Structural and Functional Analysis of the Oct-6 Schwann Cell Enhancer

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List of abbreviations

AP1	The Activator Protein 1
ARIA	Acetylcholine Receptor InducingActivity
BMP	Bone Morphogenetic Protein
BDNF	Brain Derived Neurotrophic Factor
BZIP	Basic-region-Leucine Zipper Proteins
BL	Basal Lamina
CAMs	Cell-Adhesion Molecules
Casper	Contactin-associated protein
CHN	Congenital Hypomyelinating Neuropathies
CMT	Charcot Marie Tooth
CMT1B	Charcot-Marie Tooth type 1B
CNS	central nervous system
CNTF	Ciliary-Neurotrophic Factor
CTF	CCAAT-binding transcription factor
cAMP	cyclic Adenosyl Monophosphate
Cx32	Connexin32
DBD	DNA Binding Domain
Dhh	Desert Hedgehog
Dom	Dominant megacolon
DNA	Deoxy Ribonucleic Acid
DPE	Down stream core Promoter Element
DRG	Dorsal Root Ganglion
DSS	Dejerine-Sottas Syndrome
GDNF	Glial Derived Neurotrophic Factor
GHRH	growth hormone-releasing hormone
FGFs	Fibroblast Growth Factors
GH	Growth Hormone
HMG	High Mobility Group
HNPP	Hereditary Neuropathy with liability to Pressure Palsies
HR1	Homology Region 1
HR2	Homology Region 2
HSP	Heat Shock Protein
IGF	Insulin-like Growth Factor
IFNβ	interferon-β
Ig	Immunoglobulin
Inr	Initiator
JXP	Juxtaparanode
Krox-20 SCE	Krox-20 myelinating Schwann cell element
LacZ	β-galactosidase
LCR	Locus Control Regions
LIF	Leukemia Inhibitory Factor
MBP	Myelin Basic Protein
MS	Multiple Sclerosis
μm	micrometer (10^{-6} meter)
mm	millimeter (10^{-3})
NCSC	Neural Crest Stem Cells
Nf186	neurofascin-186
NF-L	neurofilament light
NF-M	neurofilament medium
NF-H	neurofilament heavy
NT3	Neurotrophin 3
PNS	Peripheral Nervous System
P	Postnatal day
P0	Myelin Protein Zero
PDGF	Platelet Derived Growth Factor
PIC	preinitiation complex
PMP22	Peripheral Myelin Protein-22
PKA	Protein Kinase A
PRL	Prolactin
PQBP-1	Poly-glutamine tract Binding Protein
POU	Pit-1, Oct1/2, Unc-86
SC	Schwann Cell
SCE	Schwann cell specific enhancer
SMDF	Sensory and Motor Neuron Derived Factor
sp	splotch
sp^d	splotch delayed
TCRα	T-cell receptor α
TGF-β	Transforming Growth Factor β
TPA	12-O-tetradecanoylphorbol-13-acetate
Trk	tyrosine receptor kinase
TRAFs	TNFR-associated factors
TSHβ	Thyroid Hormone Receptor beta Type2

Chapter 1

Introduction

Preface

A defining feature in the biology of higher vertebrates is their extended and complex nervous system that allows them to rapidly integrate and process environmental information, control body posture, regulate homeostasis of their internal organs and develop complex behaviour. The principal cell types that make up the nervous system are the neuron and glial cell. Neurons exist in a wide range of different shapes but are generally built up of a soma, containing the nucleus, its dendritic arborisation and its single axon (Figure 1).

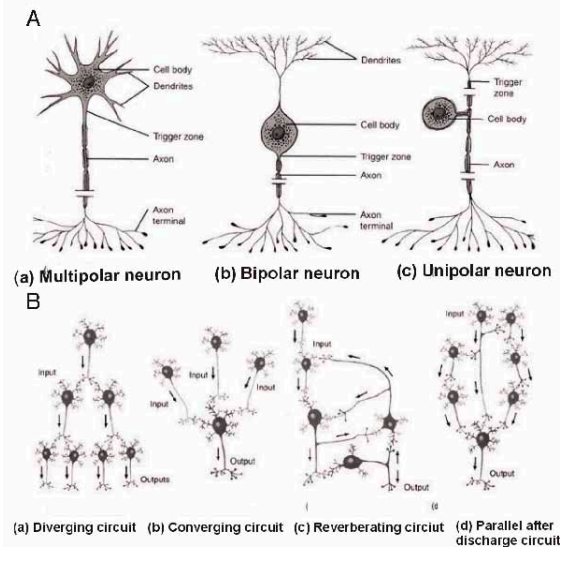

Figure 1- Neurons have a variety of shapes as shown in A. Schematic presentation of different ways of connection between neurons leading to diverting, converging, reverberating, and parallel alter-discharge circuits.

A typical neuron potentially receives input from thousands of other neurons whose axons terminate on the dendrites and soma, where they form specialised contacts called synapses. Glial cells support neurons directly and are involved in every aspect of neuronal shape and function. Communication between neurons and their peripheral targets relies on electrical impulses that travel the length of the axon, a length that can be considerable (more than one meter in humans). The speed with which an electrical impulse travels along the length of the axon depends on the internal resistance of the axon. The internal resistance inversely correlates with axonal diameter such that larger diameter axons propagate signals faster than smaller axons. It is evident that evolutionary pressure, in particular in the form of predation, calls for nervous systems with fast conducting axons. In principle,

animals can increase the diameter of their axons to allow fast escape responses. A case in point is the squid whose giant axon can be 10 cm in length, has a diameter of around 1mm and conducts impulses with a very high speed. However, there is an obvious limit to this strategy in the form of the huge energy costs associated with maintaining a resting potential over the vast axonal membrane. Most larger animals, in particular the vertebrates, have adopted an evolutionary novelty called myelin. The myelin sheath is a specialized membrane structure that spirals around the axon and is produced by glial cells: oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) (Figure 2). An important functional consequence of myelin is that axonal depolarisations are restricted to the nodes of Ranvier and that the capacitance of the internodal axonal membrane is greatly reduced. As a consequence, conductance velocities of myelinated axons are one to two orders of magnitude faster than non-myelinated axons of similar diameter. Although it is generally believed that myelin is essentially a vertebrate adaptation, recent studies in copepods (small marine animals; zooplankton) have challenged this view (Davis et al., 1999).

With this adaptation also came new vulnerabilities as instability of the myelin sheath through genetic or environmental causes results in demyelination

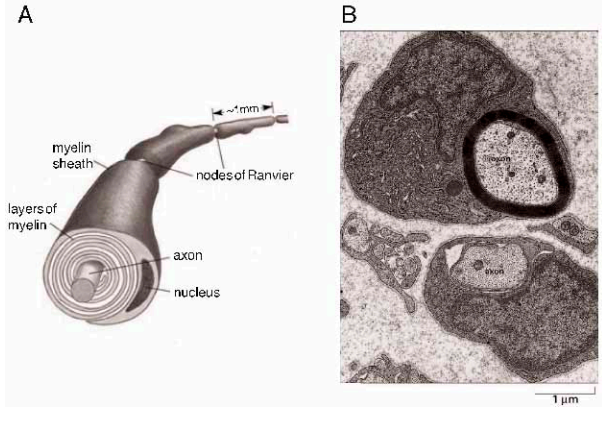

Figure 2- (A) A myelinated axon from a peripheral nerve. Each Schwann cell wraps its plasma membrane concentrically around the axon to form a segment of myelin sheath about 1 mm long. For clarity, the layers of myelin in this drawing are not shown compacted together as tightly as they are in reality. (B) An electron micrograph of a section from a nerve. Two Schwann cells can be seen; one near to bottom is just beginning to myelinate its axon; the one above has formed an almost mature myelin sheath (adapted from Molecular Biology of the Cell (Alberts et al., 2002)).

and eventually, if myelin is not repaired, in axonal loss causing mild to severe neurological problems. Prime examples of such demyelinating diseases are multiple sclerosis and Charcot Marie Tooth (CMT) disease. In fact, CMT is one of the most common inherited diseases of the nervous system with an overall prevalence of 1 in 4000. The observation that not all axons in the vertebrate nervous system are myelinated, raises the question how axons are selected to become myelinated or not. It is generally believed that during development specific cues are presented by the growing axon that induce Schwann cells to ensheath, proliferate and finally myelinate this axon. The identity of this cue or signal is still not known. Obviously, gaining a detailed understanding of the molecular mechanisms that underlie myelin formation and maintenance will provide a rational basis for clinical strategies to limit axonal damage and improve nerve regeneration. The focus of our studies has been to dissect the transcriptional cascade of myelination in the PNS. One of the transcription factors implicated in regulating the myelination program is the POU domain transcription factor Oct-6 (also referred to in the literature as SCIP, Tst-1 or pou3f1). Several studies have shown that Oct-6 is the first transcription factor to be up-regulated prior to myelination and its expression is axonal-contact dependent (Arroyo et al., 1998; Scherer et al., 1994). Oct-6 appears to function largely through the activation of a set of genes, including the zinc-finger transcription factor Krox-20, involved in myelination such as the major myelin genes and those involved in lipid metabolism (Nagarajan et al., 2001). In order to determine the exact role of Oct-6 in myelination, it is of importance to understand how the Oct-6 gene itself is regulated throughout development and regeneration. The aim of the studies described in this thesis was to gain insight into Schwann cell specific regulation of the Oct-6 gene. Given Oct-6's central role in myelination, a detailed understanding of the cis-acting sequences and the regulatory pathways that converge on them might be of immediate importance in developing strategies to combat the debilitating effects of demyelinating diseases.

1.1 General structure of myelin

Myelin is a highly organized membrane structure that is wrapped around the axon by Schwann cells in the PNS and oligodendrocytes in the central CNS. The myelin sheath can be divided into compact and non-compact myelin domains

(Scherer and Arroyo, 2002). The organization of 'unwrapped' myelin is depicted in Figure 3. Different sets of proteins are enriched in compact and non-compacted domains of the myelin sheath. Compact myelin contains myelin protein zero (P-zero), peripheral myelin protein-22 (PMP22), and myelin basic protein (MBP) (Scherer and Arroyo, 2002). P-zero is a type I transmembrane protein with a single immunoglobulin-like motif in the extracellular part and a positively charged intracellular part. P-zero is the most abundant protein of compact myelin (50% of the protein content of myelin) and plays an important role in myelin compaction through homophilic interactions in cis and in trans (Suter and Scherer, 2003).

Most of the mutations found in P-zero are associated with dominantly inherited neuropathies with a variety of phenotypes including Charcot-Marie Tooth type 1B (CMT1B), Dejerine-Sottas syndrome (DSS)/congenital hypomyelinating neuropathies (CHN), and hereditary neuropathy with liability to pressure palsies (HNPP) (Suter and Scherer, 2003).

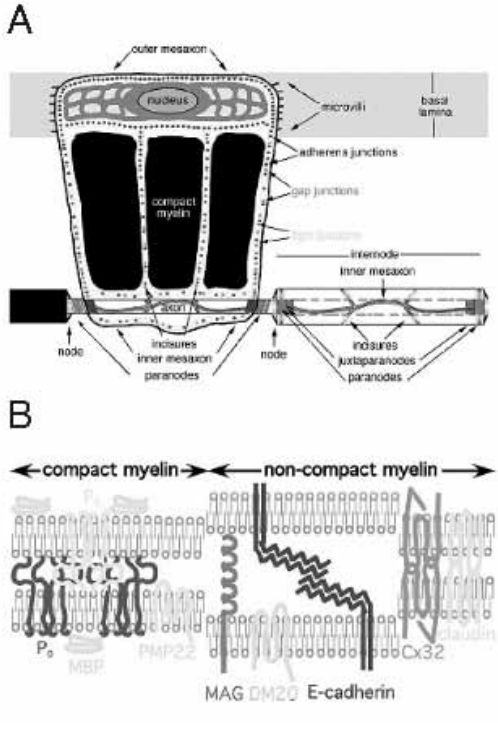

Figure 3- A and B show the schematic view of a myelinated axon in the PNS and the proteins of myelin sheathes. A- One myelinating Schwann cell has been unrolled revealing the regions that form non-compact myelin, the incisures and paranodes. Adherens junctions are depicted as two continuous lines; these form a circumferential belt and are also found incisures. Gap junctions are depicted as ovals; these are found between the rows of adherens junctions. B- In the PNS, compact myelin sheaths contain protein zero (P-zero), peripheral myelin protein 22 kDa (PMP22), and myelin basic protein (MBP). The non-compact myelin contains E-cadherin, myelin-associated glycoprotein (MAG), and connexin32 (Cx32). Note that P-zero and MAG have extracellular immunoglobulin-like domains (semicircles), and PMP22 and Cx32 have four transmembrane domains. (Adapted from Arroyo and Scherer, 2000).

PMP22 is a small trans-membrane protein and plays a structural role in compact myelin (Adlkofer et al., 1995). PMP22 is able to form dimers or multimers and interacts with P-zero (D'Urso et al., 1999; Tobler et al., 2002; Tobler et al., 1999). PMP22 was originally identified as the gene mutated in the natural mouse mutant Trembler. Two different alleles have been described for Trembler; Trembler and Trembler^{J}. Trembler and Trembler^{J} differ greatly in severity of the peripheral nerve defect reflecting the situation in human where different mutations, loss or gain of PMP22 alleles cause very different clinical phenotypes. In contrast to P-zero and PMP22, MBP is a cytoplasmic protein that mediates the fusion of the intracellular myelin membrane to form the major dense line. In mice with a complete deletion of MBP (Shiverer mice), the major dense line does not form (Kirschner and Ganser, 1980; Rosenbluth, 1980). As the PNS is not affected in Shiverer mice, it is suggested that the basic cytoplasmic domain of P-zero could effectively substitute MBP function (Martini et al., 1995).

In addition to proteins, the myelin sheath is highly enriched in specific lipids , in particular cholesterol and sphingolipids (such as galactocerebroside and sulfatide) (Kirschning et al., 1998; Schiff and Rosenbluth, 1995).

Non-compact myelin is found as paranodal loops that fold down to contact the axolemma and form the paranodal septate junction. Other regions of non-compact myelin are the Schmidt Lanterman incisures and nodal microvilli. These regions of non-compact myelin are enriched in E-cadherin, MAG, DM20, Connexin32 (Cx32) and an uncharacterized claudin (Scherer and Arroyo, 2002).

Myelin-associated glycoprotein (MAG) is a member of the Ig super gene family and is a transmembrane protein present in the apical/adaxonal membrane of the inner mesaxon. Due to this localization, it is suggested that MAG binds molecules on the axonal surface and plays a role in the stabilisation of the extracellular space, between the inner mesaxon and the axon (Collins et al., 1997; Li et al., 1994; Sawada et al., 1999).

Several junctional complexes are found in non-compact myelin. The most numerous junctions are adherens junctions, which are located in the mesaxon as well as in outermost layers of the paranodal loops and incisures (Scherer and Arroyo, 2002). Adherens junctions contain E-cadherin, α-catenin, and β-catenin, and are possibly linked to the actin cytoskeleton (Fannon et al., 1995; Scherer and

Arroyo, 2002). Gap junctions are found between the rows of adherens junctions in the paranodal loops and Schmidt-Lanterman incisures. One of the gap junction proteins is Cx32 (GJβ1) (Bergoffen et al., 1993; Chandross et al., 1996; Scherer et al., 1995). Dye transfer studies have provided evidence for the role of gap junctions in the radial transport of small molecular weight molecules across the incisures (Balice-Gordon et al., 1998). Mutations in Cx32 in human lead to X-linked CMT (CMTX) (Bergoffen et al., 1993). The pathology of this particular form of CMT is still poorly understood.

1.2 Glia cells in the peripheral nervous system

Different types of glia cells exist within the PNS. These types include myelin-forming Schwann cells, nonmyelin-forming Schwann cells, teloglia of somatic motor nerve terminals, satellite cells that envelope neuronal cell bodies in sympathetic, parasympathetic and sensory ganglia, and the astrocyte-like enteric glial cells in the autonomous ganglia in the gut wall (Gershon, 1998; Jessen and Mirsky, 1983; Pannese, 1981; Robitaille, 1998). All, or almost all, of the peripheral glia cell types originate from the neural crest, a transient embryonic structure that gives rise to a wide variety of cell types in the developing embryo (Kalcheim and Le Douarin, 1986). In addition, experiments in chicken embryos have suggested that also the ventral neural tube contributes to the peripheral glia population, in particular to Schwann cells in the ventral nerve roots (Bhattacharyya et al., 1994).

In the following paragraphs of this introduction, I will focus on the Schwann cell, which is the main glia cell type in the peripheral nerve trunks and the subject of study in this thesis. In particular, I will review how neural crest cells commit to Schwann cells lineage (determination) and develop to mature Schwann cells (differentiation).

1.3 Neural crest cells: the origin of Schwann cells

Neural crest cells delaminate from the dorsal aspect of the neural tube and migrate along several pathways to give rise to a wide variety of cell types.

Several stages can be defined in the ontology of the neural crest. These stages are: specification, delamination or migration and fate decision (Christiansen et al., 2000; Garcia-Castro and Bronner-Fraser, 1999). Neural crest cells are specified

through the interaction of surface ectoderm and neurectoderm. In addition several studies have suggested that also the lateral plate mesoderm plays an important role in neural crest formation, in specific crest precursors of the melanocytic lineage (Bonstein et al., 1998; Selleck and Bronner-Fraser, 1995). Although there are many unanswered questions about the identity of the signals involved in neural crest cell generation, members of the Wnt, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families have been implicated (Ikeya et al., 1997; Liem et al., 1995; Ruffins and Bronner-Fraser, 2000). During the next phase of development neural crest cells emigrate form the dorsal neural tube and adopt mesenchymal characteristics such as their ability to migrate (delamination). What factors trigger this epithelial to mesenchymal transition is still unknown. However a number of markers have been identified that are expressed in pre-migratory crest cells and might be involved in this transition. These are the transcription factor Slug and two cadherins, c-Cad-6B and cadherin-7 (Mayor et al., 1995; Nakagawa and Takeichi, 1998). In addition, it has been suggested that the coordinated activity of Noggin and BMP4 in the dorsal neural tube triggers delamination of specified, slug-expressing neural crest cells (Sela-Donenfeld and Kalcheim, 1999). Once neural crest cells delaminate, they migrate along specific pathways and produce diverse cell lineages. Powerful fatemapping techniques, in particular in the chick-quail transplantation system developed by Nicole LeDourain and colleagues, have allowed the identification of the diverse cell lineages that originate from the neural crest. (Johnston, 1966; Johnston et al., 1973; Nichols, 1981; Teillet and Le Douarin, 1983). These fate-mapping studies demonstrated that some lineages derive from a specific population of crest cells originating from a discrete section along the neuraxis while other lineages derive from all levels of the neuraxis. For example, the enteric nervous system derives from the vagal crest, which originates from the neural tube at the level of somite 1-7. In contrast, Schwann cells originate from crest cells at all levels of the neuraxis (Anderson, 1997) (Figure 4A). A number of growth factors have been implicated in the determination of neural crest cell fate in vitro (Figure 4B). For instance, it has been shown that glial growth factor (GGF) diverts neural crest stem cells (NCSC) towards a glial fate (Shah et al., 1994), while transforming growth factor β (TGF-β) promotes smooth muscle cell differentiation. In addition, bone morphogenic proteins 2 and 4 (BMP2/4) promote differentiation

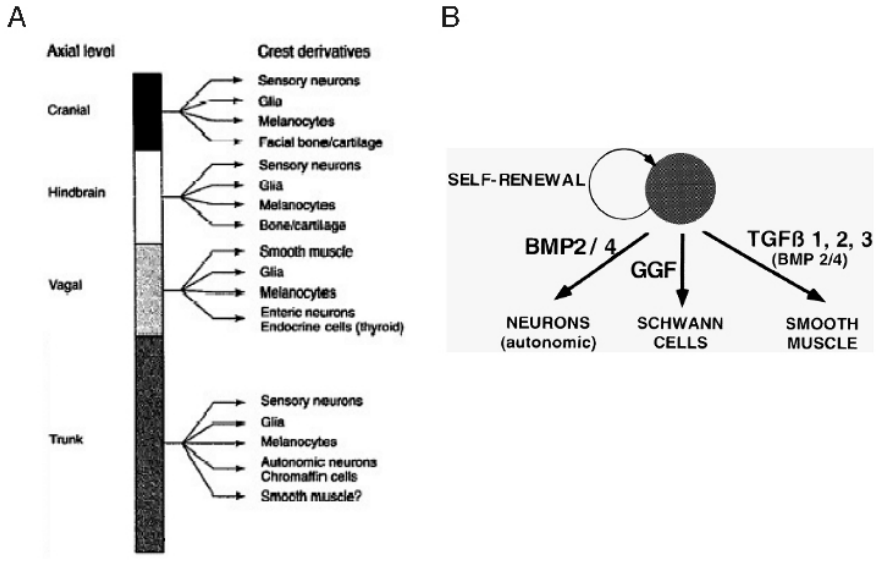

Figure 4- (A) Variations in neural crest derivatives produced at different levels along the rostrocaudal extent of the neuraxis. Only major subdivisions of the neuraxis and a simplified subset of crest derivatives are shown. The results are based primarily on fate-mapping experiments in avian embryos. (Adapted from Anderson, 1997). (B) Summary of instructive effects of growth factors on rodent neural crest cell lineage commitment in vitro. Individual factors that promote self-renewal of the neural crest cells have not yet been identified. The illustration should not be taken to imply that the three differentiated fates shown are the only ones available to NCSCs, not that all three fates are necessarily available to the cells at every division. (Adapted from Shah et al., 1994).

of autonomic neurons and to lesser extent, smooth muscle in these NCSC cultures (Anderson, 1997; Shah et al., 1996). The importance of these factors for neural crest development has been underscored by expression studies in mice (Bitgood and McMahon, 1995; Carraway and Burden, 1995; Millan et al., 1991). For example, a reduction of glial cell numbers is observed in the peripheral nerves of GGF knock out embryos, underscoring the importance of GGF for glial cell formation in the PNS. Additionally, the importance of TGF-β for cardiac crest development is demonstrated by heart defects in developing TGF-β knockout mice (Dickson et al., 1995; Meyer and Birchmeier, 1995). An assessment of the role of BMP2 and 4 in neural crest development has been precluded by the fact that these factors serve essential functions at an earlier stage of embryonic development and as a consequence BMP2 and 4-knockout mice die early during development (Lawson et al., 1999; Zhang and Bradley, 1996).

The neural crest represents a stem cell population that gives rise to several lineages depending on the local embryonic environment to which the stem cells are exposed. However, some of the fate-restricted neural crest precursors are able to change their fate by transplanting them back into younger host embryos (Le Douarin, 1986; Weston, 1991). These and later experiments from David Anderson's laboratory gave rise to the idea that neural crest stem cells gradually undergo restrictions in their development. Additionally, clonal analysis of neural crest stem cells suggests that crest cells are heterogeneous at different stages of development, even at the onset of migration (Dupin et al., 1998; Liu et al., 1990). These studies

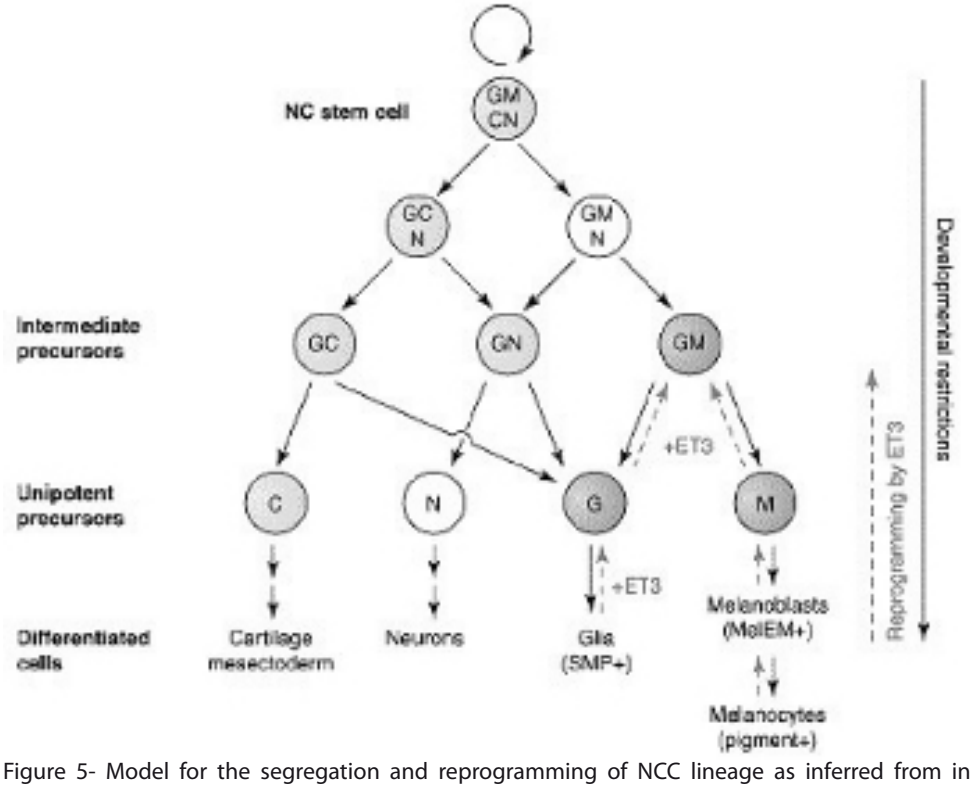

Figure 5- Model for the segregation and reprogramming of NCC lineage as inferred from in vitro clonal analysis of quail NCCs. The progenitors endowed with the capacity to give rise to cartilage, neurons (N), glial cells (G), and melanocytes (M) have been classified according to the number of cell phenotypes in their progeny. Data are consist with progressive developmental restrictions from 'totipotent"cells to unipotent precursors (arrows). Those able to yield mesectodermal cartilage cells were identified in clones derived from mesencephalic NC and are excluded from the trunk NC. Those able to yield mesectodermal cartilage cells (in yellow) were identified in clones derived from mesencephalic NC and are excluded from the trunk NC. The main target cells of ET3 are shown in pink. Differentiated glia and melanocytes could be reprogrammed by ET3 and reverse to GM bipotent NCCs. (Adapted from Le Douarin and Dupin, 2003).

demonstrated that neural crest cells represent a true stem cell population that is capable of self renewal (Stemple and Anderson, 1992) (Figure 5).

