On the role of Oct-6 in Schwann cell differentiation

(De rol van Oct-6 tijdens Schwann cel differentiatie)

Proefschrift

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Wilhelm Johannes Mandemakers

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Promotor:	Prof. Dr. D. Bootsma
Promotor:	Prof. Dr. F.G. Grosveld
Overige leden:	Prof. Dr. C.I. De Zeeuw
	Prof. Dr. C.P. Verrijzer
	Dr. A.W.M van der Kamp
Co-promotor:	Dr. Ir. D.N. Meijer

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Ter nagedachtenis aan mijn vader

Voor mijn moeder

Voor Manja

Contents

List of abbreviations

A	Adenine
A-P	Anterior-Posterior
ARIA	Acetylcholine Receptor Inducing Activity
ATPase	Adenosine Triphosphatase
Ax	Axon
BDNF	Brain Derived Neurotrophic Factor
BL	Basal Lamina
BMP	Bone Morphogenetic Protein
C	Cytosine
Ca^{2+}	calcium ion
cAMP	cyclic Adenosyl Monophosphate
Caspr 1	Contactin associated protein 1
cDNA	complementary DeoxyriboNucleic Acid
CDR	Coding Region
clp	claw paw
CMT	Charcot Marie Tooth
CNP	2', 3'-cyclicnucleotide 3'phosphodiesterase
CNTF	Ciliary-Neurotrophic Factor
CNS	Central Nervous System
CpG	Cytosine - Guanine pair
CT-1	Candiotrophin-1
D-V	Dorsal-Ventral
Dhh	Desert hedgehog
dm	dermatome
DNA	DeoxyRibonucleic Acid
dom	dominant megacolon
DRG	Dorsal Root Ganglion
DSS	Dejerines-Sottas Syndrome
dpp	decapentaplegic
E	Embryonal day
EGF	Epidermal Growth-like Factor
Ep	Epineurium
epi	epidermis
En	Endoneurium
FACS	Fluorescence Activated Cell Sorter
FGF	Fibroblast Growth Factor
G	Guanine
Galc	Galactocerebroside
GAP43	Growth associated Protein 43
GNDF	Glial Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GFR	GDNF Family Receptor
GGF	Glial Growth Factor
HNPP	Hereditary Neuropathy with liability to Pressure Palsies
Hox	Homeobox
HSP	Heat Shock Protein
HSS	Hyper Sensitive Site
icl	inner collar
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IL-6	Interleukin 6
K^+	potassium ion
L	Lumbal
LI	Immunoglobulin related adhesion molecule
LacZ	β-galactosidase
LIF	Leukaemia Inhibitory Factor
MAG	Myelin Associated Glycoprotein
Mb	Mega base
MBD	Methyl-CpG-Binding Domain
MBP	Myelin Basic Protein
µm	micrometer (10^{-6} meter)
mm	millimeter (10^{-3} meter)
mV	milliVolt (10^{-3} Volt)
my	myelin
N	node of Ranvier
Na^{2+}	sodium ion
NAB	NGFI-A-Binding Protein
nc	neural crest
NDF	Neu Differentiation Factor
N-CAM	Neural Cell Adhesion Molecule
neur	neurectoderm
NGF	Nerve Growth Factor
NGFR	Nerve Growth Factor Receptor
NRG	Neuregulin
NF3	Neurotrophin 3
ocl	outer collar
p	paranodal region
P	Postnatal day
P0	Protein zero
PDGF	Platelet Derived Growth Factor
Pe	Perineurium
PI3K	Phosphoinositide-3-kinase
PKA	Protein Kinase A
PMP22	Peripheral Myelin Protein 22
PNL	Paranodal Loops
PNS	Peripheral Nervous System
POU	Pit-1, Oct-1/2, Unc-86
Ptc	Patched
Pu	Purine
Py	Pyrimidine
RER	Rough Endoplasmatic Reticulum
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
S	Sacral
Sc	Schwann cell
sc	sclerotome
SCE	Schwann Cell Enhancer
SCIP	Suppressed cAMP-Inducible POU
Sc nucl.	Schwann cell nucleus
sGALC	Galactosulfatide
SH-3	*src* homology domain 3
Shh	Sonic hedgehog
SLI	Schmidt Lanterman Incisures
SMDF	Sensory Motor neuron Derived Factor
sog	short gastrulation
sox	sry box
sp	splotch
spd	splotch delayed
T	Thymine
TGF	Transforming Growth Factor
trk	tyrosine receptor kinase
UDP	Uridine diphosphate
UV	Ultra Violet

Chapter1

Introduction: Structure, function and development of the peripheral nerve.

1.1 General introduction and aim of this thesis

The complex nervous system of higher animals has its evolutionary origins in the simple neuro-effector system of lower animals. In these simple nervous systems only receptor-effector loops exist to respond to the ever-changing environment. Higher, and larger, animals have evolved a highly complex nervous (and endocrine) system to deal with the challenge to maintain a physiological steady state, correct body posture and, at the same time, allow the animal to respond quickly to acute changes in the environment.

One absolute requirement for the evolutionary success of these higher animals was the introduction of fast conducting nerve fibres. Some animals have responded to this challenge by increasing the diameter of their axons, thereby lowering the longitudinal resistance and increasing the speed of impulse propagation. These animals include present day invertebrates such as squid, jellyfish and molluscs. However, there is a limit to the increase of diameter of the axon and therefore to the conduction velocities that can be obtained in this way. Moreover, even rather simple neural circuits connected by large diameter axons require a considerable investment in space and energy. The real evolutionary breakthrough came with the 'invention' of the myelin sheath. The myelin sheath is a highly specialised laminar membranous organelle laid down in segments around axons. Myelinated axons propagate nerve impulses with a speed that is approximately two orders of magnitude faster than non-myelinated axons of comparable diameter. All present day higher vertebrates have adopted this innovation and have myelinated axons.

However, with this new invention also new vulnerabilities came, exemplified by the appearance of myelin related diseases in most well studied vertebrates (domesticated animals and man him/herself). In humans, destruction of the myelin sheath leads to severe neurological problems. Well-known examples are the inflammatory diseases such as multiple sclerosis and Guillain Barré syndrome and hereditary neuropathies, such as Pelizaeus Merzbacher disease, Charcot Marie Tooth disease and Dejerine Sottas syndrome.

Two different glial cell types produce myelin: the oligodendrocyte which myelinates axons in the central division of the vertebrate nervous system and the Schwann cell which myelinates axons in the peripheral division. Work described in this thesis focuses on the biology of Schwann cells.

Schwann cells are not only of functional importance in elaborating the myelin sheath but also play a crucial role during peripheral nerve development and regeneration [1]. Schwann cells in injured nerves produce factors that stimulate axonal regrowth aiding in the structural and functional recovery of peripheral nerves. This is in stark contrast to central glia (oligodendrocytes) cells, which do not support the regeneration of central nerve tracks (reviewed in [2-5]). The potential of Schwann cells to support regeneration of demyelinated or lesioned central nerves is under active clinical exploration. Thus, it is of importance to understand the cellular events and molecular mechanisms that drive Schwann cell differentiation and myelination in health and disease.

The continuous dialogue with the axon is pivotal for the underlying mechanism of the diverse functions of Schwann cells in development maintenance and regeneration of the peripheral nerves. Although a number of questions related to different aspects of this dialogue have been answered in some detail, many questions remain (reviewed in [1]). One main outstanding question in the field is how axons instruct Schwann cells to myelinate or not. Whatever the exact nature of the axonal signal(s), it must be relayed to the Schwann

cell nucleus where transcription factors respond by co-ordinate regulation of sets of genes that mark and drive myelination. One such factor is the POU domain class transcription factor Oct-6 [6]. Two characteristics of the Oct-6 expression profile point to an important role of this protein in rendering a Schwann cell competent to start myelination. First, Oct-6 expression is dependent on axonal contact during peripheral nerve development and regeneration in Schwann cells that will start the myelination process [6-8]. Second, Oct-6 is, until now, the first factor activated upon this axonal contact, preceding expression of the major myelin genes [6,9]. The precise function of Oct-6 in driving the myelination process in Schwann cells remains to be determined. Furthermore, since Oct-6 is, so far, the first factor activated upon axonal contact, it is of importance to study how Oct-6 itself is regulated. This will lead to the elucidation of Schwann cell intra-cellular signals and eventually to the characterisation of the axonal signal(s) that induce myelination. Therefore, the principal objective of this thesis is to gain insight in the function and regulation of Oct-6 during Schwann cell differentiation and myelination.

1.2 Outline of this thesis

A general introduction on peripheral nerve structure, function and development will be given in **Chapter 1**. In the subsequent chapters I will describe experiments, which provide more insight in the function and regulation of the POU transcription factor Oct-6 during Schwann cell differentiation. **Chapter 2** describes the targeted deletion of the Oct-6 gene in mice by homologous recombination. Data presented in this chapter demonstrate that Oct-6 is required for the timely initiation of myelination. Subsequently, in **Chapter 3**, we describe the expression profile of Oct-6 homologues in species other than mice. Data in this chapter demonstrate that Oct-6 expression is conserved in the Schwann cell lineage of chicken and zebrafish, pointing to a functional conservation of this gene in the Schwann cell lineage. **Chapters 4 and 5** will address the question of how Oct-6 itself is regulated. In **Chapter 4** we give a detailed characterisation of the Oct-6 promoter region and present evidence that the Oct-6 promoter by itself is not sufficient to mediate Schwann cell specific expression of the Oct-6 gene. **Chapter 5** describes the identification of distal cis-acting regulatory elements in the Oct-6 locus that are required for Schwann cell specific expression of Oct-6 during development. Finally, in **Chapter 6** these data and some future prospects will be discussed.

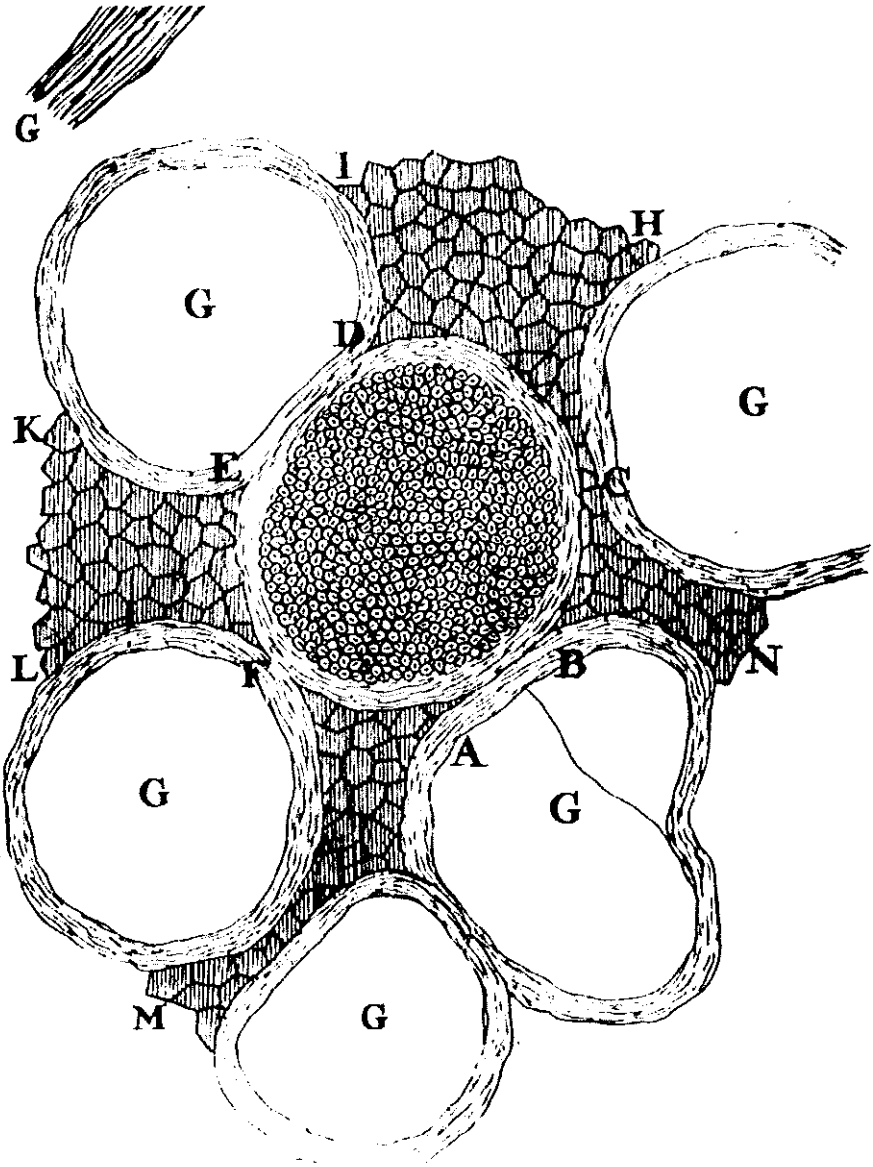

Figure 1. Representation of a transverse section through a "small nerve" (surrounded by BCDEF), which is composed of a multitude of vessels, in which the lines or strokes denote the cavities in those vessels. This nerve is surrounded by five other nerves (GGGGG), of which only the external membranes are represented. (From: Van Leeuwenhoek; 1718)

1.3 Structural aspects of the peripheral nerves

1.3.1 The peripheral nerve - a few historical notes

> *"I have often, with great pleasure, observed the texture of the nerves, which are composed of very minute vessels of an incredible thinness, and which, running along by the sides of each other, constitute a nerve; the cavity of each of these small vessels is about two-thirds its diameter; and in order to examine them I directed the spinal marrow of three cows and sheep to be brought to my house, that I might extract from thence the nerves. ... And I not only saw the size of circumference of these vessels, (some hundreds of which go to the composition of a nerve no longer than a hair of a man's beard), but some of their cavities I could as plainly distinguish, as if we were to pierce many holes in a paper with a small needle, and hold them up against the sun."*

The epigraph to this section is a translated quotation taken from Antoni van Leeuwenhoeks' paper published in 1718 [10]. Based on his microscopic observations, using a simple one lens microscope of his own making he gave the first morphologic description of nerve fibres (Fig. 1). The invention of the light microscope and its use to study biological samples by many investigators caused a revolution in biology. Nevertheless, it took more than a century, before the "vessels of incredible thinness" as described by van Leeuwenhoek, were actually identified as axons connected to a cell body, which together formed a single cell, the neuron.

In the beginning of the nineteenth century the French scientist Dutrochet (1824) described nerve cell bodies (neurons), and found them attached to individual nerve fibres (axons). However, mainly by the observations made by Purkinje on the cerebellum and Remak on sympathetic ganglia (1837 and 1838) these two structures were demonstrated to belong to one entity. A year later, this entity was dubbed the cell when Theodor Schwann together with Matthias Schleiden formulated their cell theory, which basically states that all tissues are composed of cells. Both Remak and Schwann described the presence of a medullary sheath surrounding the axons, which we know today as the myelin sheath [11,12]. In addition, Remak described differences between individual fibres consisting either of tubili primitivi (myelinated axon fibres), or fibrae organicae (unmyelinated fibres). He also described nucleated corpuscles (nuclei) along the fibres, but did not associate these with cells. A year later, the observation of nuclei was confirmed by Schwann, who proposed that these nuclei did belong to a chain of cells along the axon who had coalesced to form a syncytium. Although not correct, his description gave him the credit, instead of Remak, when these cells were named after him: the Schwann cell. In 1872 Ranvier published a paper in which he described that cells in the medullar sheath are separate entities and do not form a syncytium [13]. Further support for Ranviers theory came in 1889 when Vignal was

able to dissect individual Schwann cells from the axon and demonstrated that they ensheath the axon [14](Fig. 2). Vignal also proposed that the Schwann cells actually produce the myelin sheath. This was a rather controversial issue, since another school of thought proposed that myelin was produced by the axon, a theory supported by Ramon y Cajal [15]. The invention of the electron microscope in the middle of the twentieth century put an end to this debate, and demonstrated that the myelin sheath is a tongue of the Schwann cell wrapped around the axon [16]. As for light microscopy, the introduction of electron microscopy caused a giant leap forward in our knowledge about the fine structure of the peripheral nerves.

In the following section I will describe the various components of the peripheral nerve, as we know them to date. I will start with a description of the connective tissues of the nerve followed by a description of the glial cells in the peripheral nerve.

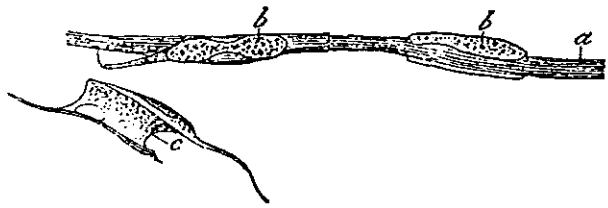

Figure 2: The earliest depiction of an individual Schwann cell (Vignal, 1889). The axon (a) is shown ensheathed by two Schwann cells (b). Vignal dissected individual Schwann cells (c) off the axon, showing that they do not form a syncytium. (From M.Jacobsen, Developmental neurobiology, Plenum Press, New York, 1991)

1.3.2 Histo-architecture of the peripheral nerve

Individual nerve fibres are protected from the environment by a compact connective tissue matrix, consisting of three distinguishable layers. These layers have been termed epineurium, perineurium, and endoneurium. The use of this terminology can be traced back to the work of Key and Retzius (1876) and Ranvier (1878) [17,18](Fig. 3). I will begin my description of the histo-architecture of the nerve with the nerve sheath.

1.3.2.1 Epineurium

The epineurium is a condensation of connective tissue loosely surrounding the fascicles in the peripheral nerve (Fig. 3). This loose fit makes the nerve trunks relatively mobile. The epineurial sheath is thicker at regions were the nerve is exposed to pressure points, such as at the joints. The amount of epineurial tissue is also greater when the number of fascicles increases. The cellular part of the epineurium mainly consists of

fibroblasts, which points to its mesodermal origin. The non-cellular part of the epineurium consists mainly of collagen (type I and III), which are orientated longitudinally. Also, variable amounts of fat are present, which might have a function in cushioning the fascicles, and protecting them against damage by compression, especially at the joints. Blood is supplied via a network of arterioles and venules called the vasa nervorum, which has branches through the perineurium into the endoneurial layer. The epineurium is also the only part of the peripheral nerve containing a lymphatic capillary network, which is connected to regional lymph nodes.

1.3.2.2 Perineurium

The perineurium is a multilayered cellular sheath surrounding individual fascicles of the nerve (Fig. 3). During development, the epithelial cells that form these sheaths originate from the mesenchyme surrounding the nerve fibres [19] [20]. The number of cell layers in the perineurium is proportional to the size of the fascicle. The layers consist of concentric sleeves of flattened epithelial cells that interdigitate and are connected by tight junctions at their margins.

Bloodvessels transverse the perineurium and link the epineurial vasa nervorum with the endoneurial capillary network. Tight junctions are also present between the endothelial cells of these capillaries, forming a blood-nerve barrier similar to the blood-brain barrier in the CNS [21].

The non-cellular part of the perineurium consists of mainly collagen fibres, running in between the cells of the perineurial sheath. Furthermore, both the most inner and outer cell layer of the sheath produce a basal lamina, consisting of a fine network of collagen IV fibres, fibronectin, heparane sulfate proteoglycan. However, the composition of the inner and outer basal lamina differs, which points to a structural and functional polarisation of the perineurial sheath.

The presence of tight junctions in the perineurial sheath and blood vessels, together with the polarisation of this sheath are structural manifestations of the major perineurial sheath function. Which is: creating a physical barrier that limits the entry of biologically active proteins, infectious agents, and migration of blood borne cells into the nerve bundles. However, a selective transport mechanism exists, which allows transfer of molecules through the cellular sheath into the endoneurium. The presence of pinocytotic vesicles and caveolae in perineurial cells together with high ATPase and creatine kinase activity, are morphological and biochemical manifestations of this transport system.

Thus, one major function of the perineurium is to protect the nerve by forming an effective diffusion barrier. In addition, the presence of collagen in the sheath points to a cushioning function.

1.3.2.3 Endoneurium

The endoneurium mainly consists of a thick layer of collagen fibrils running parallel along the nerves. This collagenous layer takes up almost fifty percent of the intrafascicular space, and increases when animals age [22]. Embedded within the endoneurium, we find axons ensheathed by glial cells (Schwann cells) (Fig. 3). Schwann

cells together with endoneurial fibroblasts are the major cellular component of the intrafascicular space. These two cell types are the main producers of the collagens present in the endoneurium.

The function of the endoneurium is mainly supportive and protective.

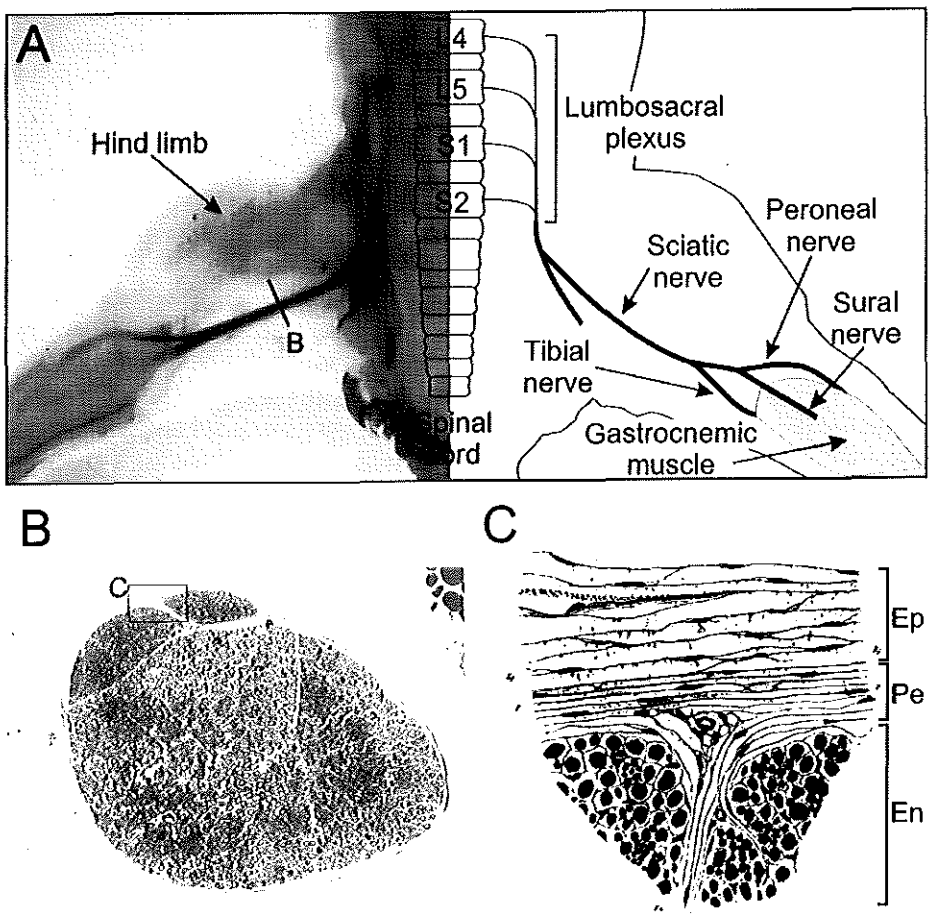

Figure 3: A. Schematic representation of peripheral nerves visualised by an enzymatic staining method in the caudal part of a postnatal day (P) 1 mouse embryo. Both sensory and motor axons, which respectively enter or leave the CNS at lumbar levels (L) 4 and 5, and sacral level (S) 1 and 2of the spinal cord, are joined in the spinal nerves of the Sacral plexus. These nerves converge into the Sciatic nerve in the upper hind limb, which is the largest peripheral nerve in vertebrates. Subsequently, more distal from the spinal cord, the Sciatic nerve bifurcates into the Peroneal, Sural and Tibial nerve. B represents a transverse section through the nerve presented in A. **B.** The nerve is filled with numerous axons surrounded by a myelin sheath (black circles). **C.** Schematic representation by Key and Retzius (1876) of region comparable to box C in Figure 1B. Portion of a transverse section through part of a peripheral nerve, showing the disposition of the epineurium, perineurium, and endoneurium. Abbreviations: Ep, epineurium; Pe, perineurium; En, endoneurium. (From Key A. and Retzius, G.: Studien in der anatomie des nervensystems und des Bindesgewebes. Stockholm, Samson & Wallin, 1876.)

1.4 Glial cells in the peripheral nerve

During evolution, glial cells appeared independently in the phyla of Protostomata (invertebrates) and Deuterostomata (vertebrates) (reviewed in [23]). Two types of glial cells can be distinguished in both phyla. First, non-myelinating glial cells are present in nerves of all members of both phyla. These cells separate the individual axons by embedding them into the glial cell plasma membrane (see Fig 4). The second glial cell type spirals several times around the axon and produces a specialised insulating structure, which is called the myelin sheath (see Fig. 5).

All vertebrates except agnatha (lamprey, hagfish) have myelin forming glial cells, while invertebrates do not possess truly myelinating cells. Although less thoroughly investigated, at least the higher invertebrates, such as annelids (earthworms), crustaceans (shrimps), and insects (flies) display multiple layers of glial ensheathments. Comparison of protein composition of the glial ensheathments from vertebrates and invertebrates suggests that evolution of glial sheath proteins in both phyla occurred independently [23].

Curiously, families, in each phylum, having myelinated nerves dominate over families lacking myelination. A striking example of this has recently been described in crustaceans [24]. Escape responses were compared between two families of copepods, which did or did not posses axons with a myelin-like sheath in their antennal nerves. The escape response of copepods with axons containing a myelin-like sheath is three folds faster than that of comparable axons lacking such a sheath in other copepod families. This improved performance of the copepod family having this myelin-like sheath reduces their reaction time to predatory attack. This results in an increased survival rate and allows them to dominate in open ocean communities [24].

This remarkable observation provides a pointed illustration of how evolution of the myelin sheath allowed the development of fast conducting low calibre axons culminating in the complex compact nervous system of higher vertebrates.

In the following section I will discuss the structure of non-myelinated and myelinated fibres as observed in most mixed nerves of the somatic division of the PNS.

1.4.1 Non-myelinated peripheral axons

Most peripheral nerves in vertebrates consist of a mix of myelinated and non-myelinated fibres. In contrast, nerves of the autonomic (sympathetic and para-sympathetic) nervous system contain predominantly non-myelinated nerve fibres.

Non-myelinating Schwann cells segregate a variable number of small calibre axons, from a single axon to up to twenty, by sending out cytoplasmic processes in between the aligning axons (Fig. 4). These processes might just be able to contact each other and seal off the axonal membrane from the endoneurial space, or they stay apart and thus leave the axonal membrane and endoneurial space only to be separated by Schwann cell basal lamina (Fig. 4)[25]. Non-myelinating Schwann cells overlap with neighbouring Schwann cells (Fig 4A). This is probably the reason why they were erroneously thought to form a syncytium [25].

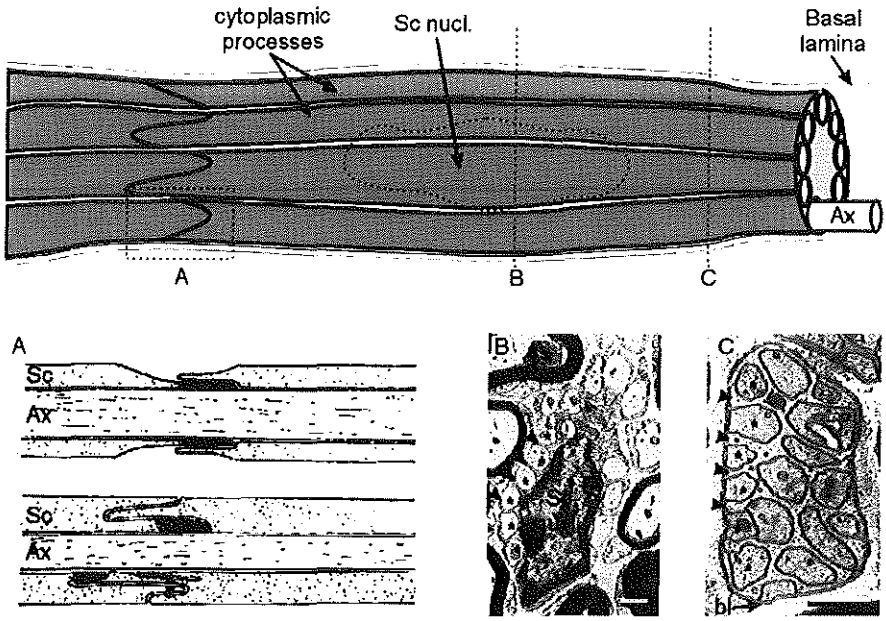

Figure 4: Schematic representation of a non-myelinating Schwann cell (Sc) separating several small calibre axons (Ax) with its cytoplasmic processes. A longitudinal section corresponding to region A and transverse sections through regions B and C are presented below the scheme. **(A)** Two examples of overlapping cytoplasmic processes of neighbouring Schwann cells (Sc) along Axons (Ax). **(B)** Section through a non-myelinating Schwann cell at the level of the Schwann cell nucleus (SC nucl.). Arrowheads indicate the cytoplasmic processes separating the axons (Asterisks). **(C)** Similar section as in B, only away from the nucleus. Some axons are completely engulfed by the Schwann cell processes, while others are only separated from the endoneurial space by the Schwann cell basal lamina (BL). Scale bar: 0,5 µm.

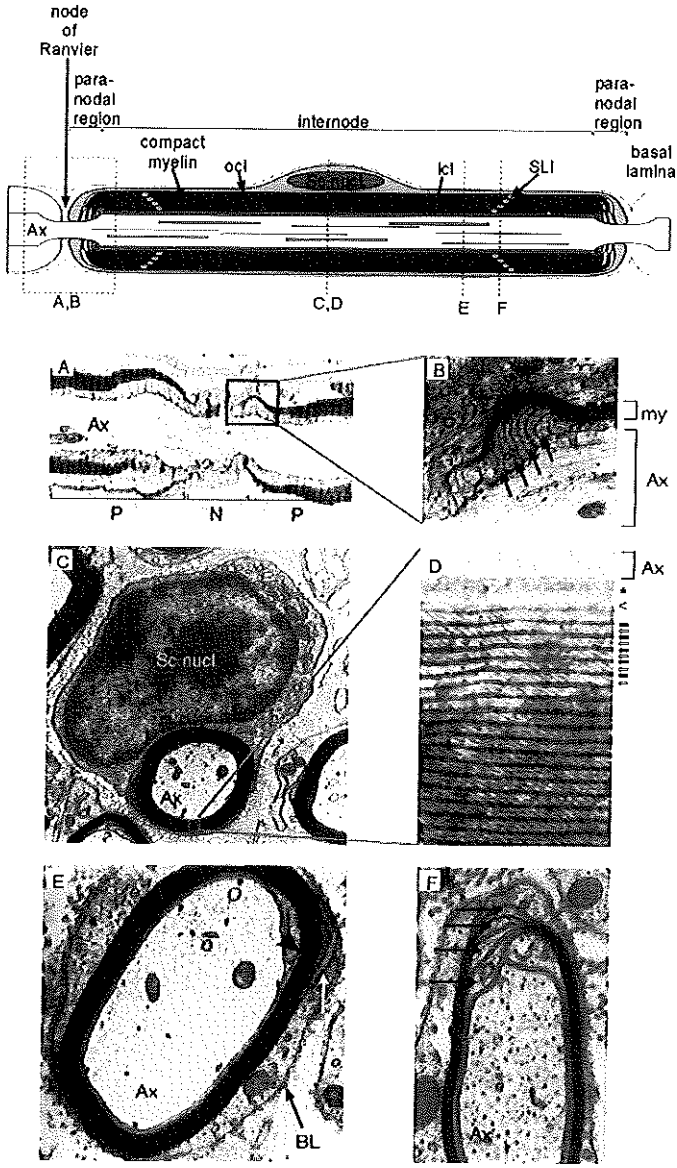

Fig. 5: Schematic representation of a myelinating Schwann cell associated with its axon (Ax). Two distinct areas can be distinguished in the Schwann cell membrane, which are compact and non-compact myelin. Non-compact myelin, the area still containing cytoplasm, can be subdivided into the paranodal regions, the outer collar (ocl), the inner collar (icl), and the Schmidt-Lantermann incisures (SLI). Neighbouring myelinating Schwann cells do not overlap with each other, generating a naked axonal membrane, which is only separated by the basal lamina from the endoneurial space. This region is called the node of Ranvier. Electron micrographs of sections through the nerve fibre corresponding to areas A-F are presented below the drawing.

1.4.2 Myelinated peripheral axons

Large calibre axons (> 1 µm diameter) are usually myelinated and these axons are fast conducting (Fig. 5). What immediately becomes evident upon studying micrographs of nerves is that, for unknown reasons, myelinating Schwann cells always associate with only one axon. A single myelinating Schwann cell associated with its axon forms an internode (Fig. 5). This configuration stands in contrast to non-myelinating Schwann cells and their myelinating counterparts in the CNS, oligodendrocytes, which both can associate with multiple axons. Oligodendrocytes are capable of myelinating up to 30 internodes. During postnatal growth of an animal, the number of internodes in the peripheral nerves does not change, but as the nerve elongates, the internodal segments become lengthened. This lengthening might occur via passive stretching or by active remodelling of the myelin sheath [26,27].

In a short region in between two internodes, the naked axon is exposed to the endoneurial environment. This is the node of Ranvier, named after its discoverer (Fig. 5A). The regions of the internode flanking the node of Ranvier are the paranodal regions (Fig. 5B). Both the node of Ranvier and the paranodal regions play an important role in the propagation of impulses along the axon (See box 1).

A fully matured internodal region is formed as the inner cytoplasmic lip of the Schwann cell has made many turns around the axon [28]. Compaction of the myelin sheath occurs as the cytoplasm is squeezed out. The compact myelin region is formed by close apposition of the intracellular layers of the plasma membrane, which generates the so-called major dense line in electron micrographs (Fig. 5D). Apposition of the two extracellular layers of the plasma membrane forms the intraperiod or intermediate line (Fig. 5D).

Cytoplasm only remains in regions of non compact myelin, such as the paranodal loops near the nodes of Ranvier (Fig. 5B), the inner collar facing the axon and outer collar containing the nucleus (Fig 5), and the Schmidt-Lantermann incisures (Fig. 5F). The latter are canals of cytoplasm that spiral together with the compact myelin around the axon and connect the inner with the outer collar. The function of the Schmidt-Lanterman incisures is still unknown.

The cytoplasmic cuffs of the paranodal loops and Schmidt-Lanterman incisures are connected via gap junctions. This allows a fast transport route for low molecular weight molecules directly through adjacent cytoplasmic cuffs, instead of the thousand fold longer circumferential route along the canals. The existence of inherited peripheral neuropathies in which a member of these gap junction proteins (connexin 32) is mutated, implies an important role for this fast transport route in myelinating Schwann cells (See section 1.5.4.5).

(Fig. 5 continued) **(A)** Section through a node of Ranvier (N) with the paranodal regions (P) of two neighbouring Schwann cells. **(B)** High power magnification of a paranodal region in A. Arrows point to the paranodal loops contacting the axon (Ax). (my: compact myelin). **(C)** Section through the nerve fibre at the level of the Schwann cell nucleus (SC nucl.). **(D)** High power magnification of the myelin sheath in C. Dark arrows point to the major dense lines. Open arrows point to the intraperiod lines. *: periaxonal space. <: inner collar. **(E)** Section through compact myelin away from the nucleus. The arrowhead points to the inner mesaxon. The white arrow points at the outer mesaxon. Both mesaxons contain tight junctions. BL: basal lamina. **(F)** Section through a Schmidt - Lantermann incisure (arrows).

The transition from non-compact myelin to compact myelin at the inner and outer collar of the Schwann cell is marked by the presence of tight junctions. These junctions separate the extracellular space between opposing Schwann cell plasma membranes in compact myelin from the intercellular space between the non-compact myelin and the axon (periaxonal space) or basal lamina (endoneurial space). These stretches of tight junctions at the inner and outer collar are termed the inner and outer mesaxon, respectively (Fig. 5E) [29].

In addition, myelinating Schwann cells produce a continuous basal lamina along the nerve fibre (also over the nodes of Ranvier) consisting of among others collagen (type IV), laminin, and fibronectin (Fig. 5E).

Recapitulating, myelinating and non-myelinating Schwann cells have highly distinct morphologies that relate to their differences in function. At the molecular level these differences manifest themselves in the expression of cell specific proteins required in each cell type (See section 1.5.3.3 and 1.5.3.4). As said, the major functional consequence of these structural differences is that impulses travel two orders of magnitude faster along a myelinated fibres than along non-myelinated ones (See box 1).

Nevertheless, both non-myelinating and myelinating Schwann cells originate from a common progenitor cell: the neural crest cell (see section 1.5.1. and 1.5.2). Moreover, Schwann cells are remarkably plastic in that they readily revert back to a more immature phenotype upon loss of axonal contact and that they can choose again either of the two adult fates when axonal contact is restored [30,31]. Thus the decision of a Schwann cell to adopt a myelinating or non-myelinating fate is influenced by the axon.

