserted as a blunt/NcoI fragment into MscI/NcoI-digested pHA-Oct-6, placing the linker just behind the triple HA tag and removing most of the Oct-6 coding sequences (HA-tag cloning vector). A 2-kb BamHI fragment containing the *Brn-2* coding sequence, isolated from pCMV-Brn-2, was subcloned into the HA-tag cloning vector linearized with BglII (HA-Brn-2). A 0.9-kb BamHI/XbaI fragment containing Brn-5 coding sequences, isolated from pCMV-Brn-5, was cloned into the HA-tag cloning vector digested with BglII/SpeI (HA-Brn-5). The NotI/SwaI fragments from HA-Brn-2 and HA-Brn-5 were then ligated into NotI/SwaI-digested R3HAOct-6-SCE vector, creating constructs SCE-HABrn-2 and SCE-HABrn-5. EcoRI restriction fragments containing the transgene construct were isolated and microinjected into fertilized FVB/N oocytes. Transgenic lines were crossed into an *Oct-6*$^{\Delta SCE/\Delta SCE}$ background.

Electron microscopy

Electron microscopy on sciatic nerves was performed as described (Jaegle et al. 1996). Mice were anesthetized with Nembutal and transcardially perfused with PBS followed by 3% paraformaldehyde (PFA) and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.5. Sciatic nerves were isolated and placed in fresh fixative overnight at 4°C. The sciatic nerves were washed in 0.2 M cacodylate buffer overnight at 4°C before being postfixed in 1% OsO_4 for 3 h, dehydrated, and embedded in Epon. Semithin sections were stained with para-phenylenediamine (ppd; Estable-Puig et al. 1965) and viewed under an Olympus microscope. Ultrathin sections were stained with uranyl acetate and lead citrate, and analyzed with a Philips CM100 electron microscope. For quantification, five random nonoverlapping electron micrographs were produced for every nerve at a final magnification of 2600×. Myelinating and promyelin figures were counted (250–450 fibers per nerve).

Whole-mount X-gal staining

Embryos were isolated and fixed by immersion for 1 h at room temperature in 2% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA at pH 8, and 0.02% NP-40 in PBS. Embryos were washed three times for 10 min in PBS with 0.02% NP-40 and stained overnight at room temperature in PBS containing 1 mg/mL Bluo-gal, 5 mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% SDS, and 0.02% NP-40. Subsequently, the stained embryos were washed twice in PBS with 0.02% NP40, postfixed overnight in 4% formaldehyde, and embedded into paraffin. Sections (8 µm) were cut, counterstained with eosin, and viewed under the microscope. Images were collected using an Olympus DP50 digital camera.

Antibodies and immunohistochemistry

Rabbit polyclonal antibodies were raised against the N terminus of Brn-2 protein (amino acids 155–271). Animals were immunized with His-tagged fused protein expressed in *Escherichia coli* and purified on Ni^{2+}-NTA-agarose beads (QIAGEN). Specificity of the Brn-2 antiserum was confirmed by EMSA (data not shown). Antibodies raised against the N-terminal portion of the Oct-6 protein have been described (Ilia et al. 2002).

Sciatic nerves were isolated, teased into single fibers, and fixed for 30 min with 4% PFA at room temperature. The tissue was blocked in 1% BSA, 0.05% Tween-20 in Tris-buffered sa-

line (TBS). Rabbit anti-Brn-2 antibodies and mouse anti-Neurofilament (hybridoma 2H3, Developmental Studies Hybridoma Bank) antibodies, both diluted 1:200, were incubated simultaneously in TBS/0.05% Tween-20 overnight at room temperature. Oregon Green-conjugated goat anti-rabbit IgGs (Molecular Probes) and Texas Red-conjugated goat anti-mouse IgGs (Molecular Probes) were subsequently used as secondary antibodies. The tissue was viewed using a Leica fluorescence microscope.

Western blotting

Sciatic nerves were isolated and directly lysed in loading buffer, followed by sonication and heating in a boiling water bath. Western blotting was performed as described (Ghazvini et al. 2002). Primary antibodies include anti-HA (rabbit polyclonal Y-11; Santa Cruz Biotechnology; used at dilution 1:2000), anti-Brn-2 (goat polyclonal C-20; Santa Cruz Biotechnology; used at 1:100), anti-Oct-6 (rabbit polyclonal; 1:1000), anti-P-zero (mouse monoclonal, hybridoma clone P07; 1:1000 (Archelos et al. 1993), and acetylated α-Tubulin (mouse monoclonal Sigma T-6793; 1:10,000). Secondary antibodies were either conjugated with alkaline phosphatase (Dako) or horseradish peroxidase (Dako) for the detection of primary antibodies.

Electrophoretic mobility shift assay

Sciatic nerve and Schwann cell extracts were prepared by placing the tissue or cells in 5–10 tissue volumes of 20 mM Hepes-KOH at pH 7.9, 400 mM KCl, 1 mM EDTA, 10 mM DTT, 10% glycerol supplemented with 1 mM PMSF, and 1× protease inhibitor cocktail (Sigma; Meijer et al. 1992). The tissue was disrupted by four cycles of snap-freezing in liquid nitrogen and thawing on ice. Cellular debris was removed by centrifugation at 14000*g* for 5 min at 4°C. The supernatant was snap-frozen and stored in aliquots at –80°C. Equal amounts of extract were used in a bandshift assay using 10 fmole of a ^{32}P end-labeled double-strand oligonucleotide probe (*GAGAGGA*ATTTG CAT*TTCCACCGACCTTCC*). Probe and protein were incubated on ice, in the absence or presence of antiserum, for 20 min in 20 mM Hepes-KOH at pH 7.9, 1 mM EDTA, 1 mM EGTA, 4% Ficoll in a total volume of 20 µL. Complexed and free probe were separated on a 4% polyacrylamide gel in 0.25× TBE electrophoresis buffer at room temperature. Gels were fixed in 10% methanol/10% acetic acid, dried, and exposed to a PhosphorImager screen (Molecular Dynamics). Relative band intensities were calculated using the ImageQuant 5.2 software.

Acknowledgments

We thank Elaine Dzierzak and Sjaak Philipsen for their comments and encouragement. John Kong a San is thanked for his expert help in microinjection, Manoussos Koutsourakis for blastocyst injection, and Matthijs Uyl and Ludo Broos for help in immunohistochemistry. The genomic *Dhh* clone was a kind gift from Dr. Andy MacMahon (Harvard University, Boston). The 2H3 monoclonal antibody, developed by T.M. Jessell and J. Dodd, was obtained from the Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. The monoclonal P07 against the extra-cellular domain of P-zero was a kind gift from Dr. Juan Archelos (Graz University, Austria). This work was supported in part by grants from the Dutch Research Council NWO (ALW 805-17.281 and MW 903-42-195).

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MANUSCRIPT I

Defining the transcriptome of differentiating Schwann cells

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manuscript

Abstract

Schwann cell differentiation occurs in response to axonal signals *in vivo*. Oct6 and Krox20 are two transcription factors that are crucial for the regulation of Schwann cell differentiation. Many aspects of Schwann cell differentiation can be mimicked *in vitro* by elevation of intracellular cAMP. To gain a better understanding of the molecular changes that occur during Schwann cell differentiation, we have analyzed changes in gene expression profiles in differentiating primary rat Schwann cells. We have identified a number of genes previously unknown to be modulated upon Schwann cell differentiation. Furthermore, we have identified mRNAs that are differentially expressed in peripheral nerves of Oct6 null versus wildtype mice. We confirmed that some of these genes are target genes of Oct6, while others are target genes of Krox20.

Keywords: Schwann cell, differentiation, expression profile, Oct6, Krox20

Introduction

Schwann cells are embryonically derived from the neural crest and produce the insulating myelin sheath in the peripheral nervous system (PNS). Neural crest cells associate with embryonic nerves to give rise to precursors that proliferate and migrate along the nerve fibers and differentiate into bi-potent immature Schwann cells. Around birth in rodents these cells will start to exit the cell cycle and finally differentiate in myelinating and non-myelinating Schwann cells [Jessen and Mirsky, 2005].

Oct6 (also known as SCIP/Tst-1; OTF6, POU3F1) is a POU-homeodomain containing transcription factor [Svaren and Meijer, 2008] that is transiently expressed in Schwann cells. In mouse, low levels of Oct6 are observed in immature Schwann cells as early as embryonic day 14 (E14) but Oct6 expression levels increas dramatically at late fetal life and remain high during early postnatal development, coinciding with the promyelinating stage of Schwann cells [Arroyo et al., 1998; Blanchard et al., 1996; Mandemakers et al., 2000]. In the PNS, Oct6 expression is restricted to the Schwann cell lineage and depends on maintained axon-Schwann cell interactions as isolation and culture of neonatal Schwann cells will result in rapid downregulation of Oct6 [Scherer et al., 1994; Zorick et al., 1996]. During postanatal development, Oct6 expression is gradually downregulated in differentiting myelin-forming Schwann cells and is completely extinguished in myelinating Schwann cells of adult animals [Scherer et al., 1994; Blanchard et al., 1996; Zorick et al., 1996].

Deletion of the Oct6 gene in mice leads to a transient arrest at the promyelinating stage of cell differentiation resulting in delayed myelination of the

PNS [Bermingham et al., 1996; Jaegle et al., 1996]. Gene deletion experiments with the zinc finger transcription factor Krox20 (Egr2) and its interactors Nab1 and Nab2 also revealed an arrest of Schwann cell differentiation at the promyelin stage. In contrast with the transient developmental arrest in Oct6 deficient mice, Krox20-/- and Nab1-/-/Nab2-/- Schwann cells are permanently arrested at the promyelin stage [Topilko et al., 1994; Le et al 2005]. Genetic studies have shown that Oct6 acts upstream of Krox20. Moreover, the Krox20 myelinating Schwann cell enhancer contains several Oct6 binding sites that are essential for enhancer function [Ghislain and Charnay 2005]. These data suggest that Oct6 directly regulates Krox20 expression in promyelinating Schwann cells. [Ghislain et al., 2002, 2006]. Gene expression profiling in Oct6 mutant animals has identified an additional six potential Oct6 target genes [Bermingham et al., 2002]. These genes encode fatty acid transport protein P2, LIM domain protein CRP2, tramdorin and 3 proteins with unknown function [Bermingham et al., 2002].

Schwann cells withdrawn from an axonal contact through nerve dissection followed by *in vitro* culturing, adopt an immature embryonic Schwann cell phenotype. Agents elevating intracellular cAMP levels can mimic the *in vivo* requirement of myelinating Schwann cells for axonal contact [Morgan et al., 1991]. Schwann cells isolated from perinatal rat sciatic nerves can be propagated *in vitro* in the presence of neuregulin-1 (Nrg1) [Brockes et al., 1980]. In a number of aspects these cells resemble embryonic Schwann cells as their survival depends on Nrg1 signaling. Upon treatment with high concentrations of forskolin their morphology changes and they start to express high levels of Oct6 and myelin protein P0 (Mpz) [Lemke and Chao 1988; Monuki et al., 1989; Morgan et al., 1991]. Thus, this culture system provides a convenient system to study molecular changes during the differentiation of immature Schwann cells and to identify Oct6 target genes.

In this study we have used a modified *in vitro* Schwann cell culture model to identify genes whose expression is regulated during differentiation [Brockes et al., 1980]. By using microarray approach combined with molecular indexing analyses, we identified multiple targets of Oct6 that were distinct of those of Krox20. In addition, we present data of the comparison of the gene expression between postnatal day 1 Oct6 -/- and wt sciatic nerves.

Materials and Methods

Primary Schwann cell culture and transient transfection assays. All cell culture reagents were obtained from Invitrogen (USA), unless indicated otherwise. Rat Schwann cell primary culture was established as previously described [Brockes et al., 1979] with slight modifications. In brief, sciatic nerves of P3 rat pups were dissected aseptically and collected into L-15 medium, nerves were teased with fine forceps and tissue was further disrupted by enzymatic digestion using 1 mg/ml collagenase (Roche,USA) for 30 min at 37°C in L15 medium. Cells were washed once with L15/10% fetal calf serum (FCS), plated onto uncoated Primaria tissue culture dishes (Becton Dickinson, USA) in DMEM/10% FCS medium and incubated overnight at 37°C and 5%CO_2. The next day, medium was replaced with fresh medium containing 10 μM cytosine arabinoside (Ara-C; Sigma) to remove fibroblasts. Cultures were treated this way for 3 days, refreshing the Ara-C containing medium daily. Pure Schwann cell cultures were maintained and passaged in DMEM, 3%FCS, 2μM forskolin (Sigma) and 5% NDF-beta conditioned medium on Primaria tissue culture dishes [Spiegel and Peles 2009]. In the differentiation experiments, medium was replaced by a defined medium (DMEM/F12, 1xN2 supplement and 5% NDF-beta conditioned medium) and incubated overnight. The following day, medium was replaced by a defined differentiation medium containing 20μM forskolin to induce the differentiation of Schwann cells. The control Schwann cell cultures were subjected to defined medium containing 0,2% DMSO. 36 hours later, the cells were used for subsequent analysis.

Schwann cells in defined medium were transfected with pCDNA3 (Invitrogen, USA), pSCTKrox20 (kind gift from Walter Schaffner) or pEVOct6 [Meijer et al., 1992] expression vectors using Fugene6 (Roche, USA) according to manufacturers instructions. 1 μg of DNA was used per 60 mm cell culture dish using Fugene6 /DNA ratio 3:1. 12 hours post-transfection the medium was replaced with fresh defined medium and cells were harvested after 36 hours.

Western blotting and immuno-fluorescence analysis. Equal amounts of cell extracts prepared from proliferating, DMSO-treated and forskolin-treated Schwann cell cultures were resolved on a 12% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). Membranes were blocked with 3%BSA, 0.05% Tween20 in PBS for 1 hour at room temperature and probed with, anti-cyclinD3 (1:2000; SantaCruz Biotech,USA), anti-p27 (1:2000 SantaCruz Biotech,USA), anti-Oct6 [Zwart et al., 1996] and anti-Mpz (mouse monoclonal, hybridoma clone P07; 1:1000; [Archelos et al. 1993]) antibodies in blocking buffer. After incubation for 2 hours, the filters were washed 5 times with 0.5% Tween20 in PBS and incubated with secondary antibodies conjugated with horseradish peroxidase or alkaline

phosphatase (1:2000, DAKO) for 1 hour in blocking buffer. Following 5 washes with 0.5% Tween20 in PBS the antigens were visualized by luminol in case of horseradish peroxidase or NBT/BCIP in case of alkaline phospatase detection methods.

For immunofluorescence analysis, the cells were fixed with 2% PFA 20 min at RT followed by 3 washes with 1x PBS and subsequent blocking with 1%BSA, 0.05% Tween20 in 1x PBS solution for about 1 hour. Affinity purified anti-Oct6 antibody [Zwart et al., 1996] was used as a primary antibody. Anti-rabbit secondary antibody conjugated with OregonGreen (1:400, Molecular Probes) was used for immunofluorescence analysis. Cell nuclei were visualized with DNA-specific fluorophore DAPI.

Microarray analysis. RNA from undifferentiated and differentiated SCHWANN CELL cultures were prepared by using Trisol (Invitrogen, USA) and polyA+ RNA was purified with Dynabeads (Dynal Biotech) according to manufacturers' instructions. cDNA, hybridization of rat GEM2 microarray (Incyte Genomics) and data collection were performed in Incyte Genomics (USA). Briefly, random primed cDNA was generated by reverse transcriptase in the presence of Cy3 or Cy5 labeled nucleotides and competitively hybridized onto the rat GEM2 microarray. Primary data was normalized, and defective hybridization spots removed using Incyte Gemtools software and difference greater than 2 times was considered as significant. Hybridization signals were determined by laser scanning fluorography and corrected via internal controls. The baseline of differential expression was set to 2 e.g. datapoints with 2-fold or more differential signal intensity were counted as "meaningful".

Molecular Indexing. Molecular indexing was performed as described in Mahadeva et al., 1998. In brief, 20 ugs of total RNA from undifferentiated and differentiated rat Schwann cell cultures was prepared using Trisol and reverse transcribed into double strand cDNA using oligo(dT) as the first strand primer by standard techniques. cDNAs were digested with a type II restriction enzyme Bbv I (New England Biolabs) at 37°C overnight. Digested cDNAs were divided into 120 pools and a combination of 2 adaptors was ligated onto each pool in a reaction containing 3.3 nM of each adaptor pool. The volume of the ligation reaction was 30 μl. Adaptor sequences were as described in Mahadeva et al., 1998. Ligation mixtures were heated to 65°C for 5 minutes and cooled to 37°C prior to adding the T4 DNA Ligase (Promega, USA). After ligation the unincorporated adaptors were removed by using Qiaquick PCR purification colums (Qiagen).

Adaptored cDNA fragments were amplified by using primer common to all the adaptor combinations in a 10 μl reaction in the presence of [^{32}P]dATP and 0.5 μM

primer with PlatinumTaq (Invitrogen). PCR conditions used for amplification were 94°C 5 minutes, (94°C 30 seconds, 60°C 30 seconds, 72°C 2 minutes and 30 seconds) x 28, 72°C 10 minutes. Amplified PCR reactions were resolved on 6% denaturing polyacrylamide gels, and analyzed by autoradiography.