Peripheral nervous system neurons and glia derive from crest cells that arise at different levels along the rostro-caudal axis of the neural tube. One basic and important question is how neural crest cells migrating in the same environment choose a neuronal or glia fate. Previously, Shah et al., (1996) has shown that the BMP2/4 promotes neuronal cell fate in neural crest stem cell cultures through induction of proneural bHLH transcription factors such as Neurogenin and Mash-1. Later studies demonstrated that bHLH transcription factors also inhibit gliogenesis upon activation of the Notch receptors (Morrison et al., 2000). It has been hypothesized that activation of Notch, through interaction with its ligands (Delta/Jagged) promotes glial differentiation (Morrison et al., 2000). In addition, early migrating neural crest cells express ErbB2, which forms a heterodimer with ErbB3 and transduces the signal of Neuregulin. These cells differentiate into glia cells in response to Neuregulin in vitro (Shah et al., 1994). Targeted deletion of ErbB3 mice show a lack of Schwann cell precursor, further reinforcing the important role of Neuregulin signaling in gliogenesis (Riethmacher et al., 1997).

1.4 Schwann cell lineage and differentiation

The development of the Schwann cell lineage critically depends on the association of post-migratory neural crest cells with a glia fate to contact outgrowing axons. Most likely, the first contact between neural crest cells and axons takes place in the anterior part of the somites during early stages of the development (Loring and Erickson, 1987; Mirsky and Jessen, 1996). Recent studies have provided evidence for a role of neurons in diverting crest cells to the glia lineage through activation of the Notch pathway (Morrison et al., 2000).

As mentioned earlier, neuregulin-1 plays an important role in promoting the transition from neural crest cells into glia cells (Shah et al., 1994). Neuregulin-1 has been identified in a number of experimental settings and therefore carried originally a number of names reflecting these different systems. Neuregulin-1 had been identified as the ligand of the Neu oncogene and was therefore called Neu differentiation factor (NDF ;a and b isoforms). Purification and identification of a glial growth factor resulted in the name GGF. An agent that induces acetylcholine

receptor expression at the neuromuscular junction was called ARIA and identified as neuregulin-1. In a fourth system neuregulin was identified as the activity named sensory and motor neuron derived factor SMDF. The neuregulin-1 gene encodes, through alternative splicing and promoter usage, three distinct isoforms termed type I, II and III (Falls et al., 1993; Garratt et al., 2000a; Ho et al., 1995; Holmes et al., 1992; Marchionni et al., 1993; Wen et al., 1992). All isoforms of neuregulin-1 bind to different combinations of heterodimeric receptors of the ErbB tyrosine kinase receptor family. Biochemical and genetic data indicate that the functional Neuregulin-1 receptor in the neural crest cells and Schwann cells is the ErbB3/ErbB2 heterodimer and that all biological effects of Neuregulin are mediated through this receptor (Carraway and Cantley, 1994; Ho et al., 1995; Horan et al., 1995; Meyer and Birchmeier, 1995; Riese et al., 1995; Riethmacher et al., 1997). Tyrosine kinase activity is associated with the ErbB2 protein. ErbB3 does not have kinase activity by itself. As already alluded to earlier, a series of in vivo studies have demonstrated the significance of Neuregulin signaling in Schwann cell development. Most ErbB3$^{-/-}$ mice die between E11.5 and E13.5 and show lack of Schwann cells and their precursors (Riethmacher et al., 1997). Mice with an erbB2 null allele die before E11 as a result of dysfunctions associated with a lack of cardiac trabeculae (Lee et al., 1995). In addition to the cardiac phenotype, these mutant mice fail to develop neural crest derived cranial sensory ganglia (Lee et al., 1995; Meyer and Birchmeier, 1995). Rescue of the cardiac defect by crossing the transgenic erbB2 mice into an erbB2 null background, demonstrated a severe loss of both sensory and motor neurons and absence of Schwann cell precursors in the PNS (Morris et al., 1999).

Another key factor in the differentiation of neural crest cells to Schwann cells is the transcription factor Sox10. Sox10 expression is initiated in the neural crest cells as they detach from the neural tube, and its expression is maintained during neural crest cell migration (Britsch et al., 2001). Subsequently, Sox10 expression is extinguished in all neural crest derivatives except in the glial and melanocyte lineages. Sox10 expression in these cells overlaps with ErbB3 (Kuhlbrodt et al., 1998a; Kuhlbrodt et al., 1998b). Analysis of Sox10 mutants has shown a lack of Schwann cells, like in the ErbB3 mutant animals, however the defect is more severe in Sox10 mutant animals as no Schwann cells are produced at all (Britsch et al., 2001). In Sox10 mutants, the whole peripheral glia cell population including

Schwann cells and satellite cells is missing from a very early developmental stage onwards. It has been suggested that in the absence of Sox10, the neural crest cells cannot differentiate to a glia cell fate (Britsch et al., 2001).

Since only a subpopulation of Sox10 expressing crest cell will adopt the glia fate and maintain its expression, it is suggested that Sox10 alone is not sufficient for the generation of glia cells and needs co-operation with additional signals (Britsch et al., 2001). For instance, it is possible that a specific signal can modify Sox10 in cells that will form glia and induce glial differentiation. Another possibility is that Sox10 functions synergistically with other transcription factors such as Pax3, Krox-20, and Oct-6 to induce glial differentiation (Kuhlbrodt et al., 1998a). However, none of the mutations in Pax3, Krox-20 or Oct-6 affect the differentiation of neural crest cells into glia (Epstein et al., 1991; Franz and Kothary, 1993; Jaegle et al., 1996; Kuhlbrodt et al., 1998a; Topilko et al., 1994)

One of the earliest markers expressed in glial fated neural crest cells is the myelin protein P-zero (Hagedorn et al., 1999; Lee et al., 1997). These P-zero positive Schwann cell precursors are further defined by expression of a number of additional markers and growth and survival requirements. These cells can first be identified in he embryonic nerve trunks at day 12-13 of gestation in the mouse or day 14-15 in the rat. Schwann cell precursors differentiate around E14-15 (mouse; E17 rat) into bi-potential immature Schwann cells, which subsequently differentiate into either myelin forming or non-myelin forming Schwann cells found in the mature nerve. These main transitions, from neural crest cells to Schwann cell precursors, from precursor to immature Schwann cells and finally the formation of the two mature Schwann cell types, are identified based on their antigenic properties and survival abilities in vitro (Jessen and Mirsky, 1999). Some of these main differences are shown in Table 1. In the developing nerve, ongoing proliferation of Schwann cell precursors and immature Schwann cells invasion of the axon bundles followed by radial sorting of individual axonal fibers and families of small fibers results in the establishment of separate axon-Schwann cell units. Each of these units consists of a large number of axons enclosed by a family of Schwann cells (Martin and Webster, 1973; Webster et al., 1973). The number of axons per unit falls gradually as Schwann cells continue to proliferate because some axons degenerate.

Migrating crest cells	Schwann cell precursors	Immature Schwann cells
β-Neuregulin-1 does not promote survival[b]	β-Neuregulin-1 promotes survival[c]	β-Neuregulin-1 promotes survival[d]
P_0 negative[d,e]	P_0 positive[e]	P_0 positive
PMP22 negative[e]	PMP22 positive[e]	
PLP negative[f]	PLP positive[f]	
GAP-43 negative	GAP-43 positive	
CD9 negative[f]	CD9 positive[f]	
B-FABP negative[h]	B-FABP positive[h]	
Desert Hedgehog RNA negative	Desert Hedgehog RNA positive[i]	Desert Hedgehog RNA positive[i]
	Die by apoptosis when removed from axons and plated in vitro (absence of autocrine loops) [g, k]	Full survival under same conditions due to presence of autocrine loops[g, k]
Cytoplasmic S100 negative[g]	Cytoplasmic S100 negative[g]	Cytoplasmic S100 positive[g]
	No mitogenic response to FGF in rat[j, c, l]	Mitogenic response to FGF in rat[j, c, l]
	Flattened with extensive cell-cell contacts in vitro[g]	Bi- or tri-polar in vitro[g]
O4 antigene negative[c, m]	O4 antigene negative[c, m]	O4 antigene positive[c, m]
GFAP negative[n]	GFAP negative[n]	GFAP positive[n]
High motility[g]	High motility[g]	Low motility[g]

Table 1 - Some of the main differences between migrating crest cells, Schwann cell precursors and immature Schwann cells (adapted from (Jessen and Mirsky, 1999)). a- Migrating crest refers to crest cells in vivo that are on a level with the dorsal third of the neural tube. b- (Shah et al., 1994); c- (Dong et al., 1995); d- (Stewart et al., 1996); d- (Lee et al., 1997); e- (Hagedorn et al., 1999); f- Unpublished observation (Brennan, Calle, Mirsky, and Jessen); g- (Jessen et al., 1994); h- (Britsch et al., 2001); i- (Parmantier et al., 1999); j- in mouse FGF is a mitogen under identical condition. k- (Meier et al., 1999); l- (Dong et al., 1999); m- At present, this has been thoroughly tested in the mouse. n- (Jessen et al., 1990).

Finally, the Schwann cell reaches a 1:1 ratio with one of axons, elongates along the axon and exits the cell cycle. These cells are referred to as promyelin stage Schwann cells (Figure 6). Around birth, most of the myelin-competent Schwann cells are at the pro-myelin stage and assemble basal lamina around the axon. Final differentiation or maturation of Schwann cells consists of wrapping of Schwann cell membrane around the axon in a process referred to as myelination. This process of myelin formation requires major cellular adaptations in gene expression and metabolism to allow the formation of specialized Schwann cell-axon structures at the node and internode, Schmidt Lanterman incisures and compaction of the myelin sheath. The mature non-myelinating Schwann cell appears later than myelin forming Schwann cells (Jessen and Mirsky, 1991). In general, non-myelinating Schwann cells ensheath a number of axons with a diameter smaller than 1 μm (Friede, 1972). There are

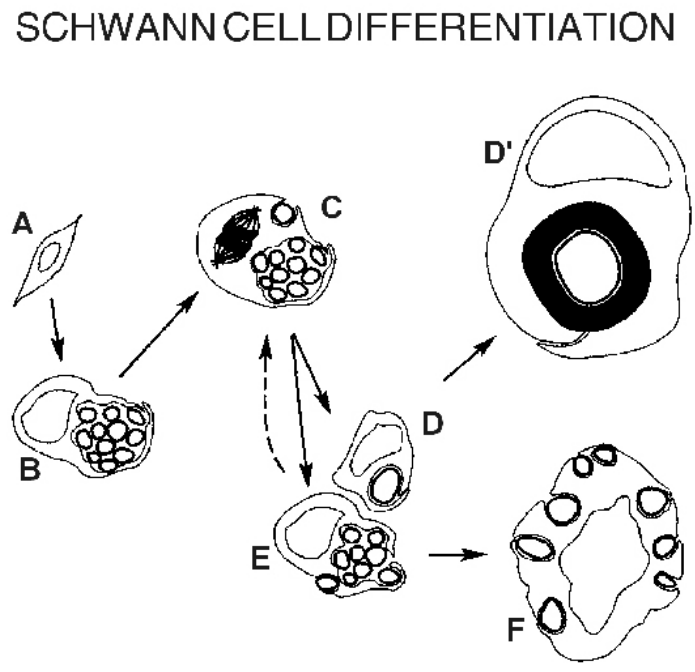

Figure 6- Schwann cell differentiation. Schwann cells originate in the neural crest (A) and migrate along the axons to the periphery. Before myelination, each Schwann cell surrounds multiple small axons (B). Myelination is preceded by Schwann cell division (C). One daughter cell isolates a single axon (D) and myelinates it (D'), while the other surrounds developing axons (E) and continues to divide (C) until all appropriate axons are myelinated. Many small diameter axons are not myelinated and are ensheated by non-myelinated Schwann cells (F). (Adapted from Lazzarini et al., 2004).

some similarities between molecular markers of non-myelinating Schwann cells and immature Schwann cells, such as GFAP and NGF receptors (Jessen and Mirsky, 1991).

1.5 Schwann cell and nerve interactions

Reciprocal interactions between axon and Schwann cells are important for the generation of the peripheral nerves. Not only Schwann cells rely on neuron-derived signals during development, in particular Neuregulin-1, but neurons also depend for their survival to a large extent on Schwann cell derived factors. This is especially true at early stages before axons have innervated their peripheral targets. In the following sections, I will briefly review some of these interactions.

1.5.1 Schwann cell precursors are dependent on axonal survival factors

It has been known for a long time that direct axonal contact stimulates proliferation of Schwann cell precursors (Salzer et al., 1980). It was also shown that glia growth factor (GGF), an isoform of Neuregulin-1, is able to prevent apoptotic death of Schwann cells in vivo (Trachtenberg and Thompson, 1996). These data suggest that direct contact is necessary for the survival of Schwann cells. The ErbB3 null mutation and rescue of erbB2 mice have given direct genetic evidence for the role of Neuregelin-1 in Schwann cell precursor maintanance, proliferation, and differentiation as I discussed in the previous section (Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999). Further, analysis of the erbB2 conditional knockout mouse using a krox20-cre allele has shown a widespread peripheral neuropathy characterized by abnormally thin myelin sheaths, containing fewer myelin wraps (Garratt et al., 2000b). This study demonstrates that Neuregulin-1, in addition to its role in establishing the Schwann cell precursor, also plays a role in myelination. These results indicate a role for Neuregulin-1 signaling during myelination of Schwann cells. Thus, Neuregulin-1 signaling has different roles during different developmental stages of Schwann cells. A recent study demonstrated a role for Neuregulin-1 signaling in regulating myelin thickness (Michailov et al., 2004). In particular, it was shown that reduction in the Neuregulin-1 gene dosage is sufficient to decrease myelin thickness, whereas no effect on myelin thickness was observed in the ErbB2$^{+/-}$ animals. Additionally, overexpression of type III Neuregulin-1 in transgenic mice (under control of the neuron-specific Thy1 promoter) induces hypermyelination. Therefore, Michailov and colleagues (2004) have suggested that axonal Neuregulin-1 represents at least one of the surface molecules regulating the amount of myelin wrapping by myelinating Schwann cells.

1.5.2 Schwann cells are the source of neurotrophic factors

Schwann cells release neurotrophic factors that are important for the survival of neurons (Jessen and Mirsky, 1999). Mice targeted for erbB3 and rescued for Erb2$^{-/-}$ showed the loss of most sensory and motor neurons besides the loss of Schwann cell precursors (Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999). The loss of neurons is not due to ablation of Neuregulin signaling

in these cells since ErbB3 is not expressed in motor neurons and is found only in a subset of cells in dorsal root ganglia (Meyer et al., 1997; Riethmacher et al., 1997). Examination of the number of neurons in these animals indicates that neurons are generated in normal numbers at first, but are gradually lost during embryonic development. In addition, absence of neuronal degeneration in erbB3$^{-/-}$ and chimeric mice indicates that the loss of neurons in erbB3$^{-/-}$ is indirect and is due to the absence of Schwann cells (Riethmacher et al., 1997). The role of Schwann cell precursors in supporting the survival of developing neurons was also evident in a study of targeted Sox10 mice, since these mice also lack Schwann cell precursors and show death of DRG and motor neurons (Britsch et al., 2001). It was reported that Schwann cells in vitro and in vivo express a variety of neurotrophic factors, including CNTF, GDNF, BDNF, LIF, PDGF, FGFs, NT3, TGF-β or IGF. Expression of these factors support maintenance of the sensory and motor neurons (Bunge, 1993; Jessen and Mirsky, 1999). In particular, these factors are strongly upregulated in denervated Schwann cells following nerve transection or damage and might thus contribute to nerve regeneration.

1.5.3 The organization of the myelinated nerve fiber

The reciprocal interaction between Schwann cell and axon directs the organization of the myelinated fiber in distinct domains. These domains include the nodes of Ranvier, the paranodal junction, the juxtaparanodes and the internodal regions, which all consist of a glial and an axonal component (Poliak and Peles, 2003) (Figure 7). The nodes of Ranvier are periodic interruptions of the myelin sheath with an interval of about 100 times the axon diameter. This nodal gap is filled with microvilli extended by the outer layer of Schwann cells and is ensheathed by a basal lamina. The nodal axolemma is characterized by a very high density of voltage-gated Na^+ channels and other transmembrane and cytoskeletal proteins, including the cell-adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily, Nrcam and neurofascin-186 (Nf186), the cytoskeletal adaptor ankyrin G and the actin-binding protein spectrin βIV (Berghs et al., 2000; Davis et al., 1996; Kordeli et al., 1995; Poliak and Peles, 2003). Recent studies have revealed the presence of two K^+ channels, $K_v3.1$ and Kcnq2, in the nodal membrane (Devaux et al., 2003; Devaux et al., 2004). Paranodal junctions are located at both sides of

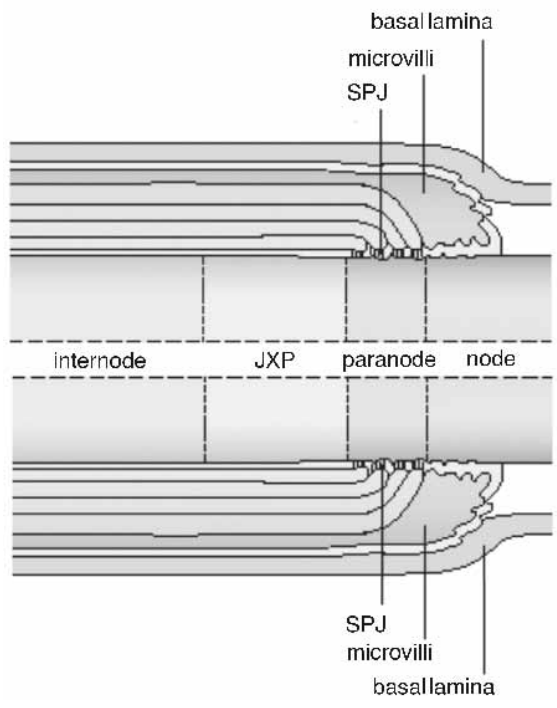

Figure 7- Schematic longitudinal cut of a myelinated fibre around the node of Ranvier showing a heminode. The node, paranode, juxtaparanode (JXP) and internode are labelled. The node is contacted by Schwann cells microvilli in the PNS. Myelinated fibers in the PNS are covered by a basal lamina. The paranodal loops form a septate-like junction (SpJ) with the axon. The internode extends from the juxtaparanodes and lies under the compact myelin. (Adapted from Poliak and Peles, 2003).

the nodes of Ranvier and are formed by the lateral sides of Schwann cell sheaths At paranodes, the axonal membrane contains a complex of two cell-adhesion molecules, contactin-associated protein (Casper) and contactin, which are essential for the generation of the axoglial junction (Poliak and Peles, 2003). Absence of these proteins at paranodal junctions results in the disappearance of septa junctions and a widening of the space between axon and the paranodal loops (Boyle et al., 2001; Poliak and Peles, 2003). The zones just beyond the paranodal junctions are called juxtaparanodes. Juxtaparanodes are the location of heteromultimers of the delayed rectifier K^+ channels of the shaker family, $K_v1.1$, $K_v1.2$, and $K_v\beta2$, which are mainly present in the small axons in a complex with Casper2 (Poliak et al., 1999; Rasband et al., 1999; Rhodes et al., 1997; Wang et al., 1993).

One of the interesting issues in the formation of the axoglial apparatus is how different compartments are established within this superstructure. Several lines of evidence have indicated that during development, the positioning of Na^+ channels at the node of Ranvier is directed by Schwann cells. During myelination, Na^+ channels are expressed at the moving edge of the developing myelin sheaths and as two neighboring Schwann cells approach each other, the two hemi-nodal

structures fuse to form the mature node (Poliak and Peles, 2003; Vabnick et al., 1996). It has been speculated that a boundary macromolecular sieve composed of the neuronal and glial transmembrane proteins bound to the cytoskeleton of both axons and myelinating cells exists. This macromolecular sieve possibly is involved in clustering of Na^+ channels when they are collected and moved as a group toward the future nodes (Pedraza et al., 2001). The importance of the myelin sheath in clustering the Na^+ channels is further underscored by the observation that demyelination leads to disruption of Na^+ clusters and spreading of channels over the demyelinated axonal membrane, which in turn, may lead to an axonal conductance block (Vabnick et al., 1996). In addition, during myelination potassium channels of the shaker type are excluded from the nodal axolemma and cluster in the juxta-paranodal region under the compact myelin (Scherer and Arroyo, 2002; Vabnick et al., 1999). Although the importance of myelin formation for the organization of the axolemma and clustering of the channels in their appropriate domains is undisputed, several studies have suggested that in the CNS axonal domains are established through induction of a soluble factor in the absence of oligodendrocytes. This factor is not produced by Schwann cells. However, the presence of some sodium channel clustering in dystrophic mice suggest that myelin independent mechanisms of axonal domain organization might play a role in the PNS as well (Deerinck et al., 1997; Kaplan et al., 1997). Additionally, it has been suggested that axoglial contact in the CNS also plays a role in the developmental switch of Na^+ channel isoforms in the nodes (Ishibashi et al., 2003; Rios et al., 2003; Ulzheimer et al., 2004). Although it is not completely clear what mechanisms operate to organize axonal domains, it is important to keep in mind that glia cells have a big impact on the distinct domains on the axolemma.

1.5.4 The role of myelinating Schwann cells on regulating axonal structure and neurofilament phosphorylation

It has long been known that myelinating Schwann cell influence the radial growth of axons (de Waegh et al., 1992). Study of nerves from Trembler mice with a defect in peripheral myelin protein 22 kDa (PMP-22), have shown three interesting points: first, clear reduction in average axonal caliber (de Waegh and Brady, 1990), second, increase in the axonal cytoskeletal elements (Low, 1976) and third decrease in axonal transport velocities (de Waegh and Brady, 1990). Grafting experiments

have indicated that these changes are spatially restricted to axon segments without normal myelination and are not observed in adjacent regions with normal myelination (de Waegh and Brady, 1990). Genetic studies have gathered adequate evidence for the role of neurofilament in determination of axonal diameter in myelinated regions (Eyer and Peterson, 1994; Jacomy et al., 1999; Ohara et al., 1993; Zhu et al., 1997). Neurofilaments are heteropolymeric intermediate filaments composed of neurofilament light (NF-L), medium (NF-M), and heavy (NF-H) chain subunits (Lee et al., 1986). It is suggested that neurofilament phosphorylation is essential for proper axonal diameter, which is regulated by Schwann cells (de Waegh and Brady, 1990; Yin et al., 1998). A recent study has demonstrated that the COOH-terminal tail domain of NF-M is an essential target for myelin-dependent axonal radial expansion (Garcia et al., 2003). It has been suggested that myelin-associated glycoprotein (MAG) plays a role in mediating the Schwann cell signal to the axon through the low affinity nerve growth factor receptor $p75^{NTR}$ in association with neuronal gangliosides GT1b and GD1a (Tcherpakov et al., 2002; Wang et al., 2002; Wong et al., 2002; Yamashita et al., 2002). In vivo support for the role of MAG dependent signaling in determining axonal caliber through neurofilament phosphorylation comes from two studies. First, peripheral nerves of MAG-deficient mice show a reduction in neurofilament phosphorylation, interfilament spacing, and internodal axonal caliber (Yin et al., 1998). Second, patients with anti-MAG demyelinating peripheral neuropathy show a significantly reduced axonal caliber (Lunn et al., 2002).

1.5.5 Formation of the perineurium

A protective epithelial sheath that is composed of two layers surrounds peripheral nerves. These two layers, called the perineurium and the epineurium respectively, surround the endoneurial compartment that contains the axons, Schwann cells, bloodvessels and resident macrophages. The peri- and epineural sheaths protect nerves and act as nerve-tissue barriers (Bunge et al., 1989; Olsson, 1990). It has been shown that Schwann cells induce the formation of perineural sheath cells by secreting Desert Hedgehog, a member of the Hedgehog family of signaling molecules (Parmantier et al., 1999). Desert Hedgehog expression can be detected in developing nerves around E11.5 by cRNA in situ hybridization, while mRNA

expression of the hedgehog receptor Patched can be detected in the mesenchyme immediately around the nerve at E15.5 (Parmantier et al., 1999). Study of the peripheral nerves in $dhh^{-/-}$ mice has shown a severely abnormal perineurium and epineurium, which are thin and patchy and permeable to proteins and migratory cells, In addition, perineural cells have abnormal tight junctions and fail to express connexin43 (Parmantier et al., 1999). These results demonstrate that Schwann cells do not only interact with neurons, but also play a role in the development of tight junctions of connective tissue sheaths of the nerves. However, it has been suggested that other signals than Desert Hedgehog are involved in the initial recruitment of mesenchymal cells around the peripheral nerve, since the generation of these cells in the peripheral nerves of Dhh null mice is not affected (Parmantier et al., 1999; Salzer, 1999).