Box 1 - Action potential propagation in peripheral nerves

The ability of neurones to transduce signals relies on unique properties of the axonal and dendritic membrane. Impulses travel along axons as a depolarisation, disturbing the axonal resting potential. In a non-stimulated situation the axonal membrane has a resting potential, which is set up by the generation of an electrochemical gradient of mainly potassium (K^+) and sodium (Na^+) ions across the membrane. Two mechanisms set up this gradient. First, a Na^+/ K^+-pump resides in the axon membrane, which actively transports two K^+ ions into the axon and three Na^+ ions out (Fig. 6(a)). This discrepancy between the number of equivalent charged ions going in and out of the cell, results in a negative charge within the axon. Secondly, this active transport mechanism is opposed by the passive diffusion of these ions along their electrochemical gradient (Fig. 6(b)). However, the permeability of the axon membrane for K^+ ions is 20 times higher than that for Na^+, resulting in a net loss of K^+ ions from the axon by passive diffusion. Together the passive diffusion and active transport set up a negative charge within the axon. This results on average in most vertebrates in a resting potential of approximately -70mV.

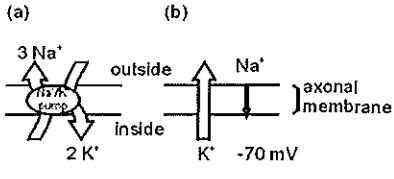

Figure 6: Active and passive ionic movements associated with the production of a negative potential inside the axon. **(a)** The Na^+/K^+ pump actively and effectively exchanges 2 K^+ ions from the outside against 3 NA^+ ions from the inside. Together with passive diffusion of mainly K^+ to the outside **(b)**, the resting membrane potential in vertebrates is set around -70 mV.

(Box 1 - continued)

Depolarisation of the axonal membrane, for instance after stimulation of one of the peripheral sensory organs results in a change in the resting potential across the membrane. If the depolarisation reaches a certain threshold value, it will evoke an action potential in an all-or-nothing event. During an action potential the negative inside value of about -70 mV rises to a positive inside value of about +40mV. This polarity change is mainly brought about by voltage gated Na^+ and K^+ channels, which are only opened after certain changes in membrane potential. Via these channels Na^+ goes in and K^+ out of the cell. Depolarisation causes the Na^+-channels to open. This influx of Na^+ ions further depolarises the membrane allowing more Na^+-channels to open up in a self-amplifying manner, until the net electrochemical driving force is zero, which is at a membrane potential of about +40 mV. At this point the Na^+ channels are inactivated. At the same membrane potential K^+ channels are opened allowing K^+ ions to run out of the axon, repolarising the membrane potential rapidly back to its resting potential, where both channels are closed again.

These events all occur in less than 0.5 millisecond. The self-amplifying depolarisation of the axon is sufficient to depolarise neighbouring regions of the axon membrane, which then go through the same cycle. Inactivation of the Na^+-channels prevents re-excitation of the repolarised region, allowing the action potential to spread only in one direction along the axon from the sensory organ to the soma of the neuron.

In non-myelinating axons the velocity of the propagated action potential depends on the longitudinal resistance of the axon cytoplasm. The resistance, in turn is related to the diameter of the axon such that the smaller the diameter the greater the resistance. This high resistance has an effect on the spread of current and reduces the length of local circuits so that only the region immediately in front of the action potential is involved in the local circuit. Many invertebrates, such as squids, have circumvented this problem by having very large diameter axons (up to 1mm), which results in lowering of the resistance, increase of length of a local circuit and subsequently a higher conduction velocity.

Vertebrates improved the speed of impulse propagation along the axon even further by myelinating their axons. In addition to the larger diameter of myelinated axons as compared to non-myelinated ones, this sheath has strong insulating capacities, which allows an increase in the length of the local circuits. The length of these circuits is extended even further by the specific placement of ion channels along the axon. As mentioned before, the myelin sheath is formed by Schwann cells, and has an interruption in between every two adjacent Schwann cells, which is called the node of Ranvier. The Na^+-channels are concentrated at these nodes. Because of this concentration of Na^+-channels at the nodes, local circuits are set up and current flows only between two adjacent nodes. This results in the effect that the action potential 'jumps' from node to node and passes along the myelinated axon two orders of magnitude faster than with a series of smaller local currents in a non-myelinated axon of the same diameter (Fig. 7). This type of conduction is called 'saltatory' conduction.

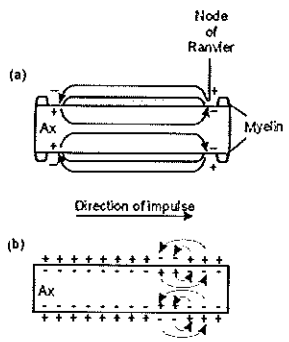

Figure 7: Diagrams showing the difference in lengths of the local circuits produced **(a)** in a myelinated axon and **(b)** a non-myelinated axon. In **(a)** conduction is described as saltatory since the action potential effectively 'jumps' from node to node.

1.5 Embryonic and postnatal development of peripheral nerve tissue

The different components that make up the peripheral nerve tissue originate from different germ layers. During embryonic development, neurons (axons) and their associated glial cells derive from the neurectoderm, while the nerve sheath and nerve vasculature derive from the mesoderm. How these germ layers contribute to form the peripheral nerve is discussed in this chapter.

First, I will review some aspects related to the biology of the neural crest. This will include a brief discussion of data related to the question of how the neural crest is specified (section 1.5.1), how delamination of the crest is initiated (section 1.5.2) and how neural crest cells choose different fates (section 1.5.3), in particular a Schwann cell fate. In subsequent sections I will review what we know about Schwann cell development and the formation of myelin (Myelogenesis)(section 1.5.4). In recent years it has become clear that Schwann cells play a central role in all aspects of nerve tissue development. These new developments will be discussed in sections 1.5.5, 1.5.6 and 1.5.7.

1.5.1 Neural crest - Specification

During vertebrate development, Schwann cells originate from a multipotent migratory cell population derived from the dorsal aspect of the neural tube: the neural crest (Fig. 8). This cell population has been first described in chicken embryos by Wilhelm His. He postulated that the neural crest is an organ forming germinal zone, containing subsets of cells with restricted fates. Over the last few decades this rather simple scheme has been revised and much more detailed information has been added. In particular, the combination of classical tissue grafting techniques and more recent molecular biological techniques have led to the identification of protein factors involved in the induction, migration, and fate determination of neural crest cells. It has become clear that, contrary to what His thought, the neural crest population is a population of multipotent cells that realise different cellular fates depending on the cellular environment in which they migrate and differentiate. In this section I will present data relating to the question how the neural crest is induced.

The neural crest cell population is formed at the border between the neural plate and the presumptive epidermis, overlying the lateral plate mesoderm in a gastrulating embryo (Fig. 8). As gastrulation proceeds, neural folds will form, bend and fuse to generate the neural tube. During this process, cells emigrate from the dorsal ridges of the neural folds to form the migratory population known as the neural crest (Fig. 8). Although, until this date, the question about how the neural crest is specified is still unanswered, several protein factors playing a role in this process have been put forward.

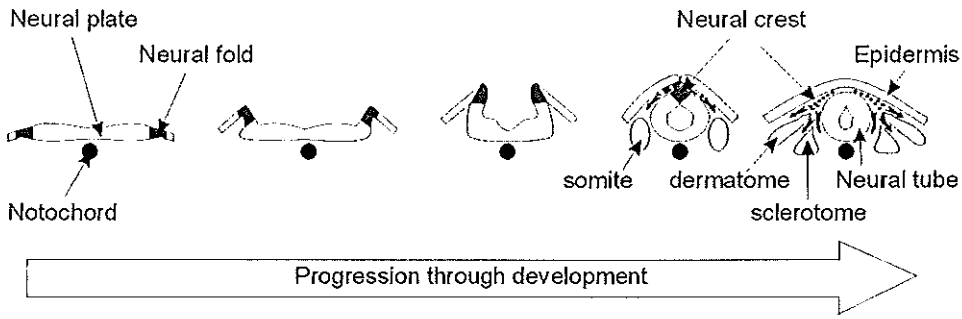

Figure 8: Schematic representation of neural tube formation. The ectodermal cells are represented either as precursors of the neural crest (dark grey) or the epidermis (light grey) or the neural tube (white). The ectoderm fuses in the most dorsal point, forming an outer epidermis, an inner neural tube, and a population of neural crest cells migrating into the embryo in between the epidermis, the neural tube, the dermatome and sclerotome. The latter two are generated from the somites, which are of mesodermal origin.

The first step in the induction of neural crest is the specification of a homogenous layer of ectodermal cells in a gastrulating embryo into the neural plate (neurectoderm) and the epidermis. A substantial body of evidence suggests that members of the bone morphogenetic proteins (BMP), belonging to the transforming growth factor (TGF) - β family, play an important role in this process. Two members of this group, BMP-4 and BMP-7 (BMP-4/7) are expressed throughout the gastrula ectoderm except in the prospective neural plate [32]. This expression pattern coincides with the presumptive epidermis.

Other factors, such as Noggin and Chordin, are produced and excreted by the notochord in a graded pattern and are only present where BMP-4/7 is absent (the presumptive neurectoderm) [33-35]. Treatment of *Xenopus laevis* animal cap explants with BMP-4/7 promoted the formation of epidermis [36]. In the same experimental setting it was shown that treatment with Noggin and Chordin induced neural tissues [33,34]. In addition, it has been demonstrated that Chordin and Noggin bind and inactivate BMP-4/7 [37]. Taken together, these data suggest that the Noggin and Chordin inactivate the epidermalising activity of BMP-4/7 by binding to it in the extracellular space. This prevents binding of BMP-4/7 to membrane-bound BMP receptors on the ectodermal cells, thereby blocking epidermalisation of the cell. Hence, counteraction of BMP signalling by Chordin and/or Noggin results in specification of ectoderm into neurectoderm and epidermis.

This model is supported by the structural and functional conservation of these molecules in invertebrates. The *Drosophila Melanogaster* homologue of BMP-4 is *decapentaplegic* (*dpp*). Flies lacking *dpp* have an expanded neurectodermal region at the expense of epidermal tissues [38]. Ectopic expression of *dpp* leads to epidermal expansion at the expense of neurectoderm [38]. Thus *dpp* suppresses neurogenesis in the fruit fly. The *Drosophila* homologue of Chordin is *short-gastrulation* (*sog*). Flies lacking *sog* have expanded epidermis at the cost of loss of neurectoderm, while ectopic *sog* expression leads to formation of neurectoderm [39,40]. Rescue of the *Drosophila dpp* phenotype by

expression of human BMP-4 and epidermalisation of *Xenopus* animal cap cells by *dpp* demonstrate that *dpp* is the functional homologue of BMP-4 [40,41]. Furthermore, the facts that *sog* neuralises animal cap cells and ectopic Chordin expression in fruit flies leads to expansion of the neurectoderm, show that *sog* and Chordin are functional homologues [40].

Genetic evidence for this model in mice is less compelling. Disruption of the BMP-4 gene resulted in defects during gastrulation and formation of the posterior body and ventral mesoderm [42]. Unfortunately, neurectodermal defects were not addressed in these experiments. Targeted deletion of Noggin demonstrated that specification of the ectoderm was unaffected, suggesting that Noggin is not essential in this process [43]. This might be explained by the fact that Chordin or another unknown protein is either essential or has a redundant function during neural induction. Targeted deletion of the mouse Chordin gene has not been reported.

However, null mutations of the Chordin homologue in zebrafish (Chordino) demonstrated that indeed Chordino is required for neurectoderm induction but is not the only neural inductive factor [44]. Noggin is not expressed in the zebrafish notochord at the time the neural plate is specified, ruling out a role for noggin in this process [45]. Other neural inducing genes in zebrafish, which are expressed at the right time and place have been identified but not characterised [46]. Thus, although the picture is not complete yet, it is likely that inhibition of BMP activity by factors such as Chordin, and Noggin play an important role in patterning the ectoderm into neurectoderm and epidermis (Fig. 9).

The second step in neural crest specification requires further patterning of the neural fold along the dorsal-ventral (D-V) axis. As gastrulation proceeds, neural folds are formed and BMP-4/7 expression fades in the epidermis and becomes restricted to the neural folds. This restricted expression of BMP-4/7 marks the (D-V) patterning of the neural tube, which eventually will lead to specification of the neural crest [32].

Elegant experiments performed in the Jessel lab demonstrated that in chicken embryos, opposing effects of dorsal BMP-4/7 expression and a ventralising signal from the notochord are required for the D-V patterning of the neural tube [47]. A good candidate for the ventralising signal is Sonic hedgehog (Shh), a secreted glycoprotein synthesised by the notochord [48]. However, Shh cannot induce neural tissues from presumptive ectoderm cells, but can change the D-V pattern of pre-existing neural tissue [49]. Nevertheless, Shh can induce expression of Noggin in ventral neural plate [50]. Although Noggin is not essential for neural induction, it is required for the D-V patterning of the neural tube [43]. Noggin -/- mice have a loss of ventral neural cell fates, despite the presence of normal Shh expression in the notochord. Together these data suggest that after neural induction, Shh dependent Noggin expression in the presumptive ventral neural tube counteracts the dorsal BMP-4/7 expression, resulting in the D-V patterning of the neural tube (Fig. 9).

It has been suggested that during D-V patterning the concentration of BMP to which a neural plate cell is exposed defines its fate. This implicates that BMP-4/7 functions as a morphogen [47]. Specifically it is thought that neural crest is generated by intermediate levels of BMP-4/7 in a BMP-4/7 gradient from high concentrations in the dorsal neural tube to low in the ventral neural tube [47,51]. Although evidence favouring this model is accumulating, other data suggests that BMP-4/7 is not the only factor involved in neural crest specification.

For instance, expression of basic fibroblast growth factor (bFGF) seems to be required for neural crest induction. It is expressed, like BMP-4, in the ectoderm in a graded fashion, inducing neural crest fate at intermediate levels. Alternatively, it is possible that bFGF renders the neural crest competent to signals such as BMPs [52].

Next, members of the Wingless family of growth factors (Wnt) Wnt-1/3A have also been implicated in neural crest induction by co-operating with other neuralising signals (such as BMP-4 or bFGF) resulting in a robust activation of neural crest marker genes [53].

Finally, the mesoderm underlying the presumptive neural crest region has been demonstrated to be a source of all these factors (BMP-4, bFGF, Wnt-1/3A)[51,54,55]. These observations suggest a role for mesoderm in the induction of neural crest.

In summary, these data indicate that induction of a neural crest cell fate might be specified by the position of a cell in a network of morphogen gradients, such as BMP-4, bFGF, Wnt1/3A along each axis (rostral-caudal, dorsal-ventral and proximal-distal) in the developing embryo (reviewed in [56]).

1.5.2 Neural crest - delamination

As gastrulation proceeds, the neural folds will fuse and form the neural tube, epidermis and cause delamination of neural crest cells (Fig. 8). To initiate migration, neural crest cells have to switch from an epithelial to a mesenchymal morphology. Recently, several factors have been identified that might be directly involved in mediating this morphological switch.

First, in *Xenopus*, chick and mouse neural crest induction is marked by the expression of the zinc-finger protein Slug, which is homologous to the *Drosophila* Snail gene. In chick and mouse embryos, dorsal midline cells express Slug before delamination of crest cells. It was hypothesised that Slug is important for the epithelial-to-mesenchymal transition that characterises the migration of neural crest, since treatment of chicken embryos with antisense oligonucleotides against *Slug* inhibited the emergence of neural crest from the neural tube [57]. However, in mice in which *Slug* (*Slugh*) is deleted, this transition occurs normally [58]. It is possible that lack of a neural crest defect in *Slug* -/- mice is the result of redundant functions of other Snail or Slug family members [59]. Thus, although Slug has proven to be a useful marker for presumptive neural crest cells, its involvement in crest induction and emigration has remained unclear.

Second, after neural fold fusion, BMP-4 and noggin are highly expressed in the dorsal aspect of the neural tube. Delamination of crest from the neural tube is initiated at the rostral end of the embryo in a rostro-caudal gradient marked by Slug positive migrating crest cells. The start of emigration coincides with the fading of noggin expression. Experiments from the Kalcheim lab have shown that ectopic noggin expression prevents crest emigration [60]. Since noggin is an efficient inhibitor of BMP-4 action, it is suggested that BMP-4 positively regulates the epithelial-mesenchymal transition that characterises crest delamination. Preliminary results from the same group further suggested that noggin expression is down regulated by signals emanating from the dissociating sclerotome (mesoderm).

Altogether, these results suggest that a co-ordinated activity of noggin and BMP-4 in the dorsal neural tube trigger delamination of specified, Slug expressing neural crest cells. Thus, BMP-4 plays multiple and discernible roles at sequential stages of neural crest ontogeny, from specification (See section 1.5.1) through delamination and later differentiation of specific neural crest derivatives (See section 1.5.3)(Fig. 9).

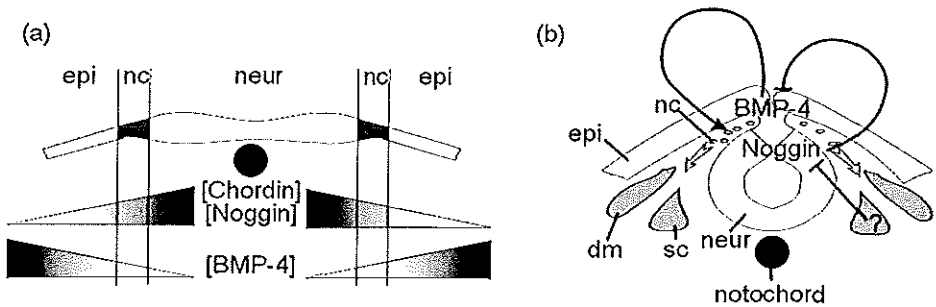

Figure 9: BMP-4 plays multiple discernible roles at sequential stages of neural crest development, from specification **(a)** to delamination **(b)**. For details see text section 1.5.1 and 1.5.2. Abbreviations: epi, epidermis; nc, Neural crest; neur, neurectoderm; dm, dermatome; sc, sclerotome.

1.5.3 Neural crest - fate determination

Classical grafting and chimera experiments, mainly performed in the quail-chick system, have generated a detailed fate map of the neural crest [61]. Transplanting neural crest cells from a certain axial level of a quail embryo into a similar level of chick neural crest (homotypic grafts) made it possible to distinguish to which tissues the grafted region contributes. These experiments demonstrated that neural crest originating from various levels along the anterior-posterior (AP) axis generated distinct but also overlapping sets of derivatives (Fig. 10). For instance chromaffin cells of the adrenal gland originate only from a small population of trunk neural crest cells, while Schwann cells can be derived from neural crest along the entire length of the AP-axis of the animal (reviewed in [61]). However, when neural crest is grafted into a different axial level than from which it originates (heterotypic grafts) it will generate, with a few exceptions, derivatives that are normally generated from the region it is grafted into. For instance, if neural crest from the trunk region is transplanted into the more rostral vagal level, it will generate enteric neurons (reviewed in [61]). In addition, neural crest cells emigrating from the same axial level along the neural tube at different developmental timepoints tend to give rise to distinct derivatives (reviewed in [62]). Nevertheless, it has also been demonstrated that both early and late migrating crest have equivalent developmental potentials *in vivo* [63]. Thus, these results highlight the plasticity of the neural crest and show that it is the environment into which they migrate primarily determines crest fate.

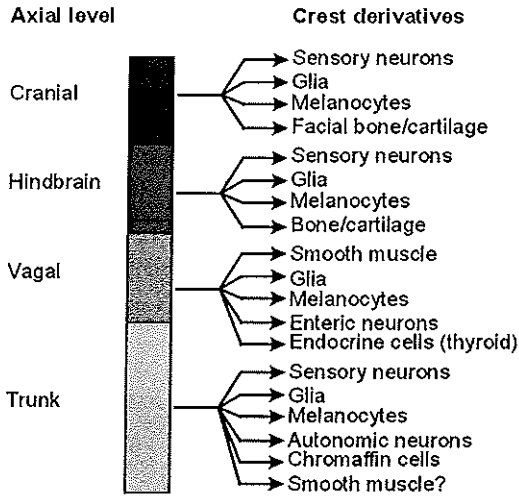

Figure 10: Variations in crest derivatives produced at different levels along the rostro-cuadal extent of the neuraxis. Only major subdivisions of the neuraxis and a simplified subset of crest derivatives are shown. The results are based primarily on fate-mapping experiments in avian embryos (adapted from [64]).

However, from these experiments it is not evident whether the environment acts instructive, on a homogenous population of neural crest stem cells, or selective, on a collection of committed crest cells. To address this issue, lineage tracing experiments have been performed, for instance by labelling single cells *in vivo* with a vital dye or with an heritable retroviral construct [65,66]. These studies demonstrate that most pre-migratory crest cells are multipotent, on the basis of the location of the daughter cells into various neural crest derivatives, such as sensory neurons and glia [67]. However, precursors generating just a single unique neural crest derivative have also been observed, indicating that some neural crest cells are already committed while still being in the neural folds. These studies characterise accurately the prospective fates of the individual cells, but do not reveal the total range of their phenotypic repertoire.

After clonal culture conditions were established of mouse and rat neural crest cells from various stages of development, it became possible to address this issue [68-70]. These studies demonstrated that most premigratory neural crest cells are multipotent self-renewing stem-like cells, which however vary in the types of derivatives they generate. For instance, in mouse it has been demonstrated that neurons, smooth muscle and Schwann cells can be produced from a single precursor *in vitro* [71].

Furthermore, these clonal culture assays have led to the identification of factors that are potentially involved in the lineage determination of the neural crest. For instance, one of the products of the neuregulin gene, glial growth factor 2 (GGF-2) causes neural crest cells to differentiate into Schwann cells [72](Fig. 11). On the other hand, neuronal development of neural crest stem cells is promoted in the presence of BMP2 or BMP-4, while smooth muscle cells are the major derivatives after treatment with TGF-β [73](Fig. 11). These signalling molecules act in an instructive manner, rather than in a selective one, since they promote development of one lineage at the expense of others. However, when these cells are treated with combinations of TGF-β, BMP-2 and GGF-2, the former two factors are dominant over GGF-2, indicating that intrinsic biases in the stem cells can

affect the outcome of lineage decisions made in the presence of multiple competing instructive signals [74].

Targeted deletion of the genes coding for these factors indicated that some of these factors might also exert these functions *in vivo*. For example, targeted mutation of the neuregulin gene, leads to a major reduction in the number of Schwann cells associated with peripheral nerves, while a TGF-β knock out interferes with cardiac and vascular development [75-78]. Mice homozygous for mutations in BMP-2 or BMP-4 die too early to evaluate a requirement for these factors in neuronal development [42,79].

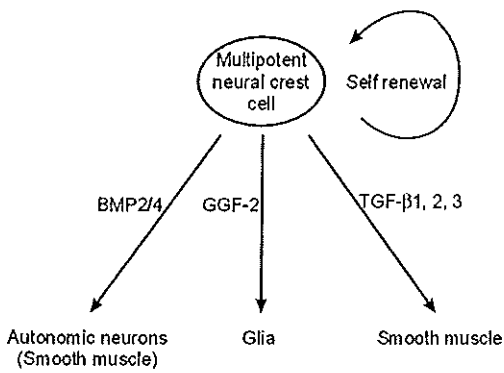

Figure 11: Individual neural crest cells are multipotent and capable of undergoing symmetric self-renewing divisions. Several growth factors have been found to instruct cultures of mammalian multipotent neural crest cells to adopt different fates. Cultures treated with TGF-β form smooth muscle, while those treated with GGF-2 form glia. Cultures treated with BMP-2/4 form autonomic neurons, and to a lesser extent smooth muscle. Adapted from [80].

Multipotent progenitors can also be derived from postmigratory neural crest populations. These progenitors, however, are more restricted in the types of derivatives they can generate when clonal cultures of these cells are challenged with the same instructive factors. For instance, progenitors derived from the developing gut are able to divide multiple times *in vitro*, but exclusively give rise to neurons. This occurs even when they are challenged with GGF-2, an agent that suppresses neural differentiation in neural crest stem cell cultures in favour of Schwann cells or smooth muscle cells [81]. Furthermore, various multipotent neural crest derived progenitors have been isolated from a rat sciatic nerve during development [82]. In this study, different populations of multipotent self renewing stem cells were isolated by FACS sorting based on the presence or absence of the surface molecules protein zero (P0), the major peripheral myelin protein, and the low affinity neurotrophin receptor (p75). Progenitors expressing p75 in the presence or absence of P0 behaved in an identical manner as the originally identified neural crest stem cells, when challenged with GGF-2, BMP-2/-4 or TGF-β. However, fate restrictions became apparent when these different clones were grown in standard culture conditions. While the majority of clones expressing p75 but not P0 exhibit multiple fates producing colonies containing glia, neurons, and myofibroblasts, clones positive for both p75 and P0 predominantly generate glia and myofibroblasts. A multipotent precursor population expressing p75, P0, and also the peripheral myelin protein 22 (PMP22), has been isolated from yet another neural crest derived tissue, the dorsal root ganglion (DRG) [83]. Strikingly, in contrast to p75

and P0 positive progenitors isolated from the sciatic nerve, these cells generate mainly glia and neurons when cultured in standard conditions. Furthermore it has been demonstrated that by a community effect groups of P0/PMP22 positive progenitors interpret instructive signals differently than single P0/PMP22 positive cells [84]. When single cells were treated with TGF-β, they are fated to generate smooth-muscle-like cells, while communities of P0/PMP22 progenitors generate neurons or go into apoptosis. It is likely that this effect also occurs *in vivo* in the DRGs, where the cellular association of these multipotent progenitors might suppress the generation of a non-neuronal fate.

Earlier studies had demonstrated P0 expression in late migratory neural crest cells. It was suggested that these P0 expressing crest cells represent a subpopulation of cells predetermined to form glia [85,86]. The data presented earlier in this section argue against this view. The fact that P0 expressing neural crest progenitor cells are able to generate other cell types (such as neurons or myofibroblasts) next to glia, either in the presence or absence of instructive signals, demonstrates that early P0 expression is not restrictive for neural crest cells to generate glia at the cost of other cell types *in vitro* [82,84]. Whether this is actually the case *in vivo* still has to be determined by transplantation experiments.

In summary, these different studies demonstrate that migrating crest cells are multipotent and that environmental cues drive them to differentiation into diverse cell types. Differentiating crest cells do not lose their multipotency. Other differentiation fates remain open. This situation is reminiscent of that in B-cell differentiation. In the absence of Pax-5, B220 positive B-cells abandon a B-cell fate and differentiate into osteoclasts when challenged with the appropriate growth factors [87,88]. The general picture that emerges from these and other studies is that lineage determination and differentiation is not a route down a one way track, but instead is highly flexible with cells retaining multipotency very far down a differentiation pathway. It also calls for caution in using so called differentiation markers to determine the fate of a cell.

1.5.4 How to become a Schwann cell

The first contact between early post-migratory neural crest cells and axons probably occurs when both start to migrate away from the neural tube through the rostral part of the somites along the rostro-caudal axis of the embryo [89]. Both neural crest cells and axons do this independently of each other [90]. This preference for the rostral site of the somite is mediated through interactions between repulsive forces in the caudal part, such as Ephrins and their receptors, F-spondin, Cytotactin-binding proteoglycan, T-cadherin, and Versican, and attractive cues, like Tenascin, butyrylcholinesterase, and Thrombospondin in the rostral part of the somite [91-100]. These cues direct the axons and the associated neural crest cells towards their target organs, resulting in segmentation of the peripheral nervous system.

Based on morphological and cell biological properties, three transitional states can be distinguished within the Schwann cell lineage. The intimate relationship between neural crest, its derivatives and axons by means of reciprocal signalling are a prerequisite for these transitions to occur. During the last decade one component of this reciprocal signalling has

been identified and was shown to play a role in at least two of these transitions and to be essential for normal peripheral nerve development. A family of epidermal growth factor (EGF)-like proteins produced by neurons, dubbed neuregulins (NRGs), are ligands for members of the EGF family of four receptor tyrosine kinases, termed ErbB1 (EGF-receptor), ErbB2 (Neu), ErbB3, and ErbB4, of which several are expressed during Schwann cell development [101]. GGF-2 is a member of this family. As discussed in the previous section this factor functions as an instructive signal for neural crest stem cells to generate glia. Other members of this family are Neu differentiation factor (NDF), heregulin, sensory and motor neuron-derived factor (SMDF), and acetylcholine receptor inducing activity (ARIA). It has been demonstrated that all these factors are products of the same gene: the neuregulin-1 gene. This family of growth factors compromises at least 12 members, which arise through alternative splicing of the 13 exons of the NRG-1 gene. All these ligands consist of different combinations of several identifiable domains: an EGF-like motif (α or β variant), a signal peptide, an immunoglobulin (Ig)-like domain, a cysteine-containing N-terminal domain, a glycosylation domain and a transmembrane domain. The presence or absence of the transmembrane domain determines whether the precursor is made as a membrane bound or a secreted protein. NRGs are grouped into three different isotypes. Members of isotype NRG I (containing NDF and ARIA) includes variants with an Ig-like domain and a glycosylation domain, which are mainly expressed at the early embryonic stages and predominate in the endocardium. Members of the NRG II group (including GGF-2) have an Ig-like domain but no glycosylation domain and are expressed in embryonic skeletal muscle. The NRG III isotypes (containing SMDF) have the cysteine-rich domain, whose main site of expressing is in the sympathetic and somatic motor and sensory neurons. As mentioned earlier, receptors for these ligands are the four members of an EGF family of receptor tyrosine kinases, ErbB1 (EGF-receptor), ErbB2 (Neu), ErbB3, and ErbB4. These proteins consist of a large extracellular ligand-binding domain, a single transmembrane segment, and an intracellular portion containing a carboxy terminal tail region and a tyrosine kinase subdomain. The latter domain of the ErbB3 receptor has diverged from the other three receptors and lacks tyrosine kinase activity. Notwithstanding this fact, together with ErbB4, ErbB3 is the actual receptor for all the NRG isoforms. ErbB2 has no affinity for NRGs, and there is no other ligand known for it. ErbB1 binds at least six other known EGF-like molecules, such as EGF, heparin-binding EGF (HB-EGF) and TGFα [101]. The lack of catalytic activity of the ErbB3 receptor, however, can be overcome by the process of ligand induced receptor dimerization, in which the three other ErbB members are recruited as coreceptors. This will lead to receptor phosphorylation and activation of downstream signalling pathways. Thus, ErbB3 signalling depends not only on NRG binding, but also requires association with other ErbB proteins, which in Schwann cells is most likely Erbb2. The other ErbB proteins share the ligand-induced formation of homo- and heterodimers, and all ten possible receptor combinations can exist. It has been demonstrated that different ligands prefer formation of specific heterodimers. Another mechanism leading to the observed multipotency of the NRGs, is the ability of the different residues in the cytosolic tail regions of these receptors to determine which intra cellular signalling proteins will be recruited to the activated receptor. Hence, heterodimerization and activation of different pairs of receptors could result in the triggering of different combinations of signalling pathways, which might also function synergistically.

In summary, different receptor-heterodimer combinations can associate with different combinations of intracellular signalling proteins, leading to diversity in the types

of pathways that are elicited by a single growth factor. This might explain the observations that NRGs can elicit different responses such as Schwann cell proliferation or survival, as will be discussed in the following sections.

1.5.4.1 Neural crest - Schwann cell precursor transition

The first transition occurs just after neural crest has invaded and populated the embryonic nerve bundles (Fig. 12). At this stage cells associated with these axons can be distinguished from neural crest cells on the basis of a number of properties, and are termed Schwann cell precursors [102,103].

First, they differ morphologically from neural crest cells. Precursors have sheath like processes, which contact each other and encircle large groups of axons into large bundle (fig. 12). These bundles can be observed in a rat E14 sciatic nerve, and two days earlier in the mouse embryo [103,104]. Second, formation of Schwann cell precursors from neural crest cells is characterised by the appearance of the differentiation markers F-spondin and the membrane associated phosphoprotein growth associated protein-43 (GAP-43) [105,106].

However, the most convincing fact demonstrating that precursors are an intermediate stage between neural crest and mature Schwann cells becomes apparent when precursors and Schwann cells are dissociated from the axons and placed in culture. In contrast to Schwann cells, which are very able to survive under defined culture conditions when deprived of axonal contact, Schwann cell precursors die through apoptosis within several hours of culture [103]. This indicates that the survival of Schwann cell precursors in developing nerves is probably supported by axon derived signals. In agreement with this

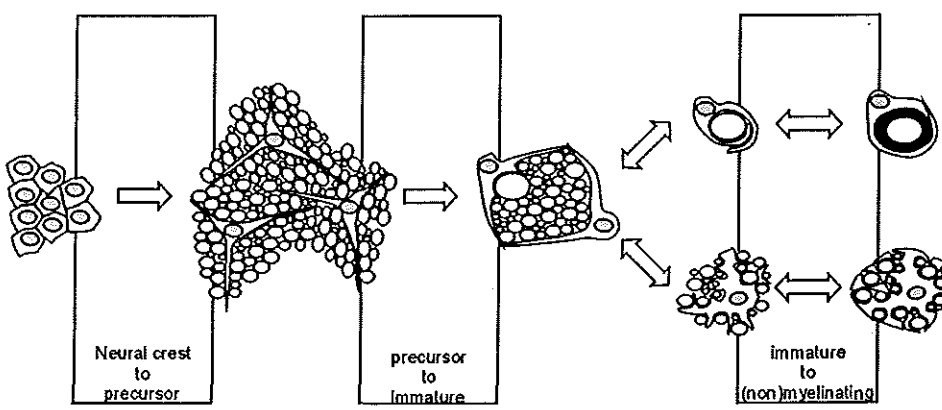

Figure 12: Three transitional stages can be identified within the Schwann cell lineage; from neural crest to Schwann cell precursor, from precursor to immature Schwann cell, and from Immature to (non) myelinating Schwann cells.

was the observation that when cultured in medium that was conditioned by DRG neurons, these precursors were able to survive and additionally differentiate into immature Schwann cells in a similar time frame as *in vivo*. The factor regulating survival and developmental progression of Schwann cell precursors has been identified as NDF, a member of the NRG growth factor family [107]. This study demonstrated that the signal in DRG conditioned medium could be blocked by addition of a soluble protein containing the extracellular domain of the ErbB-4 receptor, which is a high affinity receptor for NDF [107]. Furthermore, addition of purified NDF to defined media could mimic the effects of DRG conditioned medium.

It has also been demonstrated that NDF and its receptors are essential for precursor survival *in vivo*. Targeted deletion of the NRG-1 gene in mice resulted in an early embryonic lethal phenotype, caused by a heart defect [75]. However, just before these embryos died, it could still be assessed that there was an almost complete loss of Schwann cell precursors in the embryonic nerves. Targeted deletion of only the Ig-domain of the NRG gene leads to an isotype specific loss of type I and II, while NRG type III isoforms are normally expressed. Strikingly, mice homozygous for this mutation seem to have normal Schwann cell precursor numbers along their axons, indicating that type III NRG (= SMDF) is sufficient for precursor survival [108].

Lack of Schwann cell precursors was also observed after targeting of individual components of the NRG receptor. Animals lacking the ErbB3 receptor survive until birth, but their nerves were devoid of Schwann cells, as expected from the lack of precursors at earlier developmental stages [109]. Mice homozygous for a targeted ErbB2 allele died of heart defects at an embryonic stage, which was too early to evaluate its effect on Schwann cell development [110]. Recently, however, this heart defect has been rescued either by a transgene directing ErbB2 expression specifically to the heart via a cardiac-specific α-myosin heavy chain promoter, or by knocking in the ErbB2 cDNA into the heart specific *Nkx2.5* locus via homologous recombination [111,112]. In both cases rescued knock out mice survived until birth, and all peripheral nerves were essentially devoid of Schwann cells. This embryonic nerve phenotype is strikingly similar to the ErbB3 knockout phenotype. The ErbB4 gene has been targeted as well, but resulted in an embryonic lethal too early to investigate its role on Schwann cell precursor survival [113]. However, the fact that ErbB4 is not expressed in Schwann cells suggests that this is very unlikely [114,115]. Whether the lack of precursors is due to the fact that the neural crest is not instructed to become precursors, or precursors are formed but are not able to survive has not been demonstrated in these studies.

Nevertheless, these results underscore the importance of axonal contact, and identify the NRG-β and its receptors as essential components of this interaction. However, this dependence on axonal contact for survival will decline as precursors mature and become Schwann cells, which will be discussed in the following section.

1.5.4.2 Precursor- immature Schwann cell transition

This transition occurs quite rapidly, between E14 and E17 in the rat (E12/13-E15/16 in mice), and is accompanied by several morphological changes *in vivo*. In contrast to precursors which group axons into large bundles, immature Schwann cells start to invade this bundle and sort the axon population into smaller groups (Fig. 12). Depending on the size of the axons, immature Schwann cells will associate with only one large axon or multiple small calibre axons, and adopt a pro-myelinating and pro-non-myelinating configuration, respectively (Fig. 12). In rodents, most immature Schwann cells will have adopted this configuration just after birth.