Differentially expressed PCR fragments were eluted from gel by boiling gel-slices in TE for 5 minutes. 1/50th of the eluted PCR fragment was used for re-amplification in a 25 µl reaction. Re-amplified PCR products were cloned into T vector (Promega) and sequenced.

DNA Array hybridization. The experiments were performed by using mouse 1.2 Atlas array filters containing 1176 dotted gene fragments (BD Biosciences, Clontech). Filter hybridization and washings were all carried out following the instructions of the supplier. Hybridized filters were scanned (BAS reader, Fuji) and analyzed (peak integration, local background subtraction) by using AIDA Array Metrix 3.0 software (Raytest). Two independent experiments for each RNA pool were performed, thus generating four comparison data sets. Each signal was compared to the expression level of two reference genes: GAPDH and a virtual reference gene, which was the calculated based on the average signal intensity. Only those genes that were found to be consistently regulated among all four comparisons were considered for further analysis.

Semi-quantitative RT-PCR analysis. First strand cDNA was prepared from equal amounts of total RNA using Superscript II (Invitrogen) and oligo(dT) as a primer. cDNAs were amplified in a 50 µl reaction containing 20 nmol dNTP, 1 µCi [^{32}P]dATP, 25 pmol primers, 1x PCR buffer, 1 U Expand Polymerase mix (Roche, USA). Primer sequences are available upon request. The PCR conditions used were 94°C 2 minutes, (94°C 30 seconds, 58°C 30 seconds, 68°C 1 minute) x 36. 4 µl aliquots were taken after every 2 cycles, starting from cycle no 16, resolved using a 6% denaturing polyacrylamide gel and analyzed using Phosphoimager. The linear range of amplification differed for different cDNAs, so the total number of cycles for PCR products shown, is different in case of different cDNAs.

For non-radioactive PCR analysis, the linear range of amplification was determined by a EtBr staining of 1.5% agarose gel and for only one cyclepoint for each sample at an early log phase of amplification. Different number of cycles was used for semi-quantitative PCR analysis of different cDNAs.

Results

Modeling early Schwann cell differentiation in a serum free cell culture system. In order to identify and characterize molecular events of Schwann cell differentiation, we modified the previously established Schwann cell culture system. First, we employed serum-free conditions, to omit gene expression changes due to cell cycle exit upon serum presence. In addition, in order to diminish the effects of DMSO (as forskolin was dissolved in DMSO), we treated the control cells with volumes of DMSO that were equal to the volumes of forskolin used to induce differentiation in parallel experiments. 80% confluent Schwann cell culture grown for 36 hours in defined medium supplemented by neuregulin-1 and DMSO contained multipolar cells with a relatively small soma and long processes (Fig.1A). Upon stimulation with 20 μM forskolin the cells flattened out, indicating induction of an active extracellular matrix synthesis, and adopted mostly bipolar shape (Fig.1A).

Oct6 was initially cloned as a POU-homeodomain containing gene upregulated during cAMP induced differentiation of Schwann cells *in vitro* [Monuki et al., 1989]. However, these results were obtained in the presence of 10% fetal calf serum in the culture media. Here we show that in culture conditions where serum was omitted, Oct6 protein expression was induced in differentiated Schwann cells, but not in proliferating or DMSO treated cells (Fig.1B). Mpz showed similar pattern of expression (Fig.1B).

Despite the fact that in both conditions (control and upon differentiation) Schwann cells were found arrested in G0/G1 phase of the cell cycle (data not shown), cdk-inhibitor p27Kip1 and CyclinD3 were induced only in differentiated Schwann cells (Fig.1B).

Upon differentiation, the majority of Schwann cells adopted the flattened bipolar morphology, while rare cells with small soma and multiple processes, resembling non-differentiated Schwann cells were also observed. Next, we asked what percentage of cells expresses Oct6 upon differentiation. Using an Oct6 antibody and immunofluorescence microscopy we found that all of the differentiated Schwann cells expressed Oct6 protein (Fig.1C). In addition, all cells expressed myelin protein zero (Mpz) (data not shown).

Taken together, our data show that under serum-free conditions Schwann cells express high levels of Oct6 and Mpz following forskolin induced cell differentiation. Therefore, we conclude that our Schwann cell culture system can be used to identify changes in gene expression during early stages of Schwann cell differentiation.

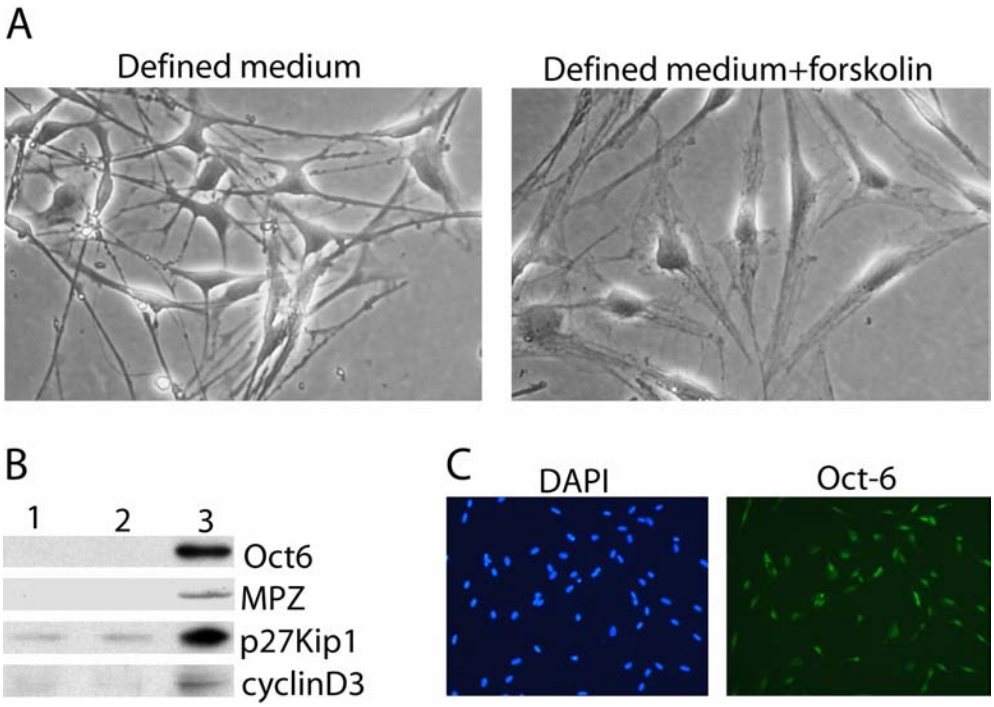

Figure 1. Primary rat Schwann cells differentiate synchronously in culture with changing cell morphology. (A) Phase contrast image of non-differentiated and differentiated rat Schwann cells grown in defined medium. (B) Expression of Oct6, Mpz, p27 Kip1 and cyclin D3 in proliferating (column 1), nondifferentiated (column 2) and differentiated Schwann cells (column 3). (C) Immunofluorescence analysis of Oct6 expression in differentiated Schwann cells indicating that all of the cells express Oct6

Analysis of gene expression changes during Schwann cell differentiation *in vitro*

To analyze changes in gene expression during Schwann cell differentiation, we used rat GEM-4 microarray chip of Incyte Pharmaceuticals, Palo Alto, CA. containing 8144 brain -enriched cDNAs as described in Materials and Methods. The experiment was performed in duplicate. The resulting hybridization profiles are depicted in supplementary figure 1. Signals from the differentiated (forskolin-treated) cells were plotted on the Y-axis and signals from the control (DMSO-treated) cells on the X-axis. For the sake of clarity, all datapoints that showed less than 2 fold difference in expression levels were omitted in the figure. We found a strong correlation between replicates within the same samples. Therefore, we concluded that these microarray hybridizations were successful and likely to provide reliable data for further analysis.

Quantification of the results of the microarray experiment shows the following: Out of 8144 datapoints, 315 are differentially regulated upon differentiation by at least 2-fold. Out of those 315 differentially expressed datapoints, 61 different annotated cDNAs are upregulated and 62 different annotated cDNAs downregulated upon Schwann cell differentiation. 53 differentially expressed datapoints give similiarities to ESTs.

All known genes and their accession numbers found to be differentially expressed during forskolin dependent Schwann cell differentiation are listed in Table I. As expected, myelin-associated mRNAs (Mpz, Mag, Pmp22, Plp, Mbp) are all upregulated during Schwann cell differentiation. Also a number of genes encoding extracellular matrix (ECM) components are upregulated upon differentiation, indicating active ECM synthesis. These data were consistent with the results from *in vivo* situation, where it has been shown that Schwann cells synthesize extensive basal lamina during myelination [Chernousov et al., 2008]. We also found that, consistent with the active basal lamina synthesis, a number of proteases and protease inhibitors are differentially regulated upon Schwann cell differentiation *in vitro* including Igfbp5, Timp-2, Timp-3, Serpine2 (GDN), serine protease inhibitor and others. In good agreement with earlier findings [Bermingham et al., 2001; Schworer et al., 2003], Nfl and Nfm mRNAs are also upregulated upon differentiation. In addition, several other nervous system-specific cytoskeletal components are found to be induced upon Schwann cell differentiation. Examples are erythrocyte protein band 4.1-like 4b (Ehm-2), Myristoylated alanine-rich C-kinase substrate Marcks, and erythrocyte protein band 4.1-like 3 (TII 4.1. brain isoform). Among transcription factors, Oct6, CoupTFII and Id2 are induced, in contrast to Myocyte enhancer factor 2C (MEF2c) and MEF2-interacting transcription repressor Mitr, that are downregulated during differentiation of Schwann cells.

Table I
A) cDNAs induced during forskolin dependent Schwann cell differentiation identified with microarray analysis.

Fold-Δ	**Gene**	**Acc. No**
Myelin genes		
-16,9	Schwann cell peripheral myelin protein (P-0)	K03242
-9	PMP-22	X62431
-4,1	CD9 mRNA for cell surface glycoprotein	X76489

-3,9	Myelin basic protein	M25889
-3	Myelin proteolipid protein (PLP)	M25888
-2,9	Myelin-associated glycoprotein (MAG)	M22357

Proteases and protease inhibitors

-7,2	Insulin-like growth factor binding protein 5 protease	AF179370
-2,2	Complement C1r	AAA51851
-2,3	Carboxypeptidase E	M31602
-6,4	Tissue inhibitor of metalloprotease 3 (TIMP-3)	U27201
-5,2	Inter-alpha (globulin) inhibitor H5-like	CAA18605
-2,8	Matrix metalloprotease inhibitor (TIMP-2)	L31884

Cell surface receptors

-6,7	Nonselective-type endothelin receptor	S65355
-2,3	Pre-PDGF receptor mRNA	X04367

Extracellular matrix proteins

-3,9	Type VII collagen	AAB66593
-3,9	Thrombospondin 2	L06421
-3,6	Laminin B1	M15525
-3	Laminin gamma 1	X94551
-2,7	Laminin-2 alpha2 chain	U12147
-2,4	Collagen alpha1 type I	Z78279
-2,3	Laminin alpha 4	Y09827
-2,2	Collagen alpha-1 type-III	M18933
-2,1	Collagen type IX alpha 3	AF237721

Cytoskeletal proteins

-10,9	Light molecular-weight neurofilament (NF-L)	AF031880
-4,8	Neurofilament protein middle (NF-M) mRNA	Z12152
-3,5	Type II brain 4.1 minor isoform	AB032828
-3,4	Myristoylated alanine-rich C-kinase substrate (MARCKS)	M60474
-2,8	Ehm2 mRNA	AB032366
-2,6	Anillin	BAA91711

-2,6	Protein 4.1G mRNA	AF044312
-2,4	Gamma-adducin	U35775
-2	Non-muscle alpha-actinin 1	AF115386

Transporters

-17,1	Ca(2+)-sensitive chloride channel 2 (Cacc)	AF108501
-4,1	ATP-binding cassette protein (Abca8) mRNA.	AF213393
-3,8	mRNA for sodium channel.	Y09164
-2	Sodium channel I mRNA	M22253
-2	Sodium channel II mRNA.	M22254
-2,2	(Na+, K+)-ATPase-beta-2 subunit mRNA	J04629
-2	Sodium-hydrogen exchanger 6 (NHE-6)	AF030409

Transcription factors

-3,7	CoupTFII	X76653
-3,1	Oct-6	M72711
-2,4	Id2	M69293

Others

-5,3	Vascular EGF repeat-containing protein EVEC (fibulin 5)	AF137350
-3,3	1-acyl-sn-glycerol-3-phosphate acyltransferase	AB005623
-3	Steroid sensitive gene-1 protein (SSG-1)	AF223677
-3	UDP-galactose:ceramide galactosyltransferase	U07683
-2,6	AIBC1	AAC39896
-2,5	Alex-3	AAF24487
-2,5	Cdc2 promoter region.	X60767
-2,4	Synaptotagmin IV	L38247
-2,4	Thymic stromal cell mRNA for TLSF-beta	D43805
-2,3	Evectin-1 (EVT1)	AF118562
-2,3	FAPP1	BAA90927
-2,3	Tyrosylprotein sulfotransferase-1	AF038008
-2,1	UDP-glucuronosyltransferase UGT1A7	U75903

-2,1	Putative tetraspan transmembrane protein L6H	AAB82947
-2,1	Inositol trisphosphate receptor subtype 3 (IP3R-3)	L06096
-2	Stathmin 1	M27876
-2	Liver alpha-2-macroglobulin	J02635
-2	ARL5 mRNA for ARF-like protein 5.	X78604
-2	DD6A4-1 mRNA	AF034237

B) cDNAs downregulated during forskolin dependent Schwann cell differentiation identified with microarray analysis.

Proteases and protease inhibitors

10,6	ADAMTS-1	AF149118
5,9	Late gestation lung protein 1 (Lgl1)	AF109674
4,9	Glia-derived nexin (GDN)	M17784
3,4	Calpastatin, clone RNCAST104	Y13588
2,1	Furin	X55660
2	Serine protease inhibitor (SPI3)	U25844

Cell surface receptors

4	Glypican	L34067
3,3	p120 (catenin delta1)	Z17804
3,2	ST7 protein	AAD44360
2,5	Thymocyte mRNA for cell surface protein (MRC OX-2)	X01785
2,4	Cadherin-10	BAA87417
2,3	NC1 protein	AJ250730
2,1	VPS10 domain receptor protein SORCS 2	NM_030889

Extracellular matrix proteins

6,3	Tenascin C	D90343
4,7	collagen, type XX, alpha 1	BAA96034
2,1	Collagen XII alpha 1	U57362

Cytoskeletal proteins

4,6	Beta-centracetin	CAA57691
3,4	Myosin X (myo 10 gene).	AJ249706
3,1	Gamma-filamin	AAF67190
3	Kinesin heavy chain (Khcs)	L27153

2,8	T-plastin.	X70706
2,6	Moesin	AF004811
2,5	E-septin long form mRNA	AF180525
2,5	MID2	Y18881
2,1	RLC-A gene for myosin regulatory light chain	X54617
2	Erythrocyte tropomodulin (E-Tmod)	AF287746

Transporters

7	Vesicular monoamine transporter	L00603
3	Ion channel homolog RIC mRNA	U72680
2,2	ATPase, H+ transporting, lysosomal accessory protein 2	CAB43210
2,2	Multidrug resistance protein 1a (Pgy1)	AF257746
2,2	Dihydropyridine-sesitive L-type calcium channel alpha-2 subunit	M86621
2	CLCN3	X78520

Transcription factors

3,6	MEF2-interacting transcription repressor (Mitr)	NM_024124
2,6	Myocyte enhancer factor 2C (Mef2c)	NM_025282
2,4	Basic transcription factor 2, 35 kD subunit	CAA82909
2	PEBP2 beta	AF087437

Others

10,75	Mouse ectropic viral integration site 2 (Evi-2) ORF	M34896
8,3	GAP-43	L21191
5,6	Developmentally-regulated cardiac factor (DRCF-5)	U95001
4,7	Cytochrome c oxidase subunit Vb	D10951
4,2	CGI-78 protein	AAD34072
4,1	Beta-galactoside-alpha 2,6-sialyltransferase	M83143
3,7	Phytanoyl-CoA hydroxylase interacting protein	BAA13402
3,4	Smooth muscle cell LIM protein (SmLIM) mRNA	U44948
3	Plasma glutathione peroxidase precursor	D00680

3,2	Platelet-derived growth factor A-chain (PDGF A)	L06894
3,1	M-Sema F	S79463
2,7	ARFGEF1	BAA91912
2,7	Peripheral plasma membrane protein CASK	U47110
2,6	Ubiquitin carboxyl-terminal hydrolase PGP9.5	D10699
2,5	Ri1	X76454
2,4	Protein phosphatase-2A catalytic subunit	X14159
2,4	Galanin	J03624
2,4	Diphosphoinositol polyphosphate phosphohydrolase type 2 (NUDT4)	AF191654
2,3	Beta-1,3-N-acetylglucosaminyltransferase	AF092050
2,3	PE31/TALLA	D26483
2,3	Phosphofurin acidic cluster sorting protein 2	BAA25528
2,3	Stress activated protein kinase alpha II	L27112
2,2	Guanine nucleotide binding protein gamma subunit 11	AF257110
2,2	Cctq-cytosolic chaperone containing TCP-1, theta subunit	Z37164
2,2	Evectin-2 (Evt2)	AF189817
2,1	Vascular protein tyrosine phosphatase-1 rDEP-1	U40790

Accession numbers given are directly taken from the microarray annotation and refer to the blast match most closely related to a datapoint. In some cases accession number given refers to a protein sequence.