1.5.6 Myelination

While it is well accepted that myelination by Schwann cells is controlled by axonal contact associated signals, the nature of these signals has remained largely uncharacterized (Mirsky and Jessen, 1999). It has been suggested that the neurotrophins, including BDNF and NT3, are important components of these signals. The neurotrophins BDNF and NT3 exert opposite effects on myelination in Schwann cells/dorsal root ganglia neuron co-cultures (Chan et al., 2001) (Figure 8). While BDNF appears to stimulate myelination, NT3 acts as an inhibitor of myelination in these cultures. Additionally, injection of NT3 in the developing sciatic nerve of newborn animals inhibits myelin formation while BDNF stimulates myelination (Chan et al., 2001). Neurotrophins act through the Trk family (TrkA, TrkB and TrkC) of tyrosine kinase receptors and the neurotrophin receptor $p75^{NTR}$. Several isoforms of TrkB and TrkC receptors exist which are generated through alternative splicing. Recently, Cosgaya and colleagues have suggested that $p75^{NTR}$, TrkB-T1 (an isoform of the TrkB receptor), and full-length TrkC receptors are the main mediators of neurotrophin signaling in PNS myelination (Cosgaya et al., 2002). They propose that activation of TrkC during proliferation, elongation and ensheathment of Schwann cells prevents inappropriate activation of the myelination program. At the time that myelination is initiated, NT3 expression levels drop releasing the inhibitory effect of NT3. In addition, they suggest that $p75^{NTR}$ is the

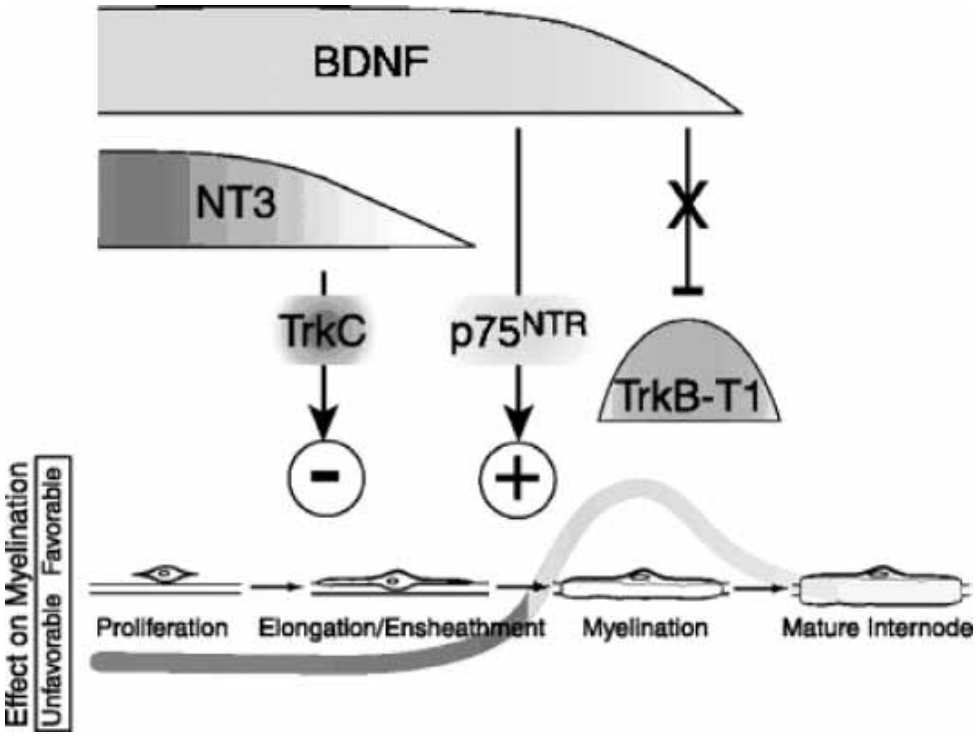

Figure 8- Actions of endogenous neurotrophins and their receptors throughout myelination. During glial proliferation, elongation, and ensheathment, NT3 levels decrease whereas TrkC and $p75^{NTR}$ remain constant. The activation of TrkC by NT3 during these phases prevents the myelination program from proceeding. When myelination is initiated, NT3 protein levels have already become undetectable, thereby removing its inhibitory action. At the same time, BDNF acts as a positive modulator of myelination through the activation of $p75^{NTR}$. Once active myelination is under way, extracellular BDNF is removed through its binding to the increased levels of TrkB-T1. After myelination is complete, all the neurotrophins and their receptors are down-regulated. (Adapted from Cosgaya et al., 2002).

functional receptor for BDNF, enhancing myelination in developing Schwann cells. The myelination process is ended by removing the extracellular BDNF through binding to the TrkB-T1 receptor, which functions as a decoy, and later through down-regulation of the neurotrophins and their receptors. Interestingly, $p75^{NTR-/-}$ knock out mice show a reduction of ≥50% in myelinated axons, which persists into adulthood (Lee et al., 1992).

A series of in vitro studies have suggested the involvement of cyclic AMP and the PKA pathway in Schwann cell proliferation and differentiation. Elevating intracellular cAMP levels in proliferating cells (through the reversible adenyl cyclase activator forskolin or dibutyryl camp) potentiates the mitogenic response of

Schwann cells to growth factors, including neuregulins, fibroblast growth factors, platelet-derived growth factors, insulin-like growth factors, and TGF-βs (Dong et al., 1997). Additionally, it is possible to promote differentiation of Schwann cells by using forskolin or other cAMP elevating reagents in absence of serum (Dong et al., 1997; Morgan et al., 1991). Several myelin related genes are upregulated in cultured Schwann cells following administration of forskolin. These genes include the myelin genes P-zero, MBP, P2, PMP-22, and connexin 32 (Lemke and Chao, 1988; Monuki et al., 1989; Scherer et al., 1995; Suter et al., 1994). Furthermore, forskolin induces expression of the Oct-6 and Krox-20 transcription factors and down-regulates AP1. Oct-6 and Krox-20 are expressed in promyelinating Schwann cells (Monuki et al., 1989; Zorick and Lemke, 1996). Therefore, cAMP might function as a second messenger of an axonal signal(s) that promote myelination (Bermingham et al., 2001). Recently, it was shown that the NF-κβ signaling pathway also plays a role in myelinating Schwann cells. It was found that NF-κβ is up-regulated in Schwann cell precursors and treatment of Schwann cell/dorsal root ganglia neuron co-cultures with inhibitors of NF-κβ leads to a differentiation arrest before Schwann cells have established a 1:1 ratio with axons (Nickols et al., 2003). In addition, knock out DRG cultures from the $p65^{-/-}$ subunits of NF-κβ show a myelin deficiency in comparison to DRG explants derived from heterozygote and wild type littermates. Interestingly, inhibition of NF-κβ in cultured Schwann cells abolishes Oct-6 gene expression, suggesting a role for NF-κβ in regulating Oct-6 expression (Nickols et al., 2003). Oct-6 plays an important role in the timely onset of myelination in Schwann cells. These data suggest that the NF-κβ pathway is essential for PNS myelination, including regulation of Oct-6 gene regulation.

1.6 Transcriptional control of cellular differentiation

Multicellular organisms develop from one single cell, the fertilized egg. During embryonic development, this cell divides and gives rise to a great diversity of different cell types. The progression of cellular differentiation requires the differential recruitment of genetic information encoded within the base sequence of DNA in the chromosomes of the cell (the genome). The expression of this genetic information is regulated at multiple levels and involves the generation of a RNA intermediate, mRNA, which is translated by the ribosomes into protein. An important regulatory decision is made at the level of initiation of transcription, the process by which the DNA sequence is transcribed by RNA polymerase into an RNA intermediate. Three different RNA polymerases exist in eukaryotic cells, each involved in the transcription of different sets of genes. RNA polymerase II transcribes protein-encoding genes, while RNA polymerase I and III transcribe ribosomal RNA genes and tRNA genes respectively. In contrast with prokaryotic polymerases, eukaryotic RNA polymerases do not bind directly to the DNA but are recruited to promoter sequences through interaction with DNA binding proteins or protein-complexes. In addition, the compaction of eukaryotic DNA into highly condensed chromatin fibers represents a major barrier for the transcription apparatus. Thus, in global terms, temporal cell-type specific expression of a particular gene requires a mechanism for overcoming this repressive barrier selectively and activation of the RNA polymerase containing complex that assembles at the gene promoter. Over the last decade it has become evident that chemical modifications of amino acids in histone proteins dramatically change the overall structure of the chromatin fiber and that these modifications correlate with gene activity. Such modifications include acetylation, methylation, phosphorylation, ubiquination and sumoylation of lysine, serine and arginine residues in the N-terminal tail of histone proteins (see for a recent review (Felsenfeld and Groudine, 2003)). Local chromatin remodeling is brought about by a host of enzymatic activities (histone acetylases, deacetylases, methylases) that are targeted to specific domains through interaction with sequence specific DNA binding proteins. Two important classes of DNA sequences can be distinguished within a gene locus. One is the gene promoter, which is operationally defined as the site at which the pre-initiation complex, including the RNA polymerase, assembles (see box).

Box-1 Basal transcription by RNA polymerase II

Transcription from a naked DNA template in the absence of regulatory elements is referred to as basal transcription. A polymerase II preinitiation complex (PIC) is assembled at the promoter of class II genes in a stepwise process. However, this process may never occur in vivo in the absence of the regulatory elements. The first step in the assembly is binding of the TATA box-binding protein (TBP) to the TATA box of pol II promoters. The majority of TBP present in a cell is associated with TBP associated factors (TAFs). This complex of TBP with TAFs that binds to the pol II promoters is called TFIID and contains at least eight TAFs with a cumulative mass of about 750 kDa. It has been shown that TFIID is also essential for transcription of TATA-less promoters and that recombinant TBP alone is not sufficient for directing transcription from TATA-less promoter containing initiator elements [Pugh and Tjian, 1990; Smale et al., 1990]. Later, it was shown that TFIID is able to bind to the initiator elements of both TATA-containing and TATA-less promoters [Kaufmann and Smale, 1994; Purnell and Gilmour, 1993].

After binding of TBP to DNA, TFIIA binds directly to TBP and stabilizes its interaction with DNA. Next, TBP bound to DNA creates a binding site for TFIIB. TFIIB itself serves as a platform for TFIIF and polymerase II (pol II), which are present in a complex. The next two factors bound to pol II are TFIIE and TFIIH and finally this mega dalton complex is able to initiate transcription. The description of assembly of the pol II complex in here is very generalized. It is important to keep in mind that the eukaryotic transcription machinery is very complex and that eukaryotic promoters are very divers. Given these complexities, there are different mechanisms involved in transcription regulation in different steps including at the chromatin level. Some of the components of the pol II complex are very conserved from yeast to human.

The second class of sequences modulates the rate of transcription from the promoter through several distinct mechanisms. These sequences include upstream promoter elements, enhancers, silencers and locus control regions. All these DNA elements can be bound by sequence specific DNA binding proteins including

transcription factors and their accessory factors, to affect local chromatin structure and transcription initiation.

1.7 Regulatory elements involved in gene expression

In general, gene expression is controlled by multiple regulatory sequences that are usually distributed at a variable distance from the gene promoter. A gene might have different regulatory elements, which contribute individually or cooperatively to expression of the gene in a temporal and/or cell-type specific fashion. These regulatory elements consist of different types of sequences including promoter elements, insulators, Locus Control Regions (LCR), and enhancers. In the following paragraphs, I will briefly discuss what is known in general about regulatory sequences, the proteins they bind and how they affect the transcriptional output of a gene.

A gene promoter is usually thought to consist of two elements: The core promoter and the promoter-proximal regions. Core promoters are the site of transcription initiation and spread from –40 to +40 nucleotides relative to the transcription start site. Several DNA sequence motifs can be identified within core promoters including the TATA box, initiator (Inr), TFIIB recognition element (BRE), and down stream core promoter element (DPE) (Butler and Kadonaga, 2002). The promoter-proximal region is located immediately upstream of the core promoter and consists of binding sites for transcription factors such as SP1 and CTF (CCAAT-binding transcription factor).

In general, enhancers are regulatory elements that can be located, at a considerable distance, either upstream or downstream of the transcription initiation site and in either orientation. Schaffner and colleagues first described enhancers in the early 1980s. They demonstrated that a 72bp tandemly repeated sequence upstream of the late viral promoter in the SV40 genome is capable of increasing the transcriptional output of a linked rabbit beta-globin gene (Banerji et al., 1981). Binding of specific combinations (dictated by the specific sequence of the enhancer) of ubiquitous and signal or cell specific transcription factors results in enhanced expression from the linked promoter. Enhancers consist of a number of transcription factor binding motifs and assembly of transcription factors on the enhancer leads to the formation of a nucleoprotein complex referred to as the “enhanceosome”

(Carey, 1998; Grosschedl, 1995; Kim and Maniatis, 1997). The assembly of the enhanceosome is dependent on the concentration of the relevant activators in a cell and their ability to engage in combinatorial interactions; subthreshold concentration, or altered positioning on the DNA prohibit cooperative binding (Carey, 1998). The binding of transcription factors to the enhancer is often cooperative; meaning that they promote the binding of each other and to DNA in a three-dimensional structure and this cooperativity results in "synergy" (Carey, 1998). The synergistic effect arises from an increase of the total free energy, translated into stability of the complex. In addition, assembly is a dynamic process as demonstrated for the formation of the interferon-β enhanceosome (IFNβ) (Munshi et al., 1998; Yie et al., 1999). Some prototypical enhanceosomes have been studied in detail, including those of the T-cell receptor α (TCRα) and interferon-β genes (Giese et al., 1995; Kim and Maniatis, 1997, Mayall et al., 1997; Merika et al., 1998). The enhancesome of IFNβ consists of the transcription factors NF-κB (p50 and p65 subunits), IRF-1, ATF-2, c-Jun, and HMG I. The enhanceosome of TCRα include a lymphoid-specific HMG-domain protein (LEF-1), Ets-1, AML-1 (CBFα2, PEB2αB), and ATF or CREB bind to TCRα (Carey, 1998). The presence of architectural proteins such as LEF-1 and HMG I in these nucleoprotein complexes is of special interest. These proteins exhibit sequence-specific DNA-bending properties and belong to the larger family of chromatin-associated high mobility group (HMG) proteins. Some of these proteins play a role in cooperativety and synergy. For example HMG I (HMGA1) facilitates binding of NF-κB to the INF-β enhancer by inducing a bend in the DNA. LEF-1 belongs to another class of HMG proteins and, unlike HMG I, has a context-dependent activation domain. Specific binding of LEF-1 induces DNA bending allowing all the other transcription factors (AML-1, ETS, ATF, and CREB) assemble on the enhancer (Hernandez-Munain et al., 1998).

Several studies have shown that the enhanceosome directly interacts with the basal transcription machinery, in particular with TFIID, TFIIA, and the USA cofactor, resulting in the synergistic recruitment of TFIIB to the promoter (Kim et al., 1998). The communication between the regulatory elements and promoter appears reciprocal. The enhanceosome recruits the pol II machinery, but the machinery reciprocally facilitates assembly of the enhanceosome (Carey, 1998; Kingston et al., 1996). Nevertheless, it is still a matter of debate how exactly enhancer-promoter

communication is established. Several models have been proposed to explain long distance gene activation by the enhancer. Most of these models explain some aspects of enhancer-promoter communication but not others and there is no direct evidence available to distinguish between them. The three most compelling models are referred to as the looping model, the tracking or scanning model, and the accessibility (linking) model. The looping model proposes the direct interaction between enhancer-promoter by formation of a DNA loop (Ptashne and Gann, 1997). The tracking or scanning model proposes that all the regulatory elements first assemble on the enhancer and then slide along the DNA until they reach the linked promoter. In the accessibility model, the recruitment of facilitator proteins to the enhancer modifies the entire chromatin domain between the enhancer and promoter and makes formation of higher order complexes along the chromatin fiber possible. Several recent studies have provided new evidence for the tracking and the looping model. Two different studies, employing different experimental approaches have shown direct interactions between enhancer and promoter in the β–globin locus (Carter et al., 2002; Tolhuis et al., 2002). However, Hatzis and Talianidis (2002) provided experimental evidence for a dynamic mechanism in HNF-4α enhancer-promoter communication such as suggested in the tracking model (Blackwood and Kadonaga, 1998).

1.8 POU domain transcription factors

The focus of research in this thesis is the role and regulation of the transcription factors Oct-6 and Brn-2. These proteins belong to the POU domain family of transcription factors. I will first review what is known about this family of proteins and then discuss the role of Oct-6 and Brn-2 in Schwann cell development. The review is further extended to include additional transcription factors known to be important for Schwann cell development and myelination.

The POU domain was originally identified as a region of high homology within the mammalian transcription factors Pit-1, Oct-1, Oct-2 and in the nematode unc-86 (Herr et al., 1988). The POU domain, which constitutes the DNA binding domain of these proteins, consists of a ~60 amino acid long homeo domain (the POU-homeodomain) connected by a short linker sequence (15-27 amino acids) to an amino terminal POU specific domain of ~75 amino acids (Ryan and Rosenfeld,

1997). More than 14 POU genes have been found in the human genome and they have been classified into six groups based on the amino acid sequence of their POU domains and on conservation of the variable linker region (Ryan and Rosenfeld, 1997; Wegner et al., 1993). A summary of expression patterns of different classes of POU proteins, in addition to their different names, is shown in table 3.

These transcription factors contribute to controlled gene expression and provide a cell type-specific pattern of gene expression during different stages of development. Most POU proteins show a spatially progressive expression

POU factor	Species	Expression		Function
		Embryo	Adult	
Class I POU1F1= Pit-1/ GHF1	rodent/ human	neural tube, pituitary	pituitary	survival of three pituitary cell types
Class II POU2F1= Oct-1	frog/ chicken/ rodent/ human	high in mid-/ forebrain anterior neural crest	ubiquitos	gastrulation, apoptosis
POU2F2= Oct-2/ OTF-2	rodent/ human	neural tube	lymphoid cells, nervous system	proliferation of maturing B-cells
POU2F3= Oct11/ Skn-1/Xlnr116/21	frog/ rodent	epidermis	thymus, testis	
Pdm-1= dPOU19/ nubbin	fruit fly	neuroectoderm, sensory organs	none	segmentation, specification of neural identity, wing patterning
Pdm2= dPOU28/ miti-mere	fruit fly	neuroectoderm, sensory organs	none	segmentation,specification of neural identity
ceh-18	nematode	muscle, epidermis	gonadal sheath cells	oocyte cell cycle arrest, gonad migration, epidermal differentiation
Class III POU3F1= Oct-6/ Tst-1/ SCIP/ XlPOU1/ zp-50	zebrafish/ frog/ rodent/ human	ES cells, nervous system, skin	brain, testis, retina, Schwann cells	Schwann cell differentiation, myelination, respiration
POU3F2= Brn-2/ N-Oct-3/XlPOU3	frog/ rodent/ human	nervous system	brain/ Schwann cells	neural induction, development of hyothalamus
POU3F3= Brn-1/ ZfPOU1	zebrafish/ rodent / human	nervous system, kidney	brain	
POU3F4= Brn-4/ RHS2/ XlPOU2	frog/ rodent/ human	nervous system	brain	neural induction, sense of hearing

cfla= drifter/ ventral veinless	fruit fly	trachea, epidermis, midline glia neurons, wing disc		migration of tracheal cells and midline glia, development of wings veins
ceh-6	nematode			
Class IV POU4F1= Brn-3/ Brn-3.0/ Brn-3A/ RDC-1	rodent/ human	sensory neurons, pituitary	brain	neurite outgrowth, coordination, suckling
POU4F2= Brn3.2/ Brn-3B	rodent/ human	sensory neurons, retina,	brain	development of retinal ganglion cells
POU4F3= Brn3.1/ Brn-3C/ XBrn3	zebrafish/ frog/ rodent/ human	sensory neurons	development of hair cells in the inner ear	
I-POU	fruit fly	ganglia, ventral nerve code supraoesophageal		
unc-86	nematode	neural lineage	neural cells	specification of neural identity
Class V POU5F1= Oct-3/ Oct-4/ OTF-3/ OTF-4	rodent/ human	ES/ EC cells, primordial germ cells	ovary, testis	pluripotency
sprm-1	rodent		male germ cells	
pou2	zebrafish	blastomeres, epiblast, hindbrain		gastrulation
Oct-25	Frog	gastrula	oocytes (low)	
Oct-60= XlPOU91	Frog	Blastula, gastrula: mesoderm, ectoderm	oocytes	
Oct-91= XLPOU91	Frog	gastrula	oocytes	
Class VI POU6F1= Brn-5/ POU[C]/ Emb/ mPOU/TCFβ1/	zebrafish / rodent/ human	widespread high in nervous system	ubiquitous	
RBF-1	rodent/ human	retina, midbrain, spinal cord	retina, medhabenula, hypothalamus	

Table 2 - Classes of the POU domain genes and their expression sites and function. Adapted from (Veenstra et al., 1997). Systematic designation and frequently used names are listed.

pattern early in development while later in development they are restricted to distinct patterns (He et al., 1989). Even Oct-1, a protein that is expressed in every cell-type and tissue tested (Scholer et al., 1989; Staudt et al., 1986; Sturm et al., 1988), is subject to differential regulation (Hsu and Chen-Kiang, 1993; Veenstra et al., 1995). Veenstra et al., (1995) observed differential expression of Oct-1 RNA and

protein in ectodermal and mesodermal cell lineages of Xenoups embryos. During later stages of development, Oct-1 expression becomes restricted to specific brain structures and cranial neural crest derivatives. Based on these results, it has been suggested that Oct-1 plays a role in the specification and differentiation of neuronal and neural crest cells (Veenstra et al., 1995).

One important way in which the activity of a transcription factor can be regulated is by sequestration in the cytoplasm. The maternally inherited Oct-1 is retained in the cytoplasm during early development, and gradually translocates to the nucleus around the mid-blastula transition (Veenstra et al., 1999). Another example is the Oct-6 protein, which is expressed, in different cerebral and hippocampal subpopulations. It is reported that the Oct-6 expression profile changes with developmental time (postnatal) such that by postnatal week 28 expression is exclusively cytoplasmic before it finally disappears two weeks later at postnatal week 30 (Ilia et al., 2003). Posttranslational modifications of POU factors, such as phosphorylation and acetylation, are also known to play a role in regulating DNA binding activity or dimerization with other transcription factors (Augustijn et al., 2002; Caelles et al., 1995; Tanaka and Herr, 1990).

As can be concluded from the expression patterns summarized in Table 2, many POU factors are expressed in overlapping patterns during development. Additionally, some of these factors have a similar DNA-binding preferences suggesting functional redundancy. This was shown for Oct-1 and OCA-B in Oct-2 deficient B cells, Brn-1 and Brn-2 genes in cortical neuron development and recently in class III factors Oct-6 and Brn-2 in Schwann cell differentiation (Jaegle et al., 2003; Luo and Roeder, 1995; McEvilly et al., 2002; Sugitani et al., 2002). It was demonstrated that mPOU binds the octamer motif (ATTTGCAT) and other POU protein target sites. Another possibility for generating a cell type-specific pattern of gene expression is the interaction between POU proteins. For example Pit-1 and Oct-1/ mPOU (Fumoto et al., 2003) or with transcription factors from other families such as vitamin D receptors, N-CoR, and GATA-2 through the homeodomain of Pit-1 (Augustijn et al., 2002). This results in either synergistic activation or repression of the target genes (Veenstra et al., 1997).

1.9 POU domain factors in development of the nervous system

Many of the known mammalian POU domain genes are expressed in the nervous and the neuroendocrine system. The only member of class I, Pit-1, is expressed from E14.5 onwards in the caudomedial region of the pituitary gland. In the adult Pit-1 regulates expression of the genes for growth hormone (GH), prolactin (PRL), growth hormone-releasing hormone (GHRH receptor), and thyroid hormone receptor beta type 2 (TSHβ) in three distinct cell types of the anterior pituitary gland; somatotropic, lactotropic, and thyrotropic cells (Rhodes et al., 1994). Pit-1 gene expression in the pituitary is maintained, at least in part, through a positive feedback loop that involves Pit-1 binding to its own promoter and to a distal enhancer (DiMattia et al., 1997; Rhodes et al., 1993). The study of mice homozygous for the Snell allele (a Pit-1 point mutation) or a Pit-1 null allele revealed that Pit-1 is necessary for the differentiation and survival of TSH-positive cells (Lin et al., 1994). Similarly, dominant and recessive mutations in Pit-1 were identified in human pituitary diseases (Cushman et al., 2002).