In addition to these changes *in vivo*, interesting differences between these two populations become apparent, when these cells are put into culture. What is immediately apparent is the difference in morphology. Precursor cells are flattened cells with high motility and extensive cell-cell contacts, while immature Schwann cells have low motility and are bi- or tri-polar cells. Furthermore, immature Schwann cells can be distinguished from precursors as the former start to express a set of molecular markers, such as the Ca^{2+} binding protein S100, Galactocerebroside (GalC), and the sulphatide lipid antigen O4 [102].

However, the most striking difference between precursors and immature Schwann cells is that the latter have the ability to survive when cultured in high density under defined conditions, without the addition of NRG-β supplemented or DRG conditioned medium [103,104]. These results indicate that these immature Schwann cells have acquired an autocrine survival loop.

First, in vitro experiments have demonstrated that survival of these cells is dependent on the density of the cultures. Low-density cultures go into apoptosis, while high-density cultures survive. Second, low-density cultures can survive when grown in medium conditioned by high density immature Schwann cell cultures [107].

Potential components of this autocrine survival signal were identified when low-density cultures were grown in defined medium in the presence of various growth factors. Three factors, insulin-like growth factor 1 or 2 (IGF-1/-2), platelet derived growth factor BB (PDGF-BB), and neurotrophin-3 (NT-3) were able to individually and synergistically block immature Schwann cell death under these conditions [116]. In contrast to NRG-β, these factors are not mitogenic and do not promote survival of Schwann cell precursors.

Mature (non-myelinating and myelinating) Schwann cells probably fully depend on the autocrine survival signal. This assumption is based on experiments that described the expression pattern of the factors that are mediating the NRG-β and autocrine survival routes. Although NRG-β is present in adult nerves, the ErbB-2 receptor is downregulated in Schwann cells of adult peripheral nerves [107,115,117]. The lack of ErbB-2 disables the formation of a functional heterodimer ErbB receptor on the Schwann cell surface (See section 1.5.4) and hence prevents activation of the NRG-β mediated survival route.

In contrast, IGF-2, PDGF-BB, and NT-3 and their corresponding receptors are upregulated in Schwann cells during peripheral nerve development and regeneration, and are all present in mature Schwann cells [116,118]. This would suggest that in adult nerves, Schwann cells depend on autocrine signals for survival.

This switch from axon dependent survival in precursors, to an axon-independent autocrine survival in mature Schwann cells occurs gradually during peripheral nerve development. This is demonstrated when axonal contact is lost after nerve transection at

perinatal stages. At that stage Schwann cells go into apoptosis, which could be prevented by the application of exogenous NRG-β [119]. However, in nerves that were transected only two weeks later Schwann cells were already able to survive with very little programmed cell death occurring [115,119,120].

Two intracellular signal transduction pathways are involved in mediating both the NRG-β and autocrine signals that lead to survival of Schwann cells. First, the mitogen activated protein (MAP) kinase pathway is involved in the regulation of both precursors and immature Schwann cell survival. In precursors, this pathway is activated by NRG-β [107]. However, NRG-β does not utilise this pathway in immature Schwann cells, since addition of agents that specifically block this pathway did not lead to a loss of NRG-β mediated survival. Nevertheless, these MAP kinase pathway blocking agents, were able to interfere with the survival of cultures supplemented either with the IGF-1/-2, PDGF-BB and NT-3, or high-density culture conditioned medium [116]. This indicates that NRG-β mediates immature Schwann cell survival via another signalling pathway.

The second intracellular signal transduction pathway that has been demonstrated to be involved in Schwann cell survival is the phosphoinositide-3-kinase (PI3K) pathway [104]. Recently, it has been shown that both precursors and immature Schwann utilise this pathway in NRG-β mediated survival [104]. In this study, NRG-β mediated survival could be prevented by addition of a specific PI3K pathway inhibitor in both precursor and low-density immature Schwann cell cultures.

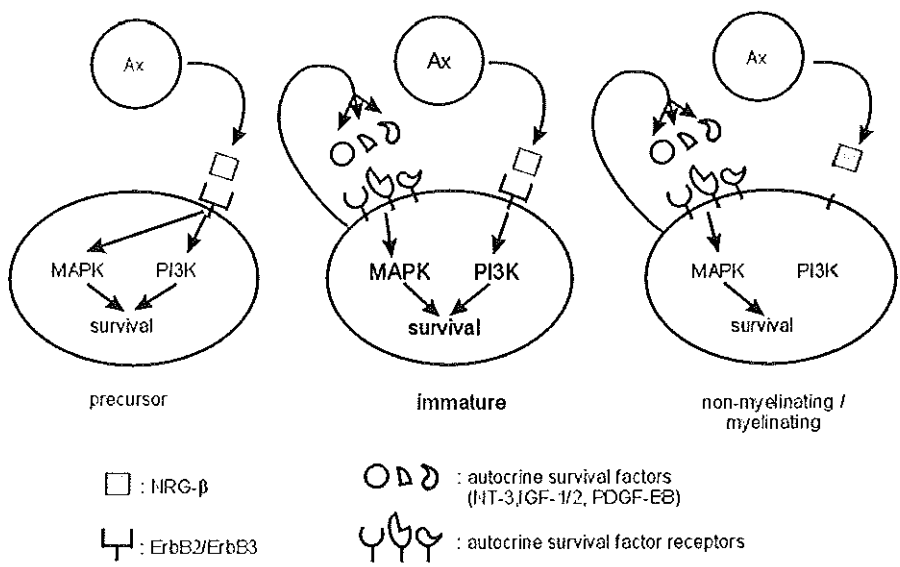

Figure 13: During peripheral nerve development, the Schwann cell gradually changes its survival potential from being completely dependent on axonal contact (NRG-β) in Schwann cell precursors to being self-supporting in mature Schwann cells via an autocrine survival loop. This autocrine survival loop is acquired by immature Schwann cells, which rely on both axonal and autocrine survival signals. The MAPK and PI3K intracellular signalling pathways are involved in mediating these signals.

In summary, during peripheral nerve development, Schwann cells gradually switch their survival potential from being completely dependent on axonal contact (NRG-β mediated survival) in Schwann cell precursors, to self supporting in non-myelinating and myelinating Schwann cells (the autocrine survival loop) (Fig. 13). These changes in survival potential during Schwann cell development can be very well reconciled with the *in vivo* situation. The dependence of precursors on axonal contact is likely involved in the matching of precursor numbers to axon numbers, preventing survival of precursors not in contact with axons at aberrant locations [120,121]. On the other hand, axon independent survival of Schwann cells is a prerequisite for axonal regeneration to occur after nerve damage (See section 1.5.5) [122].

1.5.4.3 Immature to (non-) myelinating Schwann cell transition

At this final transitional stage in Schwann cell ontogeny, which in rodents occurs perinatally, Schwann cells differentiate into a myelinating or non-myelinating phenotype (Fig. 12). The choice between either of the two fates is determined by the axon. The identity of the axonal signal is still not known although it does correlate with the axonal diameter. Axons above a certain diameter (>1μm) will be myelinated, while smaller axons will be accommodated in shallow troughs along the surface of non-myelinating Schwann cells (See section 1.4.1). If the latter option occurs, only few morphological and biochemical changes can be detected.

First, non-myelinating Schwann cells express several surface markers which distinguish them from myelinating Schwann cells, such as p75, neural cell adhesion molecule (N-CAM), glial fibrillary acidic protein (GFAP), and an immunoglobulin-related adhesion molecule (L1). However, these are also already expressed by the pro-non-myelinating Schwann cells. So far only the absence or presence of the glycolipids galactocerebroside (GalC) and galactosulfatide (sGalC) in the non-myelinating Schwann cell membrane distinguishes the immature stage from the mature stage, respectively. However, GalC is also present in the lipid bilayer of myelinating Schwann cells.

The scarcity of molecular changes during the pro-non-myelinating to non-myelinating switch is directly correlated to the number of studies performed on non-myelinating Schwann cells. Only the role of L1 in non-myelinating Schwann cells has been addressed thoroughly. Targeted deletion of the L1 gene in mice leads to a reduced association of non-myelinating Schwann cells with axons [123]. However, since neurons were also affected, it could not be addressed whether this defect was Schwann cell autonomous. Recently, it was observed that in unmyelinated sensory nerve fibres, both axons and Schwann cells express the L1 gene [124]. In nerve fibres of unmyelinated sympathetic neurons, only Schwann cells express L1. In L1 knockout mice, only non-myelinating Schwann cells of the sensory fibres were affected, while Schwann cells of the sympathetic fibres appeared normal [124]. Furthermore, transplantation experiments between nerves of wildtype and L1-deficient mice demonstrated that L1-deficient Schwann cells transplanted on wildtype axons generated apparently normal unmyelinated axons, while wildtype Schwann cells brought into contact with L1-negative axons reproduced the L1-deficient phenotype [124]. These data establish that L1 expression on axons but not on non-

myelinating Schwann cells is essential for the formation of unmyelinated axons. Probably heterophilic interactions between L1 on the axonal membrane and other components on the non-myelinating Schwann cell membrane are required for the formation of functional non-myelinated nerve fibres.

1.5.4.4 Myelogenesis

In contrast to non-myelinating Schwann cells, an overwhelming number of studies have been performed on its myelinating counterpart.

The myelin sheath is composed of two distinct domains, compact and non-compact myelin, each of which is characterised by a unique set of proteins (Fig.14). Compact myelin, forming the typical major dense and intraperiod lines as observed by electron microscopy (see section 1.4.2), contains the major myelin proteins P0, PMP-22 and myelin basic protein (MBP). Non-compact myelin are regions where the intracellular surface of the membrane are separated by cytoplasm as in the Schmidt-Lanterman incisures and paranodal loops (see section 1.4.2). These regions contain proteins such as myelin-associated glycoprotein (MAG), E-cadherin, $\alpha 6\beta 4$ integrin, and connexin 32 (Fig. 14). GalC and sGalC are the dominant glycolipids in the myelin membrane (Fig. 14). All these elements are highly upregulated as soon as the Schwann cell is instructed to myelinate, and concomitantly most immature Schwann cell markers are downregulated. Thus a transcriptional programme must exist that initiates and modulates the myelination programme in response to an axonal signal. The role of myelin proteins and glycolipids in the formation of the myelin sheath has been investigated in various experimental settings. I will first discuss the proteins present in compact myelin.

P0 is by far the most abundant protein present in the peripheral myelin accounting for approximately 50% of the total amount of protein in the sheath. P0 is a transmembrane protein belonging to the immunoglobulin class of proteins. Recently, a structural model based on X-ray diffraction data has been proposed. These data suggest that P0 molecules form tetramers that are arranged parallel to the Schwann cell membrane [125,126]. These tetramers form a functional unit that interacts with P0 tetramers on the opposing membrane, forming a lattice that holds compact myelin together. The intracellular components of P0 might also be involved in the adhesive property of the protein, by interacting with each other or acidic lipids or cytoskeleton proteins [127-129].

PMP22 is a tetraspan transmembrane protein, predominantly expressed in compact myelin. Its extracellular domain has been implicated in intracellular recognition and adhesion, while on the other hand, it also structurally resembles an ion channel [130]. The precise role of PMP22 in the compaction of myelin is not clear.

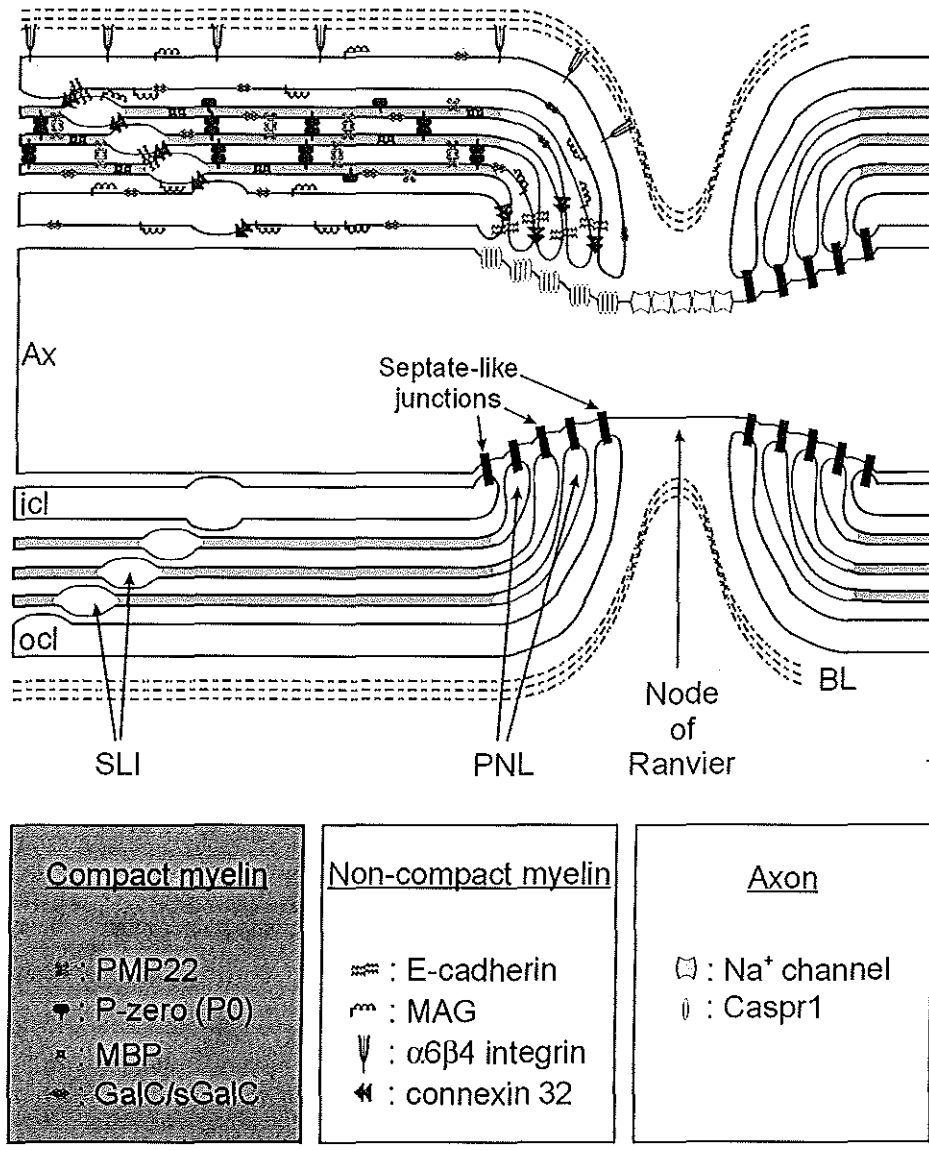

Figure 14: Location of Schwann cell membrane associated and axonal proteins in a myelinated nerve fibre. Abbreviations: Ax, axon; icl, inner collar; ocl, outer collar; SLI, Schmidt-Lantermann incisures; PNL, paranodal loops; BL, basal lamina.

Third, MBP is present in the compact myelin regions of both oligodendrocytes in the CNS and Schwann cells. In contrast to P0 and PMP22, which are transmembrane proteins, MBP is localised in the cytoplasm. The association of MBP with membranes is primarily ionic, by virtue of its high positive charge. MBP probably mediates apposition of the myelinplasma membrane by reacting with acidic lipids, undergoing conformational changes, or with other proteins on the opposite site of the intracellular plasma membrane (reviewed in [131]).

I will now further elaborate on the proteins present in the non-compacted regions. Both MAG (a member of the Ig super gene family) and E-cadherin are transmembrane proteins present in the uncompacted myelin compartment. In contrast to MAG, E-cadherin can interact homophilically and probably serves to hold the layers of non-compact myelin together at adherens junctions, which join actin bundles of two opposing Schwann cell sheaths and are found in non-compact myelin [132]. MAG has been suggested to stabilise the extracellular space between the inner mesaxon and the axon, which correlates with the adaxonal localisation of MAG during myelination [133,134].

The abaxonal side of the Schwann cell is where it contacts the basal lamina. Several Schwann cell membrane associated proteins have been identified which interact with the basal lamina. One of those factors is β4 integrin, which is expressed together with its heterodimeric partner α6 integrin on the abaxonal surface of the myelinating Schwann cell membrane. It probably functions as a receptor for laminins present in the Schwann cell basal lamina. Interactions between laminin and α6β4 integrin might potentially be linked to the *ras*-MAP kinase signal transduction pathway [135]. Other factors that have been identified, which might play a role in the interaction between the outer membrane of the myelinating Schwann cell and the basal lamina, are dystroglycan and N-syndecan.

Connexin 32 is a member of the gap junction protein family, which is expressed in these non-compacted regions of the Schwann cell membrane, such as the Schmidt-Lanterman incisures and paranodal loops [136]. Gap junctions allow fast transport of low molecular weight proteins between adjacent non-compacted myelin regions, such as paranodal loops (see section 1.4.2).

Summarising, the highly structured positioning of these molecules in the myelinating Schwann cell membrane suggests that they execute particular functions in the myelin sheath. That this is indeed the case can be concluded from the fact that mutations in, or altered levels of, most of these factors will results in the dysfunction of the myelin sheath, as discussed in the following section.

1.5.4.5 Myelinopathies

The importance of the structural Schwann cell proteins mentioned in the previous section is demonstrated by the peripheral nerve phenotype of many mice carrying targeted deletions or naturally occurring mutants of these genes. Alterations in the structure or copy number of the homologous genes in humans result in several peripheral neuropathies (Fig. 15). In this section I will discuss the consequences of these alterations on myelination in mice and humans.

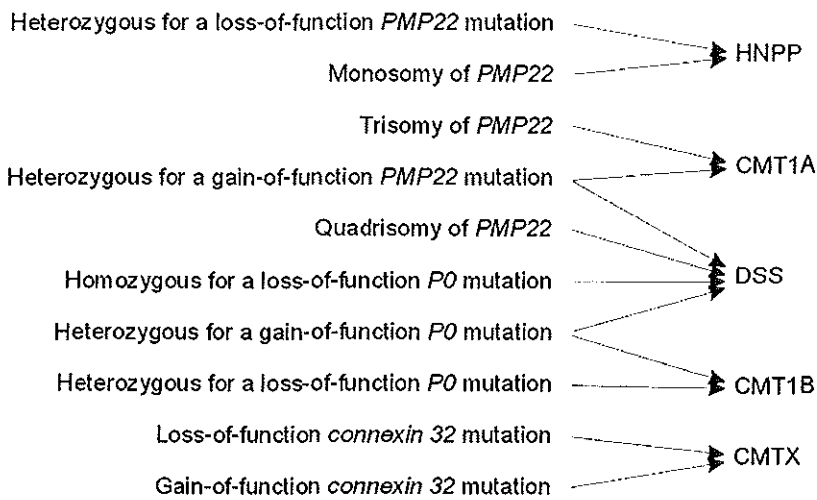

Figure 15: The relationship between mutations in PMP-22, P0, connexin 32 and inherited demyelinating neuropathies. Abbreviations: HNPP, hereditary neuropathy with liability to pressure palsies; CMT, Charcot-Marie-Tooth disease; DSS, Dejerine-Sottas syndrome. (Adapted from [137])

Targeted deletion of the P0 gene in mice results in a severe loss of myelin in the PNS [138]. Schwann cells do establish a one-to-one relation with the axon, but the subsequent process of forming compact myelin is greatly disturbed. Furthermore, young mice heterozygous for the P0 deletion appear normal, but develop progressive demyelination with age [139]. These observations suggest that myelin compaction can occur normally with only one P0 allele, but that the accurate dosage of functional P0 is required for maintenance.

Strikingly, the formation of the major dense line in P0 knockout animals is still present, while the intracellular part of P0 has been thought to be important for the formation of this structure. This led to the conclusion that other factors present in the compact myelin region might contribute, in addition to P0, to the formation of the major dense line. This became evident when the mice doubly mutated for P0 and MBP were generated. A natural occurring null-mutant of MBP, the *shiverer* mouse, was used for the generation of double mutant mice. The *shiverer* mouse has hypomyelination in the CNS, where major dense lines are lacking. PNS myelin however does not show major abnormalities, which has been attributed to the presence of P0 [140]. The combination of P0-deficient knock outs and MBP-deficient shiverer mice, generated thin myelin sheaths which lack major dense lines, indicating that both molecules contribute to the fusion of the cytoplasmic surfaces [141]. Combinations of P0 targeted mice crossed with knockout mice either deficient for PMP22, MAG, or connexin32 only gave subtle additional phenotypes when compared to mice only homozygous for the P0 deletion [142-144]. The defect of the compact myelin regions in these double knockout mice were indistinguishable from single P0-deficient knockouts,

indicating that P0 together with MBP are the major determinants of compact myelin structure.

Phenotypes of mice with mutations in the P0 gene are reminiscent to human neuropathies. This group of disorders includes Charcot-Marie-Tooth disease type 1B(CMT1B), Dejerine-Sottas syndrome (DSS) and hereditary peripheral neuropathy with liability to pressure palsies (HNPP) reviewed in [130](Fig. 15). It is assumed that mutations leading to reduced levels of functional P0 protein cause relatively mild clinical phenotypes seen in CMT1B, whereas those mutations that perturb the multimeric assembly of P0 are responsible for more severe neuropathies such as DSS [145].

Secondly, PMP22 is linked to the most prevalent form of hereditary peripheral neuropathy in humans, CMT1A, which has incidence of 1 in 4,000 new-borns. Also other peripheral neuropathies are linked to PMP22 with distinct clinical parameters. CMT1A is most frequently associated with a duplication of a 1.5Mb region of chromosome 17 resulting in a triple gene dose of PMP22. Homozygosity of this 1.5Mb duplication, leading to a quadruple gene dose or other specific point mutations in the gene result in the more severe demyelinating neuropathy DSS. Deletion of the same 1.5Mb region, resulting in a monosomy, gives rise yet to another phenotype, which is HNPP. Therefore, clinically different syndromes are mostly associated with varying gene doses of the PMP22 gene.

Changing the number of PMP22 gene copies in mice and rat generates similar phenotypes as compared to human PMP22 related syndromes having the same gene dosage. For instance, transgenic mice or rats having an extra copy of the gene develop a neuropathy which clearly resembles the CMT1A neuropathy related with a triple gene dose of PMP22 in humans [146,147]. Furthermore, mice heterozygous for targeted deletion of the PMP22 gene (one gene dose) develop pathological features reminiscent of HNPP, which again is in agreement with the corresponding gene dosage in humans [148].
Mice homozygous for targeted deletion of the PMP22 gene develop a severe polyneuropathy caused by delayed myelin formation and focal hypermyelination (tomacula)[148]. This phenotype resembles a CMT1-like demyelinating neuropathy, but human families lacking PMP22 have not been described yet. Originally, PMP22 was found to be mutated in the natural occuring mouse mutant *Trembler* [149]. *Trembler* and *Trembler*^{J} mice, have mutations which correspond to mutations at the same positions of the PMP22 gene in human CMT1A and DSS syndromes (reviewed in [137]).

Switching to proteins present in the non-compacted myelin, the X-linked gene connexin 32 has been associated with the second most common human neuropathy (occurrence 1 in 25,000), CMT1X [150]. CMTX is best characterised as an X-linked *dominant* disease. Affected males are invariably affected with moderate to severe manifestations, whereas heterozygous females tend to be more mildly affected and may be asymptomatic. The variable manifestations in females may be due to variation in X inactivation, although this has not been proven.

Mutation of connexin 32 in CMTX vary from point mutations to partial and complete deletions in the coding region, but also point mutations outside the coding regions have been identified. These mutations have been demonstrated to interfere with connexin 32 function in several ways. First, cell culture experiments demonstrated that after transfection of mutated connexin 32 genes, mutant protein was localised to the plasma membrane but did not form functional channels [151]. Strikingly cotransfection of mutated

and normal connexin 32 demonstrated that mutated connexin 32 has a dominant negative effect on the formation of channels by wildtype connexin in this system [151].
Second, mutations in the gene have been demonstrated to interfere with the trafficking of the protein to the plasma membrane. Most mutations seem to result in accumulation of mutant protein in the Golgi apparatus [152].

The effects of connexin 32 mutations and deletions have also been addressed in mouse models. Initial observations of mice with targeted disruption of the connexin 32 gene did not show any obvious signs of peripheral nerve pathology [153]. However, subsequent studies demonstrated that these mice develop a late-onset demyelinating neuropathy, similar to that seen in CMTX patients [153-155]. In female heterozygotes the loss of expression was segmental and cell autonomous, consistent with random X-inactivation [155]. Transgenic mice expressing mutant forms of connexin 32 present with a pathology that depends on the nature of the mutation. In a missense mutation, dominant effects are seen in transgenic mice [156]. These mice develop a late-onset demyelinating neuropathy despite the presence of endogenous connexin 32. Interestingly, normal and mutant connexin 32 are retained in the cytoplasm, indicating a dominant negative effect of mutant connexin 32 on the trafficking of the endogenous protein [156]. This effect of mutant connexin 32 on the normal protein is unlikely to occur in CMTX patients carrying this mutation, where, by X-inactivation, only one allele is expressed.

Although previous data suggested that MAG plays an important role in the formation of myelin, targeted deletion of MAG in mice demonstrated that MAG is not essential for myelination in rodents [133,134,157,158]. However, subtle defects in the myelin sheath developed over time in MAG -/- animals [159]. In older MAG -/- animals myelin degenerates and Schwann cells express genes that are typical for non-myelinating nerves [160]. These data indicate that MAG is important for maintenance of the myelin sheath. Natural occurring mouse mutants or human neuropathies linked to the MAG gene have not been identified.

Two other factors present in non-compact myelin are $\alpha 6\beta 4$ integrin and E-cadherin. The function of the latter in Schwann cells has not been addressed in a gene knockout or transgenic mouse paradigm. Mice homozygous for deletion of the $\beta 4$ gene have been generated, but die too early to asses its effect on myelination [161]. However, few myelinated fibres are present in femoral nerves of newborn $\beta 4$ integrin deleted animals suggesting that $\beta 4$ is not essential for initiation of myelination. In addition, in vitro myelination studies using explants from $\beta 4$ -/- animal show that Schwann cells are capable to myelinate in the absence of $\beta 4$ integrin [162].

Finally, during myelination Schwann cells synthesise myelin-enriched lipids. The dominant lipids in the myelin bilayer are the glycolipids, which include GalC and sGalC (sulfatide). These glycolipids have the unusual property that their fluid/solid phase transition temperature is so high that they almost behave as crystals at body temperature (reviewed in [163]). The identification and targeted deletion of the key enzyme in the synthesis of galactolipids, UDP-galactose-ceramide galactosyltransferase (cgt) has provided insights into the pivotal function of these glycolipids in the normal myelin sheath. Mice lacking the cgt gene are completely devoid of GalC and sGalC [164,165]. Instead these mice

incorporate glucocerebroside in their myelin membrane. Expression of P0, MBP, and MAG in these mice was normal, in agreement with the finding that myelination was unaffected in these mice. However, a striking phenotype developed from 10 days after birth, which included whole-body shivering, seizures, increasing loss of motor activity, reduced growth and death from 20 days onwards. Nerve conduction measurements demonstrated that, despite normal myelination, the nerve conduction velocity in homozygous cgt knockout mice was reduced from saltatory conduction of myelinated nerves to the much slower continuous conduction of unmyelinated nerves [164]. These results suggest that GalC and sGalC maybe involved in the maintenance of an ion permeability barrier in the myelin sheath. More recently, defects in the paranodal loops of these mice have been described. It is thought that these defects contribute to the phenotype [166].

1.5.5 Schwann cells support neuronal survival

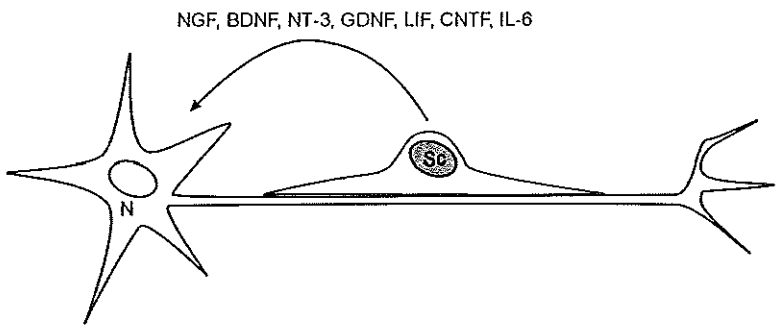

Figure 16: Schwann cells (Sc) support neuronal (N) survival during development and regeneration by the production of the neurotrophins NGF, BDNF, NT-3 and GDNF and the neurokines LIF, CNTF and IL-6. For details see text section 1.5.4.

In the intimate axon-Schwann cell relationship, the axon seems to be the main driving force of peripheral nerve development, directing Schwann cells towards their final differentiated state (myelinating or non-myelinating Schwann cell). However, numerous studies have suggested that axon-Schwann cell interactions are reciprocal. Over the last few years it has become clear that neurons do not only depend on target derived neurotrophic support, but also on support provided by the Schwann cells. In addition, much evidence has accumulated to support a role for Schwann cells in all aspects of peripheral nerve development, including the formation of a functional perineurial sheath (see section1.5.5) and positioning of the nodes of Ranvier (see section 1.5.6). In this section I will focus on the role of Schwann cells in the control of neuronal survival during development and regeneration

The involvement of Schwann cells in the regulation of sensory and motor neuron survival became apparent after detailed examination of neuregulin, ErbB2, or ErbB3 deficient mice. As mentioned earlier (see section 1.5.4.1), peripheral nerves in these mice are completely devoid of Schwann cells. Strikingly, mice homozygous for the ErbB3 mutation lost 80% of their sensory neurons at E14 while at E12 this number was still comparable to wildtype embryos. Furthermore, the number of knockout and wildtype motoneurons at E16 were similar, but at E18 the number of motoneurons in knockout animals was only 20% of the number counted in wildtype embryos [109].

Sensory neurons already died before most of them had reached their target tissue, indicating that cell death was not caused by the lack of contact between neurons and their targets, but seemed completely attributable to the lack of Schwann cells and their precursors. In contrast, survival of motoneurons seems to depend on survival signals from both Schwann cells and target organs, since motoneurons die after they have reached their target. These data also demonstrate that motoneurons depend on signals derived from Schwann cells but not from Schwann cell precursors as most have already differentiated into Schwann cells in mouse E16 peripheral nerves [104].

The generation of chimeric mice by injecting ErbB3-/- ES cells into wildtype blastocysts demonstrated that these cells contribute to sensory and motoneuron lineages, but not to the Schwann cell lineage [109]. This result established that ErbB3 function is Schwann cell autonomous.

Schwann cell dependent neuronal survival was also observed in mice lacking the ErbB2 gene after rescuing the early embryonic lethal phenotype by restoring the ErbB2 expression specifically in the developing heart [111,112]. As in the ErbB3 knockout mice, Schwann cells were completely absent in peripheral nerves of rescued ErbB2 lacking mice. Consequently, motoneuron apoptosis occurred in a comparable time frame and the same extend as in ErbB3 deficient mice. In addition, loss of sensory neurons in ErbB2 knockout mice was also observed, but the time span and extend to which this cell death occurred was not reported. By and large these experiments demonstrate that both motoneurons and sensory neurons depend on Schwann cells for their survival.

Several lines of evidence have demonstrated that Schwann cells promote sensory neuron and motoeuron survival by the production of neurotrophic factors [167]. Thus, in the absence of Schwann cells neurotrophic support is reduced and consequently neuronal cell death increased.

In addition to Schwann cells, target organs also produce neurotrophic factors. During development of the nervous system excess numbers of neurons are produced. Only those making contact with their target organs will survive, suggesting that neurons compete for a limiting supply of these target-derived factors.

The last few decades a large number of factors demonstrated to support neuronal survival have been identified [168]. They can be divided into several groups according to primary sequence similarity and to the type of receptors they bind to. Thus, a specific neurotrophic factor will only support survival of a subset of neurons expressing the corresponding receptor.

The first neurotrophin identified in 1952 was nerve growth factor (NGF) (reviewed in [169]). NGF belongs to a family of factors, including brain-derived-neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT4/5 and NT-6. These factors bind with high affinity to members of tyrosine receptor kinase (trk) family. These receptors

heterodimerize with the low-affinity receptor p75 to form a functional receptor [170]. Three receptors have been identified, each specific for a different neurotrophin. NGF binds to trkA, while trkB is specific for BDNF and NT-4/5, and NT-3 specifically interacts with trkC [170]. All three receptors are distributed to discrete but partially overlapping subpopulations of primary sensory neurons, while trkB and trkC are present on spinal motoneurons. Consistently, the target tissue innervated by these neurons express the relevant neurotrophins.

However, some sensory neurons do not express trk receptors. This population has been found to express a receptor complex for another neurotrophin termed glial-derived-neurotrophic factor (GDNF) [171]. This receptor complex consists of the Ret receptor, combined with GFRα-1 or GFRα-2. In addition to its trophic effect on this trk deficient sensory neuron population, GDNF also supports survival of motoneurons and autonomic neurons [172,173].

Other factors that have been demonstrated to promote neuronal survival, are the neurokines. They differ from the neurotrophins by their interaction with multicomponent receptors, which are different from the neurotrophin receptor tyrosine kinases. Members of the neurokine family are ciliary-neurotrophic-factor (CNTF), leukaemia inhibitory factor (LIF), and interleukin-6 (IL-6). LIF and IL-6 are able to mediate survival when the receptor is composed of two components, which are LIF receptor β (LIFRβ) and gp130. CNTF requires an additional component, CNTF receptor α (CNTFRα), to be functional [174].

The dependence of subsets of neurons to interact with specific neurotrophins and neurokines to survive underscores the complex interactions that are involved in setting up a functional network between the nervous system and the tissues it innervates. The observation that neurotrophin dependence of specific subsets of neurons varies during development sets yet another level of regulation on the formation of this network [175]. For instance, sensory neurons in the trigeminal ganglion are dependent on BDNF and NT-3 for their survival, while growing towards their targets. However, after reaching these targets, they lose their dependency on BDNF and NT-3 and now require NGF for their survival [176]. This became apparent after targeted deletion of members of the neurotrophin family and their receptors. A large number of trigeminal ganglion neurons die before reaching their targets in mice lacking NT-3 or the BDNF receptor trkB, while the same neurons die later during development in mice lacking the NGF receptor trkA (reviewed in [177]).

As in the ErbB3 and ErbB2 deficient mice, death of these neurons before they reach their targets, indicates that cells along the trajectory of the developing nerve are required for trophic support. It is likely that Schwann cells are the cells producing these factors. Indeed, most of the neurotrophins and neurokines mentioned above are expressed in Schwann cells during different stages of development (Fig 16). For instance, BDNF secreted from Schwann cells has been demonstrated to play a role in the survival of motoneurons during embryonic development [178]. It complements the motoeuron survival promoting effect of CNTF, which is only expressed in mature myelinating Schwann cells from two weeks after birth onwards [179].

NT-3 has been demonstrated to be produced by Schwann cell precurors and mature Schwann cells [116]. Although NT-3 seems to be a component of an autoregulatory survival loop for mature Schwann cells, it does not support survival of Schwann cell

precursors. This would indicate that Schwann cell precursors do not produce NT-3 to support their own survival but that of the neurons they are associated with. This could explain the death of neurons before they have reached their targets in NT-3 knockout mice [180]. Neurons in turn produce NRG, which is essential for Schwann cell precursor survival [75,107]. This reciprocal survival signalling between neurons and non-neuronal cells has also been demonstrated between sensory neurons and satellite cells in sympathetic ganglia [181].

The neurotrophin GDNF is also produced by Schwann cells and their precursors. Lack of GDNF in knockout mice results in 20% reduction of the number of motoneurons at birth [172,182].

Since the number of neurons that die in mice which are completely devoid of Schwann cells (like in ErbB3 knockout mice) is higher than in mice that lack only a single neurotrophin or neurokine, indicates that concert action of several of these factors is required for neuronal survival during development. For instance, Schwann cell derived GDNF and muscle derived cardiotrophin-1 (CT-1), in culture synergistically regulate motoneuron survival in vitro [183]. Moreover, as mentioned before, mice lacking CNTF loose a number of motoneurons during postnatal development [179]. Although, mice lacking LIF have no significant motoneuron loss, double knockout mice of LIF and CNTF produces an earlier and more severe motoneuron apoptosis, than in single CNTF targeted mice [184].