As a complement and alternative to the microarray analysis we used modification of a differential display method called molecular indexing [Mahadeva et al., 1998] to compare cDNA populations derived from non-differentiated and differentiated Schwann cells. The results of molecular indexing are listed in Table II. We identified a total of 50 cDNAs differentially expressed in non-differentiated versus differentiated Schwann cells. Eight of them (Mpz, Nfl, Col1a1, Igfbp5, gamma filamin, Tnc, Catenin delta1 and Serpine2) were also identified by microarray analysis (see Table 1). As for the rest, we found that mRNAs encoding 3 different protein kinases (p90 S6 kinase, PTEN induced kinase and brain creatine kinase) are induced during Schwann cell differentiation. We also identified a

number of additional cDNAs encoding cytoskeletal proteins that are modulated during Schwann cell differentiation. In particular, utrophin mRNA level go up whereas talin and vimentin mRNA levels drop, indicating broad rearrangements in the cyoskeletal compartment of Schwann cells during differentiation.

In addition, levels of transcription factors CP2 and Tle3 mRNAs are upregulated upon differentiation, whereas expression Atf3 and Zfand5 mRNAs is downregulated. Interestingly, although both cell populations analyzed were in G1/0 phase of the cell cycle (data not shown), the expression of Cyclin D3 mRNA is upregulated during Schwann cell differentiation. The induction of Cyclin D3 was further confirmed by Western blot analysis (Fig.1B).

Table II

cDNAs induced during forskolin dependent Schwann cell differentiation.

Gene	**Accession no.**
Myelin protein zero (Mpz)	NM_017027
Neurofilament, light polypeptide (Nefl)	NM_031783
Lamin A (Lmna)	NM_021755
Utrophin (Utrn)	NM_013070
Alpha-spectrin 2 (Spna2)	X90845
Collagen, type V, alpha 3 (Col5a3)	NM_021760
Collagen, type VI, alpha 1 (Col6a1)	XM_001079629
Collagen, type I, alpha 1 (Col1a1)	Z78279
Plexin B3 (Plxnb3)	NM_001135878
Transforming growth factor, beta receptor III (Tgfbr3)	NM_017256
Fibulin 5 (Fbln5)	AF137350
Sperm associated antigen 9 (Spag9)	NM_027569[1]
Insulin-like growth factor binding protein 5 (Igfbp5)	AF139830
Ferritin, heavy polypeptide 1 (Fth1)	NM_012848
Rho-guanine nucleotide exchange factor (Rgnef)	NM_001108542
Fibroblast growth factor (acidic) intracellular binding protein (Fibp)	NM_172334
Sequestosome 1 (Sqstm1)	Y08355
Ribosomal protein S6 kinase polypeptide 2 (Rps6ka2)	D83013
Brain creatine kinase (Ckb)	NM_012529
PTEN induced putative kinase 1 (Pink1)	XM_216565
Cyclin D3 (Ccnd3)	NM_012766
Transcription factor CP2 (Tcfcp2)	NM_001134714

Transducin-like enhancer of split 3 (Tle3)	NM_053400
Ribosomal protein S4 (Rps4x)	X14210
Ribosomal protein L5 (Rpl5)	NM_031099
Inscuteable homolog (Insc)	NM_001106285

cDNAs down-regulated during forskolin dependent Schwann cell differentiation.

Gene	**Accession no.**
Gamma C filamin (Flnc)	XM_342653
Talin 1 (Tln1)	NM_001039025
Vimentin (Vim)	NM_031140
Serine (or cysteine) peptidase inhibitor, clade B, member 5 (Serpinb5)	U58857
Non-erythrocyte beta-spectrin (Sptbn1)	AF337905
Dystonin (Dst)	NM_001108208
Peroxidasin homolog (Pxdn)	XM_001053103
Tenascin C (Tnc)	NM_053861
Catenin (cadherin associated protein), delta 1 (Ctnnd1)	NM_001107740
Sialic acid binding Ig-like lectin E (RGD1563073)	XR_009371
Transforming growth factor, beta induced (Tgfbi)	AF305713
Guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1 (Gnb2l1)	U03390
Serine (or cysteine) peptidase inhibitor, clade E, member 2 (Serpine2)	M17784
Guanine nucleotide-binding protein G-s alpha subunit (Gnas)	NM_019132
Meningioma expressed antigen 5 (Mgea5)	NM_131904
Procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1 (Plod1)	L25331
Suppressor of Ty 5 homolog (Supt5h)	U88539[1]
Activating transcription factor-3 (Atf3)	NM_012912
Zinc finger, AN1-type domain 5 (Zfand5)	NM_001106356
Heterogeneous nuclear ribonucleoprotein K (Hnrnpk)	NM_057141
Ribosomal protein L10 (Rpl10)	NM_031100
Ribosomal protein L13 (Rpl13)	NM_031101
Acidic ribosomal protein P0 (Arbp)	NM_022402
Tetratricopeptide repeat domain 1 (Ttc1)	NM_001005529

[1] refers to mouse cDNA

Expression of genes differentially regulated in the course of Schwann cell differentiation in perinatal wt and Oct6 deficient peripheral nerves

Previous data showed that inactivation of Oct6 in mice results in a transient arrest of Schwann cell differentiation at the promyelination stage [Bermingham et al., 1996; Jaegle et al., 1996]. Therefore it was of interest to examine whether the genes for which differential expression was established *in vitro* were also differently regulated in the perinatal nerves of Oct6 deficient mice as compared to peripheral nerves of wt animals.

Oct6 deficient mice die perinatally [Bermingham et al., 1996; Jaegle et al., 1996]. To obtain nerves in quantities sufficient for RNA analysis, we used a mouse in which one allele of the Oct6 locus was inactivated by insertion of LacZneo reporter gene into the coding region of Oct6 and the other inactivated by deletion of the Schwann cell specific enhancer of Oct6 [Ghazvini et al., 2002]. These mice have all the characteristics of the Schwann cell phenotype observed in Oct6 null animals but live well into adulthood due to the absence of the CNS phenotype and therefore presented an excellent genetic system to address questions related to the transcriptional targets of Oct6.

We performed semi-quantitative RT-PCR analysis of 14 cDNAs with altered expression patterns during Schwann cell differentiation *in vitro*. As shown in figure 2, four cDNAs (transcription factors Id2 and CoupTFII, myelin protein Cd9 and Fibulin5) identified as upregulated upon Schwann cell differentiation *in vitro*, display similar patterns of expression during Schwann cell differentiation in $Oct6^{\Delta SCE/wt}$ and $Oct6^{\Delta SCE/\beta geo}$ mice. The levels of Krox20 and Rgnef are strongly reduced in the nerves of the mutant animal as compared to the wt (Fig.2).

Semi-quantitative RT-PCR analysis of the RNAs found to be downregulated during Schwann cell differentiation *in vitro*, revealed that the levels of two transcription factors – Zfand5 and Mitr – are regulated accordingly *in vivo*, since the levels of Zfand5 and Mitr in P1 mutant nerves are significantly higher as compared to the levels in the nerves of $Oct6^{\Delta SCE/wt}$ mice (Fig.2). In contrast, levels of other mRNAs tested, including mRNAs encoding transcription factors Mef2C and Atf3, are reduced upon Schwann cell differentiation in culture, but remain the same in nerves of both mutant and heterozygous animals (Fig.2).

In conclusion, many genes whose expression was found to be modulated during forskolin-induced Schwann cell differentiation in culture, were not expressed differentially in perinatal peripheral nerves of wt versus Oct6 deficient animals

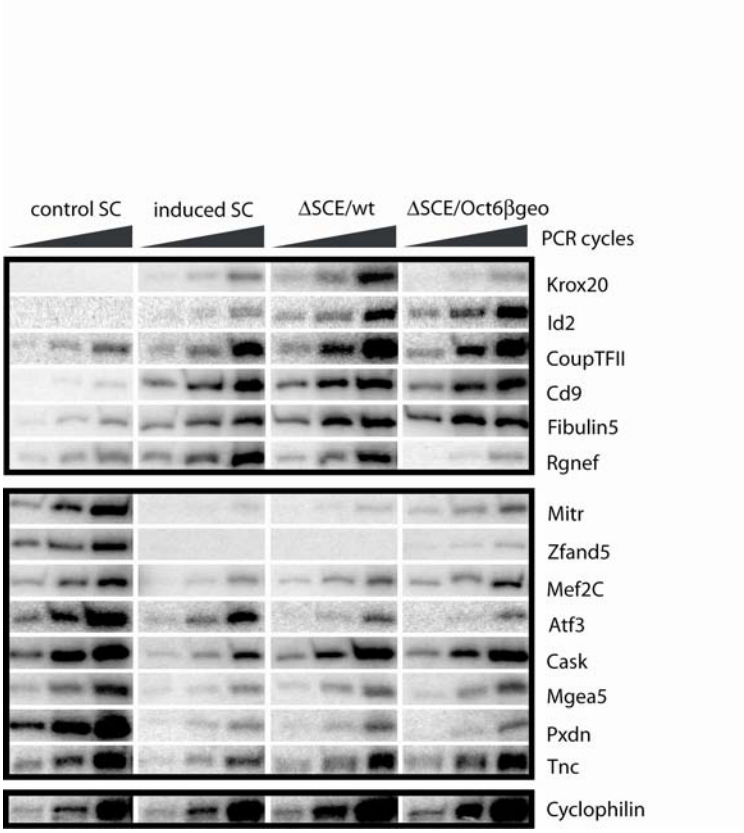

Figure 2. Semiquantitative RT-PCR analysis of gene expression differences in nondifferentiated versus differentiated Schwann cells and in postnatal day 1 sciatic nerves of *Oct6*$^{\Delta SCE/wt}$ versus *Oct6*$^{\Delta SCE/\beta geo}$ animals

Comparison of gene expression pattern in P1 Oct6 mutant and heterozygote nerves. Provided that many of the genes that we studied in cultured Schwann cells did not show differential expression in peripheral nerves of *Oct6*$^{\Delta SCE/wt}$ as compared to *Oct6*$^{\Delta SCE/\beta geo}$ mice, a direct comparison of the gene expression patterns in P1 nerves of Oct6 mutant and heterozygote animals was performed. Mouse Atlas array filters (Clontech), with 1176 individual cDNA fragments were used for gene expression analysis. The filter hybridization was repeated twice, generating 4 comparison data sets that were cross-compared. Only genes that were differentially regulated in all 4 comparisons were included for further analysis. As expected, mRNAs encoding Krox20 and Mpz and Pmp22 are more abundantly expressed in heterozygote nerves (Fig.3). Both Mpz and Pmp22 were also identified previously as differentially regulated genes in Schwann cell differentiation in our cell culture experiments (Table 1).

In addition, the levels of cdk inhibitors p27Kip1 and p57Kip2 are significantly higher in heterozygote nerves than in Oct6 mutant nerves (Fig.3). p27Kip1 was also observed to be upregulated during Schwann cell differentiation in cell culture paradigm (Fig.1B). In addition we identified that cadherin-6 and adenomatosis polyposis coli (APC) mRNAs are expressed at higher levels in heterozygote nerves as compared to Oct6 mutant nerves (Fig.3).

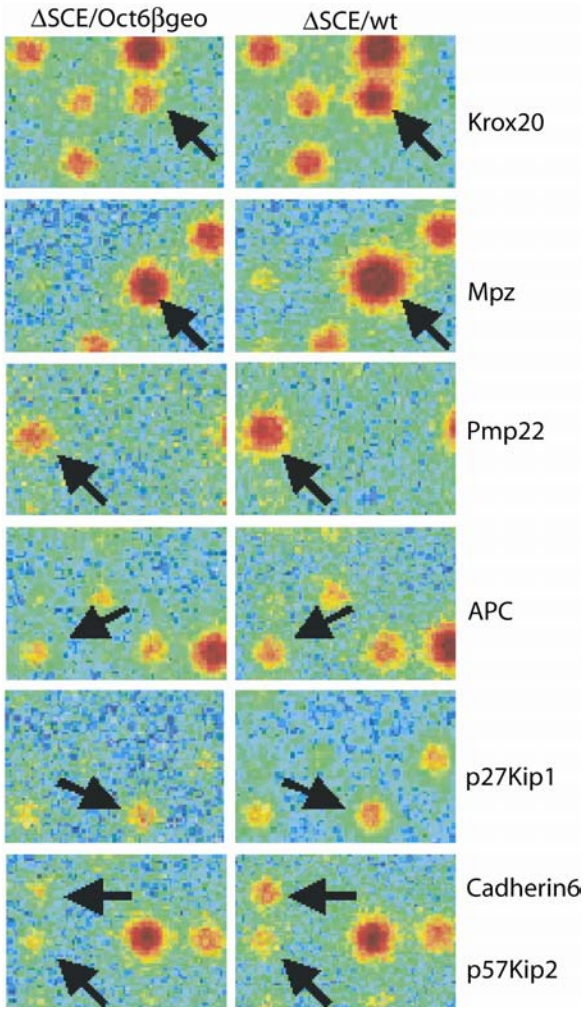

Figure 3. Comparison of gene expression in postnatal day 1 sciatic nerves of *Oct6*$^{\Delta SCE/\beta geo}$ and *Oct6*$^{\Delta SCE/wt}$ animals. Atlas array filters were used for comparison. Each spot represents a single cDNA on the filter. The loacalization of the relevant cDNAs is indicated with arrows. Left panel shows results of *Oct6*$^{\Delta SCE/\beta geo}$ and right panel *Oct6*$^{\Delta SCE/wt}$ hybridizations

Krox20, Rgnef and Fibulin5 are targets of Oct6 activity

We and others have shown that both Oct6 and Krox20 mRNAs are induced during Schwann cell differentiation. *In vivo* experiments have provided clear evidence that Krox20 functions downstream of Oct6 in the genetic hierarchy of Schwann cell differentiation, compatible with the possibility that Krox20 is a direct target gene of Oct6 [Jaegle et al., 1996] Ghislain 2005. We decided to test this possibility by transient transfection of Oct6 in undifferentiated rat Schwann cells. As shown in figure 4, over-expression of Oct6 significantly induces expression of endogenous Krox20, whereas over-expression of Krox20 has no effect on Oct6 mRNA levels (data not shown).

Next, we asked whether other differentially regulated genes that we identified in previous experiments were also direct targets of Oct6 or indirect targets through regulation by Krox20. As illustrated in Figure 4, Mpz and Cd9 mRNA levels in undifferentiated Schwann cells are upregulated by over-expressed of Krox20 but not by over-expressed Oct6. These results indicate that these myelin genes are direct targets of Krox20. In contrast, Rgnef and Fibulin5 expression is upregulated through overexpression of Oct6 but not Krox20 (Fig.5) suggesting they are indeed direct transcriptional targets of Oct6

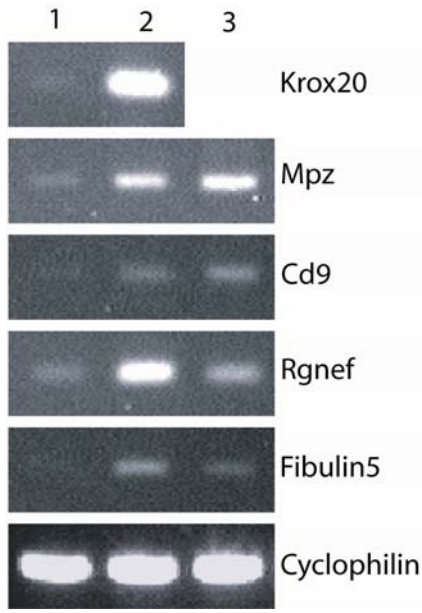

Figure 4. Semiquantitative RT-PCR analysis of the induction of endogenous Krox20, Mpz, Cd9, Rgnef and Fibulin5 mRNAs after transfection of empty vector (column1), Oct6 expression vector (column 2) and Krox20 expression vector (column 3) into nondifferentiated Schwann cells

Discussion

To identify novel genes whose expression is modulated during Schwann cell differentiation, we compared the transcriptome of undifferentiated and differentiated Schwann cells *in vitro* using microarray and molecular indexing analyses. We have identified 84 different cDNAs that are up-regulated and 83 cDNAs that are down-regulated during forskolin mediated Schwann cell differentiation *in vitro*. We further analyzed if some of the identified cDNAs were differentially expressed in the nerves of Oct6 mutant mice and compared the gene expression pattern in $Oct6^{\Delta SCE/\beta geo}$ mice and $Oct6^{\Delta SCE/wt}$ P1 peripheral nerves using ATLAS filters. Finally we showed that some of the identified genes were direct transcriptional targets of Oct6, whereas others are targets of Krox20.