Oct-1 and Oct-2 were among the first POU proteins identified and both proteins belong to sub-class II. As stated earlier Oct-1 is expressed in every cell-type examined. Oct-1 has been implicated in gonadotropin-releasing hormone (GnRH) and vasoactive intestinal peptid (VIP) gene regulation and in silencing of the TSHβ gene (Eraly and Mellon, 1995; Kim et al., 1996; Rostene, 1984). Oct-2 is prominently expressed in B-lymphocytes, as well as in the developing and adult nervous system (He et al., 1989; Stoykova et al., 1992). The Oct-2 gene is subject to alternative splicing and produces at least eight different transcripts (Wirth et al., 1991). Several studies have demonstrated that the different splice variants are expressed in different cell types and it has been suggested that the Oct-2 proteins encoded by the splice variants exercise different functions (Lillycrop and Latchman, 1992; Stoykova et al., 1992). Evidence from in vitro studies has suggested that the splice variants Oct-2.4 and Oct-2.5 act as repressors in neuronal cells, while the predominant form in the B cells, Oct-2.1, has a stimulatory effect (Lillycrop and Latchman, 1992). Oct-2 null mice develop normally but die shortly after birth (Corcoran et al., 1993). Unexpectedly, disruption of Oct-2 has no effect on the B cell development. However, B-cell maturation is disturbed in the absence of Oct-2 (Corcoran and Karvelas, 1994; Schubart et al., 2001). Although no gross structural abnormalities

were observed in the nervous system, it is suggested that functional defects in the nervous system cause the early postnatal death of Oct-2 mutant animals (Corcoran et al., 1993; Ninkina et al., 1995).

Class III POU factors were first identified through a PCR-based cloning strategy and were shown to be expressed widely in the developing nervous system (He et al., 1989). Expression of Brn-1, Brn-2, and Brn-4 is detectable from E10 in the nervous system and in the primitive endocrine hypothalamus adjacent to the third ventricle (He et al., 1989). At E14, Brn-2 and Brn-4 are colocalized in the developing paraventricular (PVN) and supraoptic nucleus (SON) regions. In addition Brn-4 is expressed in the potential precursors of the anterior hypothalamus. Further, Brn-1 is expressed dorsolaterally in the presumptive zona incerta and in a dorsoventral stripe lateral to Brn-4 expressing cells (Alvarez-Bolado et al., 1995; Andersen and Rosenfeld, 2001). In general, Brn-1 and Brn-2 show a different spatial expression pattern at the early stages of development, but an overlapped expression pattern in the adult brain. Brn-4 is widely expressed during development, but becomes more restricted in the adult (Alvarez-Bolado et al., 1995). Oct-6 exhibits very restricted expression early in the developing embryo, in particular the nervous system but its expression expands greatly at later stages of development (Alvarez-Bolado et al., 1995; Zwart et al., 1996). Homozygous deletion of the Oct-6 gene causes developmental abnormalities in the nervous system. In particular, development of the phrenic nucleus in the brain stem appears affected. As the phrenic nerve innervates the diaphragm it has been suggested that developmental defects in this brain-stem nucleus underlies the high early postnatal mortality in Oct-6 mutant animals (Bermingham et al., 1996). Additionally, Oct-6 is expressed in other nuclei involved in breathing rhythm and airway movement including the nucleus ambiguous, the nucleus tractus solitarius, and the glossopharyngeal nucleus. Furthermore, Oct-6 is expressed in the Schwann cell lineage of the PNS, which will be discussed later.

It has been suggested that class III POU factors play a role in defining different regions in the developing brain (Alvarez-Bolado et al., 1995). For example Brn-2 knock out mice show dramatic abnormalities in the hypothalamic/posterior pituitary gland and die before P6 (Nakai et al., 1995; Schonemann et al., 1995). Severely reduced neuronal cell numbers in the PVN and SON of the Brn-2 null mice

suggest a role in proliferation or survival of these neurons. In addition, decreased vasopressin and oxytocin expression levels suggest a role for Brn-2 in cell specific regulation of these neuropeptides (Nakai et al., 1995). Deletion of the Brn-4 gene in mice causes deafness in mice due to several developmental defects in the inner ear. No abnormalities in brain development were observed in Brn-4 mutant animals. Furthermore, it has been shown that mutations in Brn-4 cause non-sensory deafness in humans (de Kok et al., 1995; Minowa et al., 1999; Phippard et al., 1999). Brn-3a (Brn-3.0), Brn3-b (Brn-3.2), and Brn-3c (Brn-3.1, POU4F3) make up the subclass IV (Gerrero et al., 1993; Lillycrop et al., 1992). Expression studies of these factors imply a role for them in neuronal differentiation. Knock-out mice were generated for each of these factors. While Brn-3a null mice show widespread loss of the sensory and motor neurons and die directly after birth (McEvilly et al., 1996; Xiang et al., 1996), mice lacking Brn-3b and Brn-3c are viable and show loss of retinal neurons (Brn-3b), leading to blindness, or vestibular neurons (Brn-3c) in the inner ear leading to deafness (Erkman et al., 1996). It is suggested that differences in the phenotypes of class IV knockouts reflect their expression pattern during development and in the adult brain (Latchman, 1999; Ninkina et al., 1993; Turner et al., 1994).

The class VI POU protein Brn-5 is expressed in the developing and adult brain and some organs outside the nervous system, including kidney, lung, and testis (Andersen et al., 1993). Brn-5 is prominently expressed in post-mitotic neurons, suggesting a role in terminal neuronal differentiation (Cui and Bulleit, 1998).

1.10 Transcription factors involved in Schwann cell differentiation

As stated in previous sections, continued interactions between neuronal and Schwann cells is essential for the development of the peripheral nerve tissue. In order to understand the signaling cascades that regulate Schwann cell differentiation, we need to identify the transcription factors involved in this differentiation process. Moreover, we need to understand how these transcription factors are regulated during Schwann cell differentiation. In the next section I will discuss a number of transcription factors that have been implicated in Schwann cell differentiation (Figure 9).

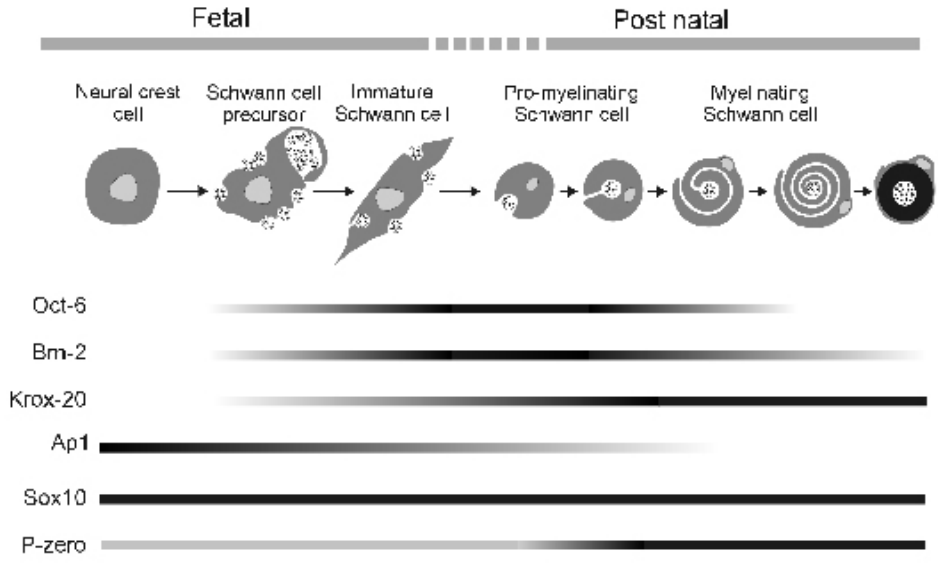

Figure 9- Transcription factor expression profile during Schwann cell development. P-zero is shown as a marker for myelination.

1.10.1 Pax-3

During embryogenesis, Pax-3 expression is detectable from embryonic day 8.5 in the small number of cells located in the dorsal region of the neural groove and in the neural tube (Kioussi and Gruss, 1996). Pax-3 expression continues in the neural crest cells at the craniofacial level in the spinal ganglia of the mouse (Goulding et al., 1993). At E12.5, Pax3 expression is observed in Schwann cell precursors that populate the embryonic peripheral nerves (Kioussi and Gruss, 1996). Kioussi and colleagues (1995) reported that Pax-3 expression is down regulated after E12.5 in proliferating Schwann cells until E18.5 (around birth in mouse) and re-expressed in terminally differentiating Schwann cells. After birth, Pax-3 expression is down-regulated in myelinating Schwann cells, but is maintained in non-myelinating Schwann cells. In contrast to these results, another group has reported continuous expression of Pax-3 in the sciatic nerve during Schwann cell proliferation (E12.5-E18.5) by RT-PCR (Blanchard et al., 1996). Due to the lack of in situ or immnuohistochemistry analysis, these data are not clear. It is also important to note that a significant number of neural crest-like cells and Schwann cell precursors are present in a developing nerve (Morrison et al., 1999), which possibly affected the results of Pax-3 expression in developing nerve.

Pax-3 acts as a repressor of myelin genes including P-zero and MBP and as an activator of non-myelinating markers including p75, the low affinity neurotrophin receptor by co-transfection studies in culture (Kioussi et al., 1995). It might be concluded from these results that Pax-3 is involved in fate decision between non-myelinating and myelinating Schwann cells.

The early role of Pax-3 in generating Schwann cell precursors is suggested by study of natural mutants splotch (sp) and a milder form splotch delayed (sp^d) in mice. splotch mice die around E13.5 and sp around E18.5 due to abnormalities in neural crest derived tissues in which Pax-3 is normally expressed including Schwann cell precursors (Epstein et al., 1991). The peripheral nerves of sp mice lack Schwann cells while sp^d mice have reduced numbers of Schwann cells (Franz, 1990; Moase and Trasler, 1990). In human, homozygous mutation in Pax-3 results in a clinical condition referred to as Waardenburg syndrome (Waardenburg, 1951).

1.10.2 SOX10

This transcription factor belongs to the large family of high mobility group proteins, containing a DNA-binding domain named as HMG domain (Laudet et al., 1993). The Sox protein family constitutes a subgroup among the high mobility group proteins as they show highest similarity (more than 50%) to the HMG box of the testis-determining gene SRY (Sry box; sox) (Wegner, 1999). Sox proteins are divided into seven subgroups (A to G groups) (Wegner, 1999). Sox10 is a member of group E and is expressed in neural crest cells and some of its derivatives including Schwann cells (Kuhlbrodt et al., 1998a). Sox10 expression is maintained during all the Schwann cell development stages and is not completely down-regulated after Schwann cell differentiation. In the CNS, Sox10 expression is detectable in the oligodendrocyte precursors and later in mature oligodendrocytes (Kuhlbrodt et al., 1998a).

The spontaneous mouse mutant Dominant megacolon (Dom) was shown to carry a mutation in Sox10 (Herbarth et al., 1998; Southard-Smith et al., 1998). In this mutant allele, a frame shift gives rise to a non-functional protein (Herbarth et al., 1998). Homozygous Dom mice die before embryonic day 13 and show a loss of neurons and glia cells in the PNS and lack of the enteric nervous system (resulting in the megacolon phenotype) (Herbarth et al., 1998; Southard-Smith et al., 1998).

Mutations in Sox10 have also been identified in patients with Shah-Waardenburg syndrome and Hirschsprung disease. In both of these diseases, the clinical phenotype results from a loss of neural crest derivatives (Herbarth et al., 1998; Southard-Smith et al., 1998). In mice with Sox10 homozygously deleted, the whole population of peripheral glia cells are missing, including satellite cells, suggesting a role for Sox10 in glia cell fate decisions (Britsch et al., 2001).

It has been demonstrated that Sox10 functions synergistically with Oct-6 as a strong transcriptional activator of promoters containing adjacent Oct-6 and Sox10 binding sites (Kuhlbrodt et al., 1998a). Interestingly, this synergistic function is specific to Oct-6 since Brn-1, closely related to Oct-6, is not able to cooperate with Sox10. Another example of synergistic function of Sox and POU proteins is between Sox2 and Oct3/4 on the FGF-4 enhancer (Yuan et al., 1995). Therefore, Sox10 not only plays a role in glia fate decisions but probably also during differentiation of these cells.

1.10.3 Transcription factor AP1

The Activator Protein (AP) is a hetero-dimeric transcription factor family that participates in the regulation of a variety of cellular processes including proliferation, differentiation, and apoptosis (Chinenov and Kerppola, 2001). AP1 can be composed of several combinations of basic-region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Maf (MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) transcription factor families These dimeric proteins bind to the 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (5'-TGAG/CTCA-3) or the cAMP response elements (CRE, 5'-TGACGTCA-3') (Shaulian and Karin, 2002).

Previously it was demonstrated that c-Jun expression levels drop in Schwann cells during myelination in vivo and after induction of differentiation by cAMP in vitro (Awatramani et al., 2002; De Felipe and Hunt, 1994; Monuki et al., 1989; Shy et al., 1996; Stewart, 1995). Prior to initiation of myelination, Schwann cells exit the cell cycle and become resistant to developmental death signals such as TGF-β. (Brown and Asbury, 1981; Friede and Samorajski, 1968; Stewart et al., 1993). TGF-β is implicated as a death signal in developing nerves, where it is believed to induce apoptosis in nonmyelinating cells, sparing cells expressing myelin

proteins (Parkinson et al., 2001). Later studies demonstrated that activated c-Jun (phosphorylation at serine-63) acts as a key downstream event in TGFβ-induced Schwann cell death (Parkinson et al., 2001).

Recently, Parkinson and colleagues showed that TGF-β death signals are inactivated through Krox-20, protecting cells from death trigger by growth factor deprivation (Parkinson et al., 2004). Furthermore, they showed that constitutive expression of Krox-20 in cultured Schwann cells results in down-regulation of the JNK-c-Jun pathway and prevents activation of this pathway by NRG-1 and TGF-β. It therefore appears the anti-proliferative function of Krox-20 is mediated through down-regulation of phosphorylated and unphosphorylated c-Jun protein and phosphorylated JNK (Parkinson et al., 2004). Interestingly, Krox-20$^{-/-}$ Schwann cells show a high rate of DNA synthesis and apoptosis during postnatal development (Topilko et al., 1994; Zorick et al., 1999). This phenotype could be explained by alteration of Krox-20 dependent cell autonomous responses to proliferative (NRG-1) and apoptotic (TGF-β) signals (Parkinson et al., 2004).

1.10.4 The POU domain transcription factor Oct-6

Oct-6, also known as POU3f1, Tst-1, and SCIP, belongs to subclass III of the POU domain transcription factor family (see Table 2) (Herr et al., 1988; Meijer et al., 1990; Suzuki et al., 1990). The Oct-6 gene is located on the distal part of mouse chromosome 4 and on the short arm of the human chromosome 1 and consists, like the other members of this subclass, of a single exon (Avraham et al., 1993; Kuhn et al., 1991; Xia et al., 1993). This led to the suggestion that this subclass arose during evolution through a retroposon event in an early vertebrate ancestor (Hara et al., 1992; Kuhn et al., 1991). Indeed, the single class III genes in the genomes of Drosophila and C elegans do contain introns (NCBI database). The four class III POU genes are not genetically linked. Therefore, the extensive overlap in expression patterns of these genes must be the result of independent acquisition of regulatory elements at the site of integration or from common regulatory sequences associated with the transposed mRNA precursor (Alvarez-Bolado et al., 1995; Suzuki et al., 1990; Zwart et al., 1996).

In the Schwann cell lineage, Oct-6 expression is first observed in Schwann cell precursors at embryonic day 12 (E12). Oct-6 expression is greatly increased

in promyelinating Schwann cells and is then rapidly down-regulated once cells have initiated myelination (Arroyo et al., 1998; Blanchard et al., 1996; Scherer et al., 1994).

In cultured primary Schwann cells, Oct-6 expression is rapidly up-regulated after treating cells with an intracellular cAMP elevating agent, such as forskolin. In addition, cAMP stimulation in cultured cells leads to expression of myelin related genes including P-zero and P2 (Monuki et al., 1989). These results have suggested a role for Oct-6 in activating myelin genes (Monuki et al., 1989). However, co-transfection studies in rat Schwann cells, using a P-zero promoter driven reporter construct and an Oct-6 expression cassette, suggested that Oct-6 represses myelin gene expression. (Monuki et al., 1993; Monuki et al., 1990). The repressive function of Oct-6 was shown to be associated with the amino-terminal domain of Oct-6. Repression of the P-zero promoter was not abolished by mutating all of the potential Oct-6 binding sites suggesting that Oct-6 represses this promoter indirectly, through titrating out a positive acting factor (squelching). However, the repressive function of Oct-6 was negated by a truncated form of Oct-6 (essentially consisting of the POU domain) (Monuki et al., 1993) These and other results led to assumption that interacting factors or adaptor molecules modulate the activity of Oct-6 in a cell type and promoter context-specific fashion (Meijer et al., 1992; Monuki et al., 1993).

In contrast to what was expected on the basis of these transfection experiments, Oct-6 knock out studies suggest that Oct-6 functions as a positive regulator of the promyelin to myelinating transition in Schwann cells. Mice homozygous for the mutated Oct-6 allele are born at normal Mendelian ratios but most of them die shortly after birth. A small percentage of Oct-$6^{-/-}$ mice (2-4%) survives for a longer period. These mice are smaller than their heterozygous littermates and exhibit occasional tremors in the second postnatal week (Bermingham et al., 1996; Jaegle et al., 1996). The high postnatal mortality in Oct-6 mutant animals has been attributed to respiratory distress and disorganization of the phrenic nucleus. Oct-6 is highly expressed in different parts of the brain, involved in breathing regulation, including the phrenic nucleus, the nucleus tractus solitarius, and the nucleus ambiguous. Apart from CNS defects, Oct-6 knock out animals demonstrate a transient arrest of Schwann cell development at the promyelin stage in the PNS (Bermingham et al., 1996; Jaegle et al., 1996). Microscopic examination of Oct-6 mutant nerves at

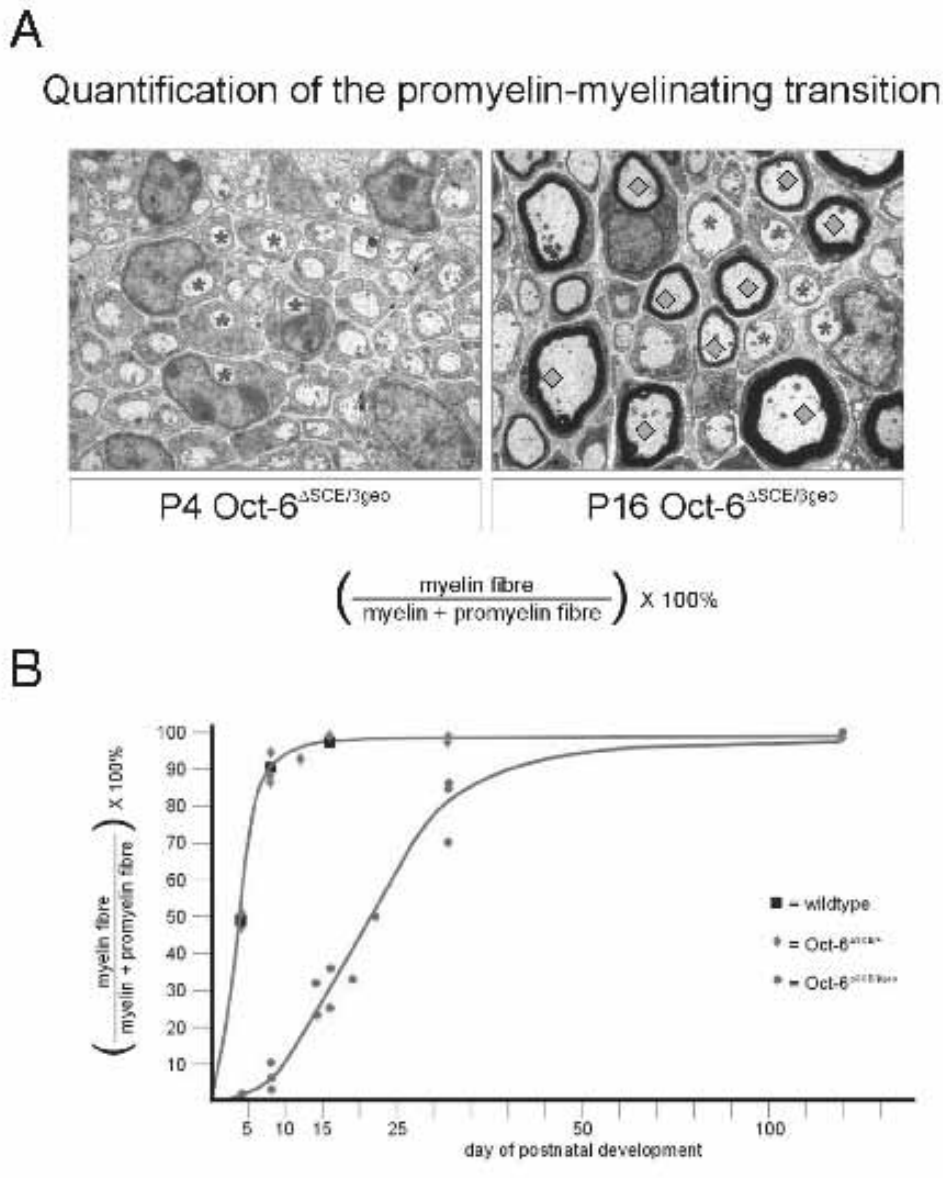

Figure 10- (A) Electron microscopic sections of Scitic nerves of p4 and p16 Oct-6 $^{\Delta SCE/\beta geo}$. (B) Quantification of the promyelin-myelinating transition in percentages. The transition of the promyelin-myelinating transition is shown in wildtypes, Oct-6$^{\Delta SCE/+}$, and Oct-6$^{\Delta SCE/\beta geo}$ during days of postnatal development.

postnatal day 1 show normal numbers of Schwann cells at the sorting and promyelin stage of differentiation. However, during the first week of postnatal development most Oct-6 mutant Schwann cells arrest at the promyelin stage of differentiation. In contrast, wild type or heterozygous Schwann cells progress to the myelinating phase during this period. Only during the second and third postnatal week do Oct-6 mutant Schwann cells initiate myelination. Thus Oct-6 appears required in promyelin Schwann cells to initiate myelination (Figure 10) (Jaegle et al., 1996). It has been suggested that down-regulation of Oct-6 in myelinating cells also depends on Oct-6. This suggestion is based on experiments, where the β-galactosidase gene expression was introduced into the Oct-6 locus and showed as a marker for Oct-6 during different developmental stages (Bermingham et al., 1996; Jaegle et al., 1996). While expression of β-galactosidase is completely lost in the adult nerve of Oct-$6^{+/-}$ animals, Oct-6 knock out animals still express β-galactosidase after completing myelination (Jaegle and Meijer, 1998). This result suggests that the Oct-6 locus is not down-regulated in the Oct-$6^{-/-}$ nerves. Thus Oct-6 itself is needed for this down-regulation.

The fact that Schwann cells, in the absence of Oct-6, do eventually initiate myelination suggested that an Oct-6 redundant factor exist in Schwann cells. Recently, such a factor (Brn-2) has been identified which is a member of the POU class III family (Jaegle et al., 2003). The Brn-2 expression pattern in Schwann cells is very similar to Oct-6. In Oct-6 deficient nerve, Brn-2 expression is normally up-regulated as in wild type nerves but the peak of expression is protracted. Brn-2 is down-regulated once myelination in Oct-6 mutant animals starts. In summary, Oct-6 gene expression in Schwann cells is under the control of axonal signals and possibly Oct-6 in turn regulates expression of down-stream genes involved in myelination, including Krox-20 (Ghislain et al., 2002; Topilko, 2001). Once myelination is underway, Oct-6 acts as a repressor by down-regulating its own expression (Jaegle and Meijer, 1998). It has been suggested that this functional change results from interaction of Oct-6 with different partners.

To uncover the identity of axonal signals regulating Oct-6 gene expression, it is needed to understand how Oct-6 gene expression is regulated. Recently, we identified a Schwann cell specific regulatory element within the Oct-6 locus (Mandemakers et al., 2000). This element is sufficient to drive the temporally correct

expression of a reporter gene or of Oct-6 in the Schwann cell lineage of transgenic mice.