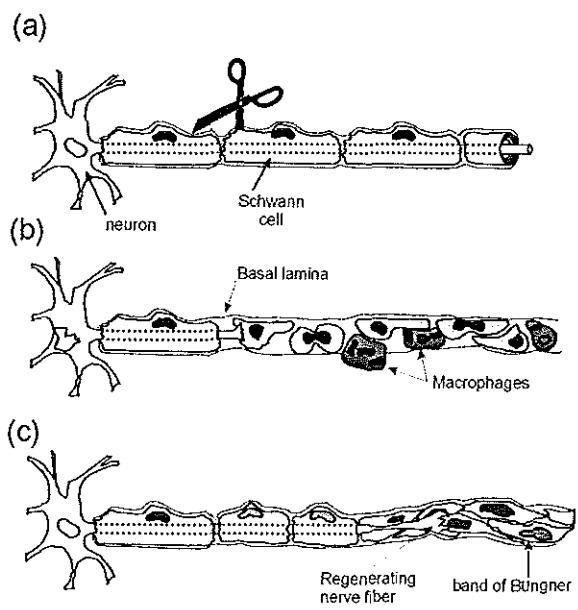

Figure 17: Changes in peripheral nerve during Wallerian regeneration. (a) An uninjured myelinated axon. The myelinating Schwann cells are surrounded by a basal lamina. The pair of scissors marks the site of injury (which in this case leaves the basal lamina intact). (b) A few days after injury, the axon and its myelin sheath degenerate distal to the site of injury. Macrophages invade the distal nerve stump, penetrate the basal lamina, and phagocytose degenerating myelin. Schwann cells proliferate. Proximal to the injury, a growth cone has formed and begins to regenerate down the bands of Büngner. (c) A few weeks after injury, the axon has regenerated further down the band of Büngner, and its more proximal portion has been re-ensheathed and subsequently remyelinated by Schwann cells. (Adapted from [185]).

Signalling from Schwann cells to neurons is also important to allow re-growth of axons following nerve damage. The first thorough description of nerve regeneration dates from the 18th century [186]. Ever since this report, the process of regeneration has been the subject of intense research. This has led to a precise temporal description of the morphological and molecular changes occurring in neurons and Schwann cells during peripheral nerve regeneration (Fig. 17).

When axonal integrity is interrupted by, for instance by crush or axotomy, degeneration will occur distal to the site of the insult in a process called Wallerian degeneration, named after its discoverer [187](reviewed in [188]). Immediately after damage, the proximal and distal nerve stumps retract, axoplasm leaks out and damaged membranes collapse. Macrophages are recruited to the site of the lesion during the first week after injury, which contribute to the lysis and phagocytosis of myelin (reviewed in [122]. Dedifferentiation of Schwann cells is accompanied by downregulation of expression of the major myelin genes (P0, PMP22, MBP) and reestablishment of many immature and non-myelinating Schwann cell markers. At this stage Schwann cells are proliferating within the distal stump and line up in their basal lamina, forming the so-called endoneurial tubes or bands of Büngner. Because these Schwann cells have obtained an autocrine survival loop (see section 1.5.4.2), they are able to survive in the absence of axonal contact. These proliferating Schwann cells form a conduit which guides regenerating axons to their target [189]. Proximal to the injury site, axons give rise to one or more sprouts, each of which contains a growth cone. These growth cones have to reach the distal part for regeneration to be successful. While both nerve crush and axotomy induce Wallerian degeneration, regeneration in the latter occurs less efficient. After a nerve crush, the continuous basal lamina provides guidance for regenerating axons, while after axotomy separation of the proximal and distal stumps can impede reinnervation. If regeneration does occur, it happens with high accuracy, in the sense that axons are able to find the targets they innervated before the injury. Upon reestablishment of axonal contact, Schwann cells stop proliferating, downregulate the immature/non-myelinating genes, and start to remyelinate the nerve sprouts. The process of remyelination is very similar to the process of myelination during normal development [25].

Schwann cells are vital during regeneration, and one of their functions is to produce neurotrophic factors to create an environment that is supportive for axonal regrowth. Following axonal injury, Schwann cells of the distal stump immediately upregulate the synthesis of NGF, and LIF, and after several days BDNF, NT-4/5, but not NT-3 (reviewed by [190]). In contrast, CNTF requires intact axon-Schwann cell contact and is reduced during Wallerian degeneration [191]. Schwann cells also upregulate the expression of IGF-I and -II, which has also been demonstrated to have neurotrophic capacities [192,193]. Furthermore, after sciatic nerve crush, there's a rapid upregulation of GDNF in the distal part of the nerve [194].

Summarising, the striking loss of sensory neurons and motoneurons before target innervation in ErbB-2 and ErbB-3 knockout mice, and the ability of neurons to regenerate after nerve damage in the absence of target derived neurotrophic factors, point to Schwann cells and their precursors as an important source of these factors. Correctly timed and combined expression of these factors by Schwann cells are essential to obtain optimal development and regeneration of sensory neurons and/or motoneurons.

1.5.6 Schwann cells and the formation of the nerve sheath

The formation of the perineurium (see section 1.3.2.2) starts during embryonal development and it arises from mesodermally derived mesenchymal cells surrounding the embryonic axons and Schwann cell precursors [19]. The recruitment of these cells occurs late during embryonic development, from about E15 onwards in the mouse sciatic nerve. Cells assemble concentrically around the developing nerve, and around birth a recognisable perineurium is present. At this stage the perineurium is still leaky, but becomes impermeable a few weeks after birth.

Recently a Schwann cell derived signalling molecule has been demonstrated to be essential in the formation of the perineurial sheath [195]. Examination of peripheral nerves of homozygous desert hedgehog (Dhh) mutant animals suggested that Dhh signalling is required for the development and function of the perineurial nerve sheath [195].

Dhh is one of the three members of the Hedgehog family of intercellular signalling molecules that play key roles in embryo patterning [196]. Dhh is expressed in testis and Schwann cells during development [197]. Mice homozygous for targeted deletion of the *Dhh* gene failed to develop mature spermatozoa, indicating a role for Dhh in spermatogenesis [198]. Strikingly, in peripheral nerves of these mice, all three layers of connective tissue were severely affected (Fig. 18). However, myelinating and non-myelinating fibres appeared morphologically normal. Not surprisingly, nerve conduction velocities in these mice were normal [195].

Expression of patched (Ptc), the cognate receptor of Dhh, was demonstrated in the presumptive perineurial cells during development in normal nerves from E15 onwards. However, Ptc expression was severely down regulated in Dhh lacking mice, indicating that Dhh regulates the expression of its own receptor. This has also been demonstrated for the other hedgehog gene family members (reviewed in [199]).

The remaining perineurial sheath consisted of only a few layers and extends abnormally within the endoneurium, forming mini-fascicles. Ultrastructural studies revealed that tight junctions of Dhh knockout perineurial cells were abnormal despite the fact the expression of several tight-junction associated proteins appeared unaffected. However, Dhh deficient perineurial cells were lacking expression of gap-junction protein connexin 43. Disruption of gap- and tight-junctions in the perineurium, suggested that the barrier function of this layer might be compromised. Indeed, marker proteins that are normally excluded penetrated the nerve of Dhh -/- animals. In addition, neutrophils moved freely into the nerve, following a focal infection outside the nerve.

The fact that still a thin layer of perineurial cells is formed in Dhh knockout mice, indicates that Dhh is not essential for the early formation of a loose perineurial sheath in embryonic nerves. The factor involved in this initial recruitment of mesenchymal cells around axon-Schwann cell units is unknown. However, the observation that a perineurial-like sheath is formed in the absence of Schwann cells in the genetically rescued ErbB2 knockout mice, suggests that the initial signal might be axon derived [112]. However, lack of markers for presumptive perineurium at these early stages makes it difficult to prove that this layer is indeed presumptive perineurium.

In summary, the data described in this section clearly indicate that in addition to supporting neuronal survival, Schwann cells are also involved in the development of a functional protective connective tissue layer surrounding the nerve.

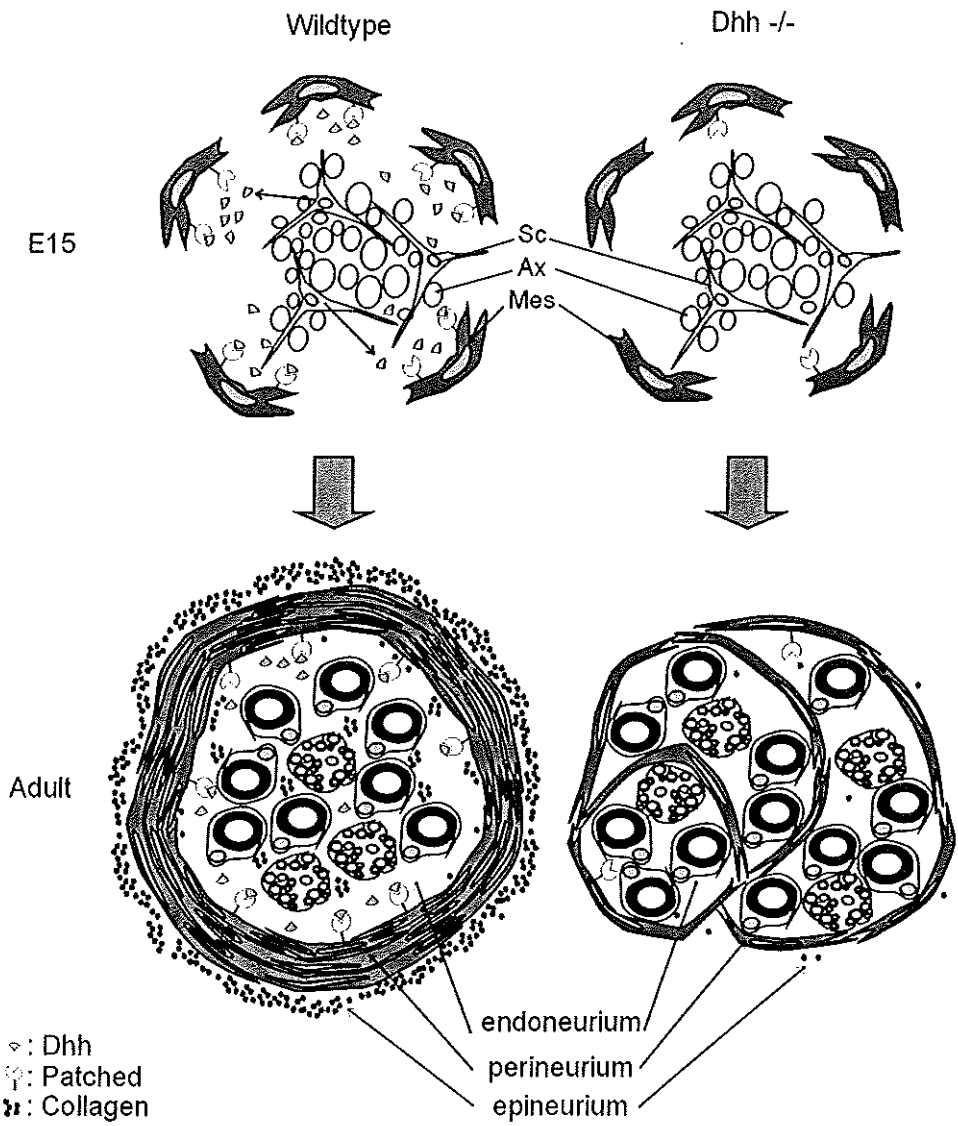

Figure 18: Schematic representation of transverse sections through peripheral nerves of wildtype and Dhh homozygous knockout animals at E15 and in adult stages of development. Nerves in both animals are morphological indistinguishable at E15. However at adult stages, the nerves of Dhh knockout mice have a very poor developed endo-, peri-, and epineurium and form minifascicles when compared to adult wildtype animals. For a more detailed description see text section 1.5.6.

1.5.7 Schwann cells and the formation of the node of Ranvier

The node of Ranvier (node) is a complex structure consisting of several specific domains that play distinct essential roles in impulse propagation and myelin stability. In the PNS, the nodes are formed by a small stretch of axonal membrane (1-2 µm), which is exposed directly to the extracellular environment and flanked on both sides by uncompacted paranodal regions of myelinating Schwann cells. This regularly spaced structure along the axon allows the rapid so-called saltatory conduction of action potentials from node to node. In concordance with its function in nerve impulse propagation, the node contains high concentrations of Na+-channels and Na+/K+-pumps. These channels are probably linked to the nodal region of the axon by interacting with a nodal specific ankyrin protein, ankyrin$_G$ 480/270 kD, which "anchors" the channels and also other transmembrane proteins to the nodal cytoskeleton [200].

Recent discoveries have demonstrated that Schwann cells play an important role in positioning of nodes along the axon. Na^+-channels are located on the axonal membrane in the middle of the node (Fig. 14). Strikingly, it has been demonstrated that the distance between Na^+-channel clusters increases as the Schwann cells elongates longitudinally along the axon during regeneration after nerve damage and during development [201-203]. Furthermore, addition of Schwann cell or oligodendrocyte (The myelin forming glia cell in the CNS) conditioned medium to glia depleted neuronal cultures, resulted in the formation of Na+-channel clusters, which in the latter case were even regularly spaced [204,205]. This would suggest that a soluble factor produced by Schwann cells and oligodendrocytes is able to induce Na^+-channel clustering along the axon. This might also explain why in dystrophic mice, which in addition to myelinated and non-myelinated axons contains amyelinated axons (lacking Schwann cells) that still have clusters of Na+-channels along the naked axons [206].

In addition to the involvement of this putative soluble factor in inducing the Na^+-channel clusters, a more physical interaction between Schwann cells and axon might be involved in the eventual positioning of the clusters. Although no direct interactions between Schwann cell and axonal proteins have been demonstrated, electron microscopic observation of longitudinal sections through a node show electron dense structures that connect the paranodal loops to the axon [207]. These structures have been termed septate-like junctions, because they resemble invertebrate septate junctions (reviewed in [208]) (Fig. 14). These junctions prevent diffusion of small molecules and ions. However the septate-like junctions do not form an impermeable barrier, since it has been demonstrated that molecules below a certain size can still pass this obstruction (reviewed in [209]). They might also physically demarcate the boundaries of the node by limiting the lateral diffusion of Na^+-channels, and thereby confining them to the node.

Recently, an axonal component of these septate-like junctions has been identified as paranodin/Contactin associated protein (Caspr1) [210,211](Fig. 14). Interestingly, the homologue of Caspr1 in *Drosophila* is the septate junction protein Neurexin IV, which has been demonstrated to be essential for axonal insulation in *Drosophila* embryos and larvae [212]. The Caspr interacting protein on the Schwann cell paranodal membrane has not been identified yet. It has been suggested that the Caspr1 interacting factor is a neuroligin related molecule, as neuroligin interacts with Neurexin IV in *Drosophila* [208].

Involvement of Caspr1 in the positioning of Na^+-channels has not been addressed yet by generation of knockout mice. Several domains in the cytoplasmic region of Caspr1 suggest that it might be connected to the nodal cytoskeleton and to several intracellular signalling pathways. The link of Caspr1 to the axonal cytoskeleton might occur via a member of the band-4.1 family of proteins, which links transmembrane proteins to the cytoskeleton [213]. Strikingly, in Neurexin IV mutant flies, the band-4.1 member Coracle is no longer restricted to the septate junctions, strongly supporting a direct interaction between Neurexin IV and Coracle [212]. Furthermore, direct interaction between band-4.1 protein and Caspr1 has been demonstrated *in vitro* [210].

The link of Caspr1 to intracellular signalling cascades, was suggested by the presence of a canonical SH3 domain binding region in the protein, which has been demonstrated to interact with several SH3 containing signalling proteins, such as fyn, src, and PI3K [214].

The ability of Caspr1 to interact with several signalling pathways at the node suggests that Schwann cells could regulate axonal structure at the node via the septate-like junctions through an unknown Schwann cell factor that interacts with Caspr1 at the node.

Together, These results underscore the role of myelinating Schwann cells in positioning of the nodes and the regulation of axonal cytoskeleton architecture.

In summary, the data presented here demonstrate that Schwann cells are involved in the regulation of many aspects of peripheral nerve function in addition to deposition of an insulating myelin. For instance the involvement of Schwann cells in the survival of neurons, in the regulation of perineurial sheath formation, and in the formation of the nodes of Ranvier stress the pivotal role of Schwann cells during peripheral nerve formation. All these processes have to be carefully controlled during peripheral nerve development and regeneration. A major point of control occurs through transcriptional regulation genes involved in these different processes. Over the last few years, a number of transcription factors have been shown to play a role at different stages of Schwann cell development. In the next section a number of these factors will be discussed

1.6 Transcriptional control in Schwann cell differentiation

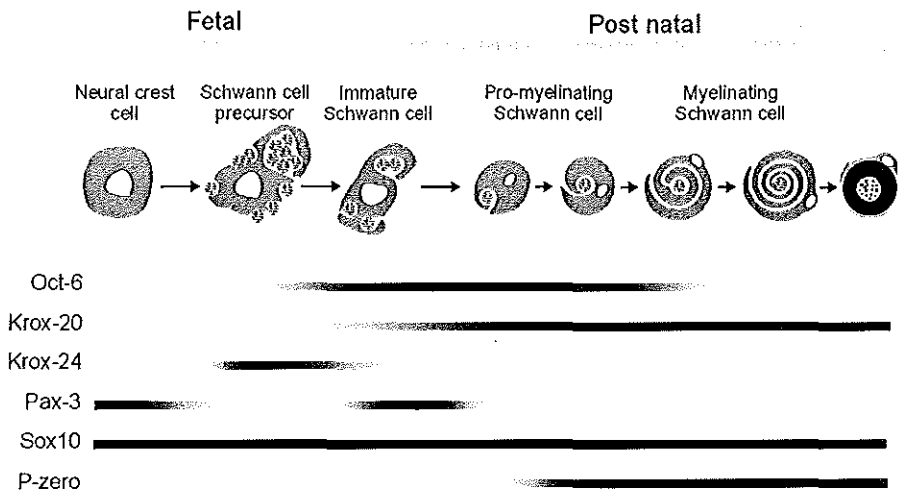

Figure 19: Transcription factor expression profile in myelinating Schwann cells and their precursors. See text for details.

1.6.1 Pax-3

One of the first detectable transcription factors, which play a role in Schwann cell development, is the paired box gene *Pax-3*. The gene is a member of the murine paired box gene family, which in vertebrates contains nine members (reviewed in [215]). These transcription factors contain a DNA-binding domain, which is homologous to the pair rule gene *paired*, in *Drosophila*. Several members of the family, also *Pax-3*, contain besides this paired domain a homeobox DNA-binding domain, which is homologous to a domain in the homeotic genes, such as *Antennapedia*, of *Drosophila*. The combination of these two DNA-binding domains allows the factor to bind to a bi-partite DNA-binding motif, which greatly increases the specificity of the interaction with the DNA target sequence, compared to members containing just the paired box. The paired domain binds a GTPyA/CPy motif while the homeodomain interacts with the TAAT sequence. The spacing between these two sequences determines the binding affinity of these factors [216].

In the mouse embryo, Pax-3 expression begins on E8.5 in the dorsal neuroepithelium from which the neural crest will arise along the antero-posterior axis [217]. A day later the expression extends to the developing dermamyotome, somitic mesoderm, and neural crest.

In the Schwann cell lineage Pax-3 is expressed in a strictly regulated pattern (Fig. 19). Based on RT-PCR data, Pax-3 is expressed in the embryonic sciatic nerve from E12 onwards at a low level, which peaks at E17 [218]. Subsequently Pax-3 levels gradually drop to a lower level at 3 days postnatal. Another report, demonstrated that Pax-3 is expressed in immature Schwann cells or precursors at E14.5 and strictly in non-myelinating Schwann cells in sciatic nerves 5 days after birth and in adult animals [219]. These data indicate that Pax-3 is associated with neural crest and the non-myelinating Schwann cell types during development.

This suggestion is further supported by the observation that Pax-3 is never coexpressed with the myelin gene MBP. Furthermore, microinjection of Pax-3 expression vector into cultured Schwann cells which had been induced by cAMP elevation to express myelin genes, led to downregulation of these markers, and upregulation of non-myelinating Schwann cell markers [219]. Cotransfection experiments have shown that Pax-3 is able to function as a general repressor of the myelin gene MBP [219]. It has also been shown by nerve crush and transection experiments that Pax-3 expression is dependent on axonal contact. In those cases, again Pax-3 is only expressed in non-myelinating Schwann cells. These results suggest that Pax-3 might be involved in determining the fate of immature Schwann cells into the non-myelinating pathway.

Mice homozygous for the natural occurring mutants of the Pax-3 gene, termed *splotch* (*sp*) and *splotch delayed* (sp^d) are embryonic lethal. Homozygosity for mutations in the Pax-3 gene in humans results in the Waardenburg syndrome (reviewed in [220]). All tissues in which Pax-3 is expressed are affected in these mutants. Strikingly, *sp* mutants die at E13.5, and their nerves are virtually lacking Schwann cells. In contrast, mice homozygous for the *spd* mutation survive until E18.5 [221]. These mice still had a small number of Schwann cells at E13.5, but already at E15.5 Schwann cells could no longer be detected [222]. There are several possibilities that could explain the absence of these cells. It could either be that neural crest delamination is affected, or that the generation of Schwann cell precursors is affected or that Schwann cell precursors die in Pax-3 knockout mice.

The potential role of Pax-3 in the specification of the non-myelinating Schwann cells can not be addressed in these mutants, because they die too early. It would require a stage and tissue specific targeting of the Pax-3 gene in mice, to elucidate this issue.

1.6.2 Sox10

Another transcription factor playing a role during Schwann cell development, which is expressed in the neural crest is the high-mobility group (HMG) domain-containing factor Sox10. Sox10 is a member of a subclass of the superfamily of HMG domain containing DNA-binding proteins, which are highly conserved in evolution. The members of the Sox family of proteins have a high amino acid similarity with the HMG domain of the testis-determining factor Sry [223,224]. This domain termed the Sry box gave the Sox proteins their name. Members of this family bind to the consensus sequence (A/T)(A/T)CAA(A/T)G (reviewed in [225]). In contrast to many other transcription factors, Sox proteins have the ability to bind to the minor groove of the DNA and are able to

strongly bend the DNA (70° - 85°) they interact with. It is therefore hypothesised that Sox proteins might perform part of their function as architectural proteins by organising local chromatin structure and assembling other DNA-bound transcription factors into biologically active, sterically defined multiprotein complexes [226].

Sox-10 transcripts can be first detected in the developing mouse embryo from E8.5 onwards in the dorsal neural tube and region of the neural plate which both correspond to the presumptive neural crest region [227]. Subsequently, expression in the CNS is confined to the oligodendrocyte precursors, which will become the myelin forming cells in the CNS. Sox-10 expression increases during embryonal development until birth, and remains high in oligodendrocytes of adult animals.

In the PNS Sox-10 can be clearly detected in the migrating neural crest after E8.5, mainly in those crest cells participating in PNS formation [227]. This restriction became clearer later during embryonal development, where Sox10 expression is detectable in enteric ganglia, glial cells of the sensory and sympathetic ganglia, and in Schwann cell precursors from E10.5 onwards. Expression of Sox10 remained at high levels in glial and Schwann cells during all subsequent stages of embryonal and postnatal development (Fig. 19).

The occurrence of natural mouse and human mutants of the gene underscore the importance of Sox10 in neural crest development. A Sox-10 mutation in mouse termed *Dominant megacolon* (*Dom*), is a valuable model system for the human mutation which results in a form of Hirschprung disease called the Shah-Waardenburg syndrome [228-230]. The *Dom* mutation leads to variable defects in all neural crest derived tissues, such as enteric aganglionosis and spotted pigmentation. Depending on the genetic background, mice homozygous for the mutation die between E13 or just after birth. Effects of the mutation on the Schwann cell lineage were not reported in these studies.

Nevertheless, the potential importance of Sox10 in Schwann cell development, became evident by its ability to specifically interact with the transcription factors Pax-3, the POU-domain factor Oct-6 and the zinc-finger protein Krox-20, and modify their transcriptional regulatory activity [227]. The latter two factors play an important role in the switch from immature-to-myelinating Schwann cell transition, as will be discussed in the following sections (1.6.3 and 1.6.4). Since Sox10 lacks a transcription activation domain, it requires interaction with these factors to regulate transcription. Cotransfection experiments demonstrated that Sox10 interacted synergistically with Pax-3 and Oct-6 to activate transcription of a reporter gene, containing a promoter with DNA binding sites for each of these factors, at levels several fold higher than when each factor was added to the culture individually [227]. Strikingly, in the same experimental set up, interaction of Sox10 with Krox-20 repressed the trancriptional activity of Krox-20.

The synergy between Sox10 and Oct-6 depended not on their DNA binding domain, but on their amino terminal region, and Sox10 was not able to synergise with other POU-domain containing proteins, such as Brn-1 or Brn-2, which closely resemble Oct-6 [231]. However, the latter two were able to synergise with another member of the Sox family, Sox11, which is not able to synergise with Oct-6 [231]. A similar interaction has also been demonstrated for yet another Sox and POU-domain protein, Sox2 and Oct-4, which synergise to activate the FGF4 promoter [232].

These observations argue for the existence of a specific partner code between members of the POU and SOX families of transcription factors. Furthermore, The synergistic effect of Sox10 on the activities of Pax-3, Oct-6 and Krox-20 suggest that Sox10 might play a pivotal role during Schwann cell development.

1.6.3 Krox-20 and Krox-24

The zinc finger transcription factors Krox-20 (Egr-2) and Krox-24 (Egr-1/NGFI-A/zif-268) belong to the family of early growth response (Egr) factors. This family also includes Egr-3 and Egr-4 (NGFI-C). The family is based on the homology within the conserved DNA-binding domain composed of three zinc-finger motifs, of which each finger binds to 3 nucleotides of a nine base pair GC-rich DNA element [233]. Both Krox-20 and Krox-24 contain, in addition to a DNA-binding domain, also an activation domain, and a domain that is able to interact with two co-repressor proteins NAB1 and NAB2 (NGFI-A-binding protein)[234,235]. Interaction of Krox-20 and Krox-24 with these co-repressors leads to repression of transcription from several promoters.

Within the Schwann cell lineage, Krox-20 and Krox-24 are expressed in reciprocal fashion [236]. Both genes are activated around E10.5 in the peripheral nerve in non-overlapping patterns (Fig. 19). Only Krox-24 is expressed in Schwann cell precursors, while both genes are expressed in the dorsal and ventral roots close to the neural tube, where it is thought that the transition from precursor to immature Schwann cell seems to occur sooner than in the peripheral nerves [237]. This situation remains until around E15, when Krox-20 is activated in the peripheral nerve, which coincides with the down regulation of Krox-24. It was suggested that this transition coincides with the switch from Schwann cell precursor to immature Schwann cell, based on study performed in the rat [103]. However, recent data demonstrated that this switch in mice already occurs between E12 and E14, with almost all Schwann cells (>90%) in the peripheral nerve being at the immature Schwann cell stage at E14 [104]. It is therefore more likely that the transition from Krox-24 to Krox-20 expression in Schwann cells coincides with the acquisition of a one-to-one relationship between Schwann cell and the axon.

Krox-24 expression in Schwann cells is strongly reactivated around birth. As myelination proceeds, Krox-24 is down regulated in myelinating Schwann cells, while it is maintained in non-myelinating Schwann cells [236]. In contrast, Krox-20 expression is maintained in the myelinating Schwann cells until adulthood.

This differential regulation of Krox-20 and Krox-24 becomes also apparent after nerve damage. During Wallerian degeneration, as Schwann cells lose their myelin sheath and dedifferentiate into an immature Schwann cell phenotype, Krox-24 is rapidly upregulated, while Krox-20 is turned off [7,236]. These complex patterns of expression coincide precisely with distinct steps of Schwann cell development and suggest antagonistic roles for both factors.

Although the Krox-24 knockout has been generated, a Schwann cell phenotype has not been reported in these mice [238,239]. On the other hand, targeted deletion of the Krox-20 did reveal its role during Schwann cell development [240]. In addition to the

development of a Schwann cell defect, which will be discussed below, Krox-20 knockout mice die soon after birth. Furthermore, Krox-20 -/- animals show defects in hindbrain segmentation and bone formation [240-244].

All Schwann cells in the peripheral nerves of Krox-20 deficient animals seem to be arrested at the promyelination stage of development [240]. They have aquired a one-to-one relation with the axon, but do not form a compact myelin sheath. This is exemplified by the strong reduction of major myelin gene expression, such as P0, MBP, and PMP22 when compared to wildtype littermates. A recent study demonstrated that Krox-20 might directly regulate P0 expression, since it is able to activate transcription of a reporter gene *in vitro* via the P0 promoter [121].

Both Schwann cell survival and apoptosis are markedly increased in the peripheral nerves of the few animals that survive after birth as compared to normal littermates [121,240]. This might be explained by the fact that promyelinating stage, Schwann cells are still proliferating [7]. This will result in an excessive amount of Schwann cells, of which the surplus will die as a result of competition for the limited amount of Schwann cell survival factors produced by the nerve. This balance between higher proliferation and apoptosis results in a more or less stable number of Schwann cells in the nerves [121].

The importance of Krox-20 in the differentiation of myelinating Schwann cells is further substantiated by the identification of Krox-20 gene in CMT1, DSS and congenital hypomyelinating neuropathy (CHN) patients [245,246]. Of the 4 reported mutations, 3 are dominant. The other mutation is recessive, and locates in the RI domain of Krox-20. This mutation prevents interaction with the NAB co-repressors and thereby increases transcriptional activity [246]. This deregulation of Krox-20 causes CHN through a gene dosage mechanism. The recessive nature of this mutation may reflect a threshold effect in which a certain increased protein level of the down stream Krox-20 target genes must be achieved for manifestation of a phenotype.

The other three mutations, which occur within the zinc-finger domain, affect DNA binding and thereby potentially influence the transcriptional activity of genes important for myelination. The variation in severity (CMT1<DSS<CHN) seen with the zinc finger mutation appears to correlate with the level of residual DNA binding [246]. The most severe phenotype (CHN) is associated with the mutant, which confers the greatest level of DNA binding and transcriptional activation, while the least severe phenotype (CMT1) is observed in the mutant which shows no binding. The dominant nature of these human mutations is interesting given that heterozygous Krox-20 knockout mice appeared phenotypically normal [241]. This suggests that rather than acting as loss-of-function alleles, the zinc finger mutations may instead be acting as dominant negative or gain-of-function alleles. Elucidation of this issue would require introduction of these zinc finger mutants via transgenesis specifically into the Schwann cell lineage of Krox-20 null mice. If this would generate a (partial) rescue of the phenotype, then the mutation would be a gain of function allele. If there would be no rescue, it would be a dominant negative allele.

Summarising, these results suggest a role for Krox-24 in Schwann cell precursors and non-myelinating Schwann cells, while Krox-20 is essential in Schwann cells to progress from a promyelinating to a myelinating phenotype.

Nerve crush experiments have demonstrated that regulation of both genes is dependent on interaction between Schwann cells and the axon. Evidence of an axonal

derived signal regulating Krox-20 expression, came from a study in which Krox-20 induction in Krox-20 heterozygous knockout Schwann cells was tested in vitro under several culture conditions [237]. These experiments reveal that Krox-20 expression is lost upon dissociation from the axon. Furthermore, Krox-20 expression could be restored when these Schwann cells were grown in contact with sensory dorsal root ganglia (DRG). However, this restoration of expression depended on the developmental age of the Schwann cells rather than that of the neurons. Only Schwann cells of E15 and older were able to induce Krox-20 expression when brought into contact with DRGs of any developmental timepoint. Thus, the stage at which Schwann cells are able to activate Krox-20 expression *in vitro* corresponds perfectly with the stage at which Krox-20 is induced *in vivo*. This indicates that Krox-20 induction in Schwann cells requires a modification in the Schwann cells, which allows them to respond to a pre-existing axonal signal. In the same study it was determined that the signal is brought about by a diffusible factor. This signal could be mimicked by growing pure Schwann cells in medium conditioned with Neuregulin or a combination of CNTF and bFGF. Furthermore, the activity of the diffusible factor could be blocked by addition of a soluble protein containing the extracellular domain of the ErbB-4 receptor, which is a high affinity receptor for neuregulin [107]. These results strongly implicate a role of the neuregulin pathway in Krox-20 activation, and hence in the generation of the promyelinating Schwann stage.

1.6.4 Oct-6

The last factor to be discussed here is the POU domain transcription factor Oct-6. As this factor is the main subject of this thesis, only those studies that relate to its role in Schwann cell differentiation will be thoroughly discussed. Oct-6 (POU3f1/SCIP/Tst-1) belongs to class III of the POU (Pit-1, Oct-1/2, unc-86) domain transcriptional regulator family [9,247-249]. The POU DNA-binding domain consists of two conserved regions, which are connected by a less conserved linker region. The aminoterminal POU-specific (POU_{sp}) domain of ~ 75 amino acids and the carboxyterminal POU-homeodomain (POU_{hd}) of ~ 60 amino acids, which show high homology with other homeodomain proteins [250,251]. The two domains constitute a bipartite DNA binding domain, which recognises the classical octameric sequence ATTTGCAT. The POU_{hd} interacts mainly with ATTT sequence, while the POU_{sp} domain interacts with the GCAT sequence and derivatives thereof [252].

Genetic experiments have demonstrated that different members of all the six classes of the POU domain transcription factor family play important roles during proliferation and/or differentiation of various cell types during embryonic and postnatal development [253,254].

The Oct-6 gene is a single exon gene located on chromosome 4 in the mouse and chromosome 1 in humans [255-257]. Based on sequence homology of the linker region, Oct-6 belongs to class III of the POU domain family, together with the as well intronless POU genes Brn-1, Brn-2, and Brn-4 [253]. Although all members are expressed in an overlapping pattern at various stages of CNS development, they are not genetically linked, in contrast to

for example the Hox genes and β-globin locus, and hence do not share transcriptional regulatory sequences [249,258,259].

During the early embryonic stages of development Oct-6 displays a highly dynamic and complex expression pattern in ectodermally derived tissues [258,259]. Later in development, this pattern becomes more restricted when Oct-6 starts to be expressed in suprabasal cells of the epidermis, proliferating oligodendrocyte precursors, and various neurons in the central nervous system (CNS) [258,260 261,262].

In the Schwann cell lineage, Oct-6 expression can be first detected at E12, at the precursor stage and is not detectable in neural crest cells (Fig. 19). Oct-6 expression is induced around E15, when immature Schwann cells start to acquire a one-to-one relation ship with large calibre axons. As myelination proceeds, Oct-6 expression is down regulated, although low levels of expression can still be seen in non-myelinating Schwann cells or some myelinating Schwann cells [6,8,218].

Oct-6 is expressed earlier than Krox-20, and their expression patterns hardly overlap, indicating that Oct-6 is upstream of Krox-20 in the genetic cascade during Schwann cell development [7]. However, recently it was shown that in Krox-20 knockout animals Oct-6 expression is not downregulated, indicating that Krox-20 might be involved in the down regulation of Oct-6 expression [121].

As Krox-20, Oct-6 expression is dependent on axonal contact, which was demonstrated by nerve crush and transsection experiments. After nerve damage, Oct-6 is induced in regenerating nerves at the stage when contact between the ingrowing axons and Schwann cells is restored (in mice between four and seven days after crush), comparable to promyelinating Schwann cells during normal development. Strikingly, in contrast to normal peripheral nerve development, complete down regulation of Oct-6 after nerve damage requires two months as compared to two weeks after Oct-6 induction during normal development. This implies that these two phases of Schwann cell morphology are not fully comparable. In transected nerves, where axonal contact is completely lost, Oct-6 expression is extinguished [6-8].

The dependence of Oct-6 expression on axonal contact can be mimicked *in vitro* by the addition of cAMP elevating reagents to cultured Schwann cells. When Schwann cell are cultured under standard conditions, they do not express Oct-6. Addition of cAMP elevating factors led to the induction of Oct-6, preceding a rise in the expression level of late myelin genes, suggesting an involvement of the adenelyl cyclase-PKA signalling pathway in the onset of myelination [9]. These results led to the assumption that Oct-6 might be directly activating the endstage myelin genes. However, cotransfection experiments in cultured Schwann cells showed that Oct-6 is a potent repressor of genes, like P0, MBP, and nerve growth factor receptor (NGFR)[263]. Furthermore, in other cell types Oct-6 has also been shown to act as an activator of transcription, suggesting that Oct-6 interacts with co-factors that can modulate Oct-6 activity in given cell type [264,265 227,231].

To investigate the role of Oct-6 during Schwann cell development *in vivo* transgenic mice were generated, which specifically express a dominant negative form of Oct-6 in the Schwann cell lineage [266]. This was achieved expressing an Oct-6 protein that lacks the first 141 amino acids under the control of the P0 promoter. This experiment was based upon the observation that Oct-6 specifically represses the P0 promoter in co-transfection experiments, as mentioned before. This repression could be prevented by co-

expression of the Oct-6 POU-domain, which by itself was not able to modulate the expression via the P0 promoter [263,266]. Therefore it was stated that Oct-6 POU expression together with normalOct-6 would interfere with the normal, repressing, function of the latter. Transgenic mice generated with this construct, named P0ΔSCIP, are viable, but demonstrated premature myelination and hypermyelination of nerve fibres with a reduced diameter. In line with this observation the major myelin gene P0 was overexpressed in these mice when compared to normal littermates. Furthermore, P0ΔSCIP transgenic mice had an increased nerve conduction velocity compared to normal littermates in axons with the same diameter [267]. Strikingly, P0ΔSCIP mice also displayed increased nerve regeneration after nerve crush [268]. These transgenic data is supported by the in vitro co-transfection data, and implicates that normal Oct-6 function is to repress the major myelin genes.