Myelin gene expression was found to be activated during forskolin mediated Schwann cell differentiation. In accordance with previous reports [Bermingham et al., 2001; Pareek et al., 1993; Lemke and Chao 1998; Jiang et al., 2000] we found that Mpz, Pmp22, Cd9, Mbp, Plp, and Mag mRNA expression was induced. Also, the expression of a number of proteases and protease inhibitors (Igfbp5, CarboxypeptidaseE, C1r, ITI-like protease, Timp2 and Timp3) was increased, while levels of other protease and protease inhibitors were decreased (Adamts1, Lgl1, Serpine2, Calpastatin, Furin, Serpinb6A). Among the latter, Serpine2 has been shown previously to be up-regulated in response to Krox20 in Schwann cells [Nagarajan et al., 2001]. Our finding that Serpine2 was downregulated during Schwann cell differentiation is therefore unexpected, since levels of Krox20 increase during differentiation. However, it has been shown that Serpine2 is downregulated upon forskolin and not NRG-1 treatment of Schwann cells [Schworer et al., 2003]. The modulation of the levels of proteases and protease inhibitors is expected, since there is active ECM synthesis and reorganization during myelination [Chernousov et al., 2008]. This assumption is further corroborated by the fact that a number of laminin and collagen genes were upregulated during Schwann cell differentiation, while others were downregulated (Tables I and II). Downregulation of Tnc has been shown before and it was found to be dependent only on forskolin and not on Nrg1 [Schworer et al., 2003].

In addition to Nfl and Nfm up-regulation and gamma filamin down-regulation that has been shown previously [Bermingham et al., 2001], we found the levels of a number of other cytoskeletal proteins to be modulated. Also, we found that there is a change in Cl^- channel repertoire during Schwann cell differentiation. Voltage-gated Clcn3 was downregulated, while calcium activated Clca1 was up-regulated (Table 1). We have shown here for the first time the expression of two types of Cl^- channels in Schwann cells.

A number of transcription factors in addition to Oct6 were found upregulated during Schwann cell differentiation. These include Id2, CoupTFII, CP2 and the

corepressor Tle3. Induction of Id2 has been observed before in dbcAMP (a membrane permeable cAMP analogue) induced primary Schwann cells. Also, the level of Id2 mRNA is elevated in postnatal day 0 sciatic nerve as compared to embryonic day 19 sciatic nerve [Stewart et al., 1997]. Tle3 is particularly interesting, since members of this family interact and regulate a variety of transcription regulatory systems such as Notch and Wnt which are known to regulate Schwann cell development [Cinnamon and Paroush, 2008] and therefore TLE-3 might be one of the global regulators of Schwann cell differentiation.

Mitr and Zfand5 were found to be downregulated during Schwann cell differentiation and the levels of these mRNAs were higher in the perinatal nerves of $Oct6^{\Delta SCE/wt}$ animals than in $Oct6^{\Delta SCE/\beta geo}$ animals. Theses genes might be negatively regulated by Oct6. Mitr is a calcium-sensitive MEF2-interacting transcriptional repressor [Zhang et al., 2001]. Intrestingly we also found MEF-2 to be downregulated during Schwann cell differentiation, but MEF-2 was expressed at normal level in Oct6 mutant nerves. It is possible that compensatory factors retain MEF-2 expression in Oct6 mutant nerves, but that Mitr is regulated negatively by Oct6. Zfand5 is particularly interesting, since it is a potent inhibitor of NfκB [Huang et al., 2004]. NfκB activity has been shown to be important for myelination in the PNS [Nickols et al., 2003]. Zfand5 interacts with the IκB kinase non-enzymatic regulatory subunit, IKKγ and prevents phosphorylation of NfκB [Huang et al., 2004]. It is possible that Oct6 is required to reduce the levels of zfp-216, therefore relieving the inhibitory action of IκB kinase.

Our comparison of gene expression between postnatal day 1 wt and Oct6 null sciatic nerve cDNA populations revealed among others cadherin-6 mRNA being expressed at higher levels in wt nerves. Cadherin-6 is also called K-cadherin. This gene was also identified as a Krox20 target gene in the study of Nagarajan and others [Nagarajan et al., 2001]. Therefore, cadherin-6 represents the first Krox20 nonmyelin target gene both *in vitro* as well as *in vivo*.

Rgnef is a RhoA interacting protein that specifically activates RhoA and stimulates cytoskeletal contraction [Gebbink et al., 1997]. Rgnef was upregulated during Schwann cell differentiation and was also found to be expressed at lower levels in $Oct6^{\Delta SCE/\beta geo}$ perinatal nerves as compared to $Oct6^{\Delta SCE/wt}$ perinatal nerves. Therefore it might be activated by Oct6. Furthermore, Rgnef colocalizes with microtubules and might provide a link between microtubule dynamics and RhoA signalling [van Horck et al., 2001].

Cyclins D3 and cdk inhibitor $p27^{kip1}$ are both induced during forskolin mediated Schwann cell differentiation. Cyclin D3 has previously been shown to be upregulated upon treatment of Schwann cell culture with a combination of neuregulin and forskolin [Schworer et al., 2003]. We also found elevated levels of cdk inhibitors p27kip1 and p57kip2 in wt nerves, as compared to the Oct6 deficient

nerves. This indicates a role for these proteins in the differentiation process, rather than proliferation of Schwann cells, since there is no proliferation defect in Oct6 mutant mice [Jaegle et al., 1996].

The induction of $p27^{kip1}$ has been also observed during the differentiation of D6P2T cell line, which is used as a model for oligodendrocyte differentiation [Friessen et al., 1997]. Therefore this might represent a general requirement for differentiation along glial lineage.

All of the cDNAs identified by our analysis might in principle be divided into 3 categories. First, they might be target genes for Oct6, second they might be target genes for Krox20. Third, they might be induced by the sharp activation of PKA pathway.

To distinguish between these possibilities we transfected nondifferentiated Schwann cells with either Oct6 or Krox20 expression vectors and examined the induction of endogenous Mpz, Cd9, Rgnef and Fibulin5. We found that in accordance with the work of Nagarajan and colleagues [Nagarajan et al., 2001] myelin gene expression is activated by Krox20, while Rgnef and Fibulin5 are activated by Oct6.

Taken together, our results give a glimpse into the transcriptional hierarchies that govern Schwann cell differentiation in the developing animal. We have shown that late myelin gene expression is activated by Krox20, which in turn is activated by Oct6. In addition to activating Krox20, Oct6 appears to regulate also genes such as Rgnef and Fibulin5.

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MANUSCRIPT II

Expression of NGF and GDNF family members and their receptors during peripheral nerve development and differentiation of Schwann cells *in vitro*

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Submitted

Abstract

Ligands of NGF and GDNF families of neurotrophic factors have important functions in the development of the vertebrate peripheral nervous system (PNS). It has been established that they also play key roles in the regeneration of PNS. Expression patterns of NGF and GDNF family members and their receptors has mostly been analyzed during regeneration, and less during development of the PNS. We describe the expression of mRNAs encoding these neurotrophic factors and their receptors during development of rat sciatic nerve and in three modes of differentiation of cultured rat Schwann cells. Our results demonstrate specific expression patterns of NGF and GDNF family ligands and their receptors during differentiation of Schwann cells *in vivo* and *in vitro*.

Introduction

Members of both NGF and GDNF families of neurotrophic factors are important in the development of the vertebrate PNS [1,2]. Neurotrophins BDNF and NGF are important positive regulators of myelination by Schwann cells, whereas NT-3 negatively regulates myelination in PNS. Exogenous administration of BDNF enhances myelination in PNS, whereas removal of endogenous BDNF inhibits myelination in mice [3,4]. Positive effect of BDNF on myelination in PNS comes through binding of BDNF to p75NTR receptor [5]. In addition, BDNF is required for re-myelination in the injured peripheral nerve in rodents *in vivo* [6]. It has been suggested that the major source of BDNF supporting myelination in DRG and Schwann cell co-cultures is neuronal cells [7]. NGF promotes myelination in DRG and Schwann cell co-cultures, whereas blocking NGF activity *in vivo* inhibits myelination [8]. GDNF promotes myelination of small caliber axons that normally do not myelinate and enhances myelination in neuron-Schwann cell co-coltures [9, 10]. In contrast, NT-3 regulates negatively myelination *in vivo* and this effect is achieved through binding of NT-3 to its high affinity receptor NTRK3 [3].

Schwann cells are the sole macroglial cells in PNS. They ensheath axons and, depending on axon caliber, differentiate to either myelinating or non-myelinating Schwann cells. Upon peripheral nerve injury they de-differentiate, start to proliferate, support regeneration of the peripheral nerve and re-differentiate upon axonal regeneration [reviewed in 11]. Expression of NGF and GDNF family members is regulated in the injured nerve with specific time course for each gene [12,13,14]. In cell culture rat Schwann cells proliferate in the presence of neuregulin and low concentrations of forskolin. Upon elevated forskolin levels, Schwann cells differentiate and induce the expression of myelin genes [15]. GDNF

and BDNF are the predominant neuregulin-releasing neurotrophic factors produced by cultured Schwann cells [16].

Despite the fact that neurotrophic factors have important roles in PNS development, expression patterns of the factors during nerve development has not been studied thoroughly. In the present study we have analyzed the expression of NGF and GDNF family members and their receptors during rat sciatic nerve development and in three differentiation stages of cultured rat Schwann cells.

Results and discussion

Expression of neurotrophins and their receptors during rat peripheral nerve development and in cultured Schwann cells

In cultured Schwann cells neurotrophin expression was analyzed in cells growing under three different conditions. Proliferating and growth arrested Schwann cells expressed low levels of differentiation marker Oct-6 and myelin genes MBP and MAG. Expression of Schwann cell differentiation marker Oct-6 and myelin genes MBP and MAG mRNAs was induced in differentiated cells (Fig.1). Proliferating Schwann cells exhibited normal cells cycle profile with 53.4% of the cells in G1, 30.3% in S and 15.5% in G2/M phase of the cell cycle as measured by propidium iodide staining. Growth arrested and differentiated Schwann cells did not proliferate with 87.4 and 88.2% of the cells arrested in G1 phase of the cell cycle respectively (Fig.2).

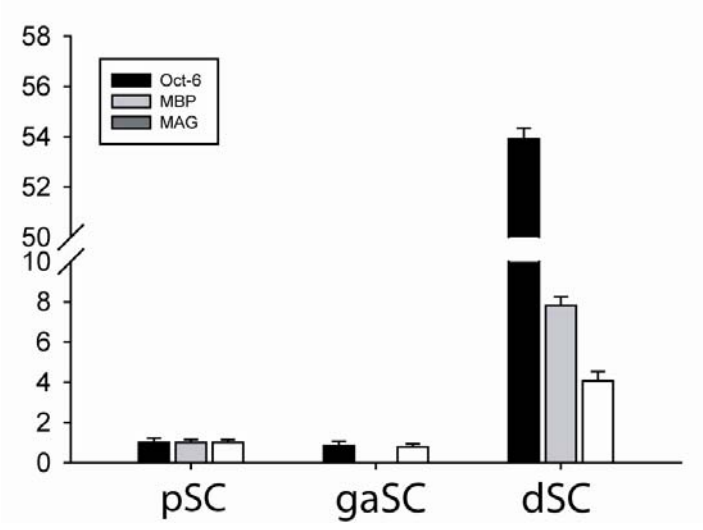

Figure 1. Quantitative RT-PCR analysis of expression of Schwann cell markers Oct-6, MBP and MAG mRNAs in proliferating (pSC), growth arrested (gaSC) and differentiated (dSC) Schwann cells.

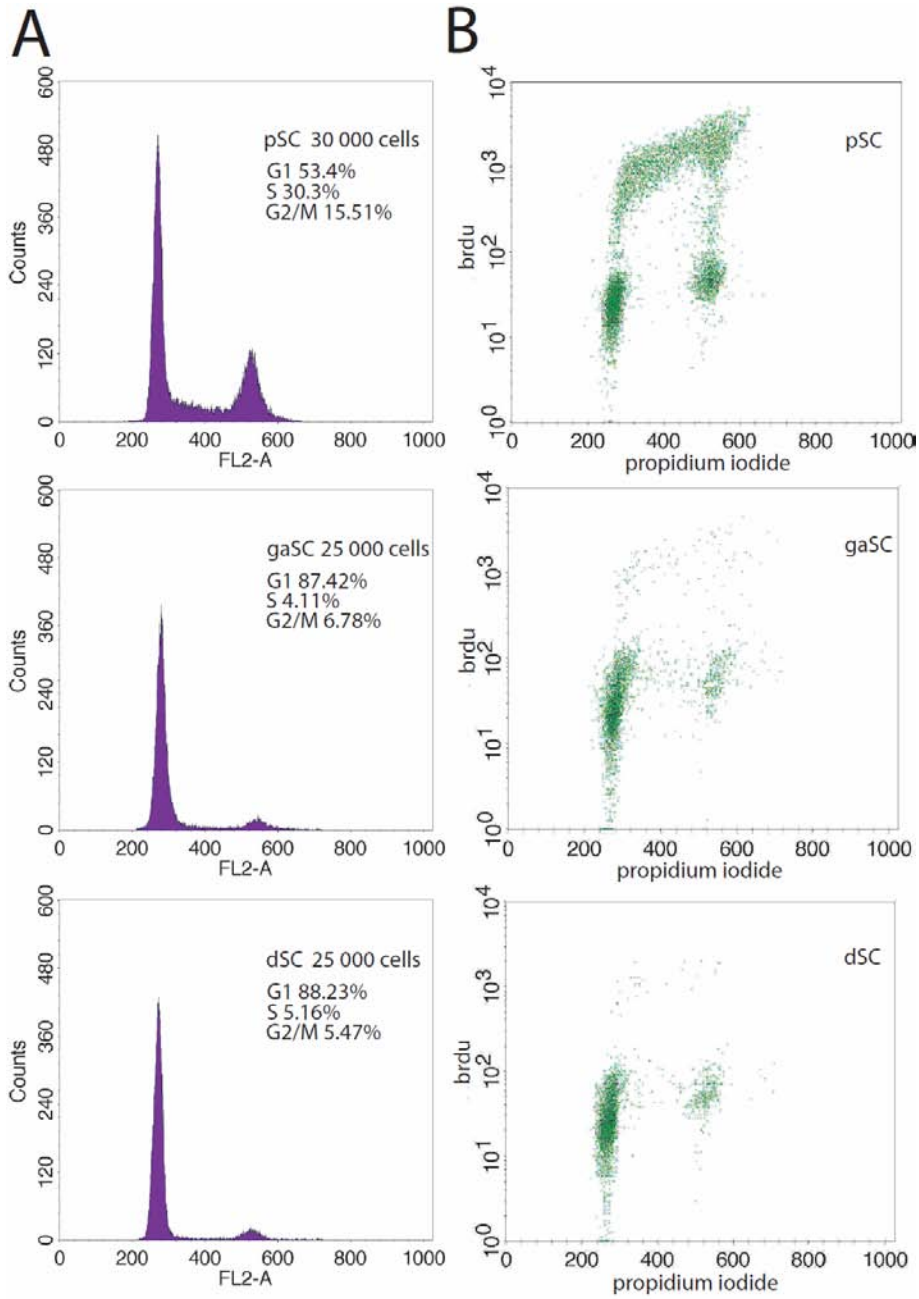

Figure 2. Cell cycle status of proliferating (pSC), growth arrested (gaSC) and differentiated (dSC) Schwann cells. A) Propidium iodide staining of pSC, gaSC and dSC. Histogram plot of DNA content in cells distinguishes between cells in G1, S and G2/M phase of the cells cycle. B) 5-bromo-2-deoxyuridine (brdu) incorporation in pSC, gaSC and dSC. Dot plot of correlation between intensity of brdu and propidium iodide signals shows the incorporation of brdu into DNA in cells situated in G1, S or G2/M phases of cell cycle