1.10.5 Zinc finger transcription factors

Krox-24 (Egr-1) and Krox-20 (Egr-2) belong to the zinc finger transcription factor family also known as Egr (early growth response factor). All members of this family have a highly conserved DNA-binding domain consisting of three zinc finger motifs. Each motif binds to three nucleotides of a nine base pair GC-rich DNA element (Pavletich and Pabo, 1991). Krox-20 is involved in hindbrain segmentation, bone formation and Schwann cell development. Most Krox-20 mutant animals die shortly after birth although some survive for up to two weeks (Levi et al., 1996; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; Topilko et al., 1994). Krox-24 is involved in pituitary development where it has a direct role in regulation of the luteinizing hormone β-subunit. Krox-24$^{-/-}$ animals show growth defect and both sexes are sterile due to severe pituitary dysfunction. Despite the fact that Krox-24 is expressed in Schwann cells, no developmental defects were observed in this cell lineage. (Lee et al., 1996; Topilko et al., 1998). Study of the Krox-24 expression pattern, using a β-galactosidase knock in reporter gene, revealed that Krox-24 is expressed in Schwann cell precursors from embryonic day 10.5 (E10.5) until E14.5 in both cranial and spinal nerves (Topilko et al., 1997). Krox-20 expression is also reported around E10.5 but it is restricted to a small glial cell population close to the neural tube. These cells form the CNS/PNS boundary and are usually referred to as boundary cap cells. Around E15.5, Krox-24 expression is down regulated while Krox-20 expression becomes activated (Topilko et al., 1997). In a mature myelinated peripheral nerve, Krox-20 is expressed in the myelinated Schwann cells while Krox-24 expression is restricted to non-myelinating Schwann cells. During degeneration/regeneration, such as in a nerve lesion experiment, Schwann cells dedifferentiate distal to the site of lesion and after removal of myelin and cell debris (Wallerian degeneration), associate to regenerate axons and differentiate once more. It has been reported that several marker genes for mature myelinating cells, including Krox-20, are rapidly down-regulated while marker genes for immature Schwann cell, such as Krox-24, are up-regulated (Topilko et al., 1997). Although it has been suggested that the switch from Krox-24 to Krox-20 may regulate the

transition from Schwann cell precursor to immature Schwann cell, there is no direct evidence for such a role (Topilko et al., 1997). In the mouse, transition from Schwann cell precursor to immature Schwann cell happens around E14-E15 and in the absence of Krox-24 this transition still occurs. Further, in Krox-24 mutant animals no Schwann cell abnormalities are reported (Lee et al., 1996; Topilko et al., 1998). Krox-20$^{-/-}$ animals have shown a clear role for Krox-20 in transition from the promyelinating to myelinating Schwann cells (Topilko et al., 1994). In the absence of Krox-20, Schwann cells show a permanent arrest at the promyelin stage in which Schwann cells establish a 1:1 ratio and ensheath axons but do not form a myelin sheath (Topilko et al., 1994). Examination of promyelin Schwann cells of Krox-20$^{-/-}$ nerves at p12 showed high proliferation as well as apoptosis still resulting in a normal number of Schwann cells in comparison to wild type nerve (Zorick et al., 1999). Oct-6 expression does not diminish in the peripheral nerve of the Krox-20 mutants and these mutants show a severe reduction in myelin protein expression, including P0, MBP, and PMP22 (Topilko et al., 1994). It is also suggested that the high expression of Oct-6 in the Krox-20 knock out nerve causes the high proliferation rate of promyelinating Schwann cells. Besides proliferation, the increase in apoptosis is explained by competition for limited axon-associated survival signals, which is in disagreement with evidence suggesting the existence of an autocrine survival pathway in promyelin Schwann cells (Cheng et al., 1998; Dowsing et al., 1999; Meier et al., 1999; Zorick et al., 1999). Recently, it has been suggested that Krox-20 is involved in altering the response of promyelinating Schwann cells to the Neuregulin and TGF-β pathways, which are involved respectively in proliferation and apoptosis in Schwann cells (Parkinson et al., 2001; Salzer et al., 1980).

Recent genetic studies demonstrated that Krox-20 expression is regulated by Oct-6 (Ghislain et al., 2002). This study has shown that Krox-20 expression is under the control of two different cis-acting regulatory elements, which independently act in immature and myelinating stages. These two elements are named as the ISE, active in immature Schwann cells and the MSE, active in myelinating Schwann cells. Both elements function in an axon-dependent manner. Interestingly, multiple potential Oct-6 binding sites are identified in the MSE, suggesting that Oct-6 directly regulates Krox-20 at the time of myelination. Subsequently, Krox-20 itself regulates multiple genes involved in myelin formation. Microarray expression analysis has

supported a role for Krox-20 in expression of genes like MPZ, PMP22, MBP, MAG, Cx32, and periaxin (Nagarajan et al., 2001). Krox-20 is also likely to be involved in regulating genes required for the synthesis of lipids and cholesterol.

Different Krox-20 mutations have been identified in patients with de- or dys-myelinating neuropathies, including congenital hypomyelinating neuropathy, Charcot-Marie-Tooth type 1 and Dejerine-Sottas syndrome. These diseases underscore the importance of Krox-20 in the myelination process. Until now, five dominant mutations have been identified in the zinc finger-binding domain of Krox-20 (Bellone et al., 1999; Timmerman et al., 1999; Warner et al., 1999). Nagarajan and colleagues (2002) have shown that the neuropathies associated with mutations in the DNA binding domain (DBD) of Krox-20 result in a dominant negative pathomechanism. The dominant negative inhibition of DBD mutations in Krox-20 can explain why Krox-20 heterozygous mice show no phenotype, while heterozygous Krox-20 DBD mutant patients are affected. In addition to dominant DBD domain mutations, a recessive mutation has been identified in the R1 domain of Krox-20. This domain serves as an interaction interface for NGFI-A-Binding Protein-1 (NAB1) and NAB2 proteins (Warner et al., 1999). These proteins negatively regulate the activity of Krox-20. It is suggested that in the absence of the inhibitory effect of NAB2, the higher amount of mutant protein in the nucleus leads to deregulation of target genes (Warner et al., 1999).

1.11. Scope of this thesis

In the introductory part of this thesis an overview of the current knowledge about Schwann cells and their development and the role of transcription factors during Schwann cell development has been provided. In part two of the introduction, several aspects of gene expression and cis-regulatory elements were discussed. In order to gain an understanding of the transcriptional regulatory networks that govern the development of the peripheral nerve tissue we analysed the cis-regulatory element within the Oct-6 locus that drive Schwann cell regulated expression. We demonstrated that this element is essential for Schwann cell regulated expression but does not contribute to other aspects of the Oct-6 expression pattern. The results of this study are described in chapter 2. Chapter 3 describes experiments in which we aimed to further define the functional domains within

the Schwann cell enhancer. The study described in chapter 4 identifies Brn-2 as an important Oct-6 redundant factor in Schwann cells. In addition, the results of the targeted deletion of Brn-2 and double Oct-6/Brn-2 knock out study are discussed in the same chapter. Finally, in chapter 5 the results obtained in the previous chapters are summarized and some future directions are discussed.

1.11 References

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Chapter 2

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

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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–Lanterman incisures and paranodal loops (Arroyo and Scherer, 2000; Poliak 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 promyelinating 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 *ΔSCE* 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 (*Hy 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 these 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-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-6*$^{\Delta SCE/\Delta SCE}$, 54 were *Oct-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-6*$^{\Delta SCE/+}$ and *Oct-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-6*$^{\Delta SCE/+}$ animals, no Oct-6 positive nuclei were observed in the nerve of *Oct-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-6*$^{\Delta SCE/\Delta SCE}$ and *Oct-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 *flgeo* 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-6*$^{flgeo/flgeo}$ 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-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

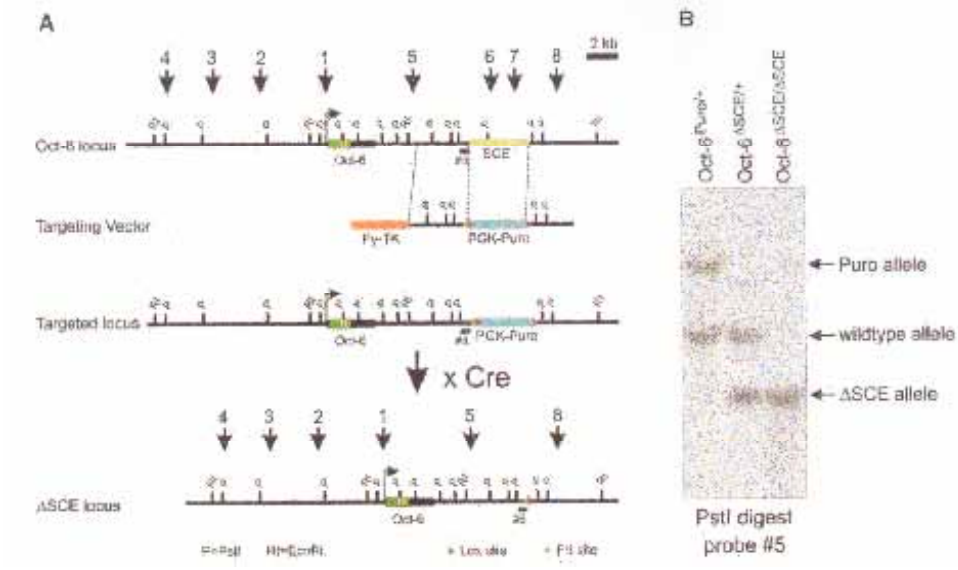

Fig. 1. Deletion of the 4.3 kb SCE from the *Oct-6* locus. (A) Gene targeting scheme for the *Oct-6* SCE. The SCE is indicated with a light green bar and is located ~12 kb downstream of the *Oct-6* gene CAP site. The intronless *Oct-6* transcription unit is indicated with a thick black line and the open 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 *HpaI–HpaI* 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 *PstI* 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 *PstI* 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 *ΔSCE* 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 neuroectoderm, 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 *ΔSCE* 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

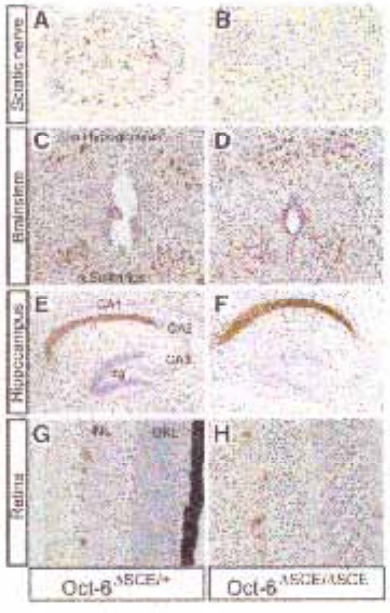

Fig. 2. The *ΔSCE* allele affects Oct-6 expression in the Schwann cell lineage only. (A) A brown precipitate identifies Oct-6 protein-expressing Schwann cell nuclei in transverse sections of sciatic nerve of *Oct-6*$^{\Delta SCE/+}$ mice at P8. (B) None of the Schwann cells in the sciatic nerve of *Oct-6*$^{\Delta SCE/\Delta SCE}$ 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); *28*]. 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.

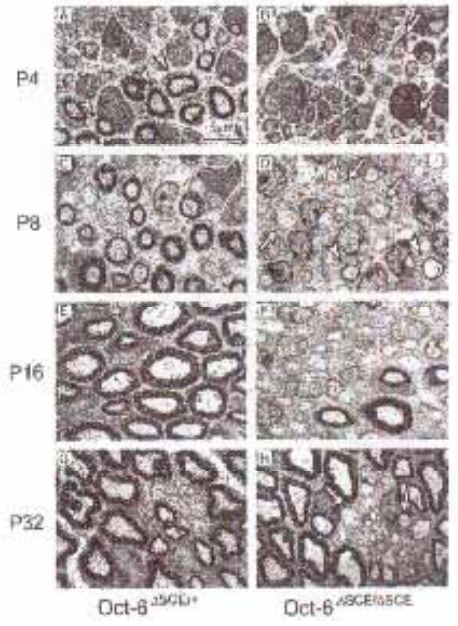

Fig. 3. Schwann cell differentiation is delayed at the promyelin stage in developing nerves of *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice relative to heterozygous mice. (A–H) Representative electron micrographs of transverse sections through the sciatic nerve of homozygous and heterozygous animals at different postnatal stages of development. While condensed myelin figures are abundantly present at P8 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 myelinogenically fated 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 *ΔSCE* 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 *ΔSCE* 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 *ΔSCE* 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 *ΔSCE* allele is a strong hypomorphic allele.

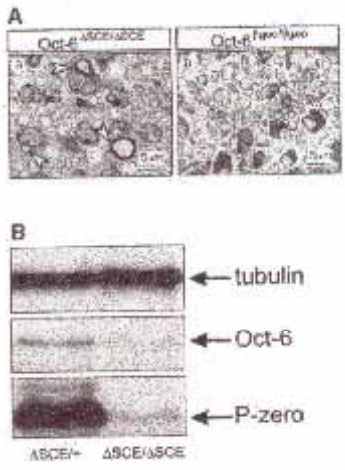

Fig. 4. The ΔSCE allele is a hypomorphic allele of *Oct-6*. (A) Comparison of sciatic nerve morphology in *Oct-6*$^{\Delta SCE/\Delta SCE}$ (a) and *Oct-6*$^{hypo/hypo}$ (b) mice at P8 reveals that a minority of Schwann cells in *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice have progressed to form compact myelin (arrowheads). In contrast, all Schwann cells are still at the promyelin stage of differentiation in sciatic nerve of *Oct-6*$^{hypo/hypo}$ mice. (B) Mice homozygous for the ΔSCE 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 is heterozygous mice is normal and myelinating Schwann cells express high levels of P-zero

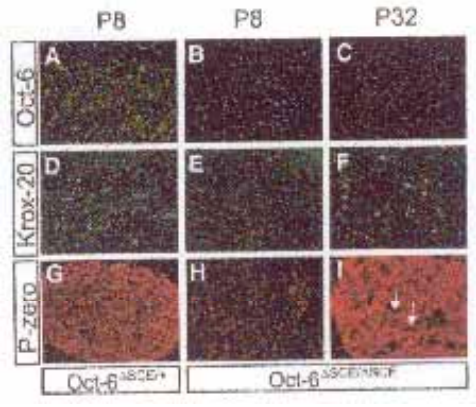

Fig. 5. Oct-6 expression is lost in Schwann cells of mice homozygous for the ΔSCE allele. (A) Heterozygous deletion of the SCE results in loss of Oct-6 expression in Schwann cells of the developing nerve and delayed appearance of Krox-20 and the major myelin protein P-zero. Transverse sections of paraffin-embedded 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).

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 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.*, 1999). 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

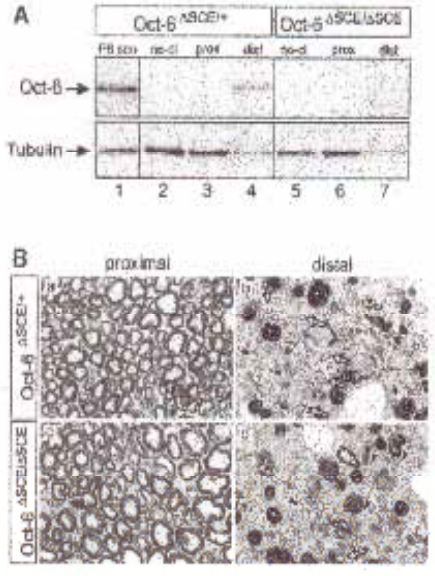

Fig. 6. Oct-6 regulation and function in the regenerating nerve. (A) Reactivation of Oct-6 expression is mediated through the SCE. Western blot analysis of sciatic nerves 12 days after crush lesion from adult *Oct-6*$^{\Delta SCE/+}$ (lanes 2–4) and *Oct-6*$^{\Delta SCE/\Delta SCE}$ mice (lanes 5–7). 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 5 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 events 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*$^{flox/flox}$ 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 *ΔSCE* 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*$^{βgeo/βgeo}$ mice, strongly supports the notion that neonatal death in *Oct-6*$^{βgeo/βgeo}$ 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 ΔSCE 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*$^{βgeo/βgeo}$ 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*$^{βgeo/βgeo}$ 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 generically, 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 *ΔSCE* 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 of 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 *ΔSCE* 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 Schwann 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 4.5 Kb *HindIII–MscI* Schwann cell enhancer fragment was subcloned from cosmid clone pTBL 6Cos. From this clone, a 3.2 kb *MscI–HpaI* fragment, containing homologous genomic sequences 5′ of the SCE, was cloned behind the negative selection cassette pgk 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 sides 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 *SwaI* si at the 5′ end of this clone. Downstream of the 3′ LoxP site, a unique *SwaI* site was used to introduce a 2.8 kb *MscI* fragment containing the genomic homologous region. The entire fragment, encompassing t PKT LoxP PGK puromycin LoxP 3′ 2.8 kb *MscI*, was excised as *SzaI–NotI* fragment and cloned in the *HpaI–NotI*-linearized Py-T plasmid. This resulted in the targeting vector, as depicted in Figure 1. The targeting vector was first linearized using *AvaI* before electroporatio into E14 ES cells. Electroporation and selection of cells in whic homologous recombination had occurred were carried out as describe G418 resistant and ganciclovir insensitive ES cell clones were screene for homologous recombination by Southern blotting of genomic DN digested with *PvuI* using a ^{32}P-labelled DNA probe derived from the homologous region (see Figure 1A). Chimeric mice were generated b injection of ES cells from the correctly targeted clone into C57BL/ blastocyst embryos. Chimeric males were crossed with FVB females an offspring were genotyped by Southern blotting of tail DNA digested wi *PvuI* using the probe described above. Offspring carrying the chromosom with the targeted SCE allele were identified (see Figure 1B) an subsequently crossed to mice carrying the *Zp3-Cre* transgene to obtai offspring in which the puromycin cassette was removed. These mice we then inter-crossed to obtain mice homozygous for the deleted *SCE* allel (see Figure 1B).

Immunohistochemistry and western blotting

For western analysis, nerves were isolated and directly lysed in Laemli buffer, followed by sonication and heating in a boiling water bath. Equ amounts of nerve extracts were resolved on a 12.5% SDS–PAGE gel an transferred to a PVDF membrane (Millipore) by electroblotting. Membranes were blocked with 5% 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 an incubated overnight at room temperature. Primary antibodies used were an Oct-6 rabbit polyclonal antiserum used at 1:300 dilution (Zorgle *et al.*, 1986); a P zero mouse monoclonal (clone 217; Archelos *et al.*, 1993) used at 1:1000 dilution, a tubulin antibody (Sigma T-9793) 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 i blocking buffer. Following five washes in 0.5% Tween-20 in PBS, th antigens were visualized by luminol (in the case of horseradish peroxidase) or NBT/BCIP (in the case of alkaline phosphatase) detection methods.

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Electron microscope analysis

Wild-type and mutant littermates (P8–P12) were anaesthetized wit pentobarbital and perfused transcardially, first with PBS 0.1 M and by 3% paraformaldehyde/1% glutaraldehyde in 100 mM cacodylate buffe pH 7.3. Sciatic nerves were dissected out and immersion fixed overnigh in the same fixative or formalin at 4°C. Nerves were then rinsed i 100 mM cacodylate and postfixed in 1% osmium tetroxide/ferricyanide i 100 mM cacodylate overnight at 4°C. Following dehydration through a ascending alcohol series, nerves were embedded in Epon resin a described. One micrometre sections were cut, mounted on a microscop slide and stained with paraphenylenediamine (ppd, Estable-Puig *et al.* 1965). Sections were examined under an Olympus BX40 ligh microscope and photographed using an Olympus DP50 digital camera. For electron microscopy, sections were cut at 50–60 nm and mounted o grids. Sections were contrasted with lead acetate and uranyl citrate, an 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 an placed on a heating pad. The sciatic nerve in the left leg was exposed an crushed for two times 15 s at the midthemoral level, using No. 5 biology forceps. Animals were killed 72 days after the operation and the lesioned and contralateral nerves were isolated for western analysis. For light an

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Chapter 3

Modular structure of the Oct-6 Schwann cell specific enhancer

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Abstract

The Oct-6 Schwann cell enhancer (SCE) is both required and sufficient to direct regulated expression of the Oct-6 transcription factor gene during Schwann cell differentiation. In this study we used a combination of functional reporter gene assays in transfected Schwann cells and transgenic mice and comparative genome analysis to identify regions within the 4.3 kb Schwann cell enhancer that are of functional importance. We identified two regions within the SCE that function as enhancers in transfected Schwann cells. These regions correspond to sequences that are highly conserved in the genomes of rat, human and dog and are named homology region 1 (HR1) and 2 (HR2). Although HR1, and to a lesser extent also HR2, functions as an enhancer in transfected Schwann cells, neither HR1 nor HR2 has significant enhancer activity in Schwann cells of transgenic mice. However, the combination of HR1 and HR2 shows full activity, demonstrating that the Oct-6 SCE consists of at least two interdependent elements. These data suggest that multiple signalling pathways are integrated at the SCE to activate Oct-6 in Schwann cells at the appropriate time and level.

Introduction

Schwann cells, the main glia cells of the vertebrate peripheral nervous system (PNS), originate from the neural crest and differentiate in close association with axons through a number of developmental stages to give rise to the two morphologically distinct Schwann cell types that can be observed in the mature peripheral nerve. Myelinating Schwann cells generate and maintain a multi-lamellar insulating sheath around an associated axon while non-myelinating Schwann cells accommodate several smaller axons in cytoplasmic cuffs. The diversion of embryonic Schwann cells along a myelinating or non-myelinating differentiation route is primarily controlled by the axons with which the cells associate (Jessen and Mirsky, 2002).

Although the nature of the axonal signal(s) that promote myelination is unknown, two Schwann cell autonomous regulators of myelination have been identified and studied most extensively over the last decade. These are the POU-domain transcription factor Oct-6/Tst-1/Scip/Pou3f1 (referred to as Oct-6 in this paper) and the zinc finger transcription factor Krox20 (Egr-2) (Topilko and Meijer,

2001). Both transcription factors are expressed dynamically in the Schwann cell lineage during development and nerve regeneration (Scherer et al., 1994; Zorick et al., 1996). Oct-6 expression is initiated in Schwann cell precursors and its regulated expression depends on continued axonal contact (Blanchard et al., 1996; Scherer et al., 1994). The highest levels of Oct-6 protein are observed at the promyelin stage of cell differentiation after which Oct-6 levels rapidly decline in actively myelinating Schwann cells (Arroyo et al., 1998). A role for Oct-6 in regulation of the myelination program is suggested by the analysis of the peripheral nerve phenotype observed in Oct-6 deficient and mutant mice (Bermingham et al., 1996; Jaegle et al., 1996; Jaegle and Meijer, 1998). In the absence of Oct-6, Schwann cells show a delay in the transition from the promyelin to the myelinating stage of cell differentiation. Similarly, Krox-20 deficient animals show a block at the promyelin stage of Schwann cell differentiation while mutations in the Krox-20 gene are associated with particular forms of hereditary motor and sensory neuropathies in man (Timmerman et al., 1999; Topilko et al., 1994; Warner et al., 1998). Genetic and cell biological studies have shown that Oct-6 and Krox-20 act in a genetic cascade (Topilko and Meijer, 2001). Oct-6 regulates the expression of a number of down-stream genes including Krox-20 (Blanchard et al., 1996; Ghislain et al., 2002). Consequently Krox-20 regulates an additional set of genes such as the major myelin genes and those involved in lipid metabolism (Nagarajan et al., 2001).

Since Oct-6 is the first transcription factor in this genetic hierarchy that is up-regulated in response to inter- and/or intra-cellular signals, it is important to understand how this gene is regulated. Previously, we identified a cis-acting regulatory element located 10 kilobases (kb) down-stream of the Oct-6 gene promoter and named it the Oct-6 Schwann cell enhancer or SCE. This 4.3 kb DNA element is characterized by the presence of two DNaseI hypersensitive sites (HSS) in chromatin of Oct-6 expressing cells (Mandemakers et al., 2000). We also demonstrated 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 gene expression pattern (Ghazvini et al., 2002). To reveal the presence of putative cis-regulatory elements within the boundaries of the 4.3 kb SCE, we compared SCE orthologous sequences in the genomes of several mammalian species. This analysis revealed the presence of two highly conserved

regions, named HR1 and HR2, one of which (HR1) corresponds with one of the DNaseI HSS's mapped in our earlier studies (Mandemakers et al., 2000). We first tested the relevance of these elements for SCE function in transfected primary Schwann cells and found that both conserved elements contribute to full enhancer activity. Subsequent analysis in transgenic mice revealed that neither HR1 nor HR2 has significant enhancer activity. However, the combination of the two elements exhibits full SCE activity, suggesting that protein complexes assembled on both DNA elements act together to regulate expression from the linked promoter in Schwann cells.

Results

Phylogenetic analysis of the SCE

Comparative genomics is a powerful tool for identifying genes, functional domains and regulatory elements (Cooper and Sidow, 2003). Previously, we reported the identification of the human SCE and showed that this fragment, like its mouse orthologue, is sufficient to direct Schwann cell specific expression of a LacZ reporter gene in transgenic animals (Mandemakers et al., 2000). This result demonstrated that the regulatory elements for the Schwann cell specific expression of the Oct-6 gene are conserved between human and mice. To further define this and other regulatory regions within the Oct-6 locus we compared the genomic sequence of the Oct-6 locus from mouse, human and rat using the Vista phylogenetic alignment tool (http://www-gsd.lbl.gov/vista) (Couronne et al., 2003; Shah et al., 2004). Figure 1A illustrates the evolutionary conservation between the mouse Oct-6 locus and the human and rat Oct-6 locus as a percentage identity plot. Percentage identity is calculated for every nucleotide within a 100 bp wide window, 50bp upstream and 50bp down-stream of that nucleotide. The homology cut off is set at 50% for the human sequence and, because of the shorter evolutionary distance between mouse and rat, at 90% for the rat. The positions of previously mapped DNaseI hypersensitive sites are indicated above the graph (Mandemakers et al., 2000). As expected, high sequence conservation among the three mammalian species is found in the Oct-6 gene itself, including its long 3'UTR sequence. Additional regions of high sequence conservation are found up-stream and down-stream of the structural

gene. Some of these conserved sequences coincide with the previously mapped DNaseI hypersensitive sites (Figure 1).