Summarising, based on the Oct-6 expression pattern, the gene seems to be involved in the transition from the promyelinating to myelinating stage of Schwann cell development. In vitro and transgenic data suggest that Oct-6 functions as a negative transcriptional regulator and as a repressor of myelin genes in proliferating Schwann cells. Hence, one would predict that in the absence of Oct-6, Schwann cells would differentiate (myelinate) prematurely and that their numbers will be reduced. In addition, induction of Oct-6 expression in vivo depends on axonal signals, which can be mimicked by addition of cAMP elevating factors to cultured Schwann cells. This suggests axonal signal(s) activate Schwann cell intracellular signalling cascades that are able to induce Oct-6 expression mediated by cAMP inducible transcription factors. These hypotheses on Oct-6 function and regulation will be addressed in the following chapters.

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Chapter 2

The POU Factor Oct-6 and Schwann Cell differentiation

Martine Jaegle, Wim Mandemakers, Ludo Broos, Ronald Zwart,
Alar Karis, Pim Visser, Frank Grosveld, and Dies Meijer.

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The POU transcription factor Oct-6, also known as SCIP or Tst-1, has been implicated as a major transcriptional regulator in Schwann cell differentiation. Microscopic and immunochemical analysis of sciatic nerves of Oct-6$^{-/-}$ mice at different stages of postnatal development reveals a delay in Schwann cell differentiation, with a transient arrest at the promyelination stage. Thus, Oct-6 appears to be required for the transition of promyelin cells to myelinating cells. Once these cells progress past this point, Oct-6 is no longer required, and myelination occurs normally.

Schwann cells are involved in the trophic support and insulation of axons and are the only glial cell type in peripheral nerve trunks. The two types of Schwann cells, myelinating Schwann cells associated with axons greater than 1 μm in diameter and nonmyelinating cells associated with multiple lower caliber axons, both differentiate from neural crest–derived Schwann cell precursors. Myelination initiation correlates with axon diameter and is governed by axonal signals that are as yet not understood (*1*).

A number of transcription factors have been proposed to be involved in Schwann cell differentiation and myelination (*2*). Prominent among those is the POU domain transcription factor Oct-6 (also known as SCIP or Tst-1) (*3*). The Oct-6 protein is expressed in the Schwann cells of the sciatic nerve and the sympathetic trunk from embryonic day 16 (E16) onward (*4*). During postnatal nerve development, the expression of Oct-6 mRNA is gradually downregulated and extinguished, with only spo-

Medical Genetics Center, Department of Cell Biology and Genetics, Erasmus University, Post Office Box 1738, 3000 DR Rotterdam, Netherlands.

*To whom correspondence should be addressed. E-mail: meijer@gen.fgg.eur.nl

radic expression in Schwann cells of the adult nerve (5). In vitro and transgenic mouse experiments have suggested that Oct-6 acts as a negative transcriptional regulator and as a general repressor of myelin genes in proliferating Schwann cells (6). By inference, Oct-6 was postulated to be involved in embryonic Schwann cell proliferation and to regulate the correct number of Schwann cells in the peripheral nerves. In the absence of Oct-6 gene function, one prediction of this model is that Schwann cells will differentiate prematurely and their numbers will be reduced.

To elucidate the role of the Oct-6 gene in Schwann cell differentiation, we interrupted the DNA binding domain of the mouse Oct-6 locus through homologous recombination in embryonic stem (ES) cells, with a β-galactosidase–neomycin fusion gene (7) (Fig. 1). Two different Oct-$6^{+/-}$ ES cell lines produced chimeric animals and had high frequencies of germline transmission. Identical results were obtained from both lines. Heterozygote animals were healthy and had no readily apparent abnormalities. Analysis of β-galactosidase activity in these animals confirmed the previously described Oct-6 expression pattern (3, 4, 8).

Mice homozygous for the mutated Oct-6 allele were produced at normal Mendelian ratios and developed to term and showed no gross anatomical abnormalities. Most Oct-$6^{-/-}$ pups died soon after birth, but 2 to 4% survived for a longer period. From postnatal day 5 (P5) onward, mutant animals could be identified by their smaller size and occasional tremors in the second postnatal week.

To determine whether Schwann cells were affected in the Oct-$6^{-/-}$ mice, immunohistochemical analysis was performed (8). Normally, myelinating Schwann cells coordinately express myelin genes such as those encoding protein zero (P_0), peripheral myelin protein (PMP-22), myelin basic protein (MBP), and myelin-associated glycoprotein

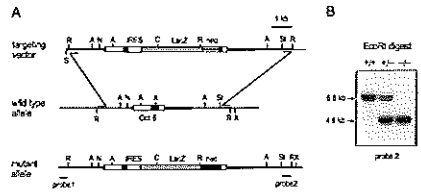

Fig. 1. Targeted disruption of the Oct-6 gene. **(A)** Targeting strategy. Schematic representation of the mouse Oct-6 gene (middle). The thick black line represents the transcribed part of the Oct-6 locus, which is encoded by a single exon. The open box represents the open reading frame, and the hatched box represents the POU domain. A DNA fragment containing a β-galactosidase–neomycin fusion gene preceded by an internal ribosome entry site (IRES) (7) (gray and striped boxes) was inserted into the Xho I site of the Oct-6 gene, thereby disrupting the DNA binding domain of the Oct-6 protein (top). A schematic representation is also shown of the mutant allele, indicating the position of the probes that were used to identify homologous recombinant ES cell lines (bottom). **(B)** Southern blot of Eco RI–restricted DNA, showing a 4.6-kb mutant allele band and a 6.8-kb wild-type allele band detected by probe 2. The mutant allele expresses a 6.9-kb bicistronic mRNA under control of the endogenous Oct-6 promoter and its regulatory sequences. No full-length Oct-6 protein can be produced from this mRNA.

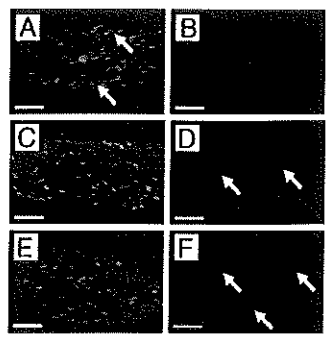

Fig. 2. Immunohistochemical analysis of wild-type versus Oct-$6^{-/-}$ mutant sciatic nerves. Sciatic nerves of 18 days postcoitum embryos (**A** and **B**) and P8 (**C** and **D**) and P14 pups (**E** and **F**) were analyzed for the expression of the Oct-6 protein (green fluorescent signal) and the myelin protein P_0 (red fluorescent signal) (9). Wild-type and Oct-$6^{-/-}$ nerves express basal levels of the P_0 protein at E18 (A and B). A few cells express already high levels of P_0 (A, white arrows). At P8 and at P14, high levels of P_0 expression are seen in wild-type nerve (C and E), whereas in mutant nerve, only a small number of Schwann cells express high levels of the P_0 protein (D and F, white arrows). Immunohistochemical analysis of paraffin sections was as described (8). The bar represents 50 μm.

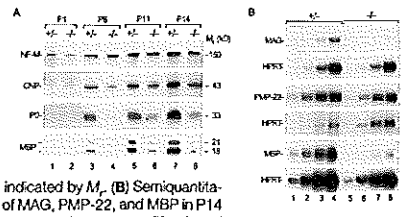

Fig. 3. Expression of early and late myelin markers in the sciatic nerves of Oct-$6^{-/-}$ and Oct-$6^{+/-}$ animals. **(A)** Protein immunoblot analysis of protein extracts from mutant and heterozygote sciatic nerves at P1, P8, P11, and P14. The blots were probed with antibodies to P_0, CNP, MBP, and NF-M. The amount of protein is normalized for the NF-M signal. The relative molecular mass is indicated by M_r. **(B)** Semiquantitative PCR analysis of expression levels of MAG, PMP-22, and MBP in P14 Oct-$6^{+/-}$ and Oct-$6^{-/-}$ sciatic nerves. For each gene, amplification of hypoxanthine phosphoribosyl-transferase (HPRT) cDNA was used as an internal control in every second panel. Samples were taken at cycles 20 (lane 1 and 5), 22 (lane 2 and 6), 24 (lane 3 and 7), and 26 (lane 4 and 8).

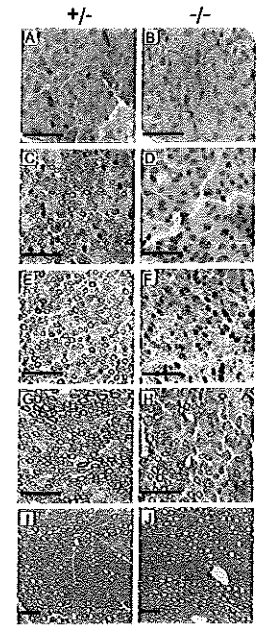

Fig. 4. Microscopic cross sections of sciatic nerves of Oct-$6^{+/-}$ and Oct-$6^{-/-}$ animals at different stages of postnatal development. The left panels were derived from Oct-$6^{+/-}$ animals, and the right panels were derived from Oct-$6^{-/-}$ animals. Semithin sections (1 μm) of Epon-embedded material were stained with methylene blue. The age of the animal is indicated on the right. The bar in each panel represents 25 μm.

(MAG). Examination of E18 nerves showed that P_0 expression levels were normal, and no abnormalities in either the number of Schwann cells or the shape of their nuclei were apparent (Fig. 2, A and B, and Fig. 4, A and B). However, P8 and P14 sciatic nerves of $-/-$ animals appeared to be defective in myelination. The nerves revealed reduced levels of P_0 expression relative to the highly P_0-positive nerves of $+/+$ littermates (Fig. 2). Some Oct-$6^{-/-}$ Schwann cells express normal, high levels of P_0 (Fig. 2D), but most express little P_0. At P14, the number of Schwann cells that express high levels of P_0 had increased (Fig. 2F).

Protein immunoblotting and semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) were used to examine the expression levels of a number of early and late myelin markers in the sciatic nerve at different stages of postnatal development (9). Less than normal P_0 and MBP levels were found in the mutant nerve (Fig. 3A). Early expression of 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) protein at P1 was not affected (Fig. 3A), but the postnatal increase was less. We also examined the mRNA levels of the genes encoding myelination markers PMP-22, MAG, and MBP (Fig. 3B) and found them to be reduced. These expression data suggest that in Oct-$6^{-/-}$ animals, nerve myelination is delayed but not completely blocked. Alternatively, it is possible that a number of Schwann cells differentiate normally, whereas the rest are blocked at an early stage of differentiation.

To distinguish between these possibilities, we examined the microscopic structure of sciatic nerves at different stages of postnatal development (*10*). Normally, only a few myelinating Schwann cells were observed at birth, with extensive myelination visible at P4 and P8 (Fig. 4, A, C, and E). At P16, myelination was well advanced, with many fully myelinated axons (Fig. 4, G and I). However, in the Oct-$6^{-/-}$ nerve, no evidence of myelination was seen until the second week of postnatal development (Fig. 4, B, D, F, and H). In adult Oct-$6^{-/-}$ nerves, myelination appeared complete (Fig. 4J).

Electron microscopic analysis of 80-nm sections of sciatic nerves from $-/-$ and $+/-$ mice revealed no differences at P1. Schwann cells were actively ensheathing axons, and promyelin figures were observed in which Schwann cells had acquired a 1:1 relation with an axon. There was a normal deposition of collagen fibrilles, and Oct-$6^{-/-}$ and Oct-$6^{+/-}$ Schwann cells produced a basement membrane (Fig. 5, A, B, and F). In wild-type P16 nerves, most large-caliber axons were myelinated, whereas Oct-$6^{-/-}$ P16 nerves showed an immature phenotype with promyelin figures with only the larger axons myelinated. Schwann cells that had myelinated a single, large caliber axon appeared normal, with an uncompacted periaxonal membrane and tightly packed myelin membranes (Fig. 5F). Very sporadically, abnormal myelin figures were observed that showed several large axons associated with a myelinating Schwann cell (Fig. 5E). These Schwann cells showed severely disrupted myelination, as evidenced by their failure to wrap myelin around the axon, resulting in disorganized myelin sheaths. Such Schwann cells started myelination before a 1:1 relation with an axon was established. Sometimes myelination was observed in the absence of an axon (Fig. 5E). In adult Oct-$6^{-/-}$ animals, nerve myelination was complete (Fig. 5, G and H).

These data demonstrate that in Oct-$6^{-/-}$ mice there is a delay in the onset of myelination and not a block in the differentiation of Schwann cells. This delay is characterized by a transient arrest of Schwann cells at the promyelin stage. Once cells progress past this stage, myelination occurs normally, which indicates that Oct-6 is not involved in the execution of the terminal differentiation program. These results are in contrast with transgenic studies in which a dominant negative form of Oct-6 under transcriptional control of the P_0 promoter results in an early onset of myelination and hypermyelination. This discrepancy may suggest separate functions for Oct-6 protein in early and late Schwann cell development, as discussed by Weinstein *et al.* (*6*, *11*). Our results suggest that Oct-6 exerts its function in Schwann cell development through the regulation of genes that are involved in embryonic Schwann cell axon interactions that govern the transition from a promyelin cell to a myelinating Schwann cell. Exactly which genes are regulated by Oct-6 and how this process mediates the progression of Schwann cell differentiation require further study.

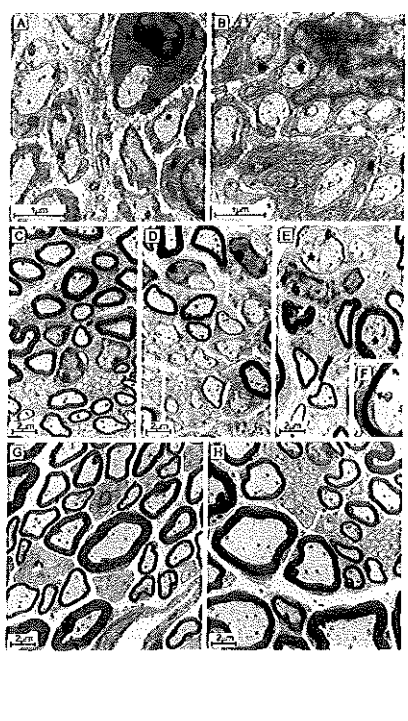

Fig. 5. Ultrastructure of sciatic nerves at different stages of postnatal development. Shown is the ultrastructural appearance of an Oct-$6^{+/-}$ **(A)** and Oct-$6^{-/-}$ **(B)** nerve at P1. Ensheathing and promyelin cells are visible in both nerves. The darker cell in the upper right part of **(A)** has made the first wrap around its axon. **(C)** Oct-$6^{+/-}$ nerve at P16. **(D** and **E)** Representative sections of an Oct-$6^{-/-}$ nerve at P16. Panel **(D)** illustrates the normal but immature appearance of an Oct-$6^{-/-}$ nerve at P16, and **(E)** illustrates the sporadic abnormalities observed in mutant nerves. The open arrow indicates myelination in the absence of an axon. The asterisks indicate large axons associated with a myelinating Schwann cell. **(F)** Mutant Schwann cell that has produced a normal myelin sheath. The solid arrows indicate an uncompacted periaxonal membrane. The white arrow points at an apparently normal basement membrane. Also shown are representative sections of adult heterozygous **(G)** and mutant **(H)** nerves.

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7. P. Mountford *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4303 (1994). Oct-6 genomic clones were isolated from a 129-genomic phage library and mapped with the use of several restriction enzymes. The targeting vector was linearized at the single Sal I site and electroporated into E14 ES cells maintained in BRL cell conditioned medium in the presence of Leukemia Inhibitory Factor. After selection in G418, correct replacement events were identified by Southern blot hybridization with the use of an internal 3' probe and an external 5' probe. Homologous recombination events had occurred in 15 of 51 G418-resistant ES cell lines. A number of these lines were karyotyped, and two cell lines with the correct number of chromosomes were injected in C57BL/6 blastocyst embryos. Both lines resulted in germline transmission. Heterozygous offspring were crossed to C57BL/6 mice and backcrossed at the F_2 generation.
8. R. Zwart, L. Broos, G. Grosveld, D. Meijer, *Mech. Dev.* **54**, 185 (1996).
9. Sciatic nerves were isolated from mutant mice and heterozygote littermates. One nerve was used for protein immunoblotting and another was used to isolate RNA. Nerves for protein immunoblotting were homogenized in sample buffer and loaded on a 15% (for P_0 and MBP) or 8% [for CNP and neurofilament medium chain (NF-M_r)] SDS-polyacrylamide gel. The gels were electroblotted on filters and further processed according to standard procedures. P_0 monoclonal antibody P07 was provided by J. J. Archelos [J. J. Archelos *et al.*, *J. Neurosci. Res.* **35**, 46 (1993)]. Other monoclonal antibodies were obtained from Boehringer Mannheim (MBP), Sigma (CNP), and Amersham (NF160). For RT-PCR, RNA was extracted with the LiCl-urea method and reverse-transcribed into cDNA with oligo(dT) and hexamer primers. Excess primers were removed on a Centricon C-30 (Amicon) concentrator. For MBP amplification, we used cDNA generated from P8 sciatic nerve RNA. After 20-fold dilution, polymerase chain reaction (PCR) amplification was performed by use of the Expand long template PCR system (Boehringer Mannheim). Each amplification cycle comprised 30 s at 94°C, 1 min at 55°C, and 1 min at 68°C. After the 10th amplification cycle, the extension time was increased with 20-s increments. Samples were taken at cycles 20, 22, 24, and 28. The following primers were used: MAG: 5'-primer GCCACGGTCATCTATGAGAGTCAGC and 3'-primer GGTGCCCAGAGATTCTGAATTCGG; HPRT: 5'-primer CACAGGACTAGAACACCTGC and 3'-primer GCTGGTGAAAAGGACCTCT; PMP-22: 5'-primer ACACTGCTACTCCTCATCAGTGAG and 3'-primer CAGGATCACATAGATGATACCACTG; and MBP: 5'-primer ACTCACACACGAGAAGTACCCA and 3'-primer CCAGCTAAATCTGCTGAGGG. The amplified fragments yielded bands of 606 bp for MAG, 316 bp for PMP-22, 249 bp for HPRT, and 170 bp for MBP. Amplification products were separated on a 4% denaturing polyacrylamide gel, and signals were measured with a Molecular Dynamics phosphoimager.
10. Animals were perfused with phosphate-buffered saline, pH 7.2, for 3 min followed by fixative (3% paraformaldehyde and 1% glutaraldehyde buffered by 100 mM cacodylate at pH 7.2) for 10 min. Nerves were dissected, cut into smaller pieces, and fixed overnight in the same fixative. After postfixation in 1% osmium tetroxide, the sample was embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate. Sections were examined and photographed with a Philips CM100 electron microscope.
11. R. Mirsky and K. R. Jessen, *Curr. Opin. Neurobiol.* **6**, 89 (1996).
12. We are grateful to D. Bootsma for his support, to E. Dzierzak for critical comments on the manuscript, and to M. Kuit, T. de Vries Lentsch, R. Koppenol, and R. Wiersma for photography. We gratefully acknowledge J. Archelos for P_0 antibodies and L. Braam for animal care. All animal experiments were carried out under license 132-95-03 (Institutional Animal Care and Use Committee, Erasmus University).

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Chapter 3

Comparison of sequence and function of the *Oct-6* genes in zebrafish, chicken and mouse

Francoise Levavasseur, Wim Mandemakers, Pim Visser, Ludo Broos, Frank Grosveld, Dana Zivkovic and Dies Meijer.

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Comparison of sequence and function of the *Oct-6* genes in zebrafish, chicken and mouse

Francoise Levavasseur[a,1], Wim Mandemakers[a], Pim Visser[a], Ludo Broos[a], Frank Grosveld[a], Dana Zivkovic[b], Dies Meijer[a,*]

[a]*MGC, Dept. of Cell Biology and Genetics, Erasmus University Rotterdam, P.O. Box 1738, 3000DR Rotterdam, The Netherlands*
[b]*Hubrecht Laboratory, NIOB, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*

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Abstract

To examine the role of the *Oct-6* gene in Schwann cell differentiation we have cloned and characterized the chicken and zebrafish homologues of the mouse *Oct-6* gene. While highly homologous in the Pit1-Oct1/2-Unc86 (POU) domain, sequence similarities are limited outside this domain. Both genes are intronless and both proteins lack the amino acid repeats that are a characteristic feature of the mammalian Oct-6 proteins. However as in mammals, the aminoterminal parts of the chicken and zebrafish Oct-6 proteins are essential for transactivation of octamer containing promoters. By immunohistochemistry we have found that the chicken Oct-6 protein is expressed in late embryonic ensheathing Schwann cells of the sciatic nerve and is rapidly downregulated when myelination proceeds. This expression profile in glial cells is identical to that in the mouse and rat. Furthermore the zebrafish Oct-6 homolog is expressed in the posterior lateral nerve at a time when it contains actively myelinating Schwann cells. Thus despite extensive primary sequence divergence among the vertebrate Oct-6 proteins, the expression of the chicken and zebrafish Oct-6 proteins is consistent with the notion that Oct-6 functions as a 'competence factor' in promyelin cells to execute the myelination program.

Keywords: *Oct-6* gene; Schwann cell differentiation; Zebrafish; Chicken; Mouse; POU genes

1. Introduction

The embryonic development of multicellular animals is a highly organized process of cellular proliferation and differentiation. Extra- and intra-cellular signals ultimately lead to the expression of specific genes that define and maintain the differentiated state of individual cells in the organism. Tissue specific transcription factors play a pivotal role in these processes. These factors are encoded by gene families defined by distinct structural characteristics of their DNA-binding domain. The POU domain genes constitutes such a family of transcription factors (for review see Ryan and Rosenfeld, 1997). The POU proteins are characterized by a 150–160 amino acid domain which can be subdivided in a carboxyterminal portion (the POU homeodomain) related to the homeodomain DNA binding proteins and a aminoterminal portion consisting of a variant helix-turn-helix motif that is a defining characteristic of this family of proteins (the POU-specific domain). More than 13 mammalian POU genes have been characterized and POU genes have been cloned from the genomes of worms, amphibians, birds and flies. Based on sequence homology and gene structure the POU genes can be subdivided into six classes (Wegner et al., 1993).

The vertebrate class III POU genes are intronless and expressed predominantly in the developing and adult nervous system where they show considerable overlap. The *Oct-6* (accession number chicken *Oct-6* gene AF064608) (*SCIP/Tst-1*) gene is one of the most intensely studied class III genes (He et al., 1989; Monuki et al., 1989; Meijer et al., 1990; Suzuki et al., 1990) and its expression pattern is highly dynamic, complex and overlapping with other members of the class III POU gene family. Oct-6 is first expressed in the primitive ectoderm of the egg-cylinder

* Corresponding author. Tel.: +31 10 4088250; fax: +31 10 4360225; e-mail: meijer@gen.fgg.eur.nl
[1] Present address: Centre de Recherche en Neurosciences, McGill University, The Montreal General Hospital Research Institute, 1650 Cedar Avenue, Montreal, PQ H3G 1A4, Canada.

stage mouse embryo. During gastrulation and subsequent neurulation expression is progressively restricted to the anterior primitive brain. There is a second wave of Oct-6 induction in the neurepithelium of the entire brain in the late headfold stage embryo. This expression becomes restricted to mid- and forebrain structures. At later stages Oct-6 is observed in specific subset of motor- and commissural neurons in the spinal cord and hindbrain, different mid- and forebrain structures, the skin and the neural crest derived Schwann cells of the peripheral nervous system (He et al., 1989; Monuki et al., 1989; Suzuki et al., 1990; Zwart et al., 1996). Genetic studies in mice have shown that the *Oct-6* gene plays an important role in the differentiation of peripheral glial cells. In the absence of *Oct-6* Schwann cells fail to mature on schedule leading to a transient arrest at the promyelin stage and a 1–2 week delay in the onset of myelination, while CNS myelination is not affected (Bermingham et al., 1996; Jaegle et al., 1996; Jaegle and Meijer, 1998).

Thus it is interesting to study the conservation of function of Oct-6 throughout evolution and take advantage of other non-mammalian vertebrate models for further analysis of this gene. The zebrafish and chicken offer several advantages among which are the accessibility of embryonic material for grafting and tracing studies and the possibility to study gene function through overexpression and ectopic expression of genes in embryos. Furthermore, comparison of *cis*-acting regulatory sequences that drive celltype-specific expression of the *Oct-6* gene in these vertebrate species will allow the identification of evolutionary conserved DNA binding sites and the nuclear factors that bind to them. Recently a zebrafish POU gene, called *zp-50*, was cloned that shows highest homology to the mouse *Oct-6* gene (Hauptmann and Gerster, 1996). Based on differences in embryonic expression patterns between this gene and the mouse *Oct-6* gene Hauptman and Gerster suggested that the *zp-50* gene might not be the functional homologue of the mammalian *Oct-6* gene. However, since genetic data obtained in the mouse suggest a critical role for *Oct-6* in the Schwann cell lineage, it is important to study the expression of putative *Oct-6* homologues in the differentiation of peripheral glia cells.

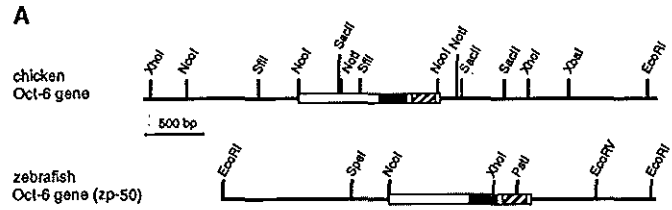
In this paper we report the cloning of the chicken and zebrafish *Oct-6* homologues and examine their transactivation potential and expression in peripheral glial cells. Within the POU domain the chicken and zebrafish are highly homologous to the mammalian Oct-6 proteins, while sequence similarities outside this domain are limited to short stretches. We show that the chicken and zebrafish Oct-6 proteins both contain a functional aminoterminal transactivation domain lacking the simple amino acid repeats which are a characteristic feature of the mammalian Oct-6 proteins. Despite sequence divergence outside the POU domain both the zebrafish and chicken Oct-6 proteins are expressed in developing peripheral nerves of both species, strongly suggesting that they indeed represent the structural and functional homologues of the mammalian *Oct-6* gene.

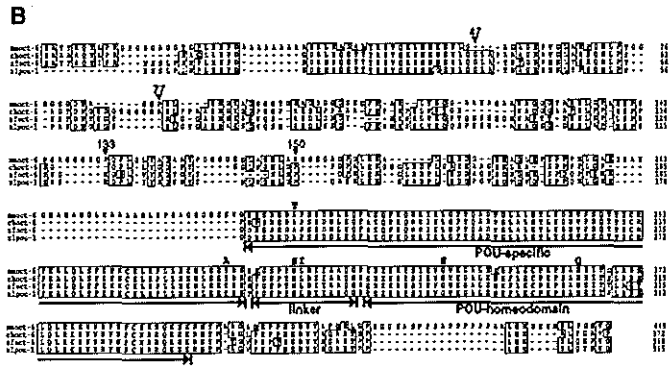
2. Results

2.1. Isolation of Oct-6 gene homologues from zebrafish and chicken genomes

Using a mouse *Oct-6* POU domain DNA probe, hybridization conditions were established at which one relatively strong hybridizing band was observed on Southern blots containing zebrafish and chicken DNA (data not shown). The probe was used under these conditions to screen genomic phage libraries of zebrafish and chicken DNA. Several phage clones were recovered from both libraries. Restriction enzyme analysis and Southern blotting revealed three independent zebrafish clones. The most strongly hybridizing clone was mapped, subcloned and partially sequenced. This zebrafish *Oct-6* homolog is identical to the *zp-50* clone described by Hauptman and Gerster (1996). The remaining phage clones were not further analyzed. Two different phage clones were isolated from the chicken library and were found to contain partially overlapping inserts. *Oct-6* hybridizing bands were mapped, subcloned and sequenced. The restriction enzyme map of the zebrafish and chicken *Oct-6* homologues genes are depicted in Fig. 1A. Both fragments contain an open reading frame not interrupted by introns. Thus, like all other vertebrate class III genes the fish and chicken *Oct-6* homologues are encoded by one exon genes.

The open reading frame (ORF) of the chicken and zebrafish (and Xenopus) (Agarwal and Sato, 1991) *Oct-6* genes predict that the homologous proteins are considerably smaller than the mouse Oct-6 protein (calculated molecular mass is 39 and 41 kDa, respectively versus 45 kDa for the mouse Oct-6 protein; Fig. 1B). This difference is due to the presence of blocks of long simple sequence repeats in the mammalian Oct-6 protein directly flanking the POU domain. The proline-rich repeat carboxyterminal of the POU domain of the mouse Oct-6 protein is encoded by an inverted repeat with a 10-bp stem. It is conceivable that this sequence was lost in all other vertebrates except mammals following a replication error over this secondary structure. Shorter simple sequence expansions in the mammalian Oct-6 protein are found in the aminoterminal half of the protein (the rat and human Oct-6 protein sequence is virtually identical to that of the mouse (Monuki et al., 1989; Faus et al., 1994) and interrupt blocks of considerable amino acid conservation (Fig. 1B). Within the POU-specific domain the proteins show 100% homology while the POU homeodomain is 99% conserved (Fig. 1B). Furthermore, all four Oct-6 proteins have a threonine residue at position 74 in the carboxyterminal helix of the POU-specific domain and a histidine residue in position 29 in helix 2 of the POU homeodomain while all other known class III proteins have an





C

GC content (%) of vertebrate Oct-6 genes

	POU domain			coding region		
Species	1st	2nd	3rd	1st	2nd	3rd
Mouse	58	39	90	77	56	85
Chicken	64	39	89	72	50	85
Zebrafish	49	39	64	54	42	69
Frog	47	38	63	56	42	68

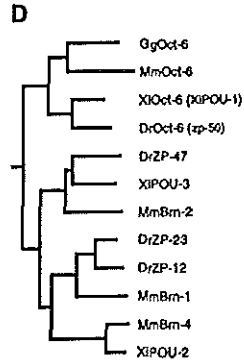


Fig. 1. (A) Schematic representation of the genomic maps of the chicken and zebrafish *Oct-6* homologous genes. The open reading frame which is not interrupted by introns is indicated as a box. The POU-specific and POU homeodomains are indicated with black and striped boxes, respectively. (B) Alignment of the amino acid sequence of the mouse, chicken, zebrafish and frog protein sequence using the clustalW program. Identical amino acids and conservative amino acid substitutions are boxed. Dashes indicate gaps in the sequence that were introduced to optimize the alignment. The POU-specific and the POU homeodomain are indicated. The starting positions of the truncated proteins used in the transactivation assay are indicated by their amino acid number and an arrow. (C) GC contents (%) of the vertebrate *Oct-6* genes at first, second and third base of codons. (D) Phylogenetic tree showing the evolutionary relationship between the known vertebrate class III POU genes. This tree was constructed using the neighbor-joining method (DNAMan software package). Abbreviations: GG, *Gallus gallus*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*.

alanine residue and a glutamine residue, respectively, in those positions. Based on these specific and overall sequence homologies (see Fig. 1D) we conclude that the chicken and zebrafish proteins are the homologues of Oct-6 as found in mouse, rat and human.

A remarkable feature of the vertebrate *Oct-6* genes is that they are encoded by DNA with very different guanine and cytosine (GC) content. The mammalian and chicken *Oct-6* genes are both encoded by very GC-rich DNA sequences (72%) while the zebrafish and Xenopus genes have a much lower GC content (55%). Analysis of the organizational principles of genomes has shown that the genomes of warm-blooded vertebrates are composed of mosaics of very long DNA segments, called isochores. Isochores are compositionally homogenous and belong to a small number of families which cover a very extended GC range (Bernardi, 1993, 1995). The base composition of genes located within isochores reflect their GC content and the compositional constraints operating on these isochores result in a bias at the third codon position. In contrast, the genomes of cold-blooded vertebrates are compositionally uniform. Although it is not known what the GC content is of the isochore in which the *Oct-6* gene resides it is of interest to compare the GC content in the first, second and third codon positions of the POU domain and the whole open reading frame of the known vertebrate *Oct-6* genes (Fig. 1C). Indeed the GC content at the third codon position is considerably higher than the average GC content in mouse, chicken zebrafish and frog suggesting a tendency towards higher GC contents in both cold-blooded and warm-blooded vertebrates. However, within the conserved POU domain there is a extreme preference for GC nucleotides at the third codon position in only the warm-blooded animals (mouse and chicken). The cold-blooded animals have a GC content that is even slightly lower than that observed at the third codon position in the entire coding region (frog and zebrafish).

2.2. Mapping the transcriptional activation domain of the chicken and zebrafish Oct-6 proteins

Simple amino acid repeats are a characteristic feature of many transcription factors. Previously, we and others have determined the transcriptional activation domain of the

mouse Oct-6 protein and found it to be located in the aminoterminal third of the protein. The contribution of the amino acid repeats to this transactivation function is not clear although Monuki et al. (1993) have shown that the polymorphic alanine stretch can be deleted without affecting the transcriptional activation potential of the protein. Since the zebrafish and the chicken Oct-6 homologues lack the amino acid repeats of mammalian Oct-6, these proteins provide an opportunity to assess their role in transactivation.

To this end we performed co-transfection experiments in HeLa cells with cytomegalovirus (CMV) promoter driven zebrafish and chicken POU protein expression vectors and a rabbit β-globin reporter construct that contains an octamer sequence just upstream of the rabbit β-globin TATA box (Westin et al., 1987; Muller et al., 1988; Matthias et al., 1989). Binding of the chicken and zebrafish Oct-6 proteins and the various aminoterminal truncation mutants to the octamer sequence (ATTTGCAT) was examined by a bandshift assay using whole cell extracts of transfected cells. As shown in Fig. 2A, full length (N1) and aminoterminal truncated (N47 and N133) zebrafish and (N77 and N150) chicken Oct-6 proteins are produced in the transfected HeLa cells and bind the octamer sequence with high affinity. This is as expected since they all contain an intact POU domain. To assess transactivation of transcription by these proteins, RNA from co-transfected cells was isolated and transcription from the reporter and reference plasmid was measured using

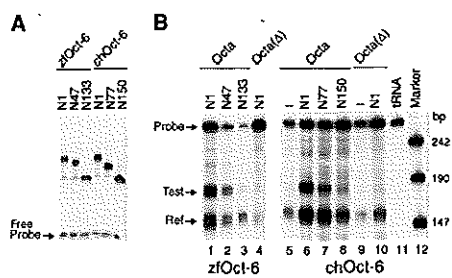


Fig. 2. Transactivation of an octamer containing promoter by zebrafish and chicken Oct-6 proteins. (A) The full length and truncated proteins are expressed at high levels in transfected cells. Bandshift experiment using a ^{32}P-labeled octamer probe with whole cell extracts from cells expressing the various effector plasmids. (B) Activation of the reporter construct OCTA was monitored by RNase protection. 'Ref' indicates the position of the reference signal that is derived from transcripts correctly initiated from the plasmid OVEC reference plasmid. The reporter plasmid and reference plasmid is described in detail in (Westin et al., 1987). 'Test' indicates the position of the correctly initiated RNA transcripts from the reporter plasmid. Lanes marked by minus signs indicate controls without transactivator plasmid. N1, N47, N133, N77, N150 refers to the amino acid position (see Fig. 1B) at which the truncated protein is fused in frame to the short leader sequence of the pEVRF vectors (Matthias et al., 1989). Octa indicates the octamer containing reporter plasmid and Octa(Δ) indicates the reporter plasmid in which the octamer sequence is mutated. Lane 11 shows the signal when the probe was hybridized to tRNA.

a RNase protection assay (Fig. 2B). Both the zebrafish and chicken proteins activate a simple promoter in a octamer dependent way (Octa; lanes 1 and 6). However, upon mutation of the octamer sequence transactivation of the reporter construct (Octa(Δ); lanes 4 and 10) is abolished. In addition, the transactivation potential of the chicken and zebrafish Oct-6 proteins is dependent upon an intact aminoterminal domain. As shown in Fig. 2B progressive deletions first slightly reduce (N47 and N77) and than abolish (N133 and N150) octamer dependent transactivation. Deletion of the first 47 amino acids in the zebrafish protein removes the largest block of homology in the aminoterminal region between the different vertebrate Oct-6 proteins, yet this does not significantly affect trans-activation. Only removal of the aminoterminal third of the protein results in a severe reduction of transactivation by both zebrafish and chicken Oct-6 proteins. Thus, like the mouse and rat Oct-6 proteins the chicken and zebrafish Oct-6 proteins have an aminoterminal transactivation domain. Despite the fact that the zebrafish and chicken proteins lack the characteristic short homopolymeric amino acid stretches present in the mammalian Oct-6 protein, they are capable of transactivating a minimal promoter in an octamer dependent way.