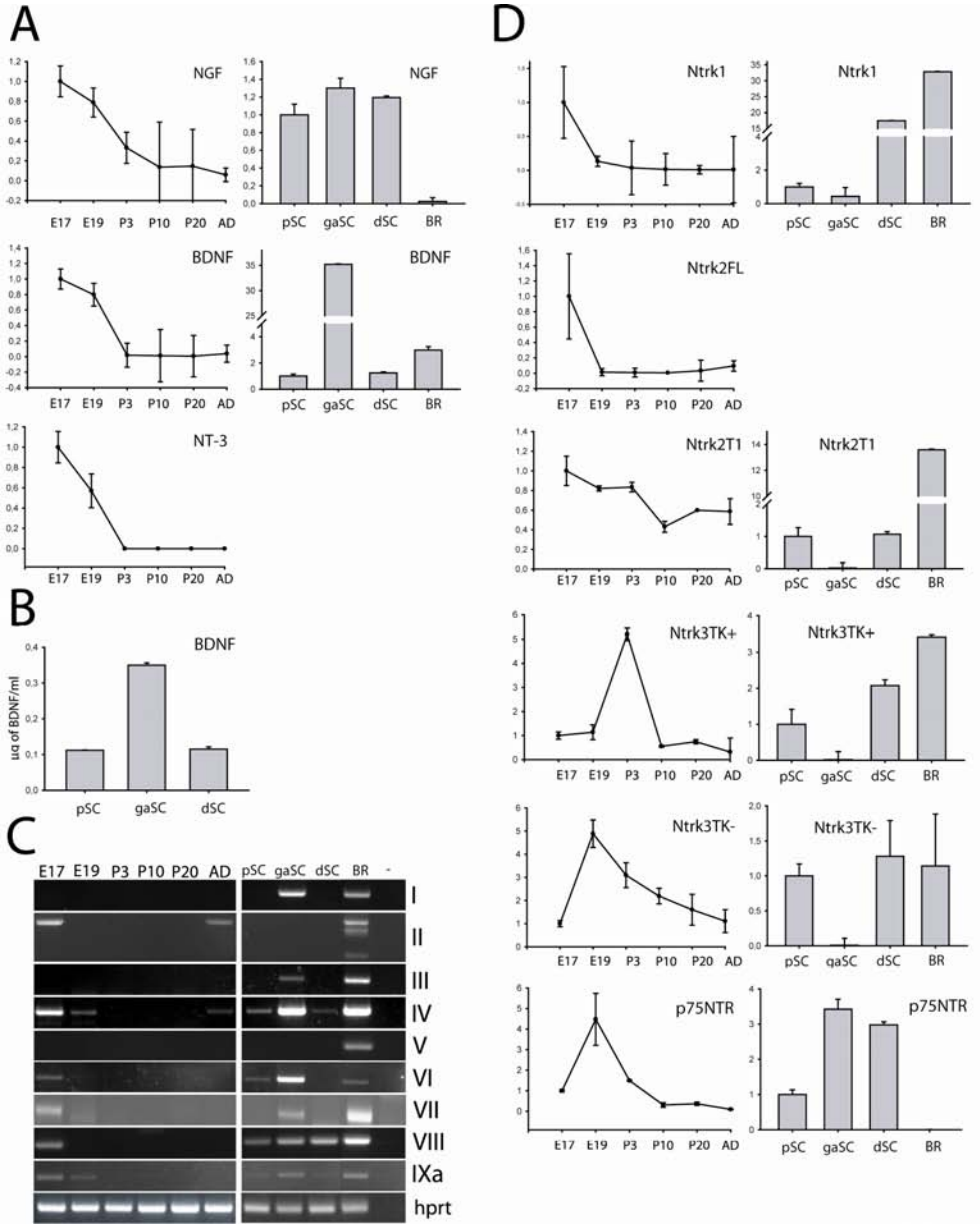

Figure 3. (A) Quantitative RT-PCR analysis of expression of neurotrophin mRNAs during rat sciatic nerve development and in three modes of Schwann cell differentiation. (B) Analysis of BDNF protein levels in three modes of Schwann cell differentiation using ELISA (C) Semiquantitative RT-PCR analysis of expression of BDNF 5' exon-specific mRNAs during rat sciatic nerve development and in three modes of Schwann cell differentiation. (D) Quantitative RT-PCR analysis of expression of neurotrophin receptor mRNAs during rat sciatic nerve development and in three modes of Schwann cell differentiation. pSC - proliferating Schwann cells, gaSC – growth arrested Schwann cells, dSC - differentiated Schwann cells, BR – adult rat brain

NGF mRNA was expressed during all three modes of Schwann cell differentiation at 50-fold higher levels than in adult rat brain. BDNF mRNA was expressed at 35- and 20-fold higher level in growth arrested Schwann cells than in proliferating and differentiated cells respectively (Fig. 3A). The 35-fold induction of BDNF mRNA levels were accompanied by a 3-fold increase in BDNF protein levels in growth arrested cells as compared to proliferating and differentiated cells (Fig. 3B). We did not detect NT-3 and NT-4 expression in cultured Schwann cells (data not shown).

We analyzed NGF, BDNF, NT-3 and NT-4 mRNA expression during rat sciatic nerve development at embryonic day (E) 17, E19, postnatal day (P) 3, P10, P20 and P60 by qPCR analysis. In general, NGF, BDNF and NT-3 expression levels were higher in embryonic sciatic nerves and downregulated upon nerve development. Expression levels of NGF, BDNF and NT-3 mRNAs were highest at E17, the earliest developmental stage analyzed, decreased slightly at E19 and were markedly downregulated during postnatal nerve development (Fig. 3A). We could not detect NT-4 mRNA expression in rat sciatic nerves (data not shown).

Expression of neurotrophins in the postnatal rodent sciatic nerve has been shown previously [12, 13]. Our results demonstrating that neurotrophin expression is high in embryonic sciatic nerves and the levels are decreasing during development suggests two possible scenarios. First, it is possible that Schwann cells in the embryonic nerves are not a homogenous population of glial cells, but rather the phenotype of a Schwann cell depends on the type of axon it is contacting with. This means that during late embryogenesis different Schwann cell populations express different set of neurotrophins. This has also been suggested previously by a study showing that Schwann cells in the regenerating peripheral nerve exhibit distinct sensory and motor phenotypes [20]. Alternatively it is possible that embryonic Schwann cells exhibit homogenous phenotype, but express higher level of neurotrophins to provide maximal support for growing axons.

Structure of BDNF gene is complex as compared to other neurotrophins. Rat BDNF gene contains nine 5’ noncoding exons and one coding exon. Each noncoding exon has its own promoter and is differentially expressed in the nervous system and in other tissues. The 5’ noncoding exons fall into two clusters, with exons I-III as a 5’ cluster and exons IV-VIII 3’cluster. In addition, a separate promoter is driving expression of exon IXa transcripts initiating in the 5’ region of exon IX [19]. Semiquantitative RT-PCR analysis showed that BDNF exon II, IV, VI, VII, VIII and IXa mRNAs were expressed in sciatic nerve, with highest levels at E17, the first developmental stage analyzed (Fig. 3C) similarly to the levels of total BDNF mRNA (Fig. 3A). In the adult sciatic nerve the expression of exon II and IV mRNAs was induced indicating that the slight upregulation of BDNF mRNA expression there could be accounted to the expression from BDNF promoters II and IV (Fig. 3C).

In cultured Schwann cells BDNF exon I, III, IV, VI, VII, VIII and IXa mRNAs were expressed and the levels were highest in growth arrested Schwann cells similarly to total BDNF mRNA (Fig. 3C). Exon VIII mRNA was expressed at lower level in proliferating Schwann cells and at higher level in growth arrested and differentiated Schwann cell suggesting that the slight increase in the expression of BDNF in differentiated as compared to proliferating Schwann cells observed in real-time PCR analysis could be attributed to exon VIII expression. The fact that we detected BDNF exon II expression in developing sciatic nerve but not in cultured Schwann cells suggests that BDNF exon II is expressed in fibroblasts or microglial cells of the developing sciatic nerve. Alternatively, exon II transcripts are expressed in Schwann cells *in vivo* but not *in vitro*. Our analysis also showed that BDNF exon III was expressed only in growth arrested Schwann cells but not in the sciatic nerve suggesting that cell culture conditions we used promoted ectopic expression of BDNF exon III in Schwann cells.

Next we investigated expression of receptors of neurotrophins during sciatic nerve development. Expression of Ntrk1 mRNA, coding the high affinity receptor for NGF, was highest at E17, decreased more than 5-fold at E19 and was almost undetectable postnatally (Fig. 3D). Previous reports have found that Ntrk1 is not expressed in the developing or regenerating rat sciatic nerve [13, 21]. It is possible that sensitivity of analyses did not allow the detection of Ntrk1 expression in these studies. Ntrk2, the high affinity receptor for BDNF and NT-4 has three major isoforms [22,23]. Full-length kinase domain containing Ntrk2FL is mainly expressed in neuronal cells, whereas kinase deficient T1 and T2 isoforms are expressed in glial cells [24]. Ntrk2FL mRNA levels were highest in E17 sciatic nerve, 70-fold decreased at E19, undetectable during early postnatal development and upregulated during later postnatal development (Fig. 3D). Ntrk2T1 mRNA level was highest at E17, and decreased thereafter reaching 2-fold lower levels in adult sciatic nerve (Fig. 3D). We did not detect Ntrk2T2 expression in the sciatic nerve (data not shown). Our results are consistent with previously published data about postnatal expression of NtrkT1 and lack of Ntrk2T2 protein expression in the sciatic nerve [5]. It has also been shown that Ntrk2FL is expressed at low level in adult rat sciatic nerve [5]. Here we show that, in addition to this, full-length Ntrk2 mRNA is expressed in E17 embryonic nerves. Ntrk3, the high affinity receptor for NT-3, has two major isoforms, full length Ntrk3 harboring tyrosine kinase domain (TK+) and truncated Ntrk3 (TK-) [23]. It has been previously shown that Ntrk3 mRNA is expressed in embryonic rat sciatic nerve from E14 to E18 [25]. Our results showed that Ntrk3TK+ mRNA was expressed at E17 and E19 at similar levels, the levels increased 5-fold at P3, decreased 10-fold at P10 and remained unchanged during later postnatal development. (Fig. 3D). Ntrk3TK- mRNA level was upregulated 5-fold at E19 as compared to E17 and gradually decreased during

postnatal development (Fig. 3D). This contrasts to previously published data showing that Ntrk3 protein level is gradually downregulated during postnatal development of rat sciatic nerve [5], suggesting that Ntrk3 mRNA and protein levels are regulated in a different manner. Expression of p75NTR mRNA, the low-affinity neurotrophin receptor increased 2.5-fold from E17 to E19 and was downregulated later during development (Fig. 3D). This is consistent with published data showing that p75 expression is downregulated during postnatal development [5]. We show here for the first time that the expression of p75 is even higher in the embryonic sciatic nerves.

Analysis of expression of neurotrophin receptors in culturd Schwann cells showed that in differentiated Schwann cells Ntrk1 mRNA expression was 17 times higher than in proliferating cells and 34 times higher than in growth arrested cells and 2 times lower than in adult rat brain. (Fig. 3D). Full length Ntrk2 and Ntrk2T2 were not expressed in cultured Schwann cells (data not shown). Ntrk2T1, Ntrk3TK+ and Ntrk3TK- mRNA was detected in proliferating and in differentiated Schwann cells, but not in growth arrested Schwann cells. Ntrk2T1 mRNA was expressed in proliferating and differentiated Schwann cells at similar level that was 14 times lower than in adult rat brain (Fig. 3D). Expression of Ntrk3Tk+ mRNA was induced 2-fold in differentiated cells as compared to proliferating cells and the level was 3.5 times lower than in rat brain. Expression level of Ntrk3TK- was similar in proliferating and differentiated Schwann cells and in adult rat brain. Expression of p75NTR mRNA was induced 3.5 and 3-fold in growth arrested and differentiated Schwann cells respectively compared to proliferating Schwann cells where the levels were still more than 300 times higher than in adult rat brain (Fig. 3D).

Expression of GDNF family of neurotrophic factors and their receptors during rat peripheral nerve development and in cultured Schwann cells

Next we analyzed GDNF, Artn, Pspn and Ntn gene expression during rat sciatic nerve development (Fig. 4A). GDNF mRNA level was highest at E17, decreased more than 30-fold at E19 and remained almost undetectable later during development. It has been previously shown that GDNF is expressed in the distal part of the lesioned adult rat sciatic nerve and in embryonic chick sciatic nerves [14, 16]. Here we show that expression of GDNF is relatively high in rat embryonic sciatic nerve and is downregulated upon nerve maturation. Pspn is expressed in two forms in mammals. Majority of the Pspn mRNA has intron retention, leading to frame-shift in the open reading frame and the minor form encodes biologically active protein [26]. Expression of the shorter functional Pspn mRNA was high at E17 and E19 and decreased postnatally to undetectable levels. As shown previously, Pspn mRNA with intron retention had higher expression

level than the spliced mRNA and was detected at all stages during sciatic nerve development, with highest levels at E17 and E19 (Fig. 4A). We did not detect Ntn and Artn mRNA expression in sciatic nerve (data not shown).

Analysis of GDNF expression in cultured Schwann cells showed that in growth arrested cells it was expressed at 2-fold higher level than in proliferating cells, and 4-fold higher than in differentiated Schwann cells (Fig. 4A). Spliced Pspn mRNA was expressed at similar level in all three modes of cultured Schwann cells (Fig. 4A). Ntn and Artn expression was not detected in cultured Schwann cells (data not shown).

Next, qPCR analysis was performed to investigate expression of receptors for GDNF family of neurotrophic factors (Fig. 4B). It has previously been shown that Gfra1 is expressed in adult sciatic nerve and its expression is upregulated in the distal part of lesion after nerve injury [14]. All members of GFR alpha receptor family, except Gfra4, were expressed in rat sciatic nerve and in cultured Schwann cells (Fig. 4B). The levels of Gfra1 mRNA increased slightly from E17 to E19, were not changed at P3 and decreased during later development. In cultured Schwann cells Gfra1 mRNA was induced 3- and 2-fold in growth arrested and in differentiated cells respectively as compared to proliferating cells, where the levels were more than 12 times higher than in adult rat brain. Gfra2 mRNA level decreased 4-fold at E19 as compared to E17, thereafter the levels increased 10-fold up to P20 and were decreased again in adult sciatic nerve. Gfra3 mRNA level was induced 6-fold at E19 as compared to E17, decreased 6-fold at P3 and and remained relatively unchanged thereafter. In cultured Schwann cells Gfra3 mRNA level decreased more than 2-fold in growth arrested and differentiated cells compared to proliferating cells where it was more than 70 times higher than in adult rat brain. Using in situ hybridization it has previously been shown that Gfra3 mRNA is expressed at similar level in sciatic nerve during embryonic development [27]. In contrast, our results showed Gfra3 mRNA levels increase 6-fold from E17 to E19 (Fig. 4B). In accordance with previously published data we detected no Ret mRNA expression in cultured Schwann cells [14]. However, in the developing sciatic nerve Ret mRNA was expressed, the levels were highest at E17, decreased 10-fold at E19, remained relatively similar up to P3 and increased thereafter (Fig. 4B). It has been shown that GDNF family of neurotrophic factors can also signal through neural cell adhesion molecule NCAM [28]. Therefore, we analyzed NCAM expression in sciatic nerve and in Schwann cells (Fig. 4B). NCAM mRNA was expressed at similar level at E17 and E19, thereafter the levels increased gradually up to P20. In the adult sciatic nerve NCAM mRNA levels were decreased to 2-fold lower levels than at E17. In cultured Schwann cells, NCAM mRNA expression levels were 3-fold higher in proliferating Schwann cells than in growth arrested and differentiated Schwann cells.

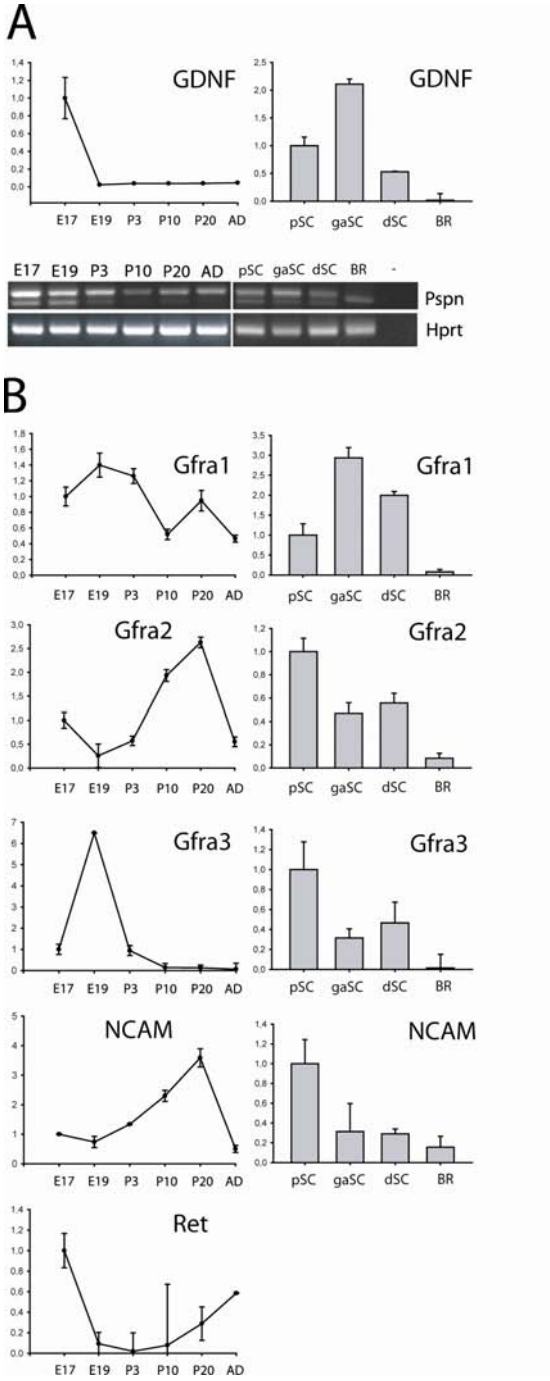

Figure 4. (A) Quantitative and semiquantitative RT-PCR analysis of GDNF and Pspn mRNA expression during rat sciatic nerve development and in three modes of Schwann cell differentiation. (B) Quantitative RT-PCR analysis of expression of receptors for GDNF family members during rat sciatic nerve development and in three modes of Schwann cell differentiation. pSC - proliferating Schwann cells, gaSC - growth arrested Schwann cells, dSC - differentiated Schwann cells, BR– adult rat brain

Taken together, our results show that all neurotrophins, except NT4, are expressed during rat sciatic nerve development and the levels decrease starting from E17, the first developmental stage analyzed. Cultured Schwann cells express NGF and BDNF, but not NT-3 and NT-4. BDNF expression is dramatically induced in growth arrested Schwann cells, while NGF levels do not change significantly in the analyzed three modes of cell culture. All receptors for neurotrophins are expressed in rat sciatic nerve. Cultured Schwann cells express all receptors for neurotrophins except full length Ntrk2. Two members of GDNF family of neurotrophic factors, GDNF and persephin, are expressed in rat sciatic nerve and in cultured Schwann cells. All Gfra receptors, except Gfra4, are expressed in cultured Schwann cells and in sciatic nerve. Ret and NCAM coreceptors are expressed in sciatic nerve, but only NCAM is expressed in cultured Schwann cells.