Within the limits of the previously defined Schwann cell enhancer element two conserved blocks of high sequence conservation are present (Figure 1B). We refer to these sequence blocks as Homology Region 1 (HR1) and Homology Region

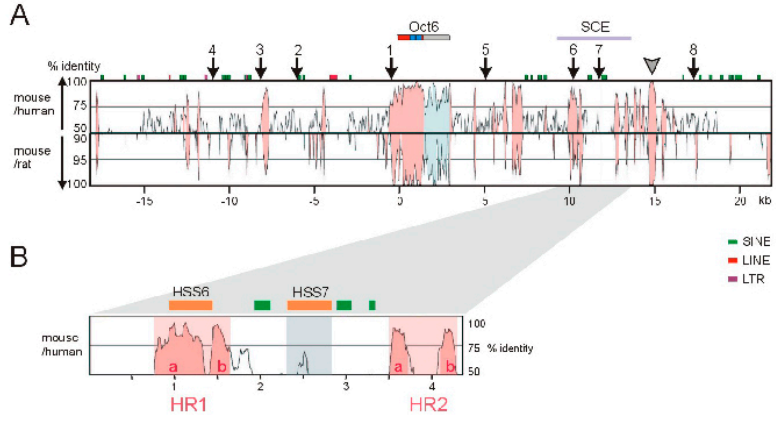

Figure 1
A. Genomic comparison of Oct-6 orthologous sequences in the genomes of mouse, human and rat using the VISTA alignment tool. Conservations levels greater than 50% are shown for the mouse versus human sequence comparison and 90% for the mouse rat comparison. The positions of the Oct-6 coding sequence, Oct-6 SCE, and DNaseI hypersensitive sites are indicated. The position of the different repeat elements such as SINE, LINE, and LTR are also shown above the plot. The homology above the 75% in mouse/human and above the 95% in mouse/rat has shown in colour.
B. VISTA graph showing an enlargement of the conserved human and mouse Oct-6 SCE region. Two conserved blocks of high sequence conservation within the Oct-6 SCE are indicated and labelled homology 1 and 2 (HR1 & HR2). HR1 and HR2 both contain two separate highly conserved regions that are marked HR1a and HR1b, HR2a and HR2b.

2 (HR2). As shown in Figure 1B, DNaseI hypersensitive site 6 maps to HR1, while DNaseI HSS7 maps to non-conserved DNA sequences. The presence of HSS7 could be associated with the promoter region of a B2 repeat element.

Deletion analysis of the SCE in transfected rat Schwann cells

To investigate which sequence elements within the SCE contribute to enhancer activity we generated a series of internal deletions and tested these for enhancer activity in transfected rat Schwann cells using a luciferase reporter assay.

Cultured primary rat Schwann cells express a number of genes that are normally expressed by non-myelinating Schwann cells in vivo. These genes include $p75^{NTR}$ (the low-affinity nerve growth factor receptor), brain derived neurotrophic factor (BDNF), and growth-associated protein 43 kDa (GAP-43) (Lemke and Chao, 1988). Elevation of intra-cellular cAMP levels results in up-regulation of myelination-associated genes such as Oct-6 and Krox-20 and down-regulation of genes associated with the non-myelinating phenotype (Figure 2A and (Arroyo et al., 1998; Monuki et al., 1989; Zorick et al., 1996). To test whether this in vitro differentiation paradigm could be used to dissect the Oct-6 SCE we first tested the responsiveness of the full enhancer. A luciferase reporter construct driven by the SV40 minimal promoter (pGL3 Promega) and the SCE cloned down-stream of the gene was transfected in rat Schwann cells in medium containing serum. Twenty-four hours later medium was removed and replaced by defined medium containing 20 μM Forskolin. Cells were harvested 24 hours later and tested for luciferase activity. As shown in Figure 2B, the SCE mediates an eight- to nine-fold activation of luciferase activity in response to the inductive signal, while a SV40 enhancer control is equally active in undifferentiated and differentiated cells. Activation of luciferase activity

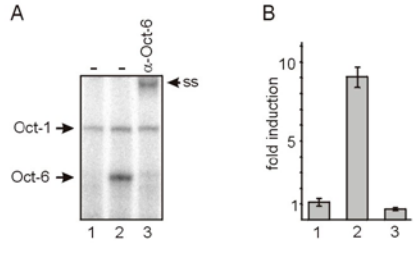

Figure 2 - A. Expression of Oct-6 in cultured rat Schwann cells, exposed to 20 mM Forskolin, is demonstrated in an electrophoretic mobility assay. Non-induced Schwann cells do not express Oct-6 (lane 1), while after induction of differentiation Oct-6 expression is detectable (lane 2). Addition of an Oct-6 polyclonal antibody results in the formation of a ternary complex with reduced electrophoretic mobility, confirming the presence of the Oct-6 protein in the complex (lane 3, SS=supershift). 2B. The Oct-6 SCE coupled to a luciferase reporter gene and a minimal SV40 promoter shows approximately ten times higher luciferase activity in differentiated Schwann cells versus non-differentiated Schwann cells. A generic enhancer, such as the SV40 enhancer is equally active in differentiated versus non-differentiated Schwann cells. Activation by the Oct-6 SCE of luciferase reporter gene expression depends on the presence of a minimal promoter, excluding the presence of a cryptic promoter within the Oct-6 SCE.

through the SCE is specific for Schwann cells, as the SCE does not mediate cAMP responsiveness in 3T3 cells (data not shown).

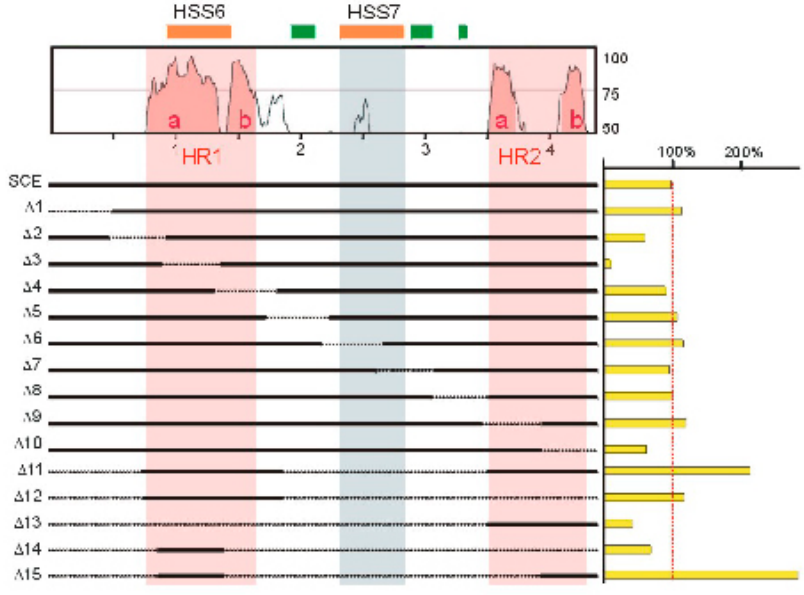

Figure 3 - Schematic representation of the luciferase activity assay of Oct-6 SCE deletion constructs in Schwann cells. The position of the conserved HR1 and HR2 domains are highlighted. The position of DNaseI hypersensitive sites 6 and & 7 are indicated above the graph. Constructs 1 to 10 are overlapping deletions, generated in the context of the whole Oct-6 SCE. Results are from one representative experiments performed in triplicate. Luciferase activity in cell extracts is normalized to β-galactosidase activity in the same cell extract. The values of luciferase activity are shown as a percentage of activity of the total SCE. The total experiment was performed at least three times and the results are reproducible.

We next tested the activity of the SCE deletion mutants in this differentiation paradigm (Figure 3).

Reporter gene activity of the various SCE deletion constructs is reported as a percentage of the full SCE activity. These results suggest that the major enhancer activity of the SCE is associated with sequences deleted in construct Δ3. Deletions in construct Δ10 result in a slight reduction of enhancer activity. The fact that Δ3 is located within the HR1, encouraged us to consider reduction in luciferase activity of deletion 10, since this deletion is also located within HR2. To determine whether the two regions HR1 and HR2 are relevant to SCE activity, we tested the luciferase activity of both regions in different combinations (Figure. 3). The combination of

both HR1 and HR2, as in construct Δ11, resulted in strong activation of reporter gene activity relative to the SCE. Also, HR1 alone (Δ12) strongly activates transcription while HR2 only (Δ13) marginally contributes to activity. These results suggest that indeed the major enhancer activity of the SCE is associated with HR1, in particular those sequences deleted in Δ3. We next tested whether these sequences (encompassing HR1a), deleted in Δ3, were sufficient to function as enhancer in Schwann cells. While HR1a sequences harbor considerable (Δ14 in Figure 3; 70% of full SCE activity) enhancer activity, its activity is further boosted when HR2a is included in the construct (Δ15 in Figure 3). Thus, HR1a and to a lesser extent HR2b function as enhancers in transfected Schwann cells and when combined, exhibit greater than additive activity. This analysis did not reveal a role for HSS7.

Deletion analysis in transgenic mice

In contrast to the data derived from the transfection studies described above, preliminary data from transgenic experiments did not reveal a role for HSS6/HR1 or HR2 in SCE function (construct 2 and 3 in Figure 3B (Mandemakers et al., 2000)). However, both these constructs terminated within HSS7, leaving open the possibility that HSS7 is required for HSS6/HR1 function in vivo. To resolve these issues we next examined the role of HSS6/HR1, HSS7 and HR2 in SCE function in vivo through the generation of transgenic mice carrying a LacZ reporter gene driven by the mouse heat shock protein 68 (hsp68) promoter (Rossant et al., 1991). LacZ reporter gene expression in neonatal Schwann cells was determined by whole mount X-Gal staining of founder transgenic mice. First we tested a construct that contains both HSS6 and HSS7. While around 80% of transgenic animals carrying a full SCE (construct 1 in Figure 4; 20 out of 26. This number is based on a summation of all transgenic animals generated with the full SCE) express the transgene in Schwann cells, only 1 out of 6 animals transgenic for construct 5 showed LacZ staining in the nerve, indicating that HSS6 and HSS7 are not sufficient to drive Schwann cell expression. Also, a construct containing HSS7 (construct 7) does not confer Schwann cell specific expression. Thus, both assays suggest that HSS7 does not play a role in activation of Oct-6 in Schwann cells.

We next tested a set of constructs in which HR1, HR2 or both sequence elements are present. Neither HR1 nor HR2 alone is capable of directing Schwann

cell specific expression of the transgene (Figure 4 constructs 4 and 7 respectively). However, when both elements are combined 3 out of 4 founders expressed the

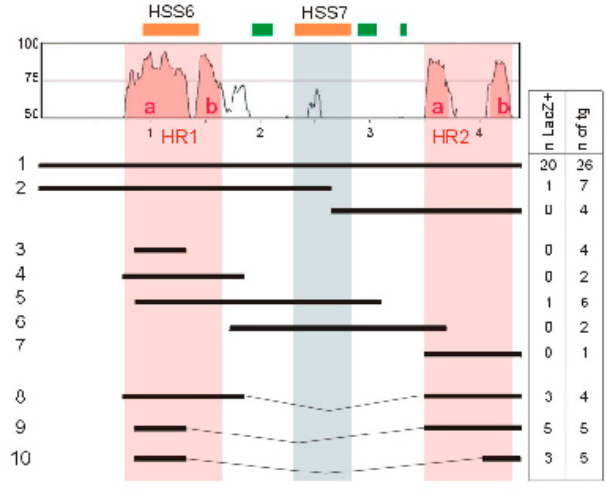

Figure 4 - Functional dissection of the Oct-6 SCE in transgenic mice. The position of the conserved domains HR1 and HR2 are highlighted. The positions of the DNaseI hypersensitive sites 6 and & 7 are indicated above the graph. Transgenic founders were examined for expression of the lac-Z reporter gene in sciatic nerve after 18 hours staining. In the columns next to the constructs, the number of the transgenics that express the reporter gene in Schwann cells are listed as #lacZ$^+$ and the total number of the transgenic founders analysed as #tg.

reporter gene (Figure 4, construct 8) in Schwann cells, indicating that both elements are needed for full enhancer activity in vivo.

Phylogenetic comparison of SCE sequences from the mouse, human and dog genome (data not shown) reveals that HR1 and HR2 can be further subdivided in two regions (HR1a and b, HR2a and b respectively) of high sequence conservation (Figure 1A). As the luciferase assay suggests that the major enhancer activity of the SCE is associated with HR1a, we tested smaller versions of HR1 and HR2 in transgenic mice. First we tested HR1a alone. This element, like the entire HR1 element, does not confer Schwann cell specific activation on the reporter gene (construct 3). However in combination with the HR2, 5 out of 5 transgenic animals now express the transgene in Schwann cells (construct 9). Next we tested the HR1a fragment in combination with the HR2b fragment and found that 3 out 5 transgenic founders express the transgene within the peripheral nerves (construct 10). Thus, the major enhancer activity within the boundaries of the SCE resides within

sequences covering HR1a and HR2b and these two elements are both required for Schwann cell specific activation of transcription.

Discussion

In the present study we have combined comparative genome analysis and functional analysis in cultured Schwann cells and transgenic mice, to identify and delineate two core elements that together make up the Schwann cell enhancer of the Oct-6 locus.

Comparative sequence analysis of mouse and human Oct-6 loci

Our initial comparative genome sequence analysis focused on approximately 50kb surrounding the Oct-6 gene, a region for which HSS mapping data were available (Mandemakers et al., 2000; Mandemakers et al., 1999). This sequence analysis included human, mouse and rat sequences. In addition to the Oct-6 gene, including its promoter and long 3' UTR, several strongly conserved sequences are found within the region analysed. Two of the conserved regions correspond with the previously mapped HSSs 3 and 6. HSS6 is located within the Oct-6 SCE, approximately 10 kb down-stream of the Oct-6 CAP site (Mandemakers et al., 2000). HSS3 corresponds to a conserved sequence that is unique and that might be part of a transcription unit as one human est, derived from a pheochromacytoma, maps to this sequence. HSS3 is present in brain and ES cells, but not in liver or MES1 cells (Mandemakers et al., 2000). Additionaly, LacZ reporter constructs carrying DNA sequences encompassing HSS3 and HSS5 are expressed in the hair follicles of the skin (Mandemakers et al., 2000). It is therefore possible that HSS3 contributes to expression of Oct-6 in the skin. Further analysis of this element in transgenic mice should reveal the importance of this element for regulated expression of Oct-6 in these tissues.

All other HSSs (2, 4, 5, 7 and 8) do not correspond to conserved sequences and most of them map to short repeat elements (SINE: see Figure 1). As many of these are actively transcribed by RNA polymerase III, it is likely that the DNaseI hypersensitivity is associated with transcriptional activity of these elements and not the Oct-6 gene (Deininger et al., 2003). Other conserved sequences could represent additional enhancer elements contributing to neuronal and skin expression of Oct-

6. Alternatively, some of these sequences might be involved in interactions with the nuclear matrix as it has been shown that up to 10% of the conserved non-coding DNA sequences in the genome share characteristics of matrix attachment regions (MARs) (Glazko et al., 2003). Of specific interest is a conserved element located downstream of the SCE (grey arrowhead in Figure 1). This element shows 100% sequence conservation between mouse and human over a stretch of approximately 250bp. This level of sequence conservation is even higher than that observed in the POU domain encoding region of the Oct-6 gene. Recently it was shown that this element is one of a family of approximately 500 DNA sequences, longer than 200bp, which are ultra-conserved in mammalian genomes (Bejerano et al., 2004). Some of these sequences are even conserved in the genomes of non-mammalian genomes. Also this ultra-conserved element in the Oct-6 locus is conserved in the genome of chicken and zebrafish (unpublished observations). Interestingly, the Brn-2 gene, which is expressed in Schwann cells in parallel with Oct-6, is also associated with an ultra-conserved sequence some 10 kb down-stream of the start codon of the Brn-2 open reading fram. Indeed, these ultra-conserved DNA sequences are frequently found in close proximity or overlapping with genes encoding RNA and DNA binding proteins, particularly those involved in early embryonic development (Bejerano et al., 2004). The functional significance of these ultraconserved sequences is not known (Bejerano et al., 2004; Boffelli et al., 2004).

Deletion analysis of SCE function

Deletion analysis in cultured Schwann cells revealed the presence of two regions within the SCE that exhibit enhancer activity. These regions correspond with sequences that are strongly conserved in mammalian genomes. Although both regions have enhancer activity in cultured Schwann cells, neither of the two have independent enhancer activity in vivo in transgenic mice. Instead, both the 526 bp HR1a and 350 bp HR2b are required for activity in Schwann cells of transgenic mice. Why these elements behave different in the two assay systems is not clear. It is unlikely that these differences reflect the use of different minimal promoters (a minimal SV40 promoter in cultured Schwann cells versus a minimal hsp68 promoter in transgenic Schwann cells) or reporter genes in the different assay systems. Most likely these differences are caused by the difference in chromatin context (episomal

versus chromosomal) and cellular context (Schwann cells in a petri dish versus Schwann cells in a developing nerve). Such difference in behaviour of enhancers has been described for many enhancers and is generally attributed to the capacity of enhancers or locus control regions to influence chromatin structure or nuclear compartmentalization in a tissue–specific fashion (Dillon and Sabbattini, 2000). Conceptually, chromatin modulating activity and enhancer activity are functionally distinct mechanisms (Dillon and Sabbattini, 2000). Experimentally it is not possible to differentiate between these activities when reporter gene activity is the only experimental read out. It would therefore be of interest to investigate the histone acetylation status of the different transgene constructs and that of the ΔSCE allele in Schwann cells (Iizuka and Smith, 2003). At present, our data are compatible with the suggestion that enhancer function and chromatin modulating activity reside in two distinct elements. Alternatively, it is possible that HR1- and HR2-nucleoprotein complexes interact directly with complexes assembled at the promoter or through formation of a holo-complex.

Enhancers are modular structures, which consists of arrays of transcription factor binding sites. Such arrays of high and low affinity binding sites are sufficient to integrate independent signals and convert them to a binary output: activation or repression of a linked gene promoter (Yuh et al., 1998). As discussed above, our data suggest that the SCE consist of at least two modules that are both required for activity in vivo. The signalling pathways that converge on these separate modules are not known yet. However, since Oct-6 mediates the initiation of myelin formation, signals that activate myelination in the PNS are likely candidates. It was shown by Lemke and colleagues that activation of PKA results in up-regulation of Oct-6 levels in cultured Schwann cells (Monuki et al., 1989). We have tested our deletion constructs in Schwann cells in which PKA was activated by cAMP or forskolin. However, activation of reporter gene activity through the PKA pathway is probably indirect or requires additional Schwann cell specific factors, as the reporter cannot be activated by cAMP through the SCE in 3T3 cells, despite the presence of a CRE related sequence in HR1 (see supplementary information). Second, it has been suggested that the timing of myelination in the PNS results from counteracting neurotrophin signalling through the neurotrophin receptors $p75^{NTR}$ and TrkC (Chan et al., 2001; Cosgaya et al., 2002). This is of particular interest as it has been

shown that signalling through $p75^{NTR}$ results in activation of NF-κβ. Recently it was demonstrated that NF-κβ is involved in the Schwann cell differentiation and that inhibition of NF-κβ results in a failure to up-regulate Oct-6 (Nickols et al., 2003). Whether NF-κβ directly regulates Oct-6 remains to be determined. No clear NF-κβ binding sites are present within the minimal SCE (HR1a and HR2b). In addition, it has been shown that the extent, and maybe the timing, of myelin formation in the PNS depends on Neuregulin-1 signalling through the ErbB2/ErbB3 heterodimeric receptor (Garratt et al., 2000; Michailov et al., 2004).

It is not known yet which transcription factors are the targets of these intracellular signals. Searching for potential transcription factor binding sites within the SCE using bio-informatics is of limited use as transcription factor DNA binding sites are notoriously degenerate. In addition, binding sites that are not represented in the database will be missed. Phylogenetic filtering for conserved binding sites has not been successful yet as the mammalian SCE sequences that have been analysed are too homologous. It is expected that inclusion of orthologous sequences from non-placental mammals such as opossum and rat kangaroo will greatly increase the power of this comparative analysis. Such comparison will prioritise potential binding sites for functional analysis in rat Schwann cells and transgenic mice.

Materials and methods

Primary Schwann cell cultures

Cultures of Schwann cells were set up from postnatal day 1 to 4 rat sciatic nerves as described before (Brockes et al., 1979; Morrissey et al., 1991) with some modification. During dissection, nerves were collected in L-15 Leibovitz. Nerves were transferred to L-15 medium containing 0.1% collagenase (Boehringer), teased by dissections needles and incubated for 30 min at 37°C by regularly pipeting up and down in order do disrupt the nerves. Collagenase reactions were stopped by washing cells in L-15, 10% FCS twice. After washing, cells were plated on PRIMARIA tissue culture dishes (Becton Dickinson) in C_B medium (Einheber et al., 1993), and incubated overnight at 37°C in presence of 5% CO_2. The next day, medium was replaced by C_B + 10μM ARA-C (Sigma). For expansion of cultures, cells were grown in proliferation medium, containing Dulbecco's modified Eagle's medium (Gibco),

3% FCS, 2μM Forskolin (Sigma) and 1% penicillin-streptomycin. Oct-6 induction is achieved by changing medium to Defined medium containing DMEM/F12 (Gibco), N2 supplement, antibiotics and + 100μM CTP-cAMP or 20μM Forskolin.

Transfection and luciferase assays

Cells were seeded in 6-well PRIMARIA plates (Becton Dickinson) and grown to 70-80% confluence. Rat Schwann cells were transfected with 1.125μg luciferase construct and 0.375μg pCMVβgal expression plasmid in the presence of fetal calf serum, using the FuGENE 6 transfection reagent (Roche). Cells were washed 18 hours after transfection, and medium was changed to DF medium + 100μM CTP-cAMP (Sigma). 72 Hours after transfection, cells were washed with PBS and lysed in Reporter lysis buffer (Promega). β-Galactosidase (β-gal) levels were measured in an activity assay using 2-nitro-phenyl-galacto-pyranoside (ONPG) substrate. Luciferase was assayed on a luminometer (TOPCount NXTv2.13, Packard) using the Steady GLO luciferase assay substrate from Promega. All the experiments were performed at least three times each in triplets.

Cloning (Plasmid constructs)

Mouse SCE sequence (4.5 kb) was amplified from a genomic subclone (129sv DNA), using primers 5'SAL SCE: ATCGCGTCGACCTTCAGGTCTCCGT GAGTAG and 3'BglII SCE: GAAGATCTGGATAAAGCCTAAAGGTTGGCCAT GAC and cloned into SalI-BglII site of pSP72 plasmid (Promega). Deletions 1 to 8 were generated by amplification of SCE-pSP72 using two primers in opposite directions:

Deletion construct	Primers
SCE Δ1	SCE1for primer (CGGAATTCGTCAAGTTCTGGGCTAAATGTAAGG) 3'BglII SCE (GAAGATCTGGATAAAGCCTAAAGGTTGGCCATGAC)
SCE Δ2	SCE1as primer (CGGAATTCAGGCTGTATCAACTATAGCTTCTTTGG) SCE2for primer (CGGAATTCCAGAATCTGGGCACAGCTGG)
SCE Δ3	SCE2as primer (CGGAATTCTCGAGCTGAGTCCTCAGGGCC) SCE3for primer (CGGAATTCAGGCAACCTCCAACCTACC)
SCE Δ4	SCE3as primer (CGGAATTCGGTCTGGCCCATCAGCCTACC) SCE4for primer (CGGAATTCCATGAAGTAGCACCAACCCTCC)
SCE Δ5	SCE4as primer (CGGAATTCAAGTTGAATATGCAGGACAGTGGC) SCE5for primer (CGGAATTCTGGCACCCCATTCTGTCTAATC)
SCE Δ6	SCE5as primer (GCGAATTCGGGAGGTTTCCAGGAGCTTGCC) SCE6for primer (CGGAATTCGGCATATGTGTTGAAGTGCATG)

SCE Δ7	SCE6as primer (CGGAATTCGAAACAGGTTTCTCTTTCTTTCCCCTC) SCE7for primer (CGGAATTCTGTCTGAAGACAGCTACAGTGTACTTAC)
SCE Δ8	SCE7as primer (CGGAATTCCCATTACAGATGGTTGTGAGCC) SCE8for primer (CGGAATTCAAGGTAGTGGGAGGGCAGAG)
SCE Δ9	SCE8as primer (CGGAATTCGTATTCGTGTGTGTCCGGCTGAC) SCE9for primer (CGGAATTCAAGAAGTGGTGAGGAGGGCC)
SCE Δ10	5'SAL SCE (ATCGCGTCGACCTTCAGGTCTCCGTGAGTAG) SCE9 as primer (CGGAATTCATCATGCTTGAAGGACTCCCCAG)

Amplified PCR products were digested with EcoRI and ligated back. Next, deletion fragments were isolated as a Sal I-Bgl II and cloned into Sal I-Bam HI sites of pGL3-promoter vector. SCE Δ11 is generated by cloning products of Sal I primer 10 (TGTCGACTTGAGGATGTCGCCTGCCCTCTAG) and EcoRI primer 11 (AGAATTCCCAGTCCTTAGCAATGAAGC) as Sal I-EcoRI fragment and SCE8for and SCE3'BglII primers as a EcoR I-Bgl II fragment into Sal I-Bam HI site of pGL3-promoter luciferase vector. Δ14SCE (HR1a) is generated by cloning product of SCE12for and SCE 12as primers as a Sal I-EcoRI into pSP72 vector. Δ15SCE (HR1a and HR2b) by cloning the Sal I- EcoRI fragment of HR1a (product of SCE12for and SCE12as primers) and EcoRI-Bgl II fragment of HR2b (generated by cloning the PCR product of primer SCE9for and 3'BglII SCE into EcoRI- Bgl II) into pGL3p vector.