2.3. Expression of the chicken and zebrafish Oct-6 proteins in peripheral nerve development

Comparison of the primary amino acid sequence of the zebrafish and chicken proteins (Fig. 1) and the ability of these proteins to transactivate transcription through the octamer consensus sequence clearly identifies them as the homologue of the mammalian *Oct-6* gene. However do these proteins actually have the same function? It is anticipated that homologous proteins perform the same crucial function in different vertebrates and therefore would be expressed in the same tissues or cell lineages in which their function is critically required. Genetic studies in mice have shown that *Oct-6* plays an important role in peripheral glia differentiation. Thus, functionally homologous chicken and zebrafish Oct-6 proteins should be expressed in differentiating peripheral glia in these species. To examine this question we raised antibodies in rabbits against bacterially overexpressed chicken and zebrafish Oct-6 protein (aminoterminal region) and used these antisera to study the developmental expression pattern of the protein in chicken sciatic nerves and in the posterior lateral nerve of zebrafish embryos.

The specificity of the chicken and zebrafish Oct-6 antiserum used for expression analysis was verified in several ways. First, the chicken Oct-6 antiserum detects only one band of 44 kDa in Western blots of protein extracts of COS cells transfected with a vector expressing chicken Oct-6 (Fig. 3A, lane Oct-6). A protein with the same electrophoretic mobility is detected in extracts of embryonic day 17 chick sciatic nerves (Fig. 3A, lane E17). Both bands are

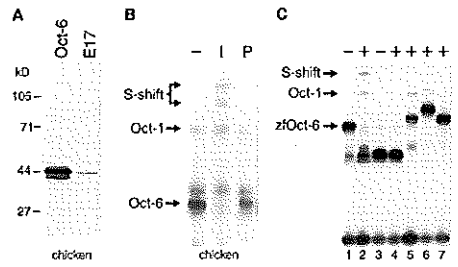


Fig. 3. The chicken Oct-6 antibody detects a 44-kDa protein in embryonic nerves. (A) Western blot. Cell extracts were prepared from COS cells expressing chOct-6 (1) and from E17 embryonic nerves. Proteins were separated on a 10% SDS polyacrylamide gel and transferred to nitrocellulose. The chicken Oct-6 antibody detects one protein of 44 kDa in embryonic nerves and Oct-6 expressing COS cells. (B) The chicken Oct-6 antibody specifically supershifts the chOct-6 complex. Electrophoretic mobility shift assay using freeze/thaw extracts of chicken E17 embryonic nerve and a radiolabeled octamer probe. The Oct-6 and Oct-1 complexes are indicated. The immune serum (lane I) specifically supershifts (sups) the Oct-6 complex but does not affect the Oct-1 complex or the unidentified complex that migrates just above Oct-6. -, no serum added to the bandshift reaction;, I, 1 μl chOct-6 anti-serum added; P, 1 μl pre-immune serum added. The free probe was run out of the gel. (C) The zebrafish Oct-6 antibody specifically supershifts the zfOct-6 complex. Electrophoretic mobility shift assay using a radiolabeled octamer probe and nuclear extracts of COS cells transfected with expression construct encoding the full length zebrafish Oct-6 protein (lanes 1 and 2), an aminoterminal truncated form of zfOct-6 (N133zfOct-6; lanes 3 and 4), mouse Brn-2 (lane 5), mouse Brn-1 (lane 6) and mouse Oct-6 (lane 7). Addition of 1 μl of zfOct-6 antiserum completely supershifts the zfOct-6/octamer complex (sup in lane 2) but does not affect the mobility of the N133zfOct-6/octamer complex (lane 4), nor does it affect the mobility of the mouse Brn-2, Brn-1 and Oct-6 complexes (lanes 5–7).

effectively competed with bacterially expressed and purified chicken Oct-6 protein (data not shown). Second, in electrophoretic mobility shift assays (EMSA) using an octamer probe and E17 embryonic nerve extracts, the antiserum selectively supershifts a protein/DNA complex that has the same electrophoretic mobility as a complex observed with chOct-6 overexpressing COS cell-extracts (Fig. 3B and data not shown). The antibody does not react with the chicken Oct-1 protein or with the protein in the unrelated complex that runs just above Oct-6. The pre-immune serum does not affect the mobility of any of the octamer complexes observed (Fig. 3B, lane P). Thus, an octamer-binding protein of 44 kDa is selectively detected in embryonic nerves with the antiserum raised against the aminoterminal domain of the chicken Oct-6 protein. The specificity of the zebrafish Oct-6 antiserum was tested in EMSAs using nuclear extracts of COS cells transfected with a full length zfOct-6 expression vector and the aminoterminal truncated form of zfOct-6 (N130zfOct-6). The antiserum completely supershifts the zfOct-6 complex (lanes 1 and 2 in Fig. 3C) but does not affect the mobility of the N130zfOct-6 complex (lanes 3 and 4 in Fig. 3C) indicating that the antiserum selectively recognizes the aminoterminal part of the zfOct-6 protein and does not recognize epitopes in the conserved POU domain of the zfOct-6 protein. Furthermore, the zfOct-6 antiserum does not cross react with the mouse POUIII proteins Brn-2 (lane 5), Brn-1 (lane 6) or Oct-6 (lane 7).

Using the chicken Oct-6 specific antiserum and a secondary fluorescent labeled antibody, Oct-6 expression in the developing sciatic nerves of chick embryos was examined by light-microscopy and was found to be high in Schwann cells (Fig. 4, lane LM). Both the level and the number of cells stably expressing Oct-6 increase dramatically from E14 to E20, while only sporadically Oct-6 expressing cells are observed in fully myelinated nerves (P21). To correlate the regulation of the *Oct-6* gene with the differentiation stage of the Schwann cells we examined the nerves using electron-microscopy (Fig. 4, lane EM). At E14 Schwann cells are ensheathing large bundles of immature axon fibers. The cells have typical cytoplasmic processes (sp in Fig. 4) invading the nerve fiber bundles and there is no evidence yet of a basal lamina. By E17 many cells have adopted a one to one relation with axon fibers and some cells have initiated myelination (arrowheads). A basal lamina is now visible. At E20 most myelinating Schwann cells have initiated myelination and some compact myelin is clearly visible. By P20 myelination is complete. Thus, the strong upregulation of Oct-6 expression coincides with the stage in which Schwann cells have adopted a one to one relation with the nerve fibers and initiate myelination. Like in the mouse, Oct-6 expression is extinguished in actively myelinating Schwann cells.

We examined the expression of the zebrafish Oct-6 protein in the lateral line nerve of the developing zebrafish at 72 h post fertilization (hpf) using zebrafish specific Oct-6 antiserum and a fluorescent labeled secondary antibody (Fig. 5C). At this stage of development Schwann cells have wrapped several rounds of uncompacted myelin around large caliber axons, while lower caliber axons are grouped in families (Fig. 5B). Some compaction of the myelin sheath is apparent in the most advanced stage of differentiation seen in Fig. 5B (arrowheads). All nuclei in this field are Schwann cells apart from one endoneural fibroblast-like cell indicated with a star. It is anticipated from the chicken and mouse data that Oct-6 will be highly expressed in the peripheral nerve at this stage. We focused on the lateral line nerve system of the zebrafish which we visualized on whole mount embryos at 72 hpf using an antibody directed against acetylated α-tubulin (Fig. 5A). The position of the section in Fig. 5B,C is indicated with a dotted line. Oct-6 positive nuclei (green fluorescent signal) are found associated with axonal tubulin stained nerve fibers of the lateral nerve, strongly suggesting Schwann cell specific expression.

Thus, the expression of the zebrafish and chicken *Oct-6* genes in the peripheral glia lineage of these species further corroborates our conclusion that these genes are the true homologues of the mouse *Oct-6* gene.

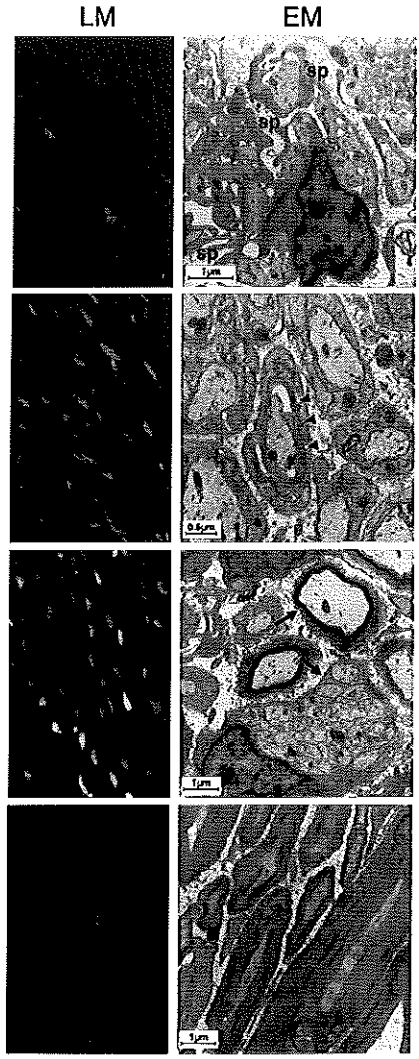


Fig. 4. Oct-6 expression during peripheral nerve development. Chicken sciatic nerves of embryonic day 14 (E14), E17, E20 and post-hatching day 21 (P21) were analyzed for Oct-6 expression. Paraffin embedded sections of sciatic nerves were incubated with chicken Oct-6 specific antibodies. Expressing cells were visualized using a FITC coupled secondary antibody and photographed using a scanning electron microscope (LM, light microscopy). The ultra structure of the contra lateral sciatic nerve was examined using transmission electron microscopy. Electron micrographs of representative fields are shown at the stages indicated. The basal lamina is clearly visible at E20 and is indicated with an arrow. The Schwann cell processes invading the nerve fibers are labeled 'sp' (E14). The formation of the first uncompacted myelin sheaths in panel E17 are indicated by arrowheads.

3. Discussion

3.1. Sequence conservation of the Oct-6 POU domain protein in evolution

High stringency screening of genomic libraries of zebrafish and chicken with a mouse *Oct-6* POU domain probe resulted in the cloning and characterization of the zebrafish and chicken orthologous genes. Not unexpectedly, both the chicken and zebrafish *Oct-6* genes, like the mammalian genes, do not contain introns in their open reading frame. Class III POU genes have been found in worms, insects and all vertebrates examined. It has been suggested that the class III POU genes arose through reverse transcription of a POU domain messenger RNA molecule and integration of the cDNA in germ line genomic DNA in the pre- or early-Cambrian period. While even flatworms have class III POU genes that do not contain introns (at least not in the POU domain for which sequence information is available (Orii et al., 1993; Stuart et al., 1995), the one class III gene present in the roundworm *C. elegans*, *Ceh-6*, has four introns (NCBI accession number z75711). Thus it is possible that the *Ceh-6* gene represents the direct descendant of the primordial class III POU gene or, alternatively, the *Ceh-6* gene has acquired introns since the gene arose.

Duplication of the ancestral class III POU gene, or multiple random integrations of its reverse transcribed mRNA, and recruitment of gene regulatory sequences in the vicinity of the integration or recombination site resulted in the four functional class III genes found in mammals (Kuhn et al., 1991; Hara et al., 1992; Avraham et al., 1993; Xia et al., 1993). The mammalian Oct-6, Brn-1 and Brn-2 proteins are encoded by GC rich genes and they contain, outside their POU domains, multiple homopolymeric stretches of proline, alanine, histidine, glycine and glutamine residues. The Xenopus and zebrafish Oct-6, Brn-1 and Brn-2 proteins lack these blocks of simple amino acid repeats and are encoded by GC-poor genes. The correlation between the presence of protein domains that consist mainly of simple homopolymeric repeats and a high GC content led Sumiyama et al. (1996) to suggest that the appearance of these new protein domains during evolution in the mammalian *Oct-6*, *Brn-1* and *Brn-2* gene has been facilitated by compositional constraints (GC pressure). Since the class III POU genes are predominantly expressed in the central nervous system of vertebrates they speculate that these newly evolved protein domains have contributed to the diversification of gene regulatory mechanisms in the central nervous system of mammals and by extension to the evolution of recent brain structures like the neocortex. Although our analysis of the transactivation potential of the chicken, zebrafish and mouse Oct-6 proteins fail to provide support for this hypothesis it is possible that the simple protein domains present in the mouse Oct-6 are important in some aspects of the regulation of complex promoters in a tissue-specific fashion. This hypothesis can now be critically tested

in an experiment in which the chicken or zebrafish *Oct-6* gene are used to rescue the phenotype of the Oct-6 knock out animals.

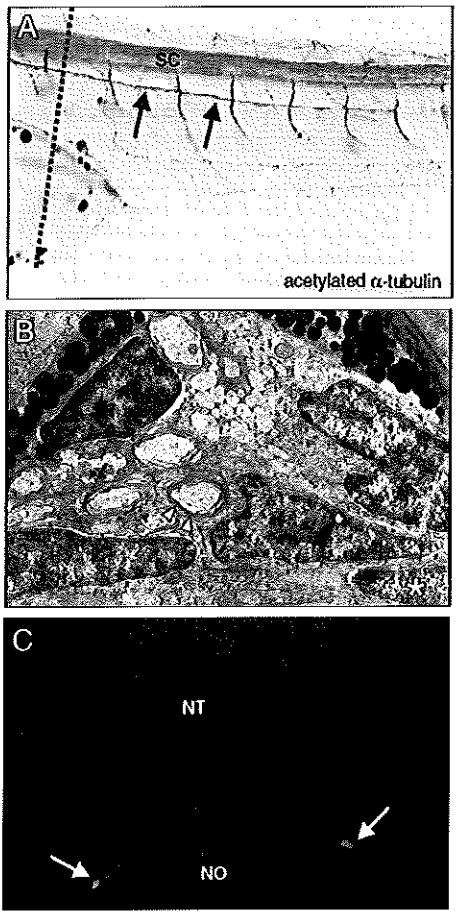


Fig. 5. Oct-6 expression in the lateral peripheral nerve of zebrafish. (A) Whole-mount immunolocalization of acetylated tubulin positive axons. Alkaline phosphatase staining visualizes the acetylated tubulin positive axons of the lateral line nerve tract (arrows) of a 72-hpf zebrafish. The plane of sectioning in (B,C) is indicated. SC, spinal cord. (B) Electron-micrograph of the posterior lateral line nerve of a 72-hpf zebrafish. Transverse section through the lateral line nerve tract of a 72-hpf zebrafish. The nucleus of an epineural cell is indicated with a star. Arrowheads point at closely apposed myelin membranes. (C) Immunolocalization of Oct-6 expressing cells. Transverse section at the anterior-posterior level indicated in (B) through a 3-day-old zebrafish. Oct-6 expressing nuclei (green fluorescent signal) are seen closely associated with acetylated tubulin positive axons (red fluorescent signal) of the lateral line nerve tract (arrows). Oct-6 positive cells are also seen in the neural tube and epidermis. NO, notochord; NT, neural tube.

Does GC pressure actually facilitate the expansion of amino acid sequences during evolution? The cloning of the zebrafish and chicken *Oct-6* genes allowed us to test this hypothesis. We compared the amino acid sequences of the zebrafish, Xenopus, chicken and mouse Oct-6 proteins and the GC content at the first, second and third codon position of the POU domain and the entire coding region (Fig. 1C). The zebrafish *Oct-6* (*zp-50*) gene is, like the Xenopus *xlpou1* (*Oct-6*), GC-poor and the predicted zebrafish Oct-6 protein lacks long simple amino acid repeats. In contrast, the chicken *Oct-6* gene is very GC-rich, like the mammalian *Oct-6* genes, but the predicted chicken Oct-6 protein lacks extensive amino acid repeats. Thus, the correlation between a high GC content in the gene and the presence of amino acid repeats in the protein sequence breaks down when the chicken gene is included in this analysis. Therefore, GC pressure alone does not seem to be sufficient to allow or to facilitate the evolution of amino acid repeats suggesting a second requirement which is met in mammals but not in birds.

3.2. Functional conservation of the Oct-6 POU domain proteins in evolution

Although the primary amino acid sequence comparison of the zebrafish, chicken and mouse Oct-6 proteins identify them as the structural homologues of the mammalian Oct-6 proteins it is not evident from such a comparison that they also represent the functional homologues. In this context it is of note that the frog and zebrafish Oct-6 proteins are more homologous to each other (85%) than the mouse and chicken proteins are (73%). This is rather surprising as the fossil record suggests that the birds and mammals have a longer shared evolutionary history than the fish and amphibians (Colbert, 1969) and is corroborated by the phylogenetic relationship of many conserved regulatory genes such as *sonic hedgehog* (*shh*) and *goosecoid* (*gsc*). The avian and mammalian shh and gsc proteins show higher homology than the amphibian and zebrafish proteins (data not shown and De Robertis et al., 1994; Fietz et al., 1994). Thus, the relationship of the vertebrate Oct-6 proteins as illustrated in Fig. 1D could suggest functional divergence of the *Oct-6* genes in the fish and amphibians which could have retained or acquired certain functions that are lost or taken over by other closely related class III POU proteins in mammals and birds or their common reptilian ancestor. Consistent with this possibility is the observation that the embryonic expression patterns of the frog, fish and mouse genes are diverged. For example the *zp-50* (*zfOct-6*) gene is expressed in hindbrain in a rhombomere 3- and 5-specific fashion while the mouse *Oct-6* gene is expressed initially throughout the hindbrain, is then progressively restricted to the posterior part and is later expressed in certain nuclei such as the facial nuclei. However, there is also substantial overlap in expression in these two vertebrates: Oct-6 is highly expressed in the rostral diencaphalon in 9.5 and 10.5 dpc mouse embryos

and zebrafish at equivalent stages, both overlapping with shh expression (Hauptmann and Gerster, 1996; Zwart et al., 1996). Also, in frogs and mice, the *xlpou1* and *Oct-6* genes are both expressed in skin (Agarwal and Sato, 1991). Thus, both the structural and the expression data are consistent with the possibility that the *Oct-6* homologues serve conserved and diverged functions in vertebrate development. Indeed, extensive overlap in expression patterns of the closely related class III POU genes does provide a fair degree of redundancy allowing for the diversification of expression patterns and function of the individual class III genes in different vertebrate species. For example, while both the frog and mouse, but not zebrafish, *Oct-6* gene is highly expressed in skin, genetic evidence in mice suggest that Oct-6 function in skin is redundant (Andersen et al., 1997). Therefore, when considering whether the chicken and zebrafish *Oct-6* genes represent functional homologs of the mammalian *Oct-6* gene it is relevant to examine and compare the expression of these genes in those cell lineages in which the gene plays a pivotal role. We therefore examined the expression of the chicken and zebrafish Oct-6 proteins in peripheral nerve development.

In all higher vertebrates, Schwann cells originate from the multipotent neural crest and proliferate and differentiate in intimate contact with the outgrowing axon (Jessen and Mirsky, 1992). The generation and survival of Schwann cells relies on axonally derived neuregulins and their receptors expressed on the Schwann cell membrane (Shah et al., 1994; Riethmacher et al., 1997). Schwann cell precursors differentiate through a series of well defined stages to give rise to the two mature phenotypes observed in the adult nerve; myelinating Schwann cells associated with one large caliber axon and non-myelinating cells esheathing multiple lower caliber axons (Mirsky and Jessen, 1996). Oct-6 is not expressed in the neural crest but is induced in the Schwann cell precursors, is upregulated in premyelinating cells reaching maximum levels when Schwann cells cease to proliferate and establish a 1:1 myelinating relationship (promyelin stage) with large caliber axons or ensheath multiple small axons. Oct-6 expression is subsequently extinguished in the terminally differentiating Schwann cell (Scherer et al., 1994; Blanchard et al., 1996; Jaegle and Meijer, 1998). Mice carrying a null mutation for the *Oct-6* gene show a marked delay in peripheral nerve maturation with Schwann cells transiently arrested at the promyelin stage suggesting that Oct-6 is a positive regulator of genes important for the transition from promyelin into actively myelinating cells (Bermingham et al., 1996; Jaegle et al., 1996). Thus like other vertebrate POU genes *Oct-6* is involved in lineage progression but not in lineage determination. The chicken *Oct-6* gene is similarly regulated as the mouse gene; the gene is induced in Schwann cell precursors, upregulated in premyelinating cells and extinguished in myelinating cells, strongly suggesting conservation of gene function and of the mechanism by which the gene is regulated. Also, the zebrafish *Oct-6* gene is highly expressed in Schwann cells of the developing peripheral nerve at a time that these cells start myelination, analogous to what is observed in mouse and chick. By what mechanism *Oct-6* expression is regulated in the Schwann cell lineage is at present unknown although in vitro experiments have suggested that the cAMP-PKA pathway is important for the upregulation of *Oct-6* in immature cells but in vivo evidence for a role of cAMP is lacking (Monuki et al., 1989). Recently we have identified a region in the mouse *Oct-6* locus that direct Schwann cell-specific expression in transgenic mice (W. Mandemakers and D. Meijer, unpublished data). The isolation of the functionally equivalent region from the chicken *Oct-6* locus and DNA sequence comparison will allow the identification of potentially important nuclear factors involved in the activation and regulation of the gene and will be helpful in further functional dissection of this DNA region. This type of phylogenetic approach to dissect gene regulatory mechanisms has been highly successful, for example, in the elucidation of the mechanism by which *Hoxb-1* expression in rhombomere 4 is regulated (Popperl et al., 1995).

On the basis of the comparative data presented here we conclude that the chicken and zebrafish *Oct-6* genes represent the homologs of the mammalian *Oct-6* gene. This work provides the basis and the tools for a further comparison in expression pattern in these different vertebrates, in particular in the brain, identification of conserved regulatory mechanisms and functional studies across large evolutionary distances.

4. Materials and Methods

4.1. Cloning of zebrafish and chicken Oct-6 genes

Genomic phage libraries of chicken (Clontech CL1012J) and zebrafish (a kind gift from Barbara Jones and Martin Petkovich) were screened with a mouse *Oct-6* fragment encompassing the POU domain. DNA from strongly positive clones was used as a template in a polymerase chain reaction (PCR) with fully degenerate primers. These primers correspond with the FAKQFK and the WFCNRR peptide sequences within the aminoterminal POU domain and the carboxyterminal homeodomain, respectively. Amplified fragments were cloned and sequenced. Clones that showed the highest homology to the mouse Oct-6 POU domain were further characterized by restriction fragment analysis and sequencing.

4.2. Transactivation assay

HeLa cells were transfected with 15 μg reporter plasmid, 2 μg reference plasmid and 3 μg of the POU factor expression plasmid using the DEAE-Dextran procedure of Holter et al. (1989). The reporter, reference and CMV based expression vectors were all obtained from the Schaffner

lab (Muller et al., 1988). The reporter plasmid consists of a consensus octamer site directly upstream of the rabbit β-globin gene (OCTA(2) (Muller et al., 1988)) and an SV40 enhancer downstream. A plasmid in which the octamer site was mutated was used as a control for octamer dependent transactivation (Octa(Δ)). Cells were harvested 30 h after transfection. RNA was extracted with the lithium/urea method of Auffray and Rougeon (Auffray and Rougeon, 1980). After the removal of any residual plasmid DNA with DnaseI, 10 μg RNA was hybridized to a ^{32}P-labeled SP6 RNA polymerase generated RNA probe that spans position −37 to +179. Hybridization products were digested with 5 units RnaseI (Promega) at 37°C for 30 min and separated on a 6% polyacrylamide/8 M urea gel.

Expression vectors encoding full length or aminoterminal truncations of zebrafish and chicken Oct-6 protein were generated by cloning different restriction enzyme fragments in frame in the appropriate pEVRF plasmid (Matthias et al., 1989). Overexpression of the proteins in COS cells and bandshift experiments were done as described previously (Meijer et al., 1992).

4.3. Generation of antibodies against chicken and zebrafish Oct-6

A 670-bp NcoI-Asp718 genomic fragment encoding the aminoterminal part of the chicken Oct-6 protein was cloned in frame in the IPTG inducible bacterial expression plasmid pQE9 (QIAGEN). This creates a fusion Oct-6 protein with 6 Histidine residues at its aminoterminus. Overexpression in E. coli M15 and purification of the protein was done as described (QIAGEN). Purified protein was resuspended in Freund's incomplete adjuvants and injected into New Zealand white rabbits. Serum was collected after the fourth immunization. The same protocol was followed to raise an antiserum against the zebrafish Oct-6 aminoterminal domain (AA 1–134).

4.4. Immunohistochemistry and electronmicroscopy

Chicken sciatic nerves were dissected from chick embryos and young chicks at different stages of development and fixed in 60% methanol/3.7% para-formaldehyde (PFA). Zebrafish embryos were allowed to develop at 28.5°C and were staged according to Kimmel et al. (1995). Embryos were fixed in 4% PFA. After dehydration and paraffin embedding 6-μm sections were cut and mounted on gelatin coated microscope slides. Antibody incubations were performed in PBS/0.05% Tween-20/5% fetal calf serum. The primary antibody was used at a dilution 1:200 (anti-chicken Oct-6 and zebrafish Oct-6) and incubated overnight at room temperature. After extensive washing the sections were incubated for 2 h at room temperature with an fluorescein isothiocyanate (FITC) coupled goat-anti-rabbit secondary antibody (Nordic). The anti-acetylated tubulin monoclonal antibody was obtained from Sigma. Sections were viewed and photographed using a Zeiss confocal laser microscope or a Leica fluorescence microscope.

Sciatic nerves to be processed for electronmicroscopy were immersion fixed in 1% gluteraldehyde, 3% paraformaldehyde in 100 mM sodium cacodylate buffer pH 7.2. After washing the nerves were incubated in a 1% osmium tetroxide solution, dehydrated and embedded in Epon. Ultra-thin sections were stained with uranyl-acetate and lead citrate. Zebrafish embryos were immersed in 2% paraformaldehyde/1% gluteraldehyde in 100 mM cacodylate buffer pH 7.2. Sections were viewed and photographed using a Philips CM100 transmission electron microscope.

4.5. Western blotting

Protein extracts of COS cells expressing chicken Oct-6 and embryonic day 17 sciatic nerves were separated on a 10% SDS-PAGE. Proteins were transferred onto nitrocellulose by electroblotting. The blot was incubated in blocking solution (1.5% BSA/1.5% milk powder in *tris*-buffered saline) for 1 h at room temperature. The first antibody incubation (anti-chicken Oct-6 serum No. 1907 1:500) was carried out overnight in blocking buffer plus 0.3% nonidet-40 (NP40). After washing the blot extensively with blocking solution, the blot was incubated with peroxidase-conjugated swine-anti-rabbit IgGs in blocking buffer for 2 h at room temperature. The staining reaction was carried out using DAB (Aldrich).

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Chapter 4.

Transcriptional regulation of the POU gene Oct-6 in Schwann cells

Wim Mandemakers, Ronald Zwart, Robert Kraay,
Gerard Grosveld, Anneke Graus, Martine Jaegle,
Ludo Broos and Dies Meijer

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Summary

Genetic evidence suggests that the POU transcription factor Oct-6 plays a pivotal role as an intracellular regulator of Schwann cell differentiation. In the absence of Oct-6 function Schwann cells are generated in appropriate numbers and these cells differentiate normally up to the promyelin stage at which they transiently arrest. During peripheral nerve development Oct-6 expression is initiated in Schwann cell precursors and is strongly upregulated in promyelin cells. Oct-6 expression is subsequently extinguished in terminally differentiating Schwann cells. Thus, identification and characterisation of the DNA elements involved in this stage specific regulation may lead us to the signalling cascade and the axon-derived signals that drive Schwann cell differentiation and initiate myelination. Here we present experiments that aim at identifying such regulatory sequences.

Introduction

Continued reciprocal signalling between axons and associated glial cells governs the proper development, maintenance and regeneration of peripheral nerves. In the Schwann cell this signalling results in modulation of transcriptional programs that direct processes like proliferation and myelination. Recently a number of transcription factors, such as Krox-20, Pax3 and Oct-6, have been implicated as important intracellular regulators of Schwann cell differentiation (Bermingham et al., 1996; Blanchard et al., 1996; Jaegle et al., 1996; Jaegle and Meijer, 1998; Kioussi et al., 1995; Topilko et al., 1994). Chief among those is the Oct-6 transcription factor.

The Oct-6 transcription factor belongs to the POU-homeodomain family of transcriptional regulators. POU proteins are characterised by a 150-160 amino acid domain which can be subdivided in a carboxyterminal portion (the POU homeodomain) related to the homeodomain DNA binding proteins and an aminoterminal portion consisting of a variant helix-turn-helix motif that is a defining characteristic of this family of proteins (the POU-specific domain; Herr et al., 1988). More than 13 mammalian POU genes have been characterised. Based on sequence similarities within the POU domain, POU genes can be subdivided into six subgroups or classes (Wegner et al., 1993). Genetic experiments have suggested important roles for the different members of this class of transcription factors in the proliferation and differentiation of distinct cell types during development (Ryan and Rosenfeld, 1997).

In early mouse development the Oct-6 gene is expressed in a highly dynamic, complex pattern, overlapping with other members of the class III POU gene family (Alvarez-Bolado et al., 1995; Zwart et al., 1996). At later, foetal stages of development Oct-6 expression is observed in specific subsets of motor and commissural neurons and oligodendrocytes in the spinal cord and hindbrain, different mid- and forebrain structures, skin and neural crest derived Schwann cells (Blanchard et al., 1996; Zorick et al., 1996; Zwart, 1996). Genetic studies in mice have shown that the Oct-6 gene has an important role in the differentiation of Schwann cells. In the absence of Oct-6 gene function Schwann cells fail to mature on schedule leading to a transient arrest at the promyelin stage and a 1-2 week delay in the onset of myelination, while CNS myelination is not affected

(Bermingham et al., 1996; Jaegle et al., 1996; Jaegle and Meijer, 1998; Weinstein et al., 1995). Oct-6 expression in the Schwann cell lineage is initiated in the Schwann cell precursor, is upregulated in premyelinating cells reaching maximum levels when Schwann cells cease to proliferate and establish a 1:1 myelinating relationship with large calibre axons or ensheath multiple small axons. Oct-6 expression is subsequently extinguished in the terminally differentiating Schwann cells (Blanchard et al., 1996; Levavasseur et al., 1998; Scherer et al., 1994). A prominent role for the adenelyl cyclase-PKA signalling pathway in the onset of terminal Schwann cell differentiation has been suggested. Oct-6 gene expression is rapidly induced in cultured Schwann cells following administration of agents that increase intracellular cAMP concentrations (Monuki et al., 1989). This induction is shortly followed by that of a number of major myelin genes such as MBP and P0. Furthermore, Oct-6 expression in oligodendroglia cell lines can be modulated by estradiol and anti-estrogens suggesting that Oct-6 is a key component in the estrogen regulation of myelination in vivo (Renner et al., 1996).

Thus, it is anticipated that the highly dynamic and diverse expression pattern of the Oct-6 gene is mediated through a mosaic of cis-regulatory elements within the Oct-6 locus that alone, and in combination, respond to different intra-cellular signalling pathways and drive or repress expression in specific cell types. In this paper we describe experiments aimed at identifying of the minimal sequence requirements for Schwann cell specific expression of the Oct-6 gene.

Results

The promoter region of the Oct-6 gene is in a DNAseI hypersensitive chromatin configuration.

The chromatin structure of promoter and enhancer regions of actively transcribed genes is often hypersensitive for nucleases, while the entire transcribed region and beyond shows an increased generalised sensitivity when compared with non transcribed genes (Gross and Garrard, 1988; Svaren and Chalkley, 1990). This DNAseI hypersensitivity reflects the accessibility of the DNA in vivo for nucleases and other molecules like transcription factors. Thus, the presence of hypersensitive sites within a region of interest is taken as a first indication of the presence of important cis-acting regulatory sequences.

The mouse Oct-6 gene is a single exon gene located on the distal arm of chromosome 4 (Hara et al., 1992; Kuhn et al., 1991). The entire transcription unit is contained within a 7kb EcoRI restriction fragment. This fragment contains 1.5 kb of upstream and 2.5 kb of downstream sequences. We probed for the presence of DNAseI hypersensitive sites within this 7 kb EcoRI fragment in chromatin of Oct-6 expressing cells versus non-expressing cells. For our analysis we choose embryonic stem (ES) cells and Tr6B (a mouse Schwannoma cell line) for Oct-6 expressing cells and MES cells (a mesoderm-like cell line) for non-expressing cells. Nuclei of these cells were incubated with increasing amount of DNAseI. DNA was extracted from the nuclei, digested with restriction enzymes and separated on agarose gels. Southern blots of these gels were

hybridised with two distinct probes for identifying hypersensitive sites upstream and downstream of Oct-6 coding sequences (Figure 1). Probe a detects an EcoRI/XhoI DNA fragment of 2.9 kb that disappears with increasing DNAseI concentration. One major band of 1.5kb and two minor shorter bands appear and disappear again with increasing DNAseI concentration. Thus, a major DNAseI hypersensitive site maps around 150bp upstream of the transcriptional start site. This hypersensitive site was also detected in the mouse Schwannoma cell line Tr6B (data not shown). Probe b detects a 4.1 kb EcoRI/XhoI restriction fragment that gradually disappears with increasing DNAseI concentration. No shorter fragments appear indicating that there are no additional hypersensitive sites in the 3"part of the Oct-6 gene up to the EcoRI site. The presence of the promoter associated DNAseI hypersensitive site detected with probe a correlates with the active state of the gene in ES and TR6B cells as it is not detected in the non-expressing cell line MES (Figure 1). No other hypersensitive sites were detected in this 7 kb restriction fragment.

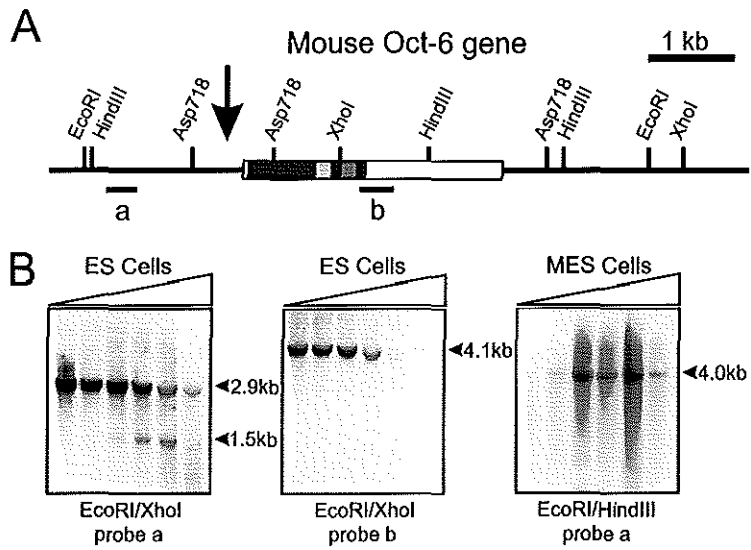

Figure 1. Mapping DNAseI hypersensitive sites within the mouse Oct-6 gene. Panel A shows the genomic organisation of the Oct-6 gene and the position of the probes used (a and b). The DNAseI hypersensitive site is indicated with a vertical arrow. Panel B DNAseI fade-outs. Southern blots of restriction enzyme digested DNA isolated from DNAseI treated ES cell nuclei were probed with the probes indicated in panel A. MES cell DNA was digested with EcoRI and HindIII.

The Oct-6 promoter is not cell-type specific.

The DNA sequence of the mouse Oct-6 gene promoter region was determined (Figure 2) and was found to be highly homologous to the rat Oct-6/SCIP gene promoter (Kuhn et al., 1991). The position of the transcription initiation site is indicated with an arrow. The sequence of the Oct-6 promoter is extremely rich in the dinucleotide CpG, as is

the entire 5' region of the Oct-6 gene. Thus the gene is located in a CpG island of approximately 1kb. Indeed this region is undermethylated in expressing and non-expressing cells (data not shown). The Oct-6 promoter has all the hallmarks of a strong basal promoter (Muller et al., 1988). Around –30bp with respect to the transcription initiation site there is a TATA-like sequence and at –90bp there is a canonical CCAAT box. A computer search identified a number of additional putative transcription factor binding sites. Not unexpectedly there are at least eight consensus binding sites for the Sp1, and Krox-20 family members. In addition there are two consensus AP-2 binding sites. No cAMP response elements are present in the promoter region. However it has been reported that cAMP induction of gene expression can be mediated through AP-2 (Imagawa et al., 1987). The presence of two AP-2 sites could therefore contribute to the cAMP inducibility of the Oct-6 gene in Schwann cells following administration of Forskolin.