Materials and Methods

Rat Schwann cell (SC) cultures from postnatal day 3 rat pups were established as described [17]. Pure SC culture was maintained and passaged in DMEM, 3%FCS, 2μM forskolin and 5% neuregulin -beta conditioned medium in Primaria (Clontech) tissue culture dishes. In the differentiation experiments the medium was replaced with defined medium (DMEM/F12, 1xN2 supplement and 5% neuregulin-beta conditioned medium) and incubated overnight. Next day the medium was replaced with defined medium containing 20μM forskolin (Sigma) to induce the differentiation of Schwann cells. The growth arrested Schwann cells were grown in defined medium containing DMSO. 36 hours later the cells were used for subsequent analysis. Cell cycle profiles were measured by propidium iodide staining and Brdu incorporation using cell sorter FacsCalibur (Becton Dickinson).

Total RNA from rat tissues and rat Schwann cells was extracted using RNAWiz (Ambion). First-strand cDNAs were synthesized with Superscript III (Invitrogen) reverse transcriptase. PCR reactions were performed using 1/50 of the first-strand cDNA reaction. Number of cycles in semiquantitative PCR analysis was determined empirically and we always analyzed the PCR product in the exponential phase of amplification. Real-time quantitative PCR (qPCR) analysis was performed in triplicates using qPCR Core Kit for SYBR Green I (Eurogentec) with Lightcycler 2.0 (Roche). Schwann cell data was normalized with housekeeping gene HPRT. Data was analysed as described previously [18]. Primers used in RT-PCR analysis are depicted in supplementary table. Exon-specific BDNF primers have been previously described [19]. BDNF protein levels were measured in cell homogenates using ChemiKine Brain Derived Neurotrophic Factor Sandwich Elisa Kit (Chemicon).

Acknowledgements

We thank Epp Väli for excellent technical assistance and Dr. Dies Meijer for providing neuregulin-beta conditioned media. This work was supported by the following grants to TT: Wellcome Trust International Senior Research Fellowship (Grant 067952), Estonian Ministry of Education and Research (Grant 0140143), and Estonian Science Foundation (Grant 7257).

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PUBLICATION III

Expression analysis of the CLCA gene family in mouse and human with emphasis on the nervous system

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BMC Developmental Biology (2009) http://www.biomedcentral.com/1471-213X/9/10

BMC Developmental Biology

Research article

Expression analysis of the CLCA gene family in mouse and human with emphasis on the nervous system

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Published: 11 February 2009

BMC Developmental Biology 2009, **9**:10 doi:10.1186/1471-213X-9-10

Received: 31 July 2008

Accepted: 11 February 2009

This article is available from: http://www.biomedcentral.com/1471-213X/9/10

Abstract

Background: Members of the calcium-activated chloride channel (CLCA) gene family have been suggested to possess a variety of functions including cell adhesion and tumor suppression. Expression of CLCA family members has mostly been analyzed in non-neural tissues. Here we describe the expression of mouse and human CLCA genes in the nervous system.

Results: We show that from the six mouse CLCA family members only Clca1, Clca2 and Clca4 mRNAs are expressed in the adult brain, predominantly in olfactory ensheathing cells. During mouse nervous system development Clca1/2 is more widely expressed, particularly in cranial nerves, the diencephalon and in the cerebral cortex. While human CLCA2 and CLCA4 genes are widely expressed in brain, and at particularly high levels in the optic nerve, human CLCA3, the closest homologue of mouse Clca1, Clca2 and Clca4, is not expressed in the brain. Furthermore, we characterize the expression pattern of mouse Clca1/2 genes during embryonic development by in situ hybridization.

Conclusion: The data published in this article indicate that within the nervous system mouse Clca1/2 genes are highly expressed in the cells ensheathing cranial nerves. Human CLCA2 and CLCA4 mRNAs are expressed at high level in optic nerve. High level expression of CLCA family members in mouse and human glial cells ensheathing nerves suggests a specific role for CLCA proteins in the development and homeostasis of these cells.

Background

Calcium activated chloride currents have been characterized in a number of cell types including smooth muscle, skeletal muscle and epithelium. Physiologically, it has been shown that activation of calcium-activated chloride current plays a prominent role in among others the maintenance of smooth muscle tone, epithelial secretion and vertebrate olfactory transduction. The precise molecular identities of the currents are still hotly debated. Proteins belonging to CLC, CLCA, bestrophin and tweety gene families have been proposed to function as calcium activated chloride channels [reviewed in [1]].

The CLCA gene family includes 4 genes in humans, 5 genes in rat and 6 genes in mouse. The nomenclature of the CLCA genes in different organisms is somewhat confusing since the numbering of different genes does not reflect the actual homologies between the genes in different organisms but rather the time of characterization [reviewed in [2]].

Although a lot of evidence shows the involvement of CLCA proteins in mediating chloride conductance, it is still unclear whether CLCA proteins are channels themselves. It has been shown that there are differences in endogenous chloride current characteristics in normal versus CLCA over-expressed cells [3]. Also, at least some of the CLCA family members appear to be secreted proteins [4,5]. Recently, using protein structure prediction, it has been proposed that CLCA proteins are membrane anchored or secreted metal-dependent hydrolases [6].

In addition to their functions as chloride channels or channel modulators, some CLCA family members function as cell adhesion molecules [7,8] and tumor suppressor proteins [9]. It has also been proposed that CLCA family members are involved in respiratory diseases like asthma [10,11] and cystic fibrosis [12].

Expression analysis by RT-PCR of mouse Clca family members, has revealed that mClca1 is expressed at high levels in spleen and bone marrow and mClca2 in mammary gland. Moderate or low expression levels of both genes were found in most tissues with only mClca1 expressed in brain tissue [13]. Expression analysis of mClca3, a secreted member of the CLCA family, has been performed by immunohistochemistry. The mClca3 protein was found only in the mucine granule membranes of the gastrointestinal and respiratory tract, uterine goblet cells and other mucin producing cells [14]. RT-PCR analysis revealed that mClca4 mRNA is also expressed in the gastrointestinal tract, as well as uterus, skeletal muscle, heart and lung [15]. RT-PCR analysis of mClca5 and 6 showed high expression of mClca5 in mouse eye and spleen, whereas mClca6 is expressed highly in the gastrointestinal tract [16].

To date, expression analysis has shown that only mClca1 is expressed in the mouse brain. However, in case of mClca3 and 4, brain tissue was not included in the expression analysis. In this study we describe the spatio-temporal expression of mClca1, 2 and 4 genes in the nervous system. We show that these genes are expressed in the olfactory ensheathing cells. In addition we also describe the expression pattern of human CLCA2 and 4 genes in the nervous system. Finally, we describe the expression pattern of mClca1/2 during embryonic development by in situ hybridization.

Methods

RT-PCR analysis

Total RNA from NMRI mouse tissues and total RNA from postmortem adult human brain regions was extracted using RNAWiz (Ambion) according to the manufacturers instructions. Total RNA from human non-neural tissues was obtained from Clontech. First-strand cDNAs were synthesized with Superscript III (Invitrogen) reverse transcriptase using 5 μg RNA as recommended by the manufacturer.

PCR reactions were performed in a volume of 25 μl, using 1/50 of the first-strand cDNA reaction. Annealing temperature for different sets of primers ranged from 55–60°C. The number of cycles used varied from 25–35 for different primer sets. Number of cycles for different primer sets was determined empirically and we always analysed the PCR product in the exponential phase of amplification. PCR with primers specific for housekeeping gene HPRT and GAPDH were used as a control to determine the variation of the amount of cDNA in different PCR reactions.

Real-time quantitative (Q) RT-PCR analysis of CLCA mRNA levels in adult mouse and human brain regions and during mouse brain development was performed in triplicates using qPCR Core Kit for SYBR Green I (Eurogentec) with Lightcycler 2.0 (Roche) according to manufacturers instructions. Data was normalized with housekeeping gene HPRT and analyzed with Lightcycler 4.05 software (Roche). Data was not normalized with HPRT in case of mClca1, mClca2 and mClca4 PCRs using equal amounts of cDNAs from different mouse brain developmental stages, since the level of HPRT mRNA is increased during development (Piirsoo and Timmusk, unpublished data).

Primers used in the experiments are the following:

hclca1 sense ACGAACAAGGACACCAGCAAA

hclca1 antisense AAGAGATCAGGTATGGGAGCAT

hclca2 sense TGCATGTCAATCACTCTCCCA

hclca2 antisense GAGTTCCTATCCATTGCTCGT

hclca3 sense GAAGGAGCTCAAACAGACGAC

hclca3 antisense ACTTTCTACTGAACCAGGCTC

hclca4 sense GCCACAGTTCATGAGGATAAG

hclca4 antisense CACAGACAATACCAGCGTAG

mclca1sense CACCAGGATCACTGGCACCAAT

mclca1 antisense GCATCGATAAGGCTGTTTAGGTC

mclca2 sense CGCCAGCATCACAGGCAAGAAG

mclca2 antisense GCGTCGATAAGGCTGCTTACATG

mclca4 sense TTCAGCAGGACAGCATCTGG

mclca4 antisense TGCCACTTGTGCGATGTTG

gapdh sense TTCCTACCCCCAATGTGTCCGTC

gapdh antisense ACCCTGTTGCTGTAGCCGTATTCA

hprt sense GATGATGAACCAGGTTATGAC

hprt antisense GTCCTTTTCACCAGCAAGCTTG

hclca2real sense AGCACCTGGAGAAGACTTTGA

hclca2real asense CTTGCTGAGGATTTCGCTTTGA

hclca4real sense AGACCTTGATGCCACAGTTCAT

hclca4real asense TGGTGACAGATCAGTAGTATTTA

mclca1 realS CACTGATAACTTGCGTATCTAC

mclca1 realAS CACAGTTGTGAACCACATTGG

mclca2 realS TCACTGATAACTTGCGTATCTAT

mclca2 realAS ACACTCGTGGACCACCTTCT

mclca4 realS AATGACAGCTCCTACCTAGC

mclca4 realAS GGCTCCACTGTGTTTGACCT

In situ hybridization

DNA fragments for riboprobe generation were subcloned into pCRII-TOPO vector (Invitrogen), sense and antisense cRNA probes were synthesized with the MAXIScript In Vitro Transcription Kit (Ambion) T7 or SP6 RNA polymerase, using [α-^{35}S]UTP (Amersham Biosciences, UK) for labeling. The hybridization specificity was confirmed using [α-^{35}S]-labeled sense riboprobes synthesized from the same templates. All sense probes resulted in the hybridization signal equivalent to the background. This shows that cRNA labeling of different CLCAs was specific. Primers used to generate probes were the following:

CLCA12 sense ATAGTATCTCTGCACTGGTG

CLCA12 antisense GAATGGATATCTAATTTCCATAG

CLCA4 sense CCTCCTGGTCTGGGTACCAA

CLCA4 antisense ATAGACGCAAATAGGAAATTTAC

Serial saggital and coronal sections (14 μm) from fresh-frozen NMRI mouse brain were analyzed by in situ hybridization analysis following the previously described protocol [17]. Emulsion-dipped sections were developed after 3 weeks using D-19 developer (Eastman Kodak, USA), fixed (sodium fixer; Kodak), and counterstained with hematoxylin (Shandon, USA).

Results

Expression pattern of mouse Clca 1, 2 and 4 in the central nervous system

Previous analyses have shown that out of six mouse Clca genes only mClca 1 is expressed in the brain and is expressed at relatively low levels compared to other tissues where the gene is expressed [1]. Our RT-PCR analysis with cDNAs from adult mouse brain showed that in addition to mClca1, mClca2 and 4 are expressed at low levels in mouse brain. In accordance with previously published data we could not detect mClca3, 5 and 6 expression in adult mouse brain (Fig 1A). All six mouse Clca genes were expressed in thymus. In addition to brain and thymus we detected mClca1 expression in spleen, kidney and testis. mClca2 was expressed in brain, thymus and kidney, mClca3 in thymus and kidney, mClca4 in brain, thymus kidney and testis. mClca5 was most widely expressed in mouse tissues, with the highest expression in thymus and lower level of expression in skeletal muscle, spleen, kidney and testis. mClca6 was expressed in thymus, skeletal muscle and testis (Fig 1A).

Quantitative real-time PCR analysis of mClca1, 2 and 4 expression during mouse brain development showed that expression of mClca1 was increasing during postnatal brain development and reached maximum levels in the adult mouse brain. mClca2 expression did not change during mouse brain development and mClca4 expression was low during embryonic development, highest around birth of the animal and the level of respective mRNA was gradually decreasing during postnatal development (Fig 1B).

In order to analyze the spatial distribution of mClca1, 2 and 4 mRNA expression in the adult brain we performed real-time PCR anaysis using cDNAs from various regions of mouse brain. Strikingly, all the mClca genes expressed in the nervous system, were highly enriched in olfactory bulb (Fig 1C). Expression levels were quantified relative to the expression in cerebral cortex. mClca1 was expressed at 98 times higher level in olfactory bulb than in cerebral cortex. mClca2 and mClca4 were expressed at 52 and 54 times higher level in olfactory bulb than in cerebral cortex. mClca2 was expressed at 10 times higher level in spinal cord than in cerebral cortex (Fig 1C).

To further analyze the cellular distribution of mClca1,2 and 4 expression in brain, we performed in-situ hybridization on adult brain sections. Since mClca1 and 2 genes share 95% identity, we were unable to design probes that distinguish between these genes. Therefore, we consider

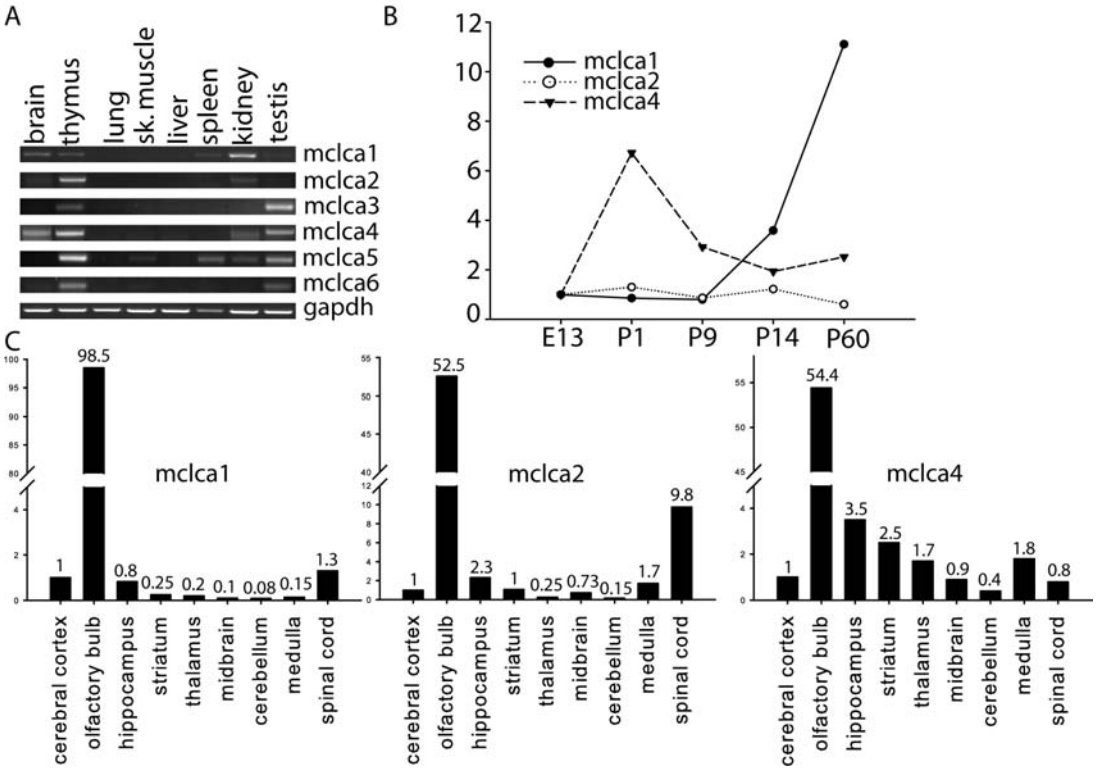

Figure 1

A) Semiquantitative RT-PCR analysis of mClca1-mClca6 and control mRNA gapdh expression in mouse tissues. B) Real-time PCR analysis of mClca1, mClca2 and mClca4 expression in the brain at indicated developmental timepoints. Expression level is shown relative to the expression level of the respective mRNA at embryonic day 13. C) Real-time PCR analysis of mClca1, mClca2 and mClca4 expression in mouse brain regions. Expression levels are shown relative to the expression level in the cerebral cortex.

the expression pattern of mClca1 and 2 together (marked mClca1/2). It should be noted however, that mClca2 was expressed at very low levels in the adult mouse brain (Fig 1A) and therefore most of the signal likely corresponds to mClca1 expression. Sense probes were used as negative controls and they did not give any signal (Fig 2, J–O). Our analysis showed that mClca1/2 and 4 genes are expressed in the olfactory nerve layer of the adult brain (Fig 2, B, C, E, F, H and 2I). High magnification imaging of mClca1/2 expression in the adult mouse brain showed expression in cells next to the glomerular layer of the olfactory bulb, namely layer of entering olfactory nerve fibers, which is populated by the olfactory ensheathing cells (Fig 2, S). We observed very low levels of mClca1/2 and mClca4 expression in hypothalamic nuclei and mClca1/2 expression in the layer II-III of the cerebral cortex (data not shown). Since mClca1 seems to be the most highly expressed CLCA family member in the mouse brain, we analyzed mClca1/2 expression also in postnatal day (P9) brain. In P9 brains the expression of mClca1/2 was more broad with the highest levels in the olfactory nerve layer of the olfactory bulb (Fig 2, A, D, G, R) and also in the layer II-III of the developing cerebral cortex containing pyramidal neurons (Fig 2.A, D, G, P). At P9 low level of mClca1/2 expression was observed in CA3 layer of hippocampus and in amygdalohippocampal area (data not shown).