Transgenic mice

The generated deletion constructs were cloned into Hsp68-LacZ vector referred as p610ZA by (Mandemakers et al., 2000) with some adaptations. DNA fragments were excised from each deletion construct using appropriate restriction enzymes and isolated from agarose gel. The isolated DNA was purified using elutip-D-mini columns (Schleicher and Schull). The DNA was dissolved in injection buffer (10mM Tris-HCl pH 7.5, 0.08mM EDTA pH 8.0) and injected into fertilised eggs derived from a FVB/N x FVB/N mating as described (Hogan, et al., 1994). Pups born at postnatal day 1 were tested for the Lac-Z expression by whole mount β-galactosidase staining and analysed for genotype by Southern blotting.

Whole mount β-galactosidase staining

Postnatal day 1 mice were sacrificed by decapitation followed by fixation in lac-Z fixative (1% Formaldehyde, 0.2% Gluteraldehyde, 2mM $MgCl_2$, 5mM EGTA,

0.02% NP40 in PBS) for 1 hour at room temperature (RT). Tissues were washed in PBS/0.02% NP40 for 3 times at RT. β-Galactosidase staining done overnight at RT in staining solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 2mM $MgCl_2$, 0.01% Nadeoxycholate, 0.02% NP40 and 1mg/ml X-gal). The staining was stopped by extensively washing in PBS/0.02% NP40 and post-fixed in a 4% formaldehyde/PBS fix.

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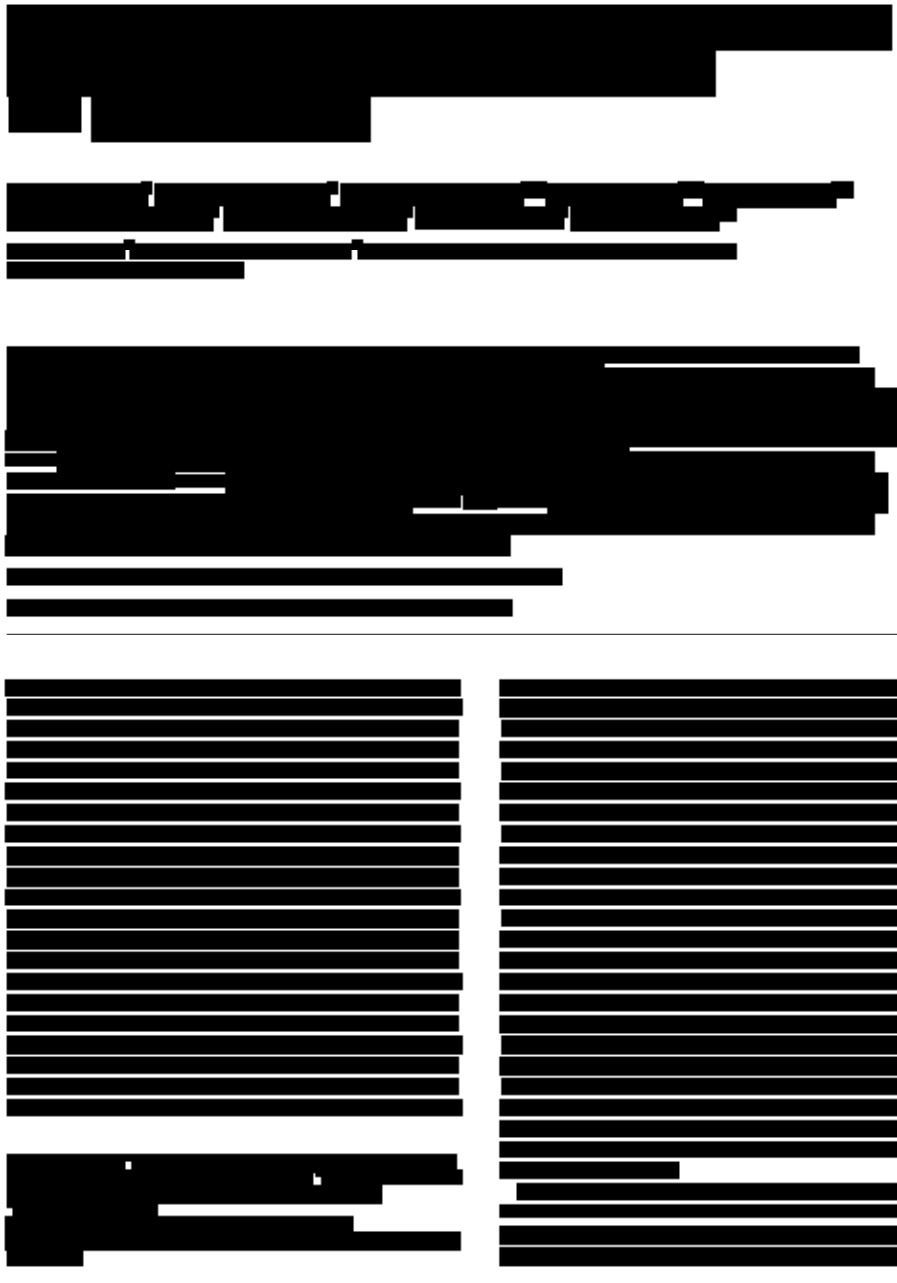
Chapter 4

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

Genes & Development 17:1380-1391, 2003



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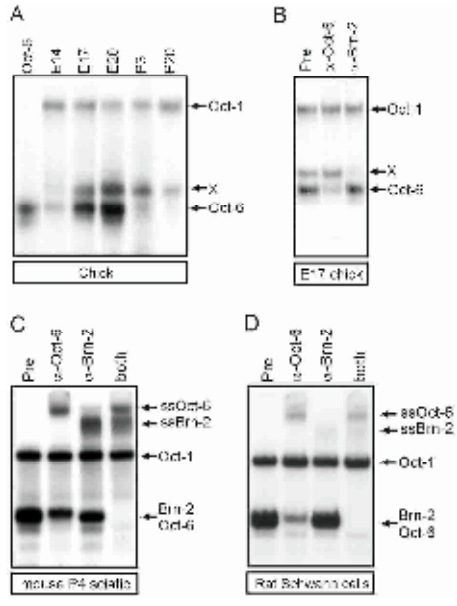

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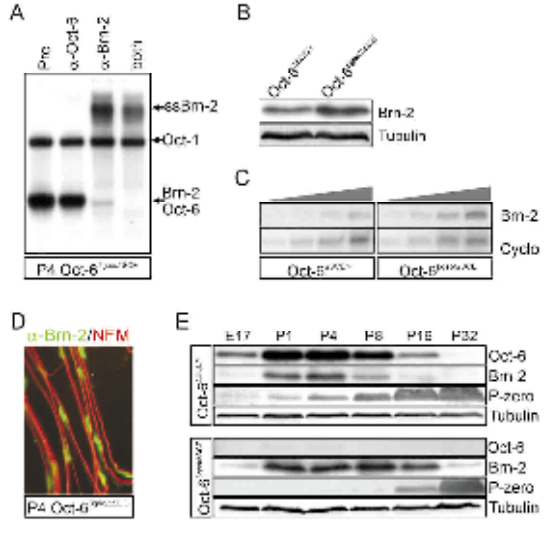
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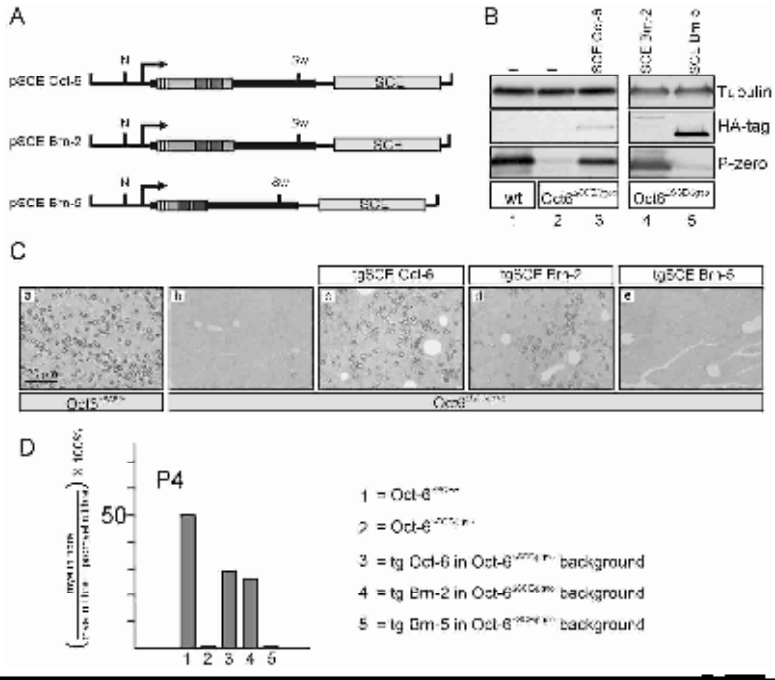
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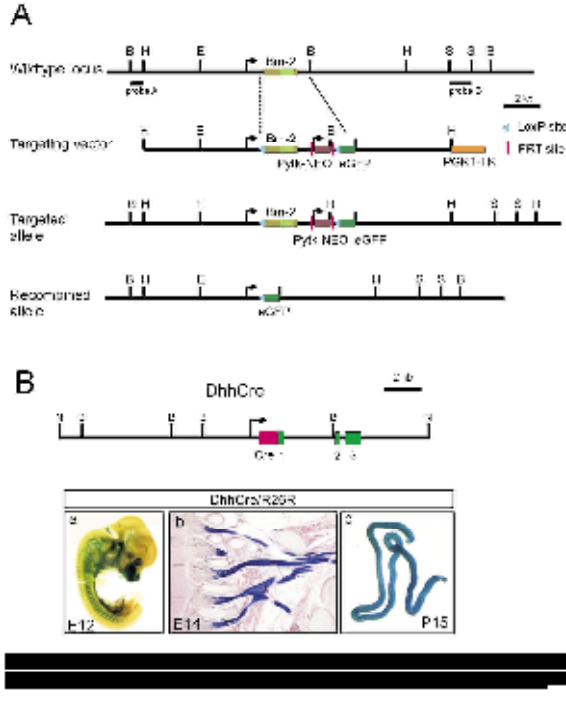

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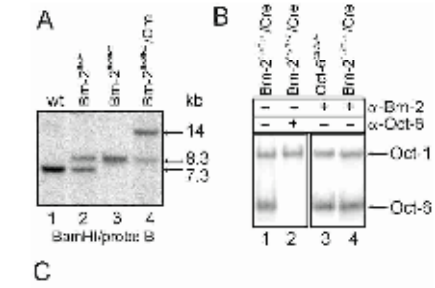

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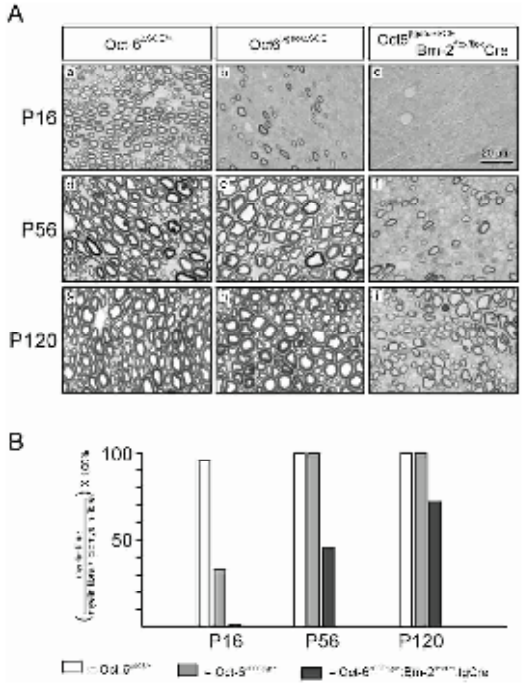

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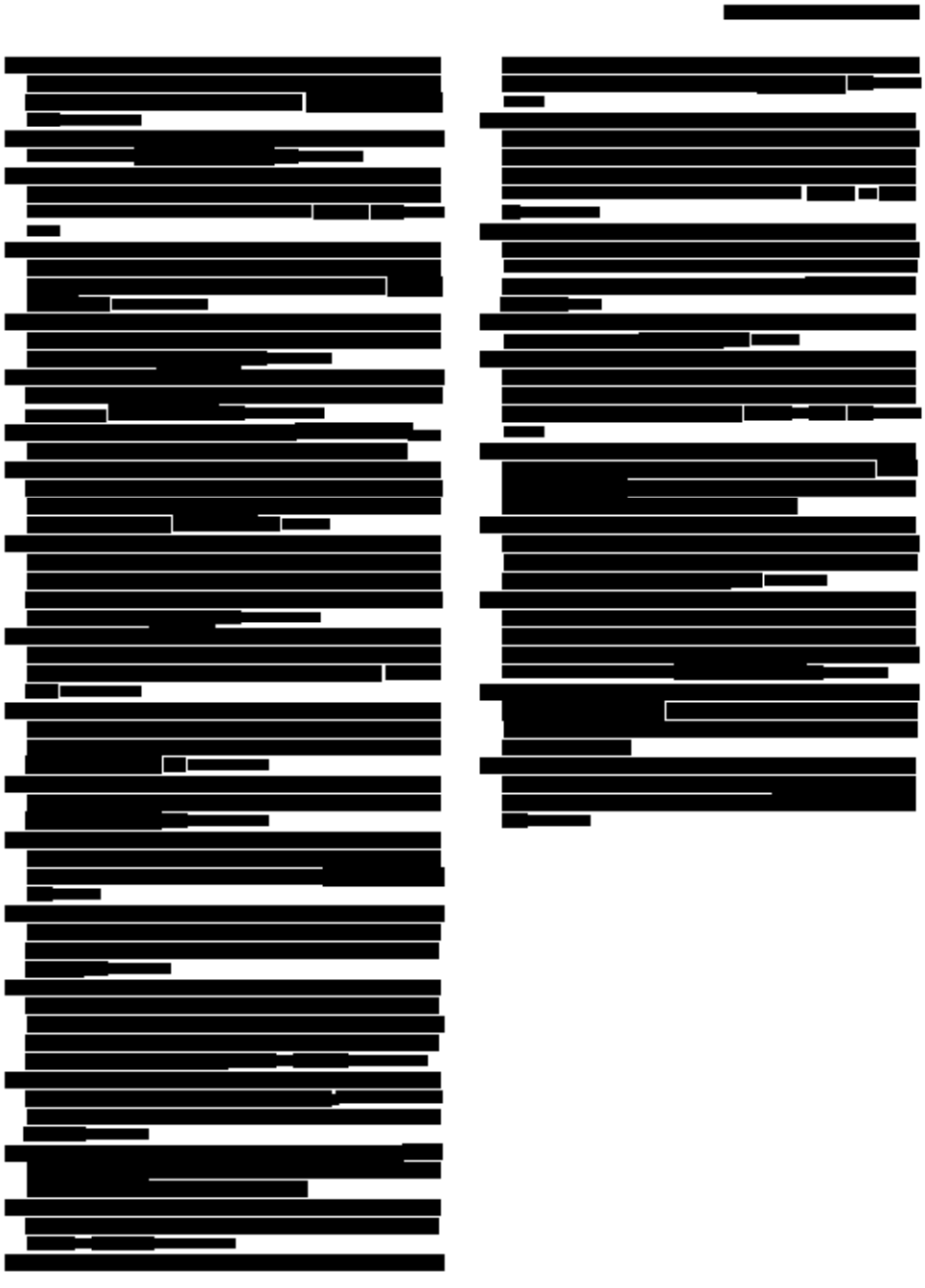

Chapter 5

Conclusions and future prospects

Although it is generally accepted that the fate decision in immature Schwann cells to adopt a myelinating or a non-myelinating phenotype results from interactions with axons, the molecular identity of these interactions and the subsequent down-stream events within the Schwann cells are still largely unknown. In the last decade, a substantial body of information has been gathered about the intracellular signaling pathways and transcription factors underlying myelination. Given the complexity of the myelination process, which involves dramatic changes in cell morphology and metabolism, it seems reasonable to expect that multiple signaling pathways are involved in this process.

Signalling pathways will converge on transcription factors involved in the gene expression program of myelination. Prime targets are the transcription factors Oct-6 and Krox20. Genetic and cell biological studies have shown that Oct-6 is the first of these two transcription factors to be strongly activated in promyelinating cells. This results in the activation of Krox20 and up-regulation of myelin gene expression (Topilko and Meijer, 2001).

In this thesis, we focus on the role of the Schwann cell specific enhancer (SCE) in regulating Oct-6 gene expression during differentiation of Schwann cells. Previous studies in our group have shown that Oct-6 SCE is sufficient for correct temporal expression of the Oct-6 gene in Schwann cells of the developing and regenerating peripheral nerve tissue. One important question, addressed in this thesis, was whether the Oct-6 SCE is also required for Oct-6 expression in Schwann cells. This question is answered in chapter 2 through the generation and analysis of mice in which the SCE is homozygously deleted. We showed that the Oct-6 SCE is the decisive cis-regulatory element governing Schwann cell specific expression. In addition, we demonstrated that the SCE does not contribute to other aspects of the Oct-6 gene expression pattern.

The peripheral nerve phenotype of Oct-6$^{\Delta SCE/\Delta SCE}$ mice is very similar to Oct-6$^{\beta geo/\beta geo}$ mice, strongly indicating that the phenotype of Oct-6 null mice is Schwann cell autonomous. As the ΔSCE allele is a Schwann cell specific hypomorphic Oct-6 allele and the peripheral nerve phenotype in Oct-6$^{\Delta SCE/\Delta SCE}$ mice is slightly less severe than in Oct-6 null mice, it is possible that the low residual amounts of Oct-6 present in the ΔSCE homozygous Schwann cells results in a moderately increased rate of Schwann cell differentiation relative to Oct-6 null Schwann cells. Therefore,

we suggested that the level of Oct-6 is important for Schwann cell differentiation. This conclusion is supported by results from another study by Weinstein and colleagues. Transgenic mice expressing a truncated Oct-6 protein (ΔSCIP) under control of the P-zero promoter in Schwann cells (Weinstein et al., 1995) show premature myelination and hypermyelination. The use of the P-zero promoter results in premature high-level expression of ΔSCIP protein in immature Schwann cells. The premature myelination, hypermyelination and frequent polyaxonal myelination all suggest that ΔSCIP functions as a dominant positive form of Oct-6 (Wu et al., 2001). Taken together, these results indicate that Oct-6 acts in a dosage-dependent manner.

In chapter 3, we focused on the structure of the Oct-6 SCE. Through pairwise sequence comparison of SCE orthologous sequences in the genomes of human, mouse, dog and rat, we identified two highly conserved regions within the boundaries of Oct-6 SCE. Using a Schwann cell transfection assay we demonstrated that homology region 1 (HR1) acts as a strong enhancer while homology region 2 (HR2) exhibits a weak enhancer activity in vitro. We also demonstrated that the major enhancer activities are contained within the HR1a and HR2b sub-regions. In contrast, neither HR1a nor HR2b exhibit any Schwann cell specific enhancer activity in transgenic animals. However, the combination of the two elements shows full SCE activity.

At present, we do not know what transcription factors bind to these elements and how they interact to form a functional enhancer. However, preliminary DNaseI footprinting experiments, using protein extracts of differentiated Schwann cells, indicated the presence of several putative binding sites for transcription factors within the HR1a sequence. The combined approach of DNAse I footprinting and using bioinformatics should produce candidate transcription factors whose relevance should study in details.

We suggest that Oct-6 SCE consists of at least two separate modules that might independently or interdependently bind to PIC (Fig. 1). Most likely, each module includes multiple binding sites to recruit specific regulatory nucleoprotein complexes and architectural proteins, like in many other eukaryotic enhancers (Carey, 1998). What are the contributions of HR1a and HR2b to full enhancer activity? The current view for transcription of any gene is that diverse regulatory

cues integrate at the promoter to adjust the level of RNA transcription (Goodrich et al., 1996). It might be expected that different signaling pathways involved in the initiation of the myelination program, converge on these separate modules and activate Oct-6 gene expression, which in turn regulates the onset of myelination. In recent years, several signaling molecules and their receptors have been implicated in myelination of the PNS, including the neuregulin, adenosine triphosphate, steroid hormones, and the neurotrophins BDNF and NT3.

Several lines of evidence suggest that neurotrophins, in particular BDNF and NT3, have important roles not only during Schwann cell development (cell survival and growth) but also in myelination (Meier et al., 1999). It is known that BDNF and NT3 can bind to different receptors from the tropomyosine-related kinase (Trk) family of tyrosine kinase receptors (Roux and Barker, 2002) and p75, the low affinity neurotrophin receptor ($p75^{NTR}$), a member of the TNF receptor superfamily (Baker and Reddy, 1998). It is assumed that during Schwann cell proliferation, NT3 activation of TrkC inhibits myelination and at the same time enhances Schwann cell migration. TrkC induces the migration of the Schwann cells through the Rac1/ Cdc42/c-jun N-terminal kinase signaling pathway (Yamauchi et al., 2003). At the onset of myelination, NT3 expression is down-regulated, while BDNF continues to be expressed during myelination (Cosgaya et al., 2002). It has been demonstrated that activation of $p75^{NTR}$ by BDNF, prior to myelination, has two striking effect on promyelinating Schwann cell. First, the interaction of BDNF and $p75^{NTR}$ stimulates the Scr kinase-dependent activation of guanine-nucleotide exchange factors Vav2 and RhoA, leading to inhibition of Schwann cell migration (Yamauchi et al., 2004). Second, this binding enhances myelination (Cosgaya et al., 2002). Schwann cells express $p75^{NTR}$ during development up to myelination, but this receptor is rapidly down-regulated at the onset of myelination. Therefore, it is proposed that $p75^{NTR}$ is required for the initiation of myelination but not myelination itself (Hempstead and Salzer, 2002). It is likely that $p75^{NTR}$ also plays a role in re-myelination following nerve injury, as it is rapidly up-regulated in reactive Schwann cells (Hempstead and Salzer, 2002). The signaling events associated with myelination down-stream of $p75^{NTR}$ are poorly understood. It is likely that $p75^{NTR}$ interacts with down-stream signaling elements through interaction with TNFR-associated factors (TRAFs). It has been demonstrated that some TRAFs link the TNF receptors to NF-κB signaling

(Bradley and Pober, 2001). Therefore, NF-κB is an attractive candidate regulator of Schwann cell myelination. Indeed, compelling evidence has been provided to suggest that NF-κB is involved in Schwann cell differentiation and possibly myelination (Nickols et al., 2003). Inhibition of NF-κB in neuron-Schwann cell co-cultures or DRG explants of $p65^{-/-}$ mice, show a significant decrease in myelination and an arrest of Schwann cell differentiation at the promyelin stage. However, it remains unclear what the exact role of NF-κB is in myelin formation. Nickols and colleagues (2003) also demonstrate that in the absence of NF-κB, Oct-6 is not up regulated in promyelinating Schwann cells. It is therefore possible that Oct-6 is a direct target of NF-κB. If so, one would expect to find NF-κB binding sites within the Oct-6 SCE, in particular within the regions defined in this thesis, namely HR1a and HR2b. However, we could not find any potential NF-κB binding site within HR1a and HR2b, using the Mathinspector or Transfac algorithms. Furthermore, a constitutively active form of NF-κB does not activate a SCE reporter construct (unpublished observations). Therefore the link between NF-κB and Oct-6 in regulating myelination remains unclear.

Neuregulin-1 (Nrg-1) is not only involved in early survival of Schwann cell precursors but also provides, at later stages of development, a signal for the regulation of myelin sheath thickness (Michailov et al., 2004). It has been known for a long time that myelin sheath thickness (the number of myelin wraps) and axonal diameter correlate positively (Friede and Samorajski, 1967). Indeed, axonal cues determine the number of myelin wraps produced by oligodendrocytes or Schwann cells (Fanarraga et al., 1998). Using a transgenic approach, it was demonstrated that the amount of axonal Nrg-1 type III determines the number of wraps of Schwann cells around the axon (Michailov et al., 2004). Neuronal overexpression of Nrg-1 causes hypermyelination of axons in vivo in transgenic animals. Furthermore, absence of ErbB2 expression in homozygous ErbB2 animals results in reduced numbers of myelin wraps. Thus, neuregulin-1 signaling is involved in the number of myelin wraps produced. It is not clear whether neuregulin-1 is also involved in the initiation of myelination. Previously, it has been shown that neuregulin-1 type III is required for survival of Schwann cell precursors during embryonic development (Garratt et al., 2000a; Garratt et al., 2000b; Lemke, 1996). In contrast, neuregulin induces demyelination and dedifferentiation of Schwann cells in myelinating

sensory neuron-Schwann cell co-cultures (Zanazzi et al., 2001). It appears that the effect of neuregulin-1 is strongly context dependent. One explanation of this phenomenon has been provided by Colognato and colleagues who suggest that the effect of Nrg-1 is modified by integrin signaling (Colognato et al., 2002). Their model suggests that interaction of Nrg-1/ErbB2 and integrins leads to activation of MAPK signaling for the Schwann cell survival, while interaction of Nrg-1 and ErbB receptors activate the phophatidyl-inositol-3-phosphate (PI3) kinase and induce differentiation.