To test whether the Oct-6 promoter region contains all the information for cell-type specific expression of the Oct-6 gene we tested several promoter deletion constructs by transient transfection of ES and MES cell lines. The promoter deletion constructs, driving a CAT reporter gene are depicted in Figure 2. The activity of the various promoter constructs was measured in a CAT assay using cell extracts of transiently transfected cells (Figure 2B). The longest promoter construct (6) tested contains all of the DNAseI hypersensitive sites and is active in ES cells. Activity is retained in mutants 4 and 5 but drops sharply (5 fold) when the promoter is further deleted to mutant 3 and is completely lost in construct 1 and 2. Thus, the region between –90 and –160 contribute significantly to the activity of the Oct-6 promoter. This region also contains the strong DNAseI hypersensitive site. However, these promoter constructs have a similarly high activity in MES cells, which do not express the endogenous Oct-6 gene. Also, these constructs do not show increased activity following Forskolin administration to the medium suggesting that the AP-2 site is not involved in cAMP induction of the Oct-6 gene (data not shown). Thus, the Oct-6 promoter constructs do not show tissue specificity suggesting that important (negative?) regulatory elements are missing or that transient assays are not suitable for analysing the Oct-6 promoter.

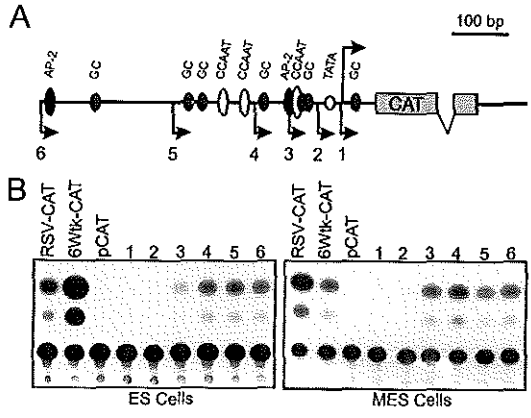

Figure 2. Functional dissection of the Oct-6 promoter. Panel A. Schematic depiction of the Oct-6 promoter CAT gene constructs. The positions of putative transcription factor binding sites are indicated. The various 5' truncations of the promoter are indicated with arrows and their numbers correspond with those in panel B. Panel B. CAT assays. RSV is the Rous sarcoma virus promoter and 6Wtk is the minimal HSV TK gene promoter as present in pBLCAT2 with a multimerized IgH enhancer upstream. The 6Wtk promoter is strongly expressed in undifferentiated ES cells but is less active in the MES cell line (Scholer et al., 1989). The reverse is true for the RSV promoter.

Regulatory sequences important for Schwann cell specific expression of the Oct-6 gene are located at considerable distance from the Cap site of the gene.

In order to further characterise the regulatory sequences of the Oct-6 gene we generated transgenic mice carrying constructs with different sequences of the Oct-6 locus (see Figure 3). To conveniently analyse tissue-specific expression of the transgene, we inserted a bacterial LacZ gene in-frame in the Oct-6 coding sequence. This allowed us to examine transgene expression using a simple enzymatic staining reaction. We limited our analysis here to the Schwann cell lineage. Two different DNA constructs were generated covering up to 32 kb of the Oct-6 gene locus. We either generated transgenic lines or analysed transgenic founder embryos at embryonic day 18. The first construct we analysed was the 7kb EcoRI fragment. Two transgenic lines and two founders were analysed. Although the transgene was expressed in all four transgenic animals, no expression was detected in the Schwann cell lineage. Expression was found mainly in the brain, in a pattern that was not consistent among the different transgenic lines. Most likely these expression patterns are a consequence of the transgene integration site in the different mouse lines. Thus, this 7 kb transgene does not contain enough sequence information to direct Schwann cell expression.

We next generated transgenic mice with a construct that contains 15 kb of 5' upstream sequences and 15 kb downstream of the gene transcription initiation site (Figure 3). Three transgenic lines were generated with this particular construct. All three lines expressed the transgene with varying expression patterns during embryonic development. Expression was mainly observed in the brain and overlapped in the midbrain area with the endogenous Oct-6 protein. However, the transgene was consistently expressed in all three lines in the Schwann cells of neonatal animals. Expression was observed in E18 embryonic sciatic nerves (Figure 3, bottom panel) but not in E16 nerves indicating that expression (as assayed here) is induced in Schwann cells between E16 and E18. Also, heterozygote Oct-$6^{+/LacZ}$ embryos show LacZ expression from E16 onwards, indicating that the transgene is expressed slightly later than the endogenous locus as assayed with X-gal staining (data not shown).

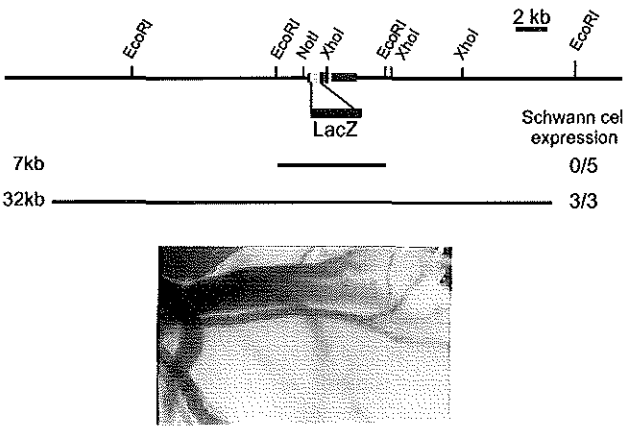

Figure 3. Schwann cell specific regulatory sequences of the mouse Oct-6 locus.The genomic organisation of the mouse Oct-6 gene and the transgenic constructs used here are schematically shown. The bacterial LacZ gene was fused in frame with aa 20 of the Oct-6 ORF. The LacZ portion does not carry any transcriptional termination sequences so that transcriptional termination takes place at the normal Oct-6 sequences. The photograph is a representative picture of hindlimb nerves of a E18 transgenic embryo stained with X-gal.

Discussion

In this report we have shown that the Oct-6 gene promoter is in a DNAseI hypersensitive chromatin configuration only in cells that express the Oct-6 gene. However, the Oct-6 gene promoter is equally active in expressing and non-expressing cells in transient transfection assays. This may not be surprising since the promoter is build up as a classical strong basal promoter with a TATA box, several CCAAT boxes and multiple GC boxes (Muller et al., 1988). These results indicate that this promoter is strongly repressed in non-expressing tissues as has been suggested previously (Zwart et al., 1996). Our DNAseI mapping data suggest that repression is achieved by sequestering the promoter region in a non-accessible chromatin configuration. Thus, a mechanism could be envisaged in which the Oct-6 promoter, in itself non-tissue specific, is made available for interaction with ubiquitous factors resulting in transcriptional activation. How the chromatin configuration of the Oct-6 locus is regulated is unknown. A number of genetic loci have been described whose transcriptional output is under control of a 'locus control region' (LCR; Fraser and Grosveld, 1998). The best studied of these locus control regions is the LCR of the β-globin domain. This LCR consists of a series of 5 DNAseI hypersensitive sites in a 10kb region upstream of the embryonic globin gene and is required for erythroid lineage-specific expression of individual genes within the locus. Strong evidence suggests that the LCR functions as holo-complex directly interacting with the individual gene promoters to direct stage-specific expression of the genes (Dillon et al., 1997). If a similar mode of regulation operates in the Oct-6 locus it is expected that additional DNAseI hypersensitive sites be present in the Oct-6 locus that are required for gene activity (see below).

To begin to delineate the sequences required for Schwann cell-specific expression of the Oct-6 gene we generated a number of transgenic lines. The analysis of these mice indicates that the cis-regulatory sequences important for Schwann cell-specific expression are located outside the 7 kb EcoRI fragment that contains the entire gene, but are included in the 32 kb cosmid clones. In preliminary experiments, we have mapped additional DNAseI hypersensitive sites within the 32kb genomic region. One of these sites corresponds to the previously described estradiol responsive enhancer located 5 kb upstream of the Oct-6 transcription initiation site. This enhancer was shown to mediate estradiol enhancement of the Oct-6 promoter driven transcription in cotransfection experiments with the estradiol receptor in oligodendroglia cell lines (Renner et al., 1996). This observation is particularly interesting since estrogens influence myelination in vivo. Therefore, the estradiol mediated enhancement of Oct-6 transcription could be a key event in estrogen regulation of myelination. Whether this element is also important for initial glia cell-specific expression of the Oct-6 gene remains to be elucidated. Further deletion analysis of the 32kb genomic fragment will answer this question and will undoubtedly lead to the identification of the cis-acting element(s) involved in Oct-6 gene activation in the Schwann cell lineage.

Expression of the 32kb transgene is correctly activated and extinguished in the Schwann cell lineage. In addition, we have previously shown that down regulation of the Oct-6 locus in terminally differentiating Schwann cells is dependent, directly or indirectly, on Oct-6 itself (Jaegle and Meijer, 1998). This suggests two things: First, down regulation of Oct-6 in terminally differentiating cells is mediated through repression of the gene and not through the gradual disappearance of an activator. Second, down regulation requires Oct-6 itself. Whether activation and down regulation are mediated through the same cis-acting elements remains to be determined. Such a mechanism has been shown to operate in

the Pit-1 locus, a Class I POU gene. Separate enhancer sequences are responsible for initial Pit-1 gene activation and for subsequent autoregulation (DiMattia et al., 1997). The autoregulatory negative feedback loop in Oct-6 regulation sets an upper limit to the levels of the Oct-6 protein in Schwann cells but does not explain how the expression of the gene is completely extinguished. One possible explanation is that Oct-6 activates expression of a factor that mediates repression through a remote silencer in the Oct-6 locus or through the Oct-6 promoter. In such a model of repression, Oct-6 gene expression is indirectly dependent on Oct-6. As repression of Oct-6 gene transcription is maintained in the adult nerve one has to assume that this repressor has either a very long half-life or, alternatively, that maintained expression of this repressor is not dependent on the Oct-6 gene product. This latter possibility seems more attractive as Oct-6 gene expression is quickly resumed in denervated Schwann cells following nerve damage. Reactivation of Oct-6 gene expression in such reactive Schwann cells could be accomplished through silencing of this hypothetical repressor. Such a mechanism could also explain the observation that Oct-6 expression is not down regulated for at least two months following nerve damage. Taken together, our data are compatible with a mechanism by which Oct-6 down regulates its own expression in an indirect way, through activation of a gene encoding a repressor that binds to an element in the 32 kb fragment. Critical testing of this hypothesis will require the identification of the silencing and cloning of the repressor that binds to it.

Materials and Methods

Mapping of DNAseI hypersensitive sites.

Cultured cells (MES and CCE ES cells) were harvested and washed twice with ice-cold PBS. Cells were resuspended in 3 ml of ice-cold HS-buffer (15mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA and 5% glycerol, supplemented with 1 mM DTT, 0.15 mM spermine and 0.5 mM spermidine, just before use). The cells were disrupted by passing 5 to 10 times through a 25G needle. Examining a drop of the suspension under the phase-contrast microscope monitored the disruption process. Nuclei were collected by a brief centrifugation step at 2500 rpm and resuspended to a final concentration of approximately 5 x 10^7 nuclei per millilitre of HS buffer (the nuclei do not pack tightly after this centrifugation step. Limited DNAseI digestion of chromatin was carried out in a final volume of 500 µl of HS buffer containing 5 x 10^6 nuclei, 5 mM $MgCl_2$ and DNAseI (Boehringer) varying in concentration from 0 to 400 units. The reaction was incubated for 15 to 30 minutes on ice, stopped by adding an equal volume of 20 mM EDTA, 1 % SDS and
100 µg/ml Proteinase K solution and the mixture was further incubated overnight at 45 ^{0}C. After phenol/chloroform extraction the DNA was precipitated with an equal volume of isopropanol. The DNA was dissolved in 200 µl of TE buffer and restricted with different restriction enzymes. Restriction fragments were resolved on 0.8% agarose gels and blotted onto a nylon membrane. The blots were hybridised to the probes indicated in Figure 1.
All other methods are described in Sambrook et al. (1989).

LacZ transgenic constructs

The LacZ reporter gene was isolated as a 3.4 kb BamHI fragment from plasmid p610ZA in which the DraI site 60 bp downstream of the LacZ stop codon had been converted into a BamHI site. This fragment was ligated into the unique NsiI site of a subclone of the Oct-6 gene. The NsiI site was modified first by blunting and addition of BamHI linkers. This procedure resulted in the creation of a fusion gene encoding the first 22 amino acids of Oct-6 followed by a threonine, introduced by the cloning procedure, and the LacZ open reading frame. The fusion gene fragment was subsequently cloned into the 7 kb EcoRI genomic fragment. This clone was extended at the 5' and 3' end with genomic fragments resulting in cosmid 2CosOct-6. The 7 kb EcoRI fragment was isolated by electroelution from agarose gels followed by purification on an elutip mini column (Schleicher and Schull). The 32kb SalI fragment from cosmid 2CosOct-6 was isolated and purified on a NaCl gradient. Transgenic mice were generated using established methods. Tissues derived from these transgenic were processed for β-galactosidase staining according to (Popperl et al., 1995)

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Chapter 5

A distal Schwann cell-specific enhancer mediates axonal regulation of the Oct-6 transcription factor during peripheral nerve development and regeneration

Wim Mandemakers, Ronald Zwart, Martine Jaegle, Erik Walbeehm, Pim Visser, Frank Grosveld and Dies Meijer

(Submitted)

Summary

The POU domain transcription factor Oct-6 is transiently expressed in the Schwann cell lineage and is a major regulator required for the timely progression of Schwann cells through the promyelin stage. During nerve development and regeneration, expression of *Oct-6* is under control of Schwann cell-extrinsic axonal signals. Identification of the cis-acting elements necessary for *Oct-6* gene regulation is an important step in deciphering the complex signaling between Schwann cells and axons governing myelination. Here we show that a fragment distal to the *Oct-6* gene, containing two DNAseI hypersensitive sites, acts as the *Oct-6* Schwann cell specific enhancer (SCE). We show that the SCE is sufficient to drive spatially and temporally correct expression, both during normal peripheral nerve development as well as during nerve regeneration. We further demonstrate that a transgene carrying a tagged version of *Oct-6*, driven by the SCE, rescues the peripheral nerve phenotype of *Oct-6* deficient mice. Thus, our isolation and characterization of the *Oct-6* SCE provides the first description of a cis-acting genetic element that responds to converging signaling pathways to drive myelination in the peripheral nervous system.

Introduction

The development and proper function of peripheral nerves in vertebrates depends on the intimate interactions and continued signaling between Schwann cells and associated nerve fibre(s). The ontogenic origin of Schwann cells, and other glial cell types in the peripheral nervous system (PNS), such as the satellite cells, the teloglia and enteric glia, is the neural crest, a transient migratory population of multipotent cells emanating from the dorsal aspect of the neural tube (Anderson, 1997; Le Douarin et al., 1991). During embryonic development neural crest cells associate with and migrate along the outgrowing axonal bundles, acquiring Schwann cell characteristics. These Schwann cell precursors proliferate, invade, bundle and sort the fibres of the embryonic nerve and this process continues until the Schwann cells ensheath individual fibres and groups of fibres. Schwann cells then cease to proliferate and commence to myelinate the larger axons, while non-myelin forming cells further ensheath multiple lower calibre axons resulting in the mature Schwann cell phenotypes observed in the nerves of adult animals (Webster, 1993).

In recent years, much progress has been made in identifying components of the cell-cell interactions necessary in early stages of peripheral nerve development (Jessen and Mirsky, 1999). In contrast, very little is known about the extracellular signals and the intracellular signalling pathways that initiate myelination, beyond the fact that axonal contact is a *conditio sine qua non*. Earlier pioneering work by Aguayo and colleagues (Aguayo et al., 1976; Aguayo et al., 1976), involving nerve transplantation and cross anastomosis experiments, have indicated that the myelination program of Schwann cells is under control of the associated axon and correlates with axonal diameter (Voyvodic, 1989). Although the formation of the basal lamina by the Schwann cell is required for myelination, the nature of the axonal signal that drives myelination by the Schwann cell remains unclear (Bunge et al., 1990; Eldridge et al., 1989). This signal must ultimately be relayed to the nucleus where transcription factors mediate this signaling by modulating of expression of distinct sets of genes, such as *periaxin*, *myelin associated glycoprotein* (*MAG*), *P-zero* (P_0) and *connexin*

32. Expression of these genes both characterises and drives the myelination process by the Schwann cell.

Of particular interest are the transcription factors Krox-20 and Oct-6 which are pivotal in the regulation of the transcriptional program that drives myelination (Bermingham et al., 1996; Jaegle et al., 1996; Topilko et al., 1994). Neither of the two genes is expressed in the neural crest. However, within the sciatic nerve of the mouse Schwann cells begin to express appreciable levels of *Oct-6* from embryonic day 16 onwards (Blanchard et al., 1996, and this paper). Expression levels peak around birth, after which expression is gradually extinguished. Fully differentiated cells do not express the gene (Levavasseur et al., 1998; Scherer et al., 1994; Zorick et al., 1996). This down regulation of *Oct-6* gene expression is dependent on the *Oct-6* gene product itself and on myelination *per se* (Jaegle and Meijer, 1998). *Krox-20* expression is induced shortly after the first appearance of Oct-6 and becomes restricted to the myelinating lineage in which expression is maintained into adulthood (Topilko et al., 1997; Zorick et al., 1996). Expression of both genes is regulated by axonal contact. Following nerve crush and Wallerian degeneration, when axonal contact is disrupted, reactive Schwann cells extinguish *Krox-20* expression in denervated nerves. During nerve regeneration, axonal contact with Schwann cells is restored and *Oct-6* expression is rapidly re-induced, and subsequently followed by *Krox-20* induction (Scherer et al., 1994; Zorick et al., 1996). In fully regenerated nerves *Oct-6* expression is downregulated while *Krox-20* expression remains high. Thus, both in development and regeneration Schwann cell differentiation from early- to late promyelinating to myelinating stages corresponds with the phenotypic progression from Oct-6$^+$/Krox-20$^-$, to Oct-6$^+$/Krox-20$^+$ to Oct-6$^-$/Krox-20$^+$.

Analysis of postnatal sciatic nerve development in *Krox-20* and *Oct-6* null mice indicates that both genes are required for normal progression of Schwann cell differentiation through the promyelin stage and initiation of the myelination program. In both genetic mutants Schwann cells appear morphologically arrested at the promyelin stage (Bermingham et al., 1996; Jaegle et al., 1996; Topilko et al., 1994). *Oct-6* null Schwann cells eventually overcome this block and myelinate their associated axon normally, while *Krox-20* null Schwann cells are permanently blocked at the promyelin stage. Induction of *Oct-6* expression in Schwann cells does not require *Krox-20* gene activity as Oct-6 is highly expressed in *Krox-20* null Schwann cells (Zorick et al., 1999). *Oct-6* and *Krox-20* gene activity represent two subsequent steps in a genetic hierarchy that regulates progression of Schwann cell differentiation through the promyelin stage. *Oct-6* is the first transcription factor in this cascade responding to axonal signaling. Hence, it is of importance to understand how this regulator itself is regulated.

As a first step in the systematic dissection of the regulation of *Oct-6* gene expression during Schwann cell development, we set out to define the cis-acting genetic elements in the *Oct-6* locus involved in the temporal and Schwann cell specific expression of the gene. Through DNAseI hypersensitive site mapping and deletional analysis in transgenic mice we identify a fragment, located approximately 12 kb downstream of the *Oct-6* structural gene, that is sufficient to activate temporally correct expression in the Schwann cells of transgenic mice. Thus, Schwann cell specificity resides solely within this DNA fragment which we named SCE for *Oct-6* Schwann cell-specific enhancer. Furthermore, we show that the *SCE/Oct-6* promoter driven transgene is downregulated in myelinating cells and re-induced in reactive Schwann cells of regenerating nerves following nerve crush. In addition, we are able to rescue the temporal delay in Schwann cell

differentiation in *Oct-6* null mice by a hemaglutinin (HA)-tagged *Oct-6* gene expressed under control of the SCE.

Results

A chromatin region spanning 45 kb of the *Oct-6* locus contains 8 DNAseI hypersensitive sites.

The mouse *Oct-6* gene is a single exon gene located on the distal arm of chromosome 4. The entire 3kb long transcription unit is contained within a 7 kb EcoRI DNA fragment and the promoter and 5' part of the gene is located in a CpG island. Previously, we have shown that the *Oct-6* gene promoter is highly active in transient transfection assays irrespective whether the transfected cell expresses endogenous *Oct-6* or not (Mandemakers et al., 1999). In addition, this promoter does not drive specific expression of a reporter gene in transgenic mice. These preliminary experiments suggested that important regulatory elements are located outside the promoter region.

To begin a systematic dissection of the regulatory sequences within the *Oct-6* locus we first cloned and mapped an extensive region flanking the mouse *Oct-6* gene (figure 1A). Single copy DNA probes were isolated from this region and used to detect DNAseI hypersensitive sites (HSSs) within chromatin of *Oct-6* expressing versus non-expressing cells and tissues. HSSs are often found in the vicinity of promoter and enhancer regions of transcriptionally active genes and represent nucleosome free sites in chromatin that are readily accessible to regulatory proteins (Felsenfeld et al., 1996; Gross and Garrard, 1988; Svaren and Chalkley, 1990). Thus, mapping HSSs within chromatin of the *Oct-6* locus will reveal the position of DNA elements potentially involved in regulated *Oct-6* gene expression.

We analysed *Oct-6* expressing embryonic stem (ES) cells, MES-68 (an adenovirus E1 region transform P19 embryonic carcinoma cell derivative), TR6B (a mouse Schwannoma cell line; Fields et al., 1975) and adult mouse brain and non-expressing mouse liver and the MES-1 (a P19 derivative) cell line. Nuclei from mouse cells were prepared and treated with DNAseI. The presence of HSSs in chromatin is revealed by the appearance of discrete hybridizing bands on Southern blots with increasing DNAseI concentration (Figure 1B-E). For example, HSS-8 is detected with probe #9 in chromatin of MES-68, ES cells and brain, all of which express *Oct-6*, but not in liver and MES-1 cells, which do not express *Oct-6*. The absence of HSS-8 in chromatin of liver cells indeed correlates with the expression status of the *Oct-6* gene in these cells and is not simply due to limited DNAseI digestion as the albumin enhancer associated HSS is readily detected in the same liver DNA samples (Figure C).

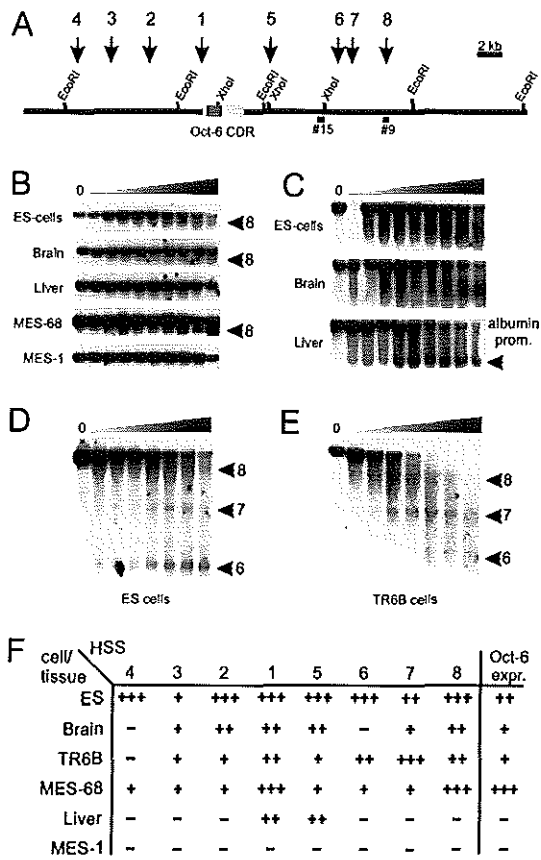

Figure 1 DNAseI hypersensitive site mapping in the mouse *Oct-6* locus. (A) Map of the mouse *Oct-6* locus. Several diagnostic restriction enzyme recognition sites used to determine the positions of the DNAseI hypersensitive sites are indicated. Black arrows and numbers above the locus indicate the positions of the corresponding HSS. The small bars below the map (#15 and #9) show the probes that were used for the HSS mappings presented in panels B, D, and E. (B) Mapping of HSS-8 in various cell lines and tissues. DNAseI treated samples were digested with StuI, separated by agarose gel electrophoresis, blotted and hybridised with probe #9. The band demonstrating the presence of HSS-8 is indicated with a black arrow. This band is absent in untreated samples (0), but emerges with increasing intensity in samples treated with increasing amounts of DNaseI (indicated by the triangle above the panels). The position of the HSS in the *Oct-6* locus was deduced from the size of the emerging fragment and the position of the probe. All positions were verified with different digests or different probes. HSS-8 could only be detected in *Oct-6* expressing cells. (C) The same samples for ES cells, brain and liver and the same procedure as described for panel B were used to verify whether any HSS could be detected in the liver series. Southern blots with EcoRI digested DNA were hybridized with a probe derived from exon 1 of the mouse *albumin* gene, to resolve the previously identified HSS coinciding with the gene promoter region (Liu et al., 1988). The band coinciding with this HSS was indeed only detected in the liver samples (black arrowhead). (D, E) These panels describe the mapping of HSS-6, HSS-7, and HSS-8 in ES-cells and TR6B cells, respectively. Samples were digested with HpaI and hybridised with probe #15. These panels show that in ES-cells HSS-6 and HSS-8 are the most prominent ones, while in TR6B cells HSS-7 and HSS-8 seem to be more sensitive than HSS-6.

In total we mapped 8 HSS within the *Oct-6* locus (Figure 1A). The degree of DNAseI sensitivity of these sites differs from cell line to cell line. For example, in ES cells HSS-6 is more pronounced than HSS-7, while in TR6B cells HSS-6 is less pronounced (also compare HSS-8 sensitivity in ES cells and MES-68 cells in Figure 1B). The relative degree of sensitivity of the sites in different cell types is summarized in Figure 1F. The relatively low sensitivity of certain sites in brain most likely reflects the great diversity of cell types in this tissue with only a small subset of cells expressing the *Oct-6* gene.

A Schwann cell specific enhancer resides in a DNA fragment containing two HSSs.

The relevance of these HSSs for *Oct-6* gene regulation was tested in transgenic mice. The bacterial *β-galactosidase* gene was inserted in frame in the *Oct-6* coding region of a 32 kb genomic DNA construct containing all 8 mapped HSSs (construct I: Figure 2). Three transgenic mouse lines were generated with this construct. Mice from all three lines expressed the *β-galactosidase* gene in the Schwann cells of their peripheral nerves. In addition, expression of LacZ was observed in many other tissues where *Oct-6* is normally expressed, such as brain and skin and in particular the hair follicles. Next we generated transgenic mice with a smaller construct, lacking all HSSs except site 1 (construct II). These mice do not express the transgene in Schwann cells, although variable ectopic expression is seen in other tissues. These results indicate that the element necessary for Schwann cell specific expression resides within the 32 kb region but outside the 7 kb EcoRI fragment containing the *Oct-6* structural gene.

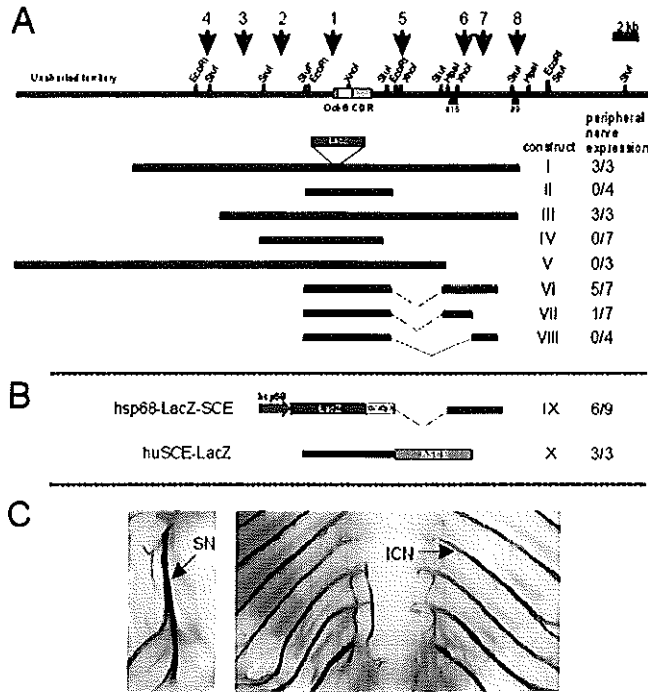

Figure 2 Deletion analysis. A. Schematic representation of the mouse *Oct-6* locus as described in Fig.1A, with the deletion constructs drawn to scale below. The *E.coli β-galactosidase* reporter gene (*lacZ*) was cloned in frame into the *Oct-6* CDR in every construct, as indicated for construct I and described in Materials and Methods. The number of transgenic animals displaying X-gal staining in the peripheral nerves out of the total number of transgenics obtained is shown to the right of each construct. The dashed lines in constructs VI, VII, and VIII indicate the deleted region between the construct II and the downstream fragments that have been linked to it. B. Schematic representation of the mouse *SCE hsp68-LacZ* construct (IX) and the human *SCE-LacZ* construct (X). C. X-gal staining of peripheral nerves. Strong blue staining (dark grey in this picture) is observed in peripheral nerves (SN = sciatic nerve; ICN = intercostal nerve) of *huSCE-LacZ* (X) transgenic animals, as shown here, and construct I, III, VI and IX transgenic animals (not shown).

Schwann cell specific expression was now tested in founder animals, containing the other deletion constructs shown in Figure 2, at 18 days post coitum (dpc), a time at which Oct-6 is highly expressed in Schwann cells of the sciatic nerve. Construct III is 8 kb shorter than construct I at the 5' end of the gene and lacks HSS-4. This site was present in ES and MES-68 cells but absent in the Schwannoma TR6B, brain and liver. All three transgenic founders obtained with construct III express LacZ in the Schwann cells of the sciatic nerve, indicating that HSS-4 is dispensable for Schwann cell specific expression. In addition, these three transgenics expressed LacZ in the hair follicles. Injections of construct IV, which contains only HSS-2 and HSS-1, yielded seven founder animals. None of these animals expressed the transgene in Schwann cells but five showed variable expression in the brain and no expression in the hair follicles. Two transgenic lines and one founder, containing construct V, in which HSS-6, HSS-7 and HSS-8 are missing, express LacZ in the hair follicles. However, none of the construct V animals expressed the transgene in Schwann cells. These results pointed to the 3' region encompassing HSS-6, HSS-7 and HSS-8, as the region containing the DNA elements necessary for Schwann cell specific expression. From this region we isolated a 4.3 kb restriction fragment containing HSS-6 and HSS-7 and cloned it downstream of the *Oct-6* structural gene in the Schwann cell non-expressing construct II. We generated five founders and two lines with construct VI. Four of the five founders and one of the two lines showed expression of the transgene in the sciatic nerve. Two of these founders expressed only in the nerves while the two other founders and the line also showed brain and neural tube expression. None of the transgenic mice expressed the transgene in the hair follicles. In an attempt to further narrow down this region, we generated two constructs that contain either HSS-6 (VII) or HSS-7 (VIII). Four founders were obtained for construct VIII, none of which expressed in the Schwann cells of the sciatic nerve. Seven founders were obtained for construct VII of which only one expressed in the sciatic nerve. In summary, these results show that a 4.3 kb fragment, containing HSS-6 and HSS-7 and located about 12 kb downstream of the *Oct-6* transcription initiation site, is sufficient to drive transgene expression from the *Oct-6* promoter. We now refer to this fragment as the *Oct-6* Schwann cell enhancer (SCE).

The Oct-6 SCE confers Schwann cell specificity on the non-cell type specific hsp68 promoter

Although the 4.3 kb SCE is sufficient for Schwann cell specific expression of the transgene, it is possible that specific *Oct-6* promoter proximal elements contribute to the Schwann cell specificity of these transgenic constructs. To test this we generated a construct in which the SCE is linked to the mouse minimal heat shock protein 68 (hsp68) gene promoter (construct IX in Figure 2B). This promoter contains a TATA-box, a SP1 site, a CCAAT box and three heat shock response elements downstream of the transcription initiation site. Others have shown that transgenic mice carrying hsp-68 promoter driven transgenes show expression patterns that differ from line to line, its specific pattern depending on the site of integration (Kothary et al., 1989; Rossant et al., 1991). We generated 3 lines and 6 founders with construct IX. One of these lines and five founders express the transgene in the Schwann cell lineage indicating that the SCE enhancer contains sufficient information to drive Schwann cell specific expression from a basic promoter. Thus, it is unlikely that *Oct-6* promoter proximal elements contribute to full enhancer function of the SCE.

The SCE is conserved in humans

The *Oct-6* gene is highly conserved among vertebrates and we have previously shown that the chicken and zebrafish *Oct-6* genes are expressed in Schwann cells of both species (Levavasseur et al., 1998). We also showed that the dynamics of Oct-6 expression in the chick Schwann cell lineage is identical to that in mouse and rat, suggesting conservation of regulatory mechanisms as well. We took advantage of the relatively short evolutionary distance between man and mice to isolate from a human cosmid library clones that cover the 3' part of the human *Oct-6* gene. We generated an extensive restriction map of this region and isolated a 5 kb DNA fragment that cross-hybridizes to probe #15 and which we tentatively named human SCE (data not shown). This fragment was cloned in construct II, to generate construct X (Figure 2B) from which three transgenic lines were established. All three lines expressed the transgene at high levels in Schwann cells of the peripheral nerves (Figure 2C) indicating that the homologous human sequence has the same functional properties as the mouse SCE, corroborating the suggestion that *Oct-6* regulatory mechanisms in the Schwann cell lineage are conserved.

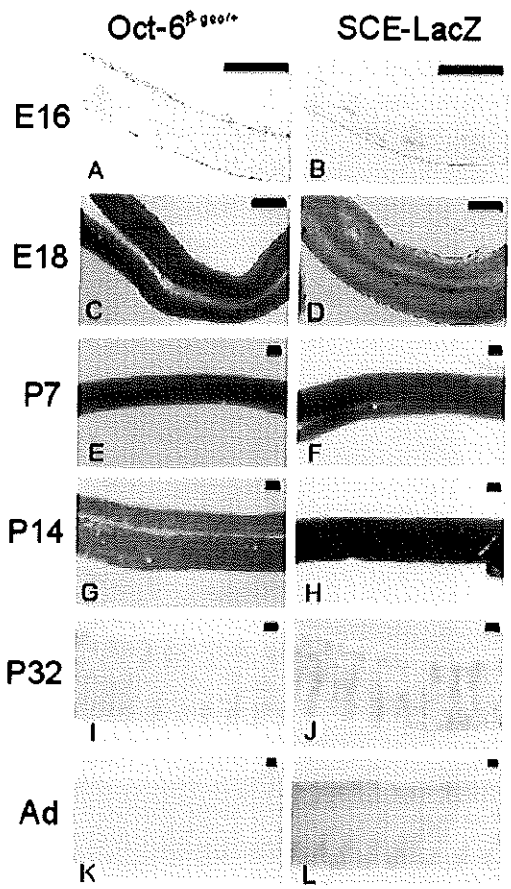

Figure 3 Developmental expression of reporter gene activity in sciatic nerves of *Oct-6*$^{\beta\text{-geo}/+}$ and *SCE-LacZ* mice. Sciatic nerves were isolated at various embryonic (E) and postnatal (P) stages, and stained for β-galactosidase activity. Whole mount X-gal stained sciatic nerves from *Oct-6*$^{\beta\text{-geo}/+}$ animals are presented in panels A, C, E, G, I, and K. *SCE-LacZ* nerves are shown in B, D, F, H, J, and L. Strong X-gal staining is dark grey; no staining is white. Low levels of expression could be detected at E16 in both mouse lines (A, B). High β-galactosidase expression is observed between E18 and P14 in both lines (C-H). At P32 few expressing cells could still be detected in the *SCE-LacZ* line (J), while X-gal staining was absent in the sciatic nerves of *Oct-6*$^{\beta\text{-geo}/+}$ animals of the same age (I). LacZ expression could not be detected in nerves of adult animals from both lines (K, L). Scale bar for each panel is 100 μm.

The SCE is sufficient to drive temporally correct expression in the Schwann cell lineage.

Thusfar, our analysis has established that the *Oct-6* SCE is sufficient to induce expression in the Schwann cell lineage. We then asked whether the SCE directed expression is also temporally correct and importantly, whether the SCE also mediates downregulation of the reporter gene in cells which have exited the promyelin stage. Within the Schwann cell lineage of the mouse, Oct-6 protein expression can first be detected, using sensitive Oct-6 antibodies, in Schwann cell precursors that populate the sciatic nerve around embryonic day 12/13 (Blanchard et al., 1996). *Oct-6* expression is strongly upregulated at later embryonic stages, peaks during early postnatal stages and is extinguished in fully differentiated cells. Previously, we and others have described the inactivation of the mouse *Oct-6* locus by insertion of a *β-galactosidase-neomycin* fusion gene (*β-geo*) (Bermingham et al., 1996; Jaegle et al., 1996). Expression of this fusion gene can be easily visualized in the developing embryo using the X-gal substrate, and recapitulates the Oct-6 expression profile as determined with Oct-6 specific antibodies and *Oct-6* cRNA *in situ* hybridization (see; Arroyo et al., 1998). As can be seen in Figure 3, essentially the same β-galactosidase expression profile is observed in nerves of transgenic mice carrying construct VI (the *SCE-LacZ* transgene) as in nerves of *Oct-6*$^{\beta\text{-}geo/+}$ mice. Thus, the *Oct-6* SCE does drive dynamic expression in the Schwann cell lineage in a temporal profile that is qualitatively identical to that of the endogenous *Oct-6* gene. Importantly, the *SCE-LacZ* transgene is also downregulated in mature nerves.