Expression pattern of mouse Clca1/2 during embryonic development

We performed in situ hybridization analysis in order to characterize mClca1/2 expression during mouse development. Our analysis showed that at embryonic day 13 (E13) mClca1/2 is expressed at high levels in the developing urethra, midgut, aorta and heart (Fig 3A). High magnification analysis showed that mClca1/2 is also expressed in cells that lie adjacent to the epithelium of the

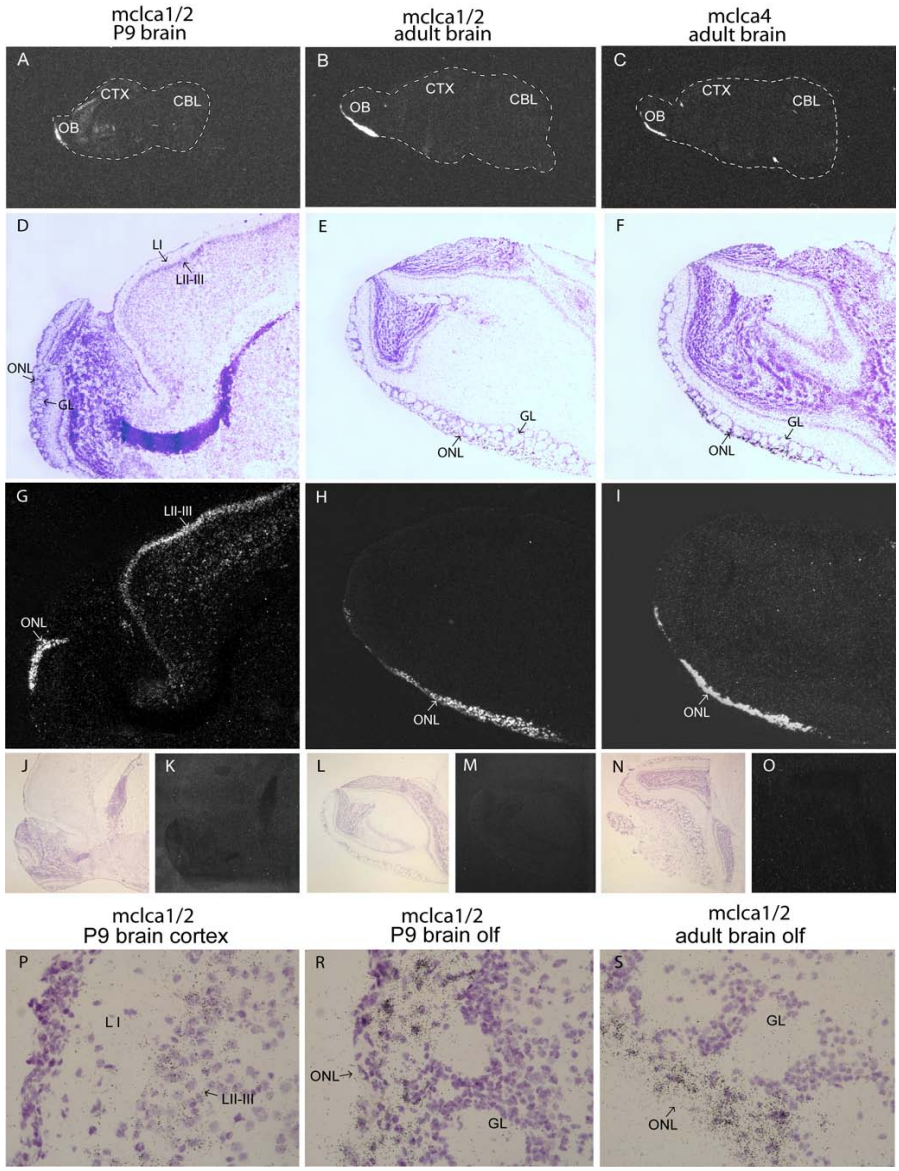

Figure 2

In situ hybridization analysis of mClca1/2 and mClca4 expression in the mouse brain. Dark-field emulsion autoradiographs of whole P9 and adult brain mid saggital sections are shown in A-C. Hematoxylin-eosin stained bright-field images (D, E and F) and corresponding dark-field emulsion autoradiographs (G, H and I) are shown at 40× magnification. Lack of in situ hybridization signal using corresponding sense probes is shown in J-O. Cellular distribution of Clca1/2 expression in P9 mouse cerebral cortex and olfactory bulb and adult mouse olfactory bulb are shown in J-L at 600× magnification. Abbreviations: CBL – cerebellum; CTX – cortex; GL – glomerular cell layer; LI – cerebral cortex layer I; LII-III – cerebral cortex layers II-III; OB – olfactory bulb; ONL – olfactory nerve layer.

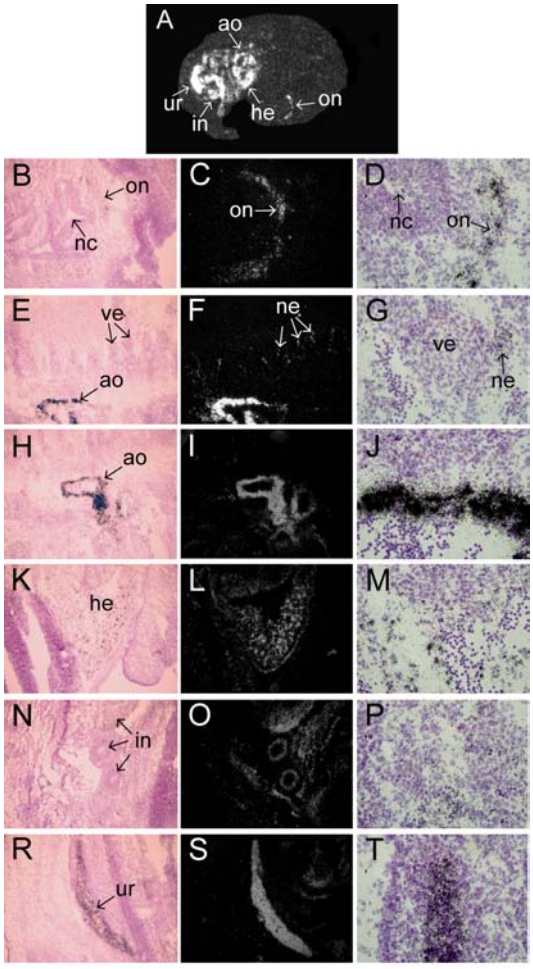

Figure 3
In situ hybridization analysis of mClca1/2 expression in E13 mouse embryo. Dark-field emulsion autoradiograph depicting whole embryo is shown in A. Hematoxylin-eosin stained bright-field images (B, E, H, K, N, R at 100× magnification and D, G, J, M, P, T at 600× magnification) and corresponding dark-field emulsion autoradiographs (C, F, I, L, O and S) are shown corresponding to various parts of the embryo. Abbreviations: ao-aorta; he-heart; in-intestine; on-olfactory nerve nc-nasal cavity; ne-spinal nerve; ur-urethra; ve-vertebrae.

nasal cavity. Most likely this expression conforms to the developing cranial nerve I (olfactory nerve)(Fig 3B–D). Low levels of mClca1/2 expression was seen in the developing spinal nerves (Fig 3E – G). In the E13 heart mClca1/2 was expressed in ventricle and atrium as well as in aorta (Fig 3H–M). High level of mClca1/2 expression was observed in E13 midgut and urethra (Fig 3N–T).

In situ hybridization analysis on E17 mouse embryos showed that high levels of mClca1/2 mRNA expression were retained in the developing urethra (Fig 4A). Expression was seen also in the brain, predominantly in the diencephalon (Fig 4B,C). Highest level of mClca1/2 expression in the nervous system was observed in optic nerve (Fig 4D,E). The expression of mClca1/2 was also detected in the olfactory nerve (Fig 4F,G) and in the trigeminal nerve (Fig 4H,I). Higher magnification images of mClca1/2 expression in the nervous system of E17 embryos are shown in Fig 5. Outside the nervous system the expression of mClca1/2 was observed in the heart (Fig 4N–R), intestine (Fig 4U,V), urethra (Fig 4W–X) and also in trachea and lung (Fig 4L–O). Lower levels were seen in liver, vertebrae (Fig 4S,T) and skin (Fig 4J,K).

Expression of human CLCA2 and 4 in the nervous system

Since three mouse Clca gene family members were expressed in brain, we were interested if any of the four human CLCA genes are expressed in the nervous system. As the numbering of CLCA family members in human and rodents is different, we performed bioinformatic analysis to reveal which rodent Clca genes have closest homology to which human family members. Schematic depiction of human, mouse and rat CLCA loci is shown in Fig 6A. We created a homology tree of the mouse, rat and human proteins using DNAMAN software (Fig 6B). Bioinformatic analysis revealed that the order of genes within the rodent and human locus was similar e.g. hCLCA2, which is the most 5' of the human genes has the highest homology with mClca5 and rClca2 (predicted gene), which also lie in the most 5' part of the mouse and rat locus respectively (Fig 6A). Our analysis also showed that the 3' part of the locus has undergone duplication in rat (rbClca and rbClca2 genes) and triplication in mouse (mClca1, 2 and 4 genes), whereas there is a single 3' gene (CLCA3) in the human locus (Fig 6).

Our bioinformatic analysis showed that human CLCA3 is the closest homologue of mouse Clca1, 2 and 4 genes (Fig 6B). It shares 74% homology at the protein level with its mouse counterparts. RT-PCR analysis revealed that neither hCLCA3 nor hCLCA1 was expressed in the nervous system. In contrast, hCLCA2 and hCLCA4 were expressed in the brain (Fig 7A). Expression of hCLCA1 was largely confined to the gastrointestinal tract, with high level of expression in the small intestine. Low level of hCLCA1 expression was detected in testis. hCLCA2 was expressed at high levels in the brain, testis and lung and at low levels in the small intestine and colon. hCLCA3 was expressed in testis, kidney and colon. hCLCA4 was expressed widely

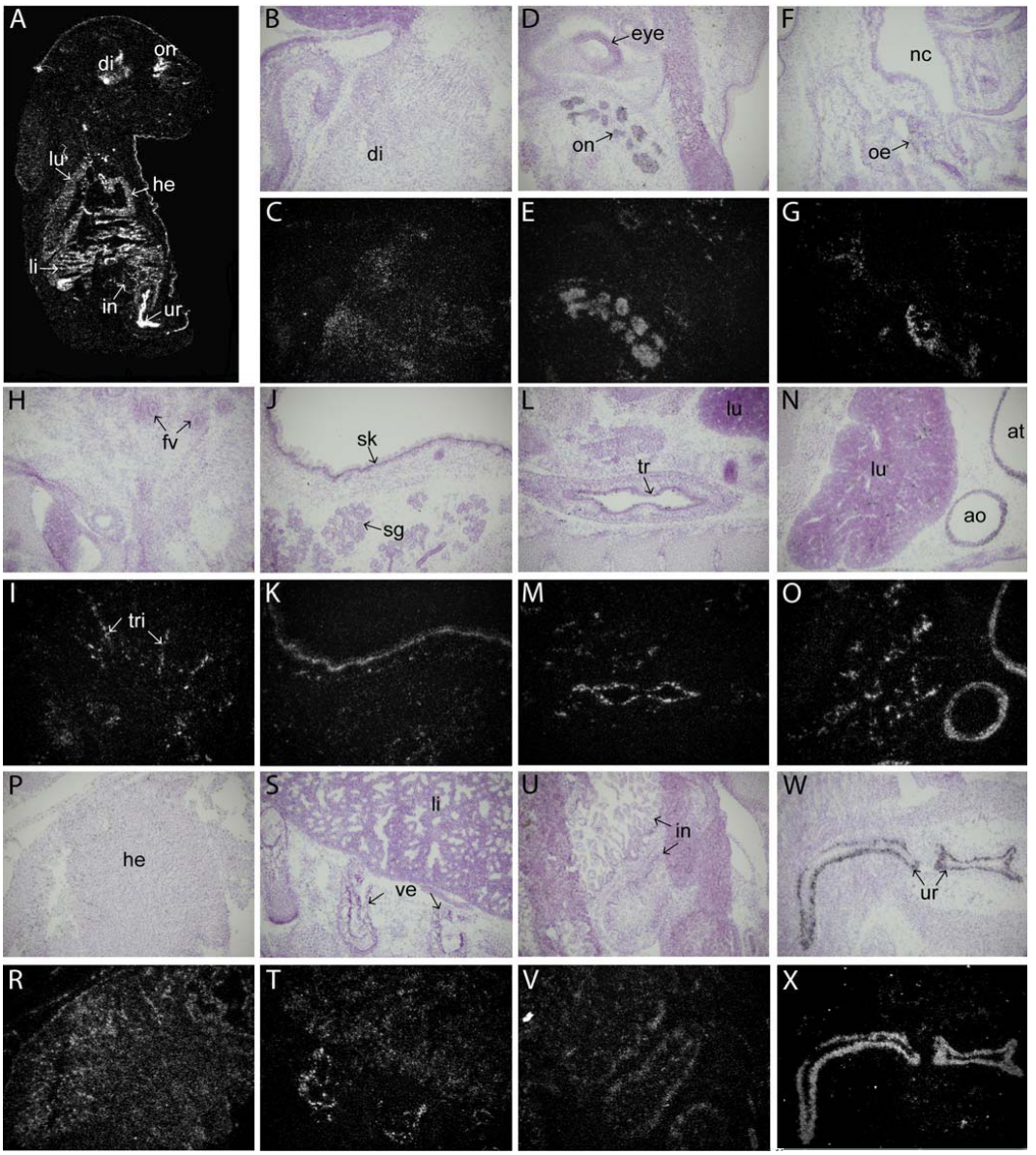

Figure 4

In situ hybridization analysis of mClca1/2 expression in E17 mouse embryo. Dark-field emulsion autoradiograph depicting whole embryo is shown in A. Hematoxylin-eosin stained bright-field images (B, D, F, H, J, L, N, P, S, U, W) and corresponding dark-field emulsion autoradiographs (C, E, G, I, K, M, O, R, T, V, X) taken at 100× magnification, corresponding to various parts of the embryo are shown. Abbreviations: di-diencephalon; fv-follicles of vibrissae; he-heart; in-intestine; li-liver; lu-lung; oe-olfactory epithelium; on-optic nerve; sg-submandibular gland; sk-skin; tr-trachea; tri-trigeminal nerve; ur-urethra; ve-vertebrae.