A third signal potentially involved in regulating the onset of myelination in the PNS is provided by electric activity of the axon. (Stevens et al., 1998). Some studies have shown that the low frequency impulse (0.1 Hz) activity of axons inhibits myelination, while higher impulse activity (1 Hz) has no effect (Stevens et al., 1998). In vitro studies showed that lower stimulation activity of axons decreases expression of the cell adhesion molecule L1-CAM. Previously, it was suggested that L1-CAM has a critical role in initiation of myelination (Seilheimer et al., 1989a; Seilheimer et al., 1989b; Wood et al., 1990). It is also suggested that during Schwann cell development, impulse activity might play a role in the onset of myelination, and that this effect is mediated through adenosine triphosphate (ATP) (Stevens and Fields, 2000). ATP activates the purinergic receptors expressed on the cell membrane of Schwann cells. As a result, Ca2+ is released from its intracellular stores, activating Ca2+/calmodulin kinase and MAP kinase. It is believed that these signals postpone the differentiation of Schwann cells until they become exposed to appropriate axon-derived signals (Stevens and Fields, 2000).

Another signaling pathway important for axonal ensheathment and myelination is laminin-2 and its receptor β1 integrin. It has been shown that β1 integrin in immature Schwann cells is important for ensheathing axons in a 1:1 manner and to progress to promyelinating stage. Disruption of β1 integrin specifically in Schwann cells leads to inhibition of radial sorting in immature Schwann cells (Feltri et al., 2002).

Taken together, the components of one or more pathways mentioned above can potentially be involved in regulation of Oct-6 through the Oct-6 SCE. Identification of the nuclear factors that bind to HR1a and HR2b will be instrumental

in unraveling the regulatory network up-stream of the Oct-6 transcription factor leading to onset of myelination.

To realize this aim it will be necessary to further narrow down the critical regions within the SCE by generating further deletions in the context of the HR1a and HR2b in a luciferase reporter construct. The fragments with significant enhancer activity might then be used in a DNA pull-down assay using biotin tagged dsDNA oligonucleotides and identification of bound proteins by mass spectrometry.

In a parallel approach, biochemical experiments such as DNaseI footprinting are required to find out what the potential protein binding sequences are and which putative factors bind to these sequences. The role of these potential binding sites could be investigated by mutagenesis. Cultured rat Schwann cells allow us to test the functionality of these mutated sites in the context of HR1a and HR2b, coupled to a luciferase reporter gene. Subsequently, constructs with a critical binding sequence can be verified in vivo by mutagenesis or deletion through homologous recombination in mice. Moreover, these sequences might be used to identify the factors that bind to them by screening of a Schwann cell cDNA library in a yeast one-hybrid system.

In another approach, similar to the one described in the previous paragraph, one could generate a double strand oligonucleotide chip, on which overlapping fragments of the Oct-6 SCE sequence are spotted and determine which DNA fragments are bound by nuclear factors derived from P1 nerves or cultured Schwann cells. In principle, this approach would allow identification of all the proteins expressed in the Schwann cells nucleus, including activators and repressors, and thus potentially involved in the regulation of the Schwann cell specific expression of the Oct-6 gene.

Further investigation of transcription factors up-regulated in Schwann cells between E18 and the day of birth, using a luciferase reporter assay might lead to identification of specific factors binding to HR1a and HR2b elements.

Another interesting question is how HR1a and HR2b elements activate transcription from the Oct-6 promoter. Using a transgenic mouse approach, described in chapter 3, we demonstrated that both HR1a and HR2b are required for enhancer activity in transgenic mice. It appears that the distance between these two elements has no dramatic effect on enhancer function, since in our transgenic

constructs these two elements are joind. At present we suggest that nucleoprotein complexes binding to HR1a and HR2b might either interact independently with components of the preinitiation complex (PIC) at the promoter (Fig 1A) or interdependently, through formation of a holocomplex (Fig. 1B).

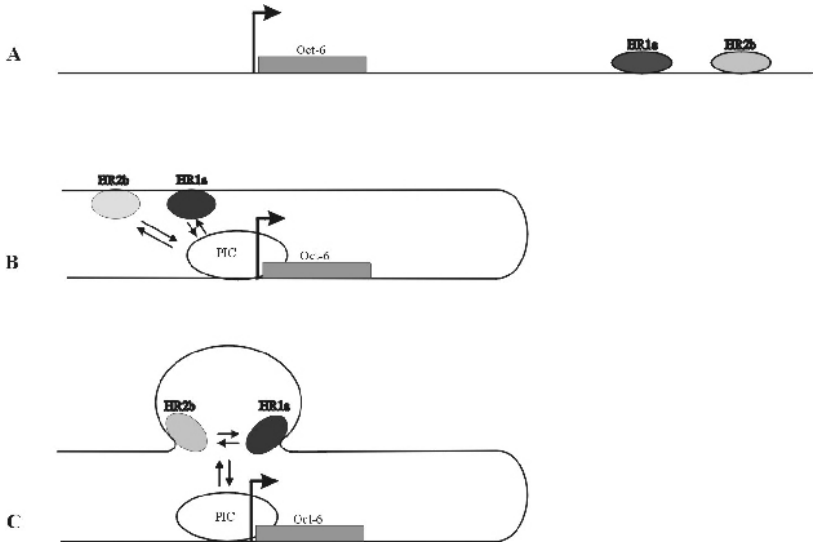

Figure 1- Schematic presentation of different mechanisms for activation of Oct-6 gene expression through HR1a and HR2b.

5.1 Identifying Oct-6 redundant factor

In chapter 4, we demonstrated that the class III POU domain protein Brn-2 functionally overlaps with Oct-6 during Schwann cell development. This conclusion is derived from two different genetic experiments. First, we showed that over-expression of Brn-2, under control of the Oct-6 SCE, in Oct-6 deficient Schwann cells resulted in an increase of the number of myelinated Schwann cells, partially rescuing the Oct-6 mutant phenotype. Second, Schwann cell-specific deletion of Brn-2 in an Oct-6 deficient background resulted in an increase in severity of the delayed hypomyelinating phenotype. Although we concluded that Brn-2 shares a role with Oct-6 during Schwann cell development, we observed that a quantitatively higher amount of Brn-2 protein is required to initiate myelination on schedule. This difference in biological function of Brn-2 and Oct-6 could arise from differences in affinity for transcriptional cofactors or other transcription factors as discussed

in chapter 4. In particular it has been suggested that a specific POU/Sox protein combinatorial code exists (Kuhlbrodt et al., 1998). Since the cooperative activation of a synthetic reporter construct by Oct-6 and Sox10 requires the N-terminal domain of both proteins, it will be of interest to investigate whether swapping the amino-terminal portions of Oct-6 and Brn-2 is sufficient to confer Sox10 cooperativity on Brn-2. This is of particular interest as Sox10 is strongly expressed in the Schwann cell lineage. Transgenic constructs in which the amino-terminal portions of the Oct-6 and Brn-2 protein are swapped have been generated in our group and are currently crossed into the Oct-6$^{\Delta SCE/\Delta SCE}$ background to study their ability to rescue the delayed myelination phenotype.

Another possible explanation for the difference in biological function between Oct-6 and Brn-2 is that Brn-2 function is modified or inhibited through interaction with another protein that does not interact with Oct-6. Such a protein has been described for Brn-2. This protein, which is called "poly-glutamine tract binding protein" or PQBP-1, was shown to interact with Brn-2 through its amino-terminal glutamine stretch of 23 residues. This interaction inhibits Brn-2's ability to activate an octamer containing reporter construct (Waragai et al., 1999). It is thus suggested that binding of PQBP-1 to polyglutamine tracts of regulatory proteins, including Brn-2, modulate transcription of target genes during differentiation. It will be of interest to investigate the role of PQBP-1 and its interaction with Brn-2 during Schwann cell differentiation. Study of the expression level of PQBP-1 by semi-quantitative RT-PCR has shown that indeed PQBP-1 is expressed in Schwann cells. However, no difference in the level of expression of the PQBP-1 gene in Oct-6 deficient Schwann cells is observed (M. Jaegle, unpublished data). If PQBP-1 does play a role in modulating Brn-2 activity it is anticipated that overexpression of PQBP-1in Schwann cells will result in a more severe Oct-6 mutant phenotype. Since both Oct-6 and Brn-2 are similarly expressed during Schwann cell development, it is possible that both of these genes are regulated through the same signaling pathways. The Schwann cell specific regulatory sequences in the Brn-2 locus are unknown. There is no obvious sequence homology between Oct-6 and Brn-2 outside the POU domain. Potential regulatory sequences could first be identified through phylogenetic sequence comparison and subsequently tested for activity in transgenic mice or in cultured rat Schwann cells.

5.2 Schwann cell specific regulatory elements

To date, several Schwann cell specific cis-regulatory elements has been identified including the Oct-6 SCE, the subject of this thesis, Krox-20 SCEmse (Krox-20 myelinating Schwann cell element) (Ghislain et al., 2002), MbpSCE1 (Forghani et al., 2001; Taveggia et al., in press), and a 10 kb region of the PMP22 (Maier et al., 2002).

Work described in this thesis demonstrated that the Oct-6 SCE is required for Oct-6 gene expression in Schwann cells during myelination and regeneration. One potential target of Oct-6 regulation is Krox20. Recently, Ghislain and colleagues have identified the cis-acting sequences that regulate Krox-20 expression during myelination and regeneration in an axon dependent manner (Ghislain et al., 2002). Interestingly, they identified multiple candidate Oct-6 binding sites within the Krox-20 MSE by biochemical studies. These results indicate that Oct-6 might directly regulate transcription of Krox-20 (Ghislain et al., 2002). However, in chapter 2 we showed that Krox-20 expression is activated through an Oct-6 independent mechanism in Schwann cells of Oct-6$^{\Delta SCE/\Delta SCE}$ mice, albeit with a delay. Although there is no evidence that the delayed activation of Krox-20 is mediated through this element, it is possible that another POU factor, related to Oct-6, can occupy Oct-6 binding sites in the Krox-20 enhancer and activate the Krox-20 gene. Previously, it was suggested that Brn-5, a class VI POU domain protein, might be a potential candidate as Oct-6 redundant factor (Wu et al., 2001). However, we demonstrated that overexpression of Brn-5, under control of the Oct-6 SCE, in Oct-6 deficient Schwann cells is not able to rescue the differentiation delay.

A recent publication about the mouse claw paw (clp) mutation indicates the existence of multiple parallel pathways for initiation of myelination in Schwann cells (Darbas et al., 2004). Similar to Oct-6 null mice, mice homozygous for the clp allele show a delay in initiation of myelination in the peripheral nerves. Although clp/clp Schwann cells express Oct-6 at high level, the expression of Krox-20 is delayed.

Krox-20 plays an important role in the up-regulation of myelin genes including P-zero, MBP and PMP22 (Nagarajan et al., 2001; Topilko et al., 1994). Schwann cells of Krox-20$^{-/-}$ animals show a strong reduction of P-zero, MBP, and

PMP-22 expression. In Oct-6 SCE deficient Schwann cells, P0 level is upregulated after activation of Krox-20 (Ghazvini et al., 2002). In addition, in vitro studies have shown that Krox-20 transactivates the P0 promoter (Zorick et al., 1999). Surprisingly, mutation analysis demonstrated that none of the potential Krox-20 binding sites in the MbpSCE1 are necessary for activation of a Lac-Z reporter gene in the peripheral nerves of transgenic animals (Taveggia et al., in press; However, these experiments do not rule out a role for Krox-20 acting through non-consensus DNA binding sites. Another possibility is that additional regulatory elements, containing Krox-20 binding sites, are involved in activation of the Mbp gene, as Forghani and colleagues have suggested (2001).

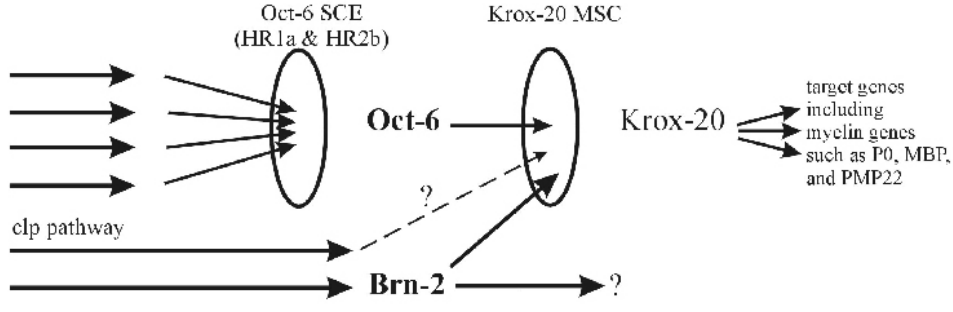

Figure 2- Model of regulatory cascade of myelination.

In summary, intracellular pathways involved in myelination converge on HR1a and HR2b elements located within the Oct-6 SCE and activate expression of Oct-6. Subsequently, Oct-6 regulates the expression of its down-stream target genes including Krox-20. Oct-6 probably acts on the Krox-20 MSE and up-regulates Krox-20 expression. Krox-20 can also be activated through an Oct-6 independent mechanism as in clp Schwann cells. Although Oct-6 appears to be required for Krox-20 activation, it is not sufficient for the activation of Krox-20 and myelination as it seen in clp animals. Krox-20 is involved in the regulation of a set of genes involved in myelination, including myelin genes P-zero, MBP, and PMP22. The Oct-6 redundant factor Brn-2 is a positive regulator of myelination. Brn-2 and Oct-6 expression are regulated independently.

5.3 References

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Summary

The reciprocal interactions between Schwann cells and their associated axons are important during development, maintenance, and regeneration of the peripheral nervous system. Schwann cells arise from the neural crest cell and develop through different transitional stages to non-myelinating and myelinating Schwann cells found in the mature peripheral nerves. In recent years, evidence has accumulated to suggest that in particular two transcription factors play pivotal roles in regulation of the myelination program. These transcription factors are Oct-6, and Krox-20. Genetic studies have shown that the POU domain transcription factor Oct-6 is a major regulator of Schwann cells assuring the timely progression of cell differentiation from the promyelinating to myelinating stage. Additionally, it was shown that expression of Oct-6 in Schwann cells is under control of axonal signals, which ultimately converge on the Oct-6 Schwann cell specific regulatory element (Oct-6 SCE). The Oct-6 SCE is sufficient to drive the correct expression of the Oct-6 gene or a reporter gene, during development or regeneration. Consequently, Oct-6 regulates a set of down-stream genes, including the zinc-finger protein Krox-20. Homozygous deletion of Oct-6 or Krox-20 in mice leads to arrest of Schwann cells at the promyelin stage. While the differentiation block of Oct-6 deficient Schwann cells is transient, this block is permanent in Krox-20$^{-/-}$ Schwann cells.

In this thesis, we demonstrated that the Oct-6 SCE is not only sufficient, but also required for correct spatial and temporal expression of Oct-6 in the Schwann cell lineage. Moreover, our results demonstrate that Oct-6 acts in a dose dependent mechanism and that Oct-6 function is required in the Schwann compartment of the developing nerve.

Our structural analysis of the SCE identified two regions that together function as an enhancer of the Oct-6 gene in vitro and in vivo. In our transgenic studies we showed that both these elements are required for full enhancer activity. Therefore, we suggested that Oct-6 SCE enhancer activity depends on the interdependent interaction of these elements with the basal transcription factors assembled at the promoter.

Further, we identified Brn-2, a POU domain transcription factor closely related to Oct-6, as an Oct-6 redundant factor in Schwann cell development. We

showed that Brn-2 expresses in Schwann cells in a similar developmental profile as Oct-6. While overexpression of Brn-2, under control of the Oct-6 SCE, can partially rescue the developmental delay of Oct-6 deficient Schwann cells, ablation of Brn-2 and Oct-6 in Schwann cells results in a more severe phenotype.

The studies presented in this thesis, deepen our insight in the molecular mechanisms of myelination and the role Oct-6 plays in this important and fascinating cellular differentiation process. It is hoped that this deepening of our understanding of the molecular processes that underlie nerve development and homeostasis will one day result in the development of rational strategies to combat the many debilitating diseases of the peripheral and central nervous system.

Nederlandse samenvatting

Meercellige organismen ontwikkelen zich uit één enkele cel, namelijk de bevruchte eicel. De eicel vermenigvuldigt zich gedurende de embryonale ontwikkeling. Celdeling leidt tot het ontstaan van een groot aantal cellen die vervolgens verschillende karakteristieke functies aannemen in een proces dat celdifferentiatie heet. Celdifferentiatie zorgt voor het ontstaan van een grote variatie van cellen, zoals bloedcellen, spiercellen, zenuwcellen, huidcellen, levercellen enz.

De genetische informatie die nodig is voor het bouwplan en het functioneren van de cellen is opgeslagen in het DNA in de vorm van genen (het genoom). Elke cel bevat hetzelfde DNA, maar door activiteit van een bepaalde set genen en onderdrukken van andere genen, ontstaan verschillen in de functie van cellen. Activiteit van een gen houdt in dat door een heel gereguleerd proces, een gen kan worden afgelezen (komt tot expressie). Hierbij ontstaat een RNA molecuul (transcriptie) dat vervolgens omgezet wordt in een eiwit (translatie). Eiwitten zijn de functionele onderdelen van de cel. Het is van belang dat elke cel de juiste set van genen op het juist moment vertaalt naar eiwitten. Dit wordt verzorgd door (eigen) controle-mechanismen in elk celtype. Een deel van de celcontrole-mechanismen wordt uitgevoerd door transcriptie factoren. Transcriptie factoren zijn speciale eiwitten die specifiek op bepaalde plaatsen aan het DNA kunnen binden en zorgen dat een gen afgelezen kan worden of juist niet.

Het onderzoek dat in dit proefschrift wordt beschreven, is gericht op de rol van een transcriptie factor genaamd Oct-6. Het Oct-6 gen komt tot expressie in verschillende cel types zoals bepaalde zenuwcellen in de hersenen, haar follikels en in Schwann cellen. Schwann cellen groeien naast de zenuwbanen (axonen) in het perifere zenuwstelsel en vormen een isolerende vetlaag rond de axonen. Deze vetlaag heet de myeline schede. De aanwezigheid van deze myeline schede is belangrijk voor het sneller geleiden van zenuw signalen in de vorm van elektrische impulsen. De aanmaak van de myeline schede is afhankelijk van contact met axonen. Echter, niet alle axonen in het perifere zenuwstelsel zijn gemyelineerd. Axonen met een kleinere diameter dan 1 μm zijn weliswaar geassocieerd met Schwann cellen, maar ze worden niet gemyelineerd. Het is niet bekend hoe de axonen Schwann cellen kunnen instrueren voor de aanleg of het niet aanlegen van de myeline schede. Het is wel bekend dat beschadiging of instabiliteit van de myeline schede leidt tot

een verslechterde geleiding van signalen langs deze zenuwbanen. Dit resulteert in ernstige neurologische problemen. Bekende voorbeelden van ziektes waarbij de myeline schede is aangedaan zijn onder andere de auto-immuun ziektes multiple sclerose (MS) en Guillain-Barré of de genetische afwijkingen Charcot-Marie-Tooth (CMT), Dejerine Sottas en Perlizaeus-Merzbacher.

Er is aangetoond dat een tijdige aanwezigheid van het Oct-6 eiwit in Schwann cellen belangrijk is voor het tijdig vormen van de myeline schede. Een van de centrale vragen in dit proefschrift is hoe het Oct-6 gen wordt gereguleerd om op het juiste moment in Schwann cellen actief te zijn. Voorafgaande experimenten binnen onze onderzoeksgroep hebben laten zien dat binnen enige afstand van het Oct-6 gen een gebied van 4000 base paren (bp), genaamd de Oct-6 Schwann Cell Enhancer (SCE), belangrijk is voor de expressie van het Oct-6 gen. Dit element is in staat om een reporter gen (dat een zichtbare blauwe kleur kan vormen) in Schwann cellen tot expressie te brengen in dezelfde periode en met hetzelfde patroon van het Oct-6 gen. Dit suggereert dat de expressie van het Oct-6 gen in Schwann cellen gecontroleerd wordt door dit element.

De proeven beschreven in hoofdstuk 2, laten zien dat de SCE inderdaad het regulerende element is voor de aanwezigheid van het Oct-6 eiwit op het juiste moment in Schwann cellen maar niet in andere type cellen waar Oct-6 wel tot expressie komt. Tevens is de SCE ook betrokken bij expressie van het Oct-6 gen tijdens regeneratie. Dit maakt de SCE aantrekkelijk met het oog op de ontwikkeling van toekomstige therapieën in ziektes die ontstaan door een defect gen in de Schwann cellen.

In hoofdstuk 3, hebben wij verder gezocht welk gedeelte binnen de SCE belangrijk is voor het binden van transcriptie factoren die vervolgens het Oct-6 gen expressie in Schwann cellen op tijd kunnen aan- en uitschakelen. Voor deze proeven hebben we drie verschillende benaderingen gekozen. Als eerste hebben we de DNA sequentie van de SCE in muizen vergeleken met die van de mens en de rat in een specifiek computer programma. Aangezien de sequentie van genen en de belangrijke regulator elementen tijdens de evolutie geconserveerd zijn gebleven, is het mogelijk dat een geconserveerde regio binnen de SCE sequentie op zulke elementen duiden. Binnen de SCE hebben we inderdaad twee zeer geconserveerde regios gevonden, genaamd HR1 en HR2. Verder hebben we de functie van deze

twee regios getest in onze gekweekte Schwann cellen met gebruik van een in vitro (op een schaal) experiment en in transgene muizen (in vivo). Met deze experimenten hebben we laten zien dat HR1 en HR2 belangrijke sequenties bevatten voor het binden van transcriptie factoren, die vervolgens het Oct-6 gen activeren. Verder hebben we verschillende deletie constructen gemaakt door de steeds weglaten van 500 base paren van de SCE sequentie in een construct inclusief een reporter gen. Vervolgens konden we de activiteit van deze deletie constructen in onze gekweekte Schwann cellen testen door te kijken naar de expressie van het reporter gen Uit deze proeven zijn twee regios naar voren gekomen die belangrijk zijn voor de activiteit van het reporter gen. Deze twee regios vallen binnen de voorgaande geïdentificeerde HR1 en HR2. Vervolgens hebben we de functie van deze twee kleinere fragmenten getest in transgene muizen en geconcludeerd dat deze twee regio samen nodig zijn voor de expressie van het Oct-6 gen in Schwann cellen.

In gewervelde dieren vormen Schwann cellen de myeline schede na de geboorte. Voorafgaande experimenten binnen onze groep en andere groepen hebben aangetoond dat de Oct-6 transcriptie factor belangrijk is voor de juiste timing van de aanleg van de myeline schede. In afwezigheid van het Oct-6 eiwit in een proefdier model, zoals de muis, wordt de myeline schede met twee weken vertraging gevormd. Hier ontstaat de vraag waarom in afwezigheid van Oct-6, Schwann cellen uiteindelijk alsnog de myeline schede kunnen aanleggen. Het is aannemelijk dat een ander eiwit in Schwann cellen aanwezig is die de functie van het Oct-6 eiwit kan overnemen. De experimenten die in hoofdstuk 4 staan beschreven hebben aangetoond dat een andere eiwit, Brn-2, inderdaad na enige tijd de functie van Oct-6 in de Schwann cellen overneemt. Wij hebben laten zien dat een verhoogde expressie van het Brn-2 eiwit in Schwann cellen van een muis met een defect Oct-6 gen, de Schwann cellen gedeeltelijk eerder beginnen met de myeline aanleg vergeleken met Oct-6 deficiënte Schwann cellen. Verder, het inactief maken van het Oct-6 en het Brn-2 gen in Schwann cellen van muizen met behulp van genetische technieken (homologe recombinatie) leidt tot veel ernstigere effecten op Schwann cellen; zelfs na 3 maanden is de formatie van de myeline schede niet compleet. Dit experiment laat zien dat Brn-2 inderdaad de functie van Oct-6 in Schwann cellen kan overnemen.

In het laatste hoofdstuk bediscussiëren we verschillende signalering routes die kunnen leiden tot Schwann cellen een myelinerende lot nemen.

Aan de hand van een model suggereren we hoe de geïdentificeerde HR1a en HR2b elementen op DNA niveau de Oct-6 gen expressie kunnen reguleren. Vervolgens hebben we verschillende vervolg-experimenten besproken voor het voorzetten van dit onderzoek.

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List of publications:

Ghazvini M, Mandemakers W, Jaegle M, Piirsoo M, Driegen S, Koutsourakis M, Smit X, Grosveld F, Meijer D. A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration. EMBO J. 2002 Sep 2;21(17):4612-20.

Jaegle M, Ghazvini M, Mandemakers W, Piirsoo M, Driegen S, Levavasseur F, Raghoenath S, Grosveld F, Meijer D. The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. Genes Dev. 2003 Jun 1;17(11):1380-91.

Dankwoord

Eindelijk ben ik dan zover; de laatste pagina van mijn boekje. Een heleboel dingen kan ik over de afgelopen jaren vertellen. De barbecue traditie van het lab in de afgelopen jaren, de Oct-6 dag, …

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