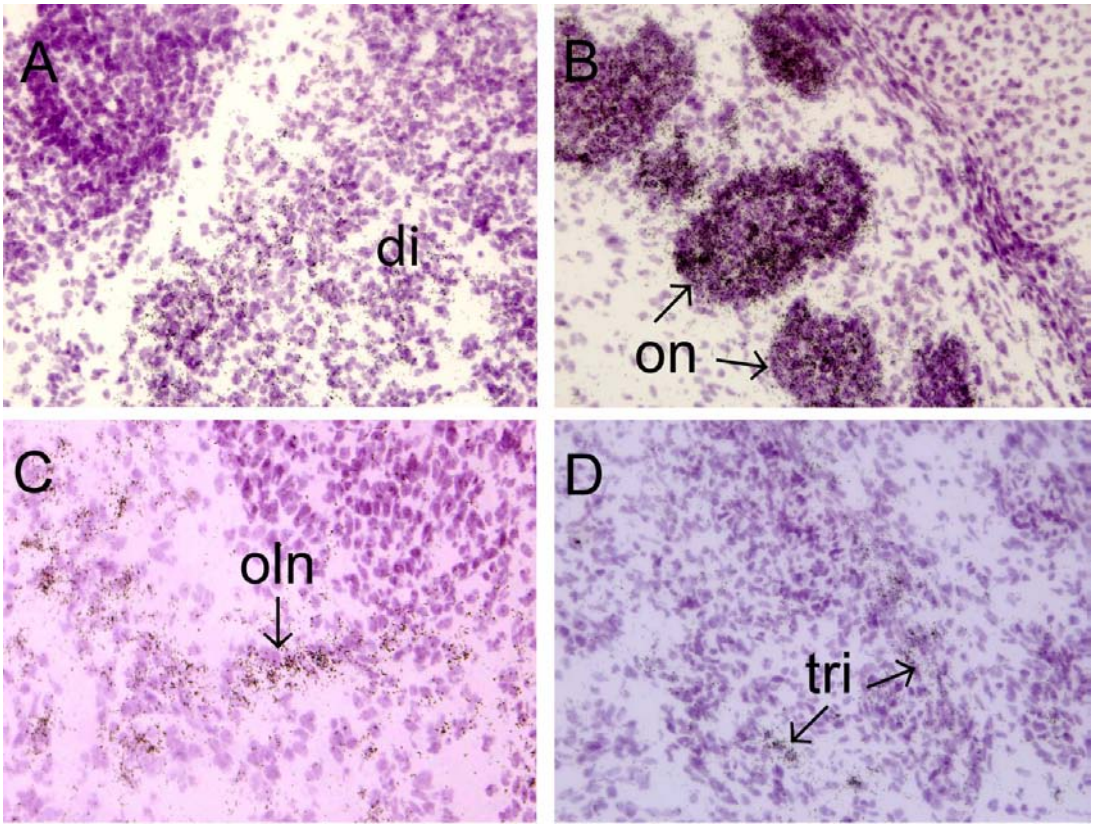

Figure 5
High magnification images of mClca1/2 expression in the nervous system at E17. Images of diencephalon (A), optic nerve (B), olfactory nerve (C) and trigeminal nerve (D) are shown at 600× magnification. Abbreviations: di-diencephalon; oln-olfactory nerve; on-optic nerve; tri-trigeminal nerve.

in human tissues. High levels of hCLCA4 were expressed in the brain, testis, small intestine, colon and lung. Lower levels of expression were detected in heart (Fig 7A).

hCLCA2 and 4 were differentially expressed in the adult brain as revealed by quantitative RT-PCR analysis (Fig 7B). The highest level of hCLCA2 and hCLCA4 expression was found in optic nerve. The expression level of hCLCA2 and hCLCA4 in optic nerve exceeded 58 and 35 times the expression level of the corresponding gene in cerebral cortex. The expression of hCLCA2 and hCLCA4 was also significantly higher in medulla and olfactory tract as compared to the expression level in cerebral cortex. Very low levels of hCLCA2 and hCLCA4 expression were found in cerebral cortex, cerebellum and spinal cord (Fig 7B).

Discussion

In this study we show novel expression sites for mouse Clca1, Clca2 and Clca4 genes. All six mouse Clca genes are located in chromosome 3 and are clustered in the same locus. Our RT-PCR and in situ hybridization analyses reveal that in the murine nervous system, mClca1, 2 and 4 genes are preferentially expressed in the olfactory ensheathing cells. In contrast to our findings, it has been previously shown that mClca2 is not expressed in the mouse brain [13]. Our analysis reveals that mClca2 is expressed in mouse brain, albeit at very low levels. The discrepancy between the results may come from the observation that at least in the gastrointestinal tract, there are marked differences in the expression level of mouse Clca genes between different mouse strains [18]. Also, it has

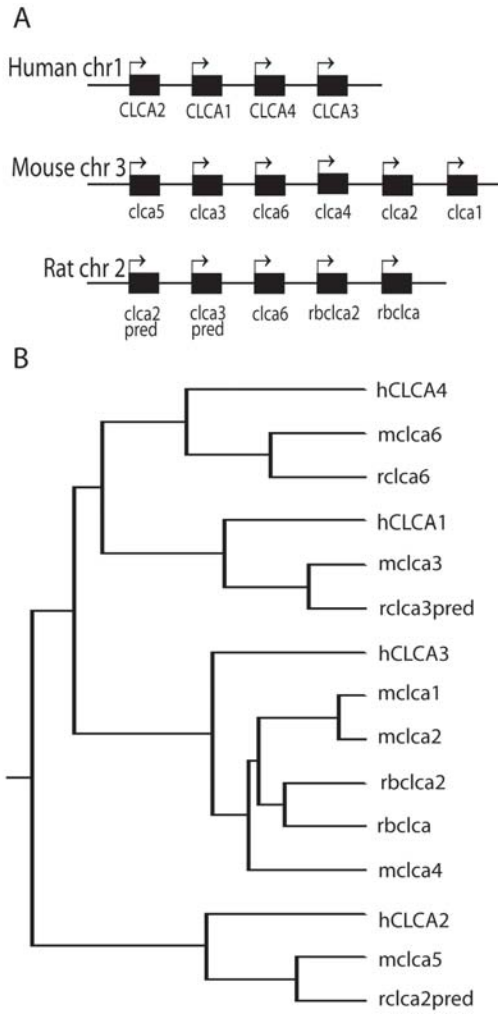

Figure 6
Structure of human, mouse and rat CLCA locus and homology between CLCA family members. Schematic depiction of order of succession of genes within human, mouse and rat CLCA locus is shown in A (the figure is not drawn in scale). Homology tree showing relationship between human, mouse and rat CLCA proteins (B). The dendrogram was generated using DNAMAN software (Lynnon Biosoft) with complete amino acid sequences of the CLCA proteins.

recently been shown that mClca2 is expressed in the cerebral cortex, albeit at very low levels as compared to mClca1 [19]. The same study also detected low levels of mClca5 expression in the dorsal root ganglia of adult mouse.

mClca 1, 2 and 4 genes share highest similarity with each other within the gene family. At amino acid level mClca1 and 2 share 95% identity and mClca4 shares 81% identity with mClca2 and 80% identity with mClca1. Moreover, these three genes lie next to each other and form a 3' gene cluster in the Clca gene locus. Given their similar expression pattern in the nervous system, it could be argued that either their olfactory ensheathing cell specific expression is driven by a common regulatory element or each of these mouse genes has retained an olfactory ensheathing cell specific promoter element following gene duplication. Other reports have shown that the rat Clca genes most homologous to mouse Clca1,2 and 4, i.e. rbClca and rbClca2 are expressed in rat brain [20,21]. Their analysis revealed that both genes are expressed at comparable levels in the cerebellum, cerebrum and spinal cord. Also, they showed by single cell PCR analysis that rbClca is expressed in both neurons and glial cells. It would be interesting to analyze the expression level of rbClca and rbClca2 in the olfactory bulb of rat.

Our analysis of mClca1/2 expression in the nervous system at early postnatal development revealed that it is expressed also in the layer II-III of the cerebral cortex. Interestingly the expression was seen only in the frontal part of the developing cortex. At E17 mClca1/2 expression was more widespread in the brain, with prominent expression in diencephalon. During embryonic development mClca1/2 expression was seen in the developing nerves of the peripheral nervous system. We could detect expression at E13 in the developing olfactory nerve and spinal nerves and at E17 in the optic nerve, trigeminal nerve and olfactory nerve. It is possible that mClca1/2 expression marks the glial cells ensheathing peripheral nerves.

In this study we have also analyzed the expression of human CLCA genes in the nervous system. Human CLCA locus contains 4 genes. The most 3' of the genes is hCLCA3, which is also most closely related to mClca1, 2 and 4. RT-PCR analysis showed that unlike its mouse homologues, hCLCA3 is not expressed in the nervous system. In contrast, our results show that two other members of the family, hCLCA 2 and 4 are expressed in various parts of human brain. It has previously been shown, using RNA dot-blot analysis, that hCLCA4 is expressed rather uniformly in the brain with striking absence in the cerebellum [22]. However, RT-PCR analysis performed in this study showed low level of CLCA4 expression in cerebellum. Unlike mouse Clca genes expressed in the nervous system, human CLCA 2 and 4 expression is not confined to olfactory ensheathing cells. It should be noted however, that both genes were expressed at higher levels in the olfactory nerve as compared to olfactory bulb. Highest expression for both genes was found in the optic nerve. Together with the data from the analysis of mouse

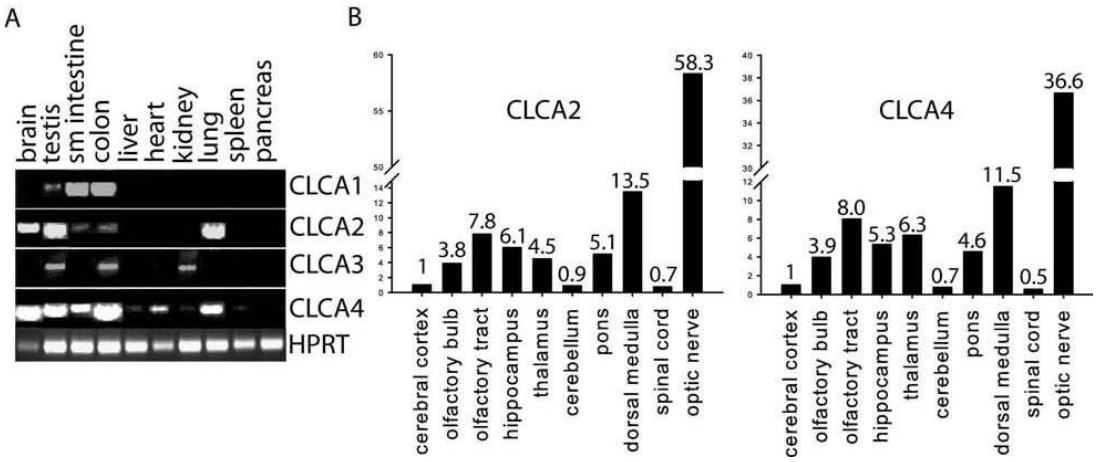

Figure 7
A) Semiquantitative RT-PCR analysis of human CLCA1-4 mRNA expression and control mRNA hprt in adult human tissues. B) Real-time PCR analysis of CLCA2 and CLCA4 expression in adult human brain regions. Expression levels are shown relative to the expression level in the cerebral cortex.

mClca1/2 expression, it could be suggested that CLCA genes could be expressed at higher levels in the cells that ensheath cranial nerves.

Conclusion

In this study we have shown that mClca1, mClca2 and mClca4 are expressed in the olfactory ensheathing cells of the adult mouse CNS. During mouse development mClca1/2 widely expressed in the CNS but at particularly high levels in cranial nerves. In addition, we found that mClca1/2 is expressed in layer II of the developing cerebral cortex at P9. Our analysis also reveals that hCLCA2 and hCLCA4 are expressed in the CNS of adult humans.

Authors' contributions

TT and DM contributed to the design of the experiments and to the preparation of the manuscript. MP performed the experiments and contributed to the design of the experiments and to the preparation of the manuscript.

Acknowledgements

We thank Enn Jõeste and Liina Kiho from North Estonian Regional Hospital, Tallinn, for help in collecting tissue samples and Epp Väli for technical assistance. This work was supported by Estonian Ministry of Education and Research (Grants 0222602 and 0140143) and Estonian Science Foundation (Grant 7257).

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ABSTRACT

Myelination in the vertebrate peripheral nervous system is carried out by Schwann cells. Schwann cells myelinate axons with diameters greater than one micrometer. Defects in the myelination process lead to demyelinating peripheral neuropathies, a heterogenous group of pathologies, with no cure at present. Understanding the molecular mechanisms leading to myelinating phenotype helps the design of measures of prevention and deceleration of the course of demyelinating neuropathies.

Myelination in the peripheral nervous system is initiated by signals from the axon and is under control of a set of transcription factors in Schwann cells that function in a hierarchical manner. One of the first transcription factors that is activated in Schwann cells in response to axonal signals is Oct-6. In the absence of Oct-6, there is a severe delay in myelination of the peripheral nervous system in mice. Our studies show that in addition to an important role in myelination during development, Oct-6 has a major role in remyelination after damage of a peripheral nerve. We further show that Oct-6 function in Schwann cells is shared by a homologous transcription factor Brn-2. Our analysis of Oct-6 downstream genes in Schwann cells show that, in addition to previously identified target gene Krox-20, there are a number of genes that are both up- and downregulated in Schwann cells by Oct-6. Beside that, we have identified multiple genes, whose expression is regulated during Schwann cell differentiation in an Oct-6 independent manner.

In addition to signals from axon, that require direct contact between Schwann cell and axon, it has been shown clearly that secreted neurotrophic factors have important functions in the Schwann cell lineage during development. It has been assumed that the source of neurotrophic factors in the developing perippheral nerve is mainly neuronal. Our analysis shows that Schwann cells express both NGF and GDNF families of neurotrophic factors and their receptors. We have showed particularly high expression of neurotrophic factors in the embryonic peripheral nerves, indicating that during development Schwann cells are another important source of neurotrophic activity, beside neurons.

We have identified CLCA, gene coding for a potential chloride channel, as being very sharply regulated during Schwann cell differentiation. We show that CLCA family members are expressed in the nervous system of both mice and humans with a specific pattern. We find particularly high expression of CLCA genes in the cells ensheathing nerves, indicating an important function of CLCA proteins in the development and homeostasis of these cells.

In conclusion, we have characterized a number of changes, taking place at molecular level, during peripheral nerve development. Outcome of these changes is subject of further investigations.

KOKKUVÕTE

Selgroogsete loomade piirdenärvisüsteemis on müeliniseerivateks rakkudeks Schwanni rakud. Schwanni rakud müeliniseerivad aksoneid, mille diameeter on suurem kui 1 mikromeeter. Defektid müeliniseerimise protsessis viivad demüeliniseeruvate neuropaatiateni, mis on heterogeenne grupp haigusi. Arusaam müeliini moodustamise regulatsiooni molekulaarsetest mehhanismidest aitab kavandada abinõusid demüeliniseeruvate neuropaatiate vältimiseks ja haiguse kulu aeglustamiseks.

Piirdenärvisüsteemi aksonite müeliniseerimine algatatakse aksonaalsete signaalide poolt, mis käivitavad Schwanni rakkudes hierarhilises kaskaadis komplekti transkriptsioonifaktoreid. Üks esimesi transkriptsioonifaktoreid, mis aktiveeritakse aksonaalsete signaalide poolt, on Oct-6. Oct-6 geeni puudumisel hiirtes on perifeerse närvisüsteemi aksonite müeliniseerimine tugevalt häiritud ja toimub arengus hiljem kui normaalsetel hiirtel. Meie tööd näitavad, et lisaks olulisusele arengus on Oct-6 ka väga oluline faktor aksonite remüeliniseerimise korral pärast närvi vigastust. Lisaks näitame oma töödes, et Oct-6-le sarnast funktsiooni Schwanni rakkudes omab transkriptsioonifaktor Brn-2. Kirjanduse andmetel on põhiliseks Oct-6 märklaud-geeniks Schwanni rakkudes Krox-20. Meie andmed näitavad, et Oct-6 on lisaks ülalnimetatud geenile võimeline reguleerima veel mitmeid geene, ning seda nii positiivselt kui ka negatiivselt. Lisaks oleme kirjeldanud geene, mille avaldumine on reguleeritud Schwanni rakkude diferentseerumisel Oct-6 sõltumatul moel.

Lisaks aksonist tulevatele signaalidele, mis eeldavad otsest kontakti Schwanni raku ja aksoni vahel, on näidatud, et sekreteeritavad neurotroofsed faktorid omavad olulist rolli Schwanni rakkude arengus. Peamiseks neurotroofsete faktorite allikaks perifeerses närvis peetakse neuronaalset rakku. Meie oleme leidnud oma töös, et Schwanni rakkudes on avaldunud nii NGF ja GDNF perekonna neurotroofsed faktorid kui ka nende retseptorid. Lisaks oleme näidanud, et eriti kõrge on neurotroofsete faktorite avaldumise tase lootelises perifeerses närvis, mis viitab sellele, et arengu käigus on Schwanni rakud olulisteks neurotroofsete faktorite allikaks.

Üks geen, mille avaldumine on tugevalt reguleeritud Schwanni rakkude diferentseerumisel, kodeerib valku CLCA, millel on võimalik kloriidi kanali aktiivsus. Oleme oma töös näidanud, et hiire ja inimese CLCA perekonna geenide avaldumine närvisüsteemis on väga spetsiifilise mustriga. Kõige kõrgem CLCA perekonna geenide avaldmise tase närvisüsteemis on gliia rakkudes, mis ümbritsevad närve. See viitab antud perekonna valkude olulisele funktsioonile nende rakkude arengus ja homeostaasis.

Kokkuvõtteks võib öelda, et antud töös on kirjeldatud mitmeid perifeerse närvisüsteemi arengus toimuvaid molekulaarseid muutuseid ja antud muutuste tagajärgede täpsem uurimine seisab alles ees.

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