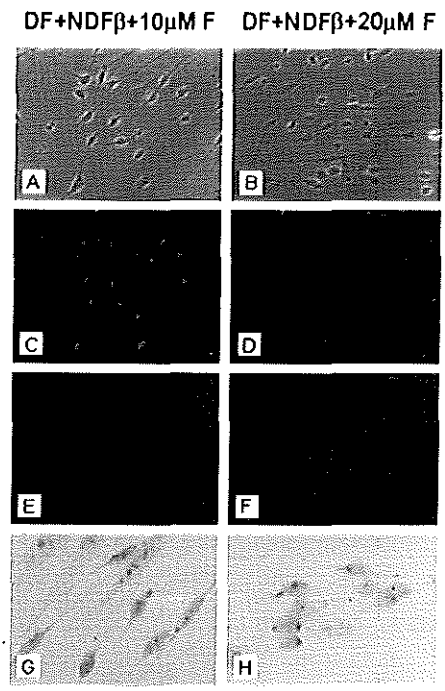

Figure 4 Induction of β-galactosidase expression in *SCE-LacZ* transgenic Schwann cells parallels induction of Oct-6 protein expression in response to Forskolin. Primary Schwann cell cultures were established from wild type and *SCE-LacZ* transgenic animals at postnatal day 4. Cells were cultured in defined medium (DF) supplemented with 5% NDF-β conditioned medium and exposed to 10 μM Forskolin (panels A, C, E and) or 20 μM Forskolin (panels B, D, F and H) for 48 hours (see Materials and Methods). (A, B) Phase contrast photographs of primary Schwann cell cultures derived from wild type animals. (C, D) DAPI staining of nuclei of the cells shown in A and B, respectively. (E, F) Many Schwann cells express Oct-6 (white spots) when exposed to 20 μM Forskolin (F) while only few Schwann cells express Oct-6 when exposed to 10 μM Forskolin (E). Fibroblasts, which are easily recognised by their flattened morphology and large nucleus, never express Oct-6. (G, H) Bright-field images of X-gal stained *SCE-LacZ* primary Schwann cells. In cultures exposed to 20 μM Forskolin (H), many cells with a Schwann cell morphology show a X-gal precipitate (dark grey spots in the cells), while only few such cells are observed in the culture exposed to 10 μM Forskolin (arrows in G). Fibroblasts, which are recognised by their flattened morphology and their pronounced nucleoli, do not express the transgene. Cells were counter-stained with Eosin.

Schwann cells express the *SCE-LacZ* transgene in response to cAMP *in vitro*

Actively myelinating Schwann cells in early postnatal peripheral nerve express high levels of *Oct-6* and myelin genes. Disruption of axonal contact, either through nerve transection or dissociation and culture of Schwann cells, results in a rapid downregulation of *Oct-6* and myelin gene expression (Scherer et al., 1994). Previously it has been shown that elevation of intracellular levels of cAMP levels in cultured Schwann cells leads to a rapid, but modest, induction of *Oct-6* gene expression shortly followed by expression of myelin genes such as P0 and P2 (Monuki et al., 1989). These and related observations led to the suggestion that axonal signals operating during nerve development and regeneration are transduced, at least in part, through the adenylyl cyclase pathway. To test whether the cAMP signalling pathway could activate expression of the *β-galactosidase* transgene through the SCE we established primary Schwann cell cultures from postnatal day 5 sciatic nerves from wild type and *SCE-LacZ* transgenic mice. Cultures were exposed to different concentrations of Forskolin, an activator of adenylyl cyclase. When Schwann cells are brought into culture they rapidly downregulate expression of the endogenous *Oct-6* gene and the transgene. However, when cultured for 48 hours in defined medium supplemented with 5% NDF-β conditioned medium, endogenous *Oct-6* gene expression is re-induced in a Forskolin concentration dependent manner (compare panel E and F in Figure 4). Under these conditions LacZ expression is similarly induced in transgenic Schwann cells (compare panels G and H in Figure 4) suggesting that cAMP induction of *Oct-6* gene expression *in vitro* is mediated through the SCE.

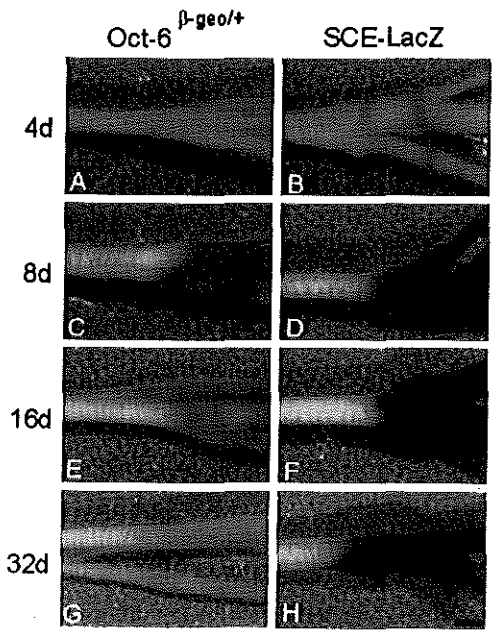

Figure 5 Axonal regulation of reporter gene expression during nerve regeneration. Whole mount X-gal staining was performed on crushed sciatic nerves of adult *Oct-6*$^{\beta\text{-geo/+}}$ and *SCE-LacZ* animals at 4, 8, 16, and 32 days (d) after crush injury. (A, B) Four days after crush, no staining was visible in the nerve of *Oct-6*$^{\beta\text{-geo/+}}$ animals, while some patchy staining (grey) could be observed distal to the site of crush in the *SCE-LacZ* nerve. (C, D) At 8 days after crush, strong staining can be detected in both mouse lines, but staining is more intense in the *SCE-LacZ* animal. (E, F) After 16 days, staining is already fading in the crushed *Oct-6*$^{\beta\text{-geo/+}}$ Sciatic nerve, while lacZ expression is still high in the *SCE-LacZ* nerve. (G, H) Only low levels of lacZ expression could be detected in the *Oct-6* heterozygous animal 32 days after crush injury. Also, staining intensity in the *SCE-LacZ* nerve at 32 days has decreased compared to 16 days after crush (F). Scale bar in (H) is 1mm, and also applies to the other panels.

Induction of *Oct-6* gene expression in reactive Schwann cells during nerve regeneration is mediated through the SCE.

Following nerve damage, axons disintegrate distal to the lesion resulting in loss of axon-Schwann cell contact and dedifferentiation of Schwann cells (Wallerian degeneration). Denervated myelinating Schwann cells cease expression of myelin related genes and reactivate expression of genes typical of immature cells such as the low affinity neurotrophin receptor p75 and GAP-43 (Scherer and Salzer, 1996). Upon regeneration axonal contact is restored and regrowing axon are ensheathed and remyelinated by Schwann cells whose differentiation recapitulates the events during normal development including the transient re-expression of *Oct-6* (Scherer et al., 1994; Zorick et al., 1996).

To test whether the SCE also mediates re-induction of *Oct-6* expression in Schwann cells during regeneration we performed X-gal staining of whole sciatic nerves at 4, 8, 16 and 32 days following crush lesion of the nerve in *Oct-6*$^{\beta\text{-}geo/+}$ and *SCE-LacZ* transgenic mice (Figure 5). Re-activation of *Oct-6* expression in *Oct-6*$^{\beta\text{-}geo/+}$ nerves occurs between day 4 and 8 post-crush and correlates with the re-ensheathment and remyelination of regenerating axons. Expression of the *Oct-6*$^{\beta\text{-}geo}$ allele is then gradually extinguished and only low levels of expression are observed in nerves 32 days post crush. In *SCE-LacZ* transgenic mice reactivation of expression is seen during the same interval as in *Oct-6*$^{\beta\text{-}geo/+}$ mice. Staining levels are most intense at 16 days postcrush after which staining levels begin to fade. The difference in staining intensity between the two different mouse lines probably reflects the difference in *β-galactosidase* copy numbers (1 in *Oct-6*$^{\beta\text{-}geo/+}$ and 7 copies in *SCE-LacZ* mice). However, qualitatively the SCE driven transgene shows the same expression profile as the *Oct-6*$^{\beta\text{-}geo/+}$ mice. Thus, the SCE also mediates reactivation of *Oct-6* expression in Schwann cells during nerve regeneration. It is therefore likely that the same intracellular signalling pathway induces *Oct-6* gene expression during development as well as during regeneration.

A SCE driven HA tagged *Oct-6* transgene rescues the peripheral nerve phenotype of *Oct-6* null mice.

Since the SCE is sufficient to mediate temporal regulation of *Oct-6* gene expression within the Schwann cell lineage it should be possible to rescue the peripheral nerve phenotype in *Oct-6* deficient mice by expressing a tagged *Oct-6* gene under control of this enhancer element. To test this hypothesis, a DNA construct in which a triple HA-tagged *Oct-6* gene is linked to the SCE was used to generate three independent transgenic mouse lines, named HA-1, HA-2 and HA-3 carrying 12, 7 and 3 copies of the transgene respectively. Using antibodies against the Oct-6 protein and the HA tag, immunohistochemistry on sciatic nerves of new born pups shows that all three lines expressed the transgene (data not shown). These lines were crossed with *Oct-6*$^{\beta\text{-}geo/+}$ mice to generate compound heterozygotes and offspring were backcrossed to *Oct-6*$^{\beta\text{-}geo/+}$ mice. Viable *HA/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals were born from each line and the survival rate of these animals was higher than *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals as previously reported. Besides a slight reduction in size, *HA/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals appeared normal.

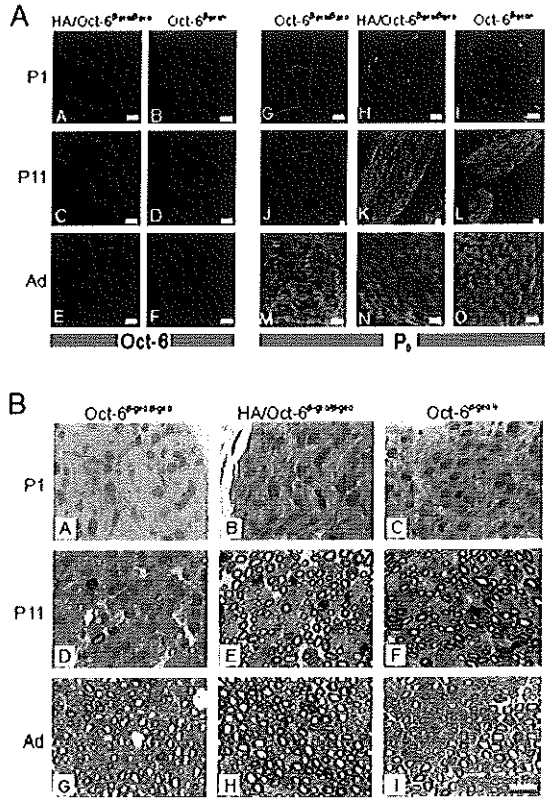

Figure 6 The SCE driven HA tagged *Oct-6* transgene is expressed in Schwann cells and rescues the congenital hypomyelination phenotype in *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals. A. Developmental expression of HA-Oct-6 in the Schwann cells of *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals (panel A, C and E) is both quantitatively and qualitatively indistinguishable from that of endogenous *Oct-6* in *Oct-6*$^{\beta\text{-}geo/+}$ animals (panel B, D and F). Both, the HA tagged Oct-6 protein encoded by the transgene and the endogenous Oct-6 protein are detected here with an Oct-6 specific antibody (light grey signal). P_0 protein (light grey signal) could not be detected in sciatic nerves of *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals at P1 (panel G), while few Schwann cells express high levels of P_0 protein in nerves of *Oct-6*$^{\beta\text{-}geo/+}$ (H) and *HA/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ (I) animals at this stage. At P11, high levels of P_0 expression are seen in *HA/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ and *Oct-6*$^{\beta\text{-}geo/+}$ nerves (K and L), whereas in the *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ only a small number of Schwann cells express high levels of the P_0 protein (J). In adult sciatic nerve high levels of P_0 protein expression are observed in Schwann cells of all three genetic backgrounds consistent with their myelination status (M, N, O; see also 6B). Scale bar represents 10 µm. B. Myelin appears on schedule in sciatic nerves of *HA-Oct-6* transgene expressing *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ mice. Myelin is clearly visible as dark grey rings around axons in micrographs of nerves stained with methylene blue. At P1 no myelin could be detected in *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ nerves (A), while few Schwann cells have elaborated a myelin sheath around axons in *HA/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ and *Oct-6*$^{\beta\text{-}geo/+}$ mice (B and C, respectively). At P11 myelin formation is well advanced in nerves of *HA/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ and *Oct-6*$^{\beta\text{-}geo/+}$ animals (E and F), while only a few myelin figures could be detected in nerves of *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals. Although with considerable delay, myelination in *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals does commence. The microscopic morphology of adult sciatic nerves of *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals is nearly indistinguishable from that of adult *HA/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ and *Oct-6*$^{\beta\text{-}geo/+}$ sciatic nerves (H and I). Scale bar represents 10 µm and applies to all panels.

Pursuing our analysis with transgenic line HA-2, we observed that the expression profile of HA-Oct-6 in sciatic nerves of *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals at P1, P11 and in adults (Figure 6A. panels A, C and E) was indistinguishable from the endogenous Oct-6 expression in *Oct-6*$^{\beta\text{-}geo/+}$ animals (Figure 6A. panels B, D and F). From P1 to P11 many Schwann cells express the transgene in the *Oct-6* null background, while in adult *Oct-6* null mice the transgene is expressed in only a small number of cells.

Previously we have reported that the expression of one of the major structural myelin proteins, P_0, is delayed in Schwann cells of *Oct-6* null mice (Jaegle et al., 1996). We therefore examined whether the P_0 protein expression would be restored to normal in nerves from *HA-2/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ mice. As is evident from Figure 6A (Panels G through O) this is

indeed the case, suggesting that the Schwann cells of *HA-2/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ animals are actively myelinating. Also, light microscopic examination and comparison of semi-thin sections of sciatic nerves derived from *HA-2/Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$, *Oct-6*$^{\beta\text{-}geo/\beta\text{-}geo}$ and *Oct-6*$^{\beta\text{-}geo/+}$ animals show that myelination is restored in *Oct-6* null animals by SCE driven HA-Oct-6 expression (Figure 6B). These data clearly demonstrate that the *Oct-6* Schwann cell enhancer is sufficient to mediate correct temporal expression at physiological relevant levels of the *Oct-6* gene during development as well as during nerve regeneration.

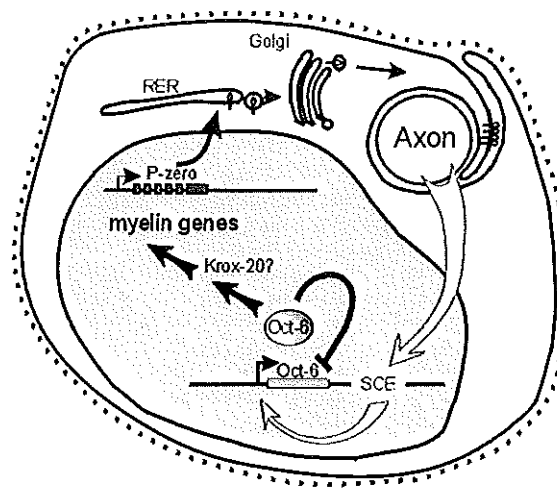

Figure 7. Schematic representation of the cascade of inductive events governing myelination. Myelination is under control of continued axonal signaling. One important target of this signaling is the *Oct-6* gene whose function is required for timely differentiation of myelinating Schwann cells (Jaegle et al., 1996; Bermingham et al., 1996). Axonal regulation of *Oct-6* gene expression is mediated through the SCE. A potential intra-cellular component of this signaling is the cAMP pathway. *Oct-6* gene expression is extinguished through a mechanism that involves Oct-6 itself and progression of myelination (Jaegle and Meijer, 1998). An important target of Oct-6 signaling in promyelin cells is *Krox-20* as *Krox-20* upregulation is delayed in the absence of Oct-6 (Mandemakers et al. in preparation). Krox-20 is required in promyelin cells for these cells to initiate myelination, possibly by directly regulating expression of the major myelin genes (Topilko et al., 1994; Zorick et al., 1999).

Discussion

Schwann cells are induced to adopt a one to one relationship with axons and initiate myelination under influence of continued axonal signaling. The exact molecular nature of this signaling is not understood. However, one major target is the POU domain gene *Oct-6*. Oct-6 function is required in Schwann cells for normal progression of cell differentiation and myelination. We describe a cis-acting element, the SCE, within the *Oct-6* locus on which intra-cellular signaling pathways converge to activate *Oct-6* gene expression in response to this elusive axonal signal (see Figure 7).

DNAseI hypersensitive sites are associated with major regulatory elements in the *Oct-6* locus

The mouse *Oct-6* gene is expressed in a highly dynamic and complex pattern during embryonic development (Alvarez-Bolado et al., 1995; Zwart et al., 1996). Our preliminary experiments had shown that the gene promoter itself did not carry sufficient information to direct regulated expression in cell lines or transgenic mice (Mandemakers et al., 1999), suggesting that remote cis-acting elements regulate the *Oct-6* expression pattern. As a first approach we used DNAseI hypersensitivity mapping to identify potential cis-acting elements in the *Oct-6* locus. DNAseI hypersensitive regions in chromatin are often associated with important gene regulatory sequences such as enhancers, promoters and locus control regions (LCR) (Felsenfeld et al., 1996; Gross and Garrard, 1988; Svaren and Chalkley, 1990). Eight such sites were mapped in the *Oct-6* locus, six of which are only observed in *Oct-6* expressing cells. The relevance of these hypersensitive sites for regulated *Oct-6* expression in the Schwann cell lineage was assessed in transgenic mice using the bacterial *β-galactosidase* gene as a reporter. Differences in copy-number between our transgenes (usually multi-copy) and the one allele in the *Oct-6*$^{\beta\text{-}geo/+}$ mice resulted in quantitative differences in β-galactosidase expression levels. Hence, β-galactosidase expression was used only as a qualitative indicator for tempero-spatial expression of *Oct-6*. We have made no attempt to correlate expression level (β-galactosidase activity) with copy-number since most most animals were analyzed as founders and thus potentially mosaic.

HSS-1 is associated with the *Oct-6* gene promoter which consists of an atypical TATA box (TTTAA) at position -23 and a GCCAAT box at -80 relative to the transcriptional start site and is embedded in a CpG island that extends well into the coding region of the gene. The presence of this site, together with HSS-5, is not strictly correlated with *Oct-6* gene expression. Both sites are also present in chromatin of cells that do not express *Oct-6*. The fact that this region is DNAseI hypersensitive might reflect the unusual chromatin conformation associated with regions of high CG content (Antequera and Bird, 1999). Alternatively, it is possible that HSS-1 is related to proliferation as it has recently been suggested that CpG islands are associated with origins of DNA replication (Antequera and Bird, 1999; Delgado et al., 1998). However, HSS-1 (and HSS-5) is also present in chromatin of non-proliferating brain and liver cells. A third possibility is that HSS-1 is part of a different unidentified transcription unit in the vicinity of the *Oct-6* gene. Recently we have identified such a transcription unit 3' of the *Oct-6* gene. This gene, which overlaps with HSS-5, is expressed in heart, ES cells, testis and brainstem but not in liver (our unpublished observations). Thus, the relationship between HSS-1 and this new unidentified gene remains obscure.

The presence of HSS-2 is correlated with *Oct-6* expression in the panel of cell lines and tissues we examined. This site contains a previously identified estrogen and TPA responsive enhancer (Renner et al., 1996). Estrogen and TPA were shown to synergistically enhance *Oct-6* expression in oligodendroglia cell lines. Subsequent cotransfection in these cell lines indicated that the synergistic effect of TPA and estrogen on *Oct-6* expression was mediated through this enhancer. Earlier studies had shown that the estrogen receptor is expressed in Schwann cells and oligodendrocytes and that estrogens have an effect on Schwann cell proliferation and myelin gene expression (Jung-Testas and Baulieu, 1998). Together these results led Renner and colleagues to suggest that this element mediate estrogen regulation of *Oct-6* gene expression and myeliogenesis. However, our results

demonstrate that this element is not required for regulated expression of the *Oct-6* gene in the Schwann cell lineage *in vivo*.

HSS-3 and HSS-5 are both present in chromatin of the Schwannoma cell line TR6B but are not sufficient or required to drive Schwann cell expression of the transgene that contains both these sites (construct V). This construct, together with construct I and III, does however express in the hairfollicles, another prominent site of *Oct-6* expression (data not shown). It is therefore possible that either of the two sites, or both sites, is necessary for this aspect of the *Oct-6* expression pattern.

Our analysis did not reveal any obvious function for HSS-4 and HSS-8. Both sites are dispensable for Schwann cell specific expression. It is possible that these sites are involved in some other aspect of the *Oct-6* expression pattern not analysed here.

Within the CNS, *Oct-6* is prominently expressed in layer 2 and 5 of the cortex, the hippocampus and brainstem. None of the constructs that we tested were consistently expressed in these areas of the CNS (data not shown). Instead, transgene expression in the brain was highly variable from transgenic animal to animal suggesting that these patterns mainly result from integration position effects. It is likely that these aspects of *Oct-6* expression are regulated by elements outside the region tested.

A 3' distal enhancer in the *Oct-6* locus mediates axonal regulation and Schwann cell specific expression in a promoter independent fashion

Schwann cell specific expression was observed with most constructs that contained HSS-6 and HSS-7. A DNA restriction fragment containing these hypersensitive sites was termed the *Oct-6* Schwann cell enhancer (SCE) and was further shown to be sufficient to drive temporally correct expression of a transgene, both during normal development as well as during nerve regeneration and *in vitro* following stimulation by Forskolin. Apparently, *Oct-6* promoter specific sequences are not required as the SCE also drives Schwann cell specific expression from the hsp68 promoter. Furthermore, an HA-tagged version of the *Oct-6* gene under control of the SCE rescues the peripheral nerve phenotype observed in *Oct-6* null animals. These results provide strong evidence that the *Oct-6* SCE is sufficient to mediate all aspects of regulated *Oct-6* gene expression in the Schwann cell lineage. Further deletion analysis of this fragment in which we separated HSS-6 and HSS-7 failed to give Schwann cell specific expression suggesting that both sites are necessary for full enhancer activity. In this respect the *Oct-6* SCE resembles other enhancers such as the 3' enhancer of the stem cell leukemia (SCL) gene which also consists of two hypersensitive sites. Both sites are required for full SCL enhancer function in mast cells and early haematopoietic progenitors (Fordham et al., 1999; Sanchez et al., 1999). In fact this is a rather common feature of many enhancers and locus control regions and reflects the modular nature of most of these elements in which bound nuclear factors need to interact to form a larger holo-complex to stimulate transcription from the linked promoter (Mannervik et al., 1999; Muller et al., 1988).

Despite our demonstration that the SCE is sufficient to drive all aspects of regulated *Oct-6* expression in the Schwann cell lineage, not all transgenes containing the SCE do actually express in Schwann cells. We generated 8 transgenic lines and 11 founders with various constructs that contained only HSS-6 and HSS-7, in addition to either the *Oct-6* promoter or the hsp68 promoter. Of these 11 founders, 9 expressed the transgene in the

Schwann cell lineage. It is possible that the two mice that do not express were in fact highly chimaeric, with no or only few transgenic cells contributing to the Schwann cell lineage. Chimaerism, however cannot explain why three out of the 8 lines we generated do not express the transgene in Schwann cells. This is not due to a partial deletion of the transgene since Southern blotting confirmed the integrity of the transgene. Most likely these transgenes integrated in an unfavourable chromatin region that is not accessible in Schwann cells. Apparently, the SCE cannot overcome this negative integration position effect. In this context it is of interest to note that all 6 transgenic lines generated with construct I and III all express in the Schwann cell lineage. These constructs, contain in addition to HSS-6 and HSS-7 several other hypersensitive sites (see figure 2). It is possible that one of these sites contributes to the SCE to overcome negative position effects. Larger numbers of transgenic animals need to be generated and analysed to test this possibility. In addition, deletion of the SCE, through the route of homologous recombination in ES cells, will reveal whether HSS-6 and HSS-7 are required for Schwann cell specific regulation or that additional Schwann cell specific elements are present in the *Oct-6* locus.

The fact that *Oct-6* gene expression in Schwann cells is under control of axonal signals is perhaps most graphically illustrated during nerve regeneration when axonal contact with reactive Schwann cells is restored and *Oct-6* gene expression is rapidly re-induced (Scherer et al., 1994 and Figure 5). Thus, axonal signals are ultimately relayed to the *Oct-6* SCE. This signaling does not involve the transcription factor Krox-20, as *Krox-20* null promyelin arrested Schwann cells express high amounts of Oct-6 (Zorick et al., 1999). It is therefore possible that *Krox-20* is regulated in parallel with *Oct-6* or *Krox-20* regulation depends on Oct-6. The latter possibility appears to be the case as *Krox-20* upregulation is delayed in *Oct-6* null mice (our unpublished observations). One possible intra-cellular signalling pathway involved in upregulation of *Oct-6* is the adenylyl cyclase-PKA pathway (Lemke and Chao, 1988; Mirsky and Jessen, 1996; Monuki et al., 1989). Elevation of cAMP, through activation of adenylyl cyclase with Forskolin, in the presence of NDF-β leads to induction of *Oct-6* expression in cultured Schwann cells and this induction is Forskolin dose dependent (Figure 6). The major target of cAMP signalling is PKA, which in turn activates the transcription factor CREB through phosporylation on position Ser-133 (Gonzalez and Montminy, 1989). Also, NDF-β signalling has been shown to result in phosphorylation of CREB through a P21ras and MAP kinase dependent pathway (Kim et al., 1997; Tabernero et al., 1998). Hence, it is possible that these pathways synergize in activation of CREB and stimulation of *Oct-6* gene expression. As CREB is expressed in the Schwann cell lineage this is a likely candidate nuclear factor to bind to the *Oct-6* SCE. Indeed, potential CREB binding sites are present in the SCE while no such sites are found in the *Oct-6* proximal promoter and this promoter is not active in Schwann cells of transgenic mice. The involvement of the cAMP pathway in this activation *in vivo* is however unclear as it has been shown that cAMP levels increase in the distal nerve stump only after activation of P_0 expression (Poduslo et al., 1995). A critical assessment of the role of the PKA pathway in myelination will require the Schwann cell and stage specific inhibition of this pathway, for example by stage specific expression of a dominant negative form of the regulatory subunit of PKA.

Further detailed characterisation of the *Oct-6* SCE will identify transcription factors that bind to this element and on which intra-cellular signaling pathways converge to regulate *Oct-6* gene expression and myelination. The identification of this unique enhancer does provide us with an important tool, not only to study those transcription factors and the

signaling pathways that regulate their activity, but also to manipulate the expression of transgenes in Schwann cells during a defined period of their differentiation. It is, in particular, this last characteristic that makes this human SCE element very attractive in future gene therapy strategies for Schwann cells to be transplanted into lesioned nerves. Also, the demonstration here that an SCE driven *Oct-6* transgene rescues the peripheral nerve phenotype of *Oct-6* null animals provides an experimental setting for the *in vivo* mapping of functional domains of the Oct-6 protein by transgene expression of various deletion mutants of the Oct-6 protein and to test possible *Oct-6* redundant genes (Jaegle and Meijer 1998).

Recently a number of families have been described with hereditary myelinopathies associated with mutations in the *Krox-20* (*EGR2*) gene (Warner et al., 1998). The search for mutations in this gene was directly motivated by the striking hypomyelination phenotype of *Krox-20* null animals. To date, no mutations in the *Oct-6* gene have been described to be associated with hereditary myelinopathies. However, in the context of our findings, it is tempting to speculate that within the group of hereditary peripheral neuropathy patients not associated with mutations in any of the known genes such as *PMP-22*, P_0 and *Krox-20*, there might be patients that carry mutations or deletions of the *Oct-6* Schwann cell enhancer.

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Materials and Methods

DNAseI hypersensitive site mapping

Nuclei were prepared from mouse tissues and cell lines essentially as described in (Lichtsteiner et al., 1987). Briefly, 1 gr of brain or liver tissue was homogenised in 12 ml of buffer A using a Teflon/glass motor driven homogeniser. (Buffer A; 2 M Sucrose, 10 mM Hepes-KOH pH 7.6, 15 mM KCl, 2 mM EDTA pH 8.0, 10 % glycerol. The following components were freshly added before use; 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT, 1 mM PMSF, 1 mM Benzamidine, 1 µg/ml Antipain, 2 µg/ml Aprotinin, and 1 µg/ml Leupeptin.) Nuclei were purified by centrifugation through a 0.5 ml cushions of buffer A for 45 minutes at 22000rpm at 0°C in SW41 Ultra clear centrifuge tubes. Nuclei (pellet) were washed once in 50 ml of ice-cold HS-buffer (15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl. 0.2 mM EDTA pH 8.0, 0.2 mM EGTA pH 8.0, and 5 % glycerol, supplemented with the same additives as for Buffer A). After centrifugation at 0°C for 10 minutes at 1500 rpm, nuclei were resuspended in HS-buffer to a final concentration of

approximately $3x10^7$cells/ml. Cultured cells (AB-1 ES, MES-68, MES-1, and TR6B cells) were collected, washed twice with ice-cold PBS and resuspended in 4 ml of ice-cold HS-buffer. The cell suspension was diluted with 4 ml of HS-buffer/0.2% NP-40 (Roche) and forced through a 25G needle 5 to 10 times to disrupt the cell membrane. Nuclei were collected by a brief centrifugation step at 2500 rpm and resuspended to a final concentration of $3x10^7$ cells/ml. From this point onwards, nuclei from tissues and cultured cells were treated the same way. Limited DNaseI digestion of chromatin was carried out in a final volume of 1ml of HS-buffer containing $1.5x10^7$ nuclei, 20 mM $MgCl_2$, 1 mM $CaCl_2$ and different amounts of DNaseI (Roche) varying from 0 to 30 units per sample. Nuclei were incubated for 30 minutes on ice. The reaction was stopped by adding an equal volume of 2x stopmix (0.2 M Tris-HCl pH 7.5, 0.4 M NaCl, 1 % SDS, 200 mg/ml Proteinase K), and further incubated overnight at 45°C. DNA was purified by phenol/chloroform extraction, precipitated with 0.6 volume of isopropanol, washed with 70% ethanol and dissolved in 200 µl of TE buffer. 10 µg of DNA of each sample was digested with different restriction enzymes. Restriction fragments were resolved on 0.8 % agarose gels and blotted onto Hybond-N+ membrane (Amersham). The blots were hybridised with single copy probes derived from the *Oct-6* locus, two of which are shown in Figure 1A. All other methods are described in (Sambrook et al., 1989).

Cloning and transgenesis.

The *LacZ* reporter gene was isolated as a 3.4 kb fragment from plasmid p610ZA (Kothary et al., 1989) in which the DraI site 60 bp downstream of the *LacZ* stopcodon was converted into a BamHI site. This fragment was ligated into a unique NsiI site of the *Oct-6* CDR on a 7kb EcoRI genomic fragment, after the NsiI site was modified by blunting and addition of BamHI linkers. This resulted in the creation of construct II (see Figure 2A), containing the *Oct-6* promoter region directing the expression of a fusion gene encoding the first 22 amino acids of Oct-6, followed by a threonine, introduced by the cloning procedure, and the lacZ open reading frame. A cosmid clone containing the *Oct-6* gene plus 15 kb upstream and 15 kb downstream was isolated from a mouse 129 genomic library. The *LacZ* reporter gene was introduced into this cosmid by replacing a 5 kb NotI/EcoRI fragment containing the *Oct-6* CDR, by an 8 kb NotI/EcoRI fragment from construct II that contains the *Oct-6/LacZ* fusion gene. The RecA-AC reaction (Koob et al., 1992) was performed to facilitate this cloning step, which resulted in the generation of construct I. Construct III was generated by a partial BglII digest and religation of construct I. The 13 kb Construct IV, containing HSS-1 and HSS-2, was isolated directly from construct I by digestion with restriction enzyme StuI to completion. Construct V was created by introducing, using the RecA-AC reaction, into cosmid 3 the 8 kb NotI/EcoRI *Oct-6/LacZ* fusion gene. Construct VI (*SCE-LacZ*) was generated by introducing a 4.3 kb HpaI/MscI genomic fragment into the unique StuI site of construct II in the same orientation as in the *Oct-6* locus. This 4.3 kb fragment (SCE) contains HSS-6 and HSS-7, which are located downstream of the *Oct-6* CDR. Constructs VII and VIII were generated by cloning a 2.7 kb HpaI/NdeI fragment and a 1.6 kb NdeI/MscI fragment, derived from the 4.3 kb SCE of SCE-LacZ into the StuI site of construct II, respectively. Hsp68-LacZ-SCE was generated by cloning the SCE into the SmaI site of p610ZA, which contains the promoter of the mouse hsp68 gene, the *E.coli LacZ* reporter gene, and an SV40 poly adenylation signal (Construct IX in Figure 2B) (Kothary et al., 1989). The human homologue of the mouse SCE was cloned as a 5 kb

EcoRV/BamHI fragment from a cosmid containing the human *Oct-6* locus on the basis of its hybridizing with #15 (see Figure 1A). The human SCE was ligated into the unique StuI site of construct II, resulting in hu*SCE-LacZ* (Construct X).
A fragment containing the sequence ; 5-'TATGACGTCCCAGATTACGCAAGTTT GCCCGGGTATGATGTTCCTGATTATGCTAGCCTCCCGGGTTACGATGTGCCCGA CTATGCCTCACTTCCAGGC-3', coding for a triple repeat of an internal region of the influenza haemagglutinin (HA) protein (YDVPDYASLPG), was ligated in frame at the start codon of the *Oct-6* CDR of the 7 kb genomic EcoRI fragment that contains the entire *Oct-6* transcription unit. This resulted in a HA tag/*Oct-6* fusion gene, which was subsequently isolated as a NotI/SwaI fragment and ligated into a NotI/SwaI digested *SCE-LacZ*, replacing the *Oct-6/LacZ* fusion gene, generating HA-Oct-6. DNA fragments were excised from construct I to X using appropriate restriction enzymes, separated on agarose gels, isolated by electro-elution and purified using elutip-D-mini columns (Schleicher and Schull). The DNA was dissolved in injection buffer (10 mM Tris-HCl pH 7.5, 0.08 mM EDTA pH 8.0) and introduced, by pronuclear injection, into fertilised eggs derived from a FVB/N x FVB/N mating as described (Hogan et al., 1994). Transgenic animals were analysed as founders or as lines. DNA samples of all animals were analysed by Southern blotting and hybridization to appropriate probes to confirm transgenesis. LacZ expression was visualised by whole mount X-Gal staining of E18 embryos or nerves dissected from transgenic animals at different stages of postnatal development.

Whole mount X-gal staining

Embryos or dissected nerves (N. ischiaticus) were fixed for 30 minutes at room temperature (RT) in 2 % Formaldehyde (BDH), 0.2 % glutaraldehyde (Sigma), 2 mM $MgCl_2$, 5 mM EGTA pH 8.0, 0.02 % NP-40 in Phosphate buffered saline (PBS). Subsequently, embryos were washed 3 times for 10 minutes at RT in PBS containing 0.02 % NP-40. Staining was done overnight at RT in PBS containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.01 % sodium deoxycholate, 0.02 % NP-40. Stained embryos were washed twice with PBS/0.02 % NP-40 and post fixed overnight in 4 % formaldehyde.

Primary Schwann cell cultures

Cultures of Schwann cells were set up as has been described before with some modifications (Brockes et al., 1979; Kleitman et al., 1991). Dissected nerves were collected in L-15 Leibovitz. Nerves were transferred to L-15 medium containing 1 mg/ml collegenase (Roche), teased with dissection needles and incubated for 30 min. at 37° C, with repeated pipetting to disrupt the nerves. Cells were washed once with L-15 containing 10% fetal calf serum (FCS), and plated on uncoated tissue culture dishes in Cb medium (Einheber et al., 1993), and incubated overnight at 37°C and 5% CO_2. The next day, Schwann cells were harvested using the "Cold jet" method (Jirsova et al., 1997), and plated on collagen coated coverslips. Cells were incubated for 48 hrs at 37°C and 5% CO_2 in either Cb medium (containing 10% FCS) or in Defined medium (DF) (Murphy et al., 1996) supplemented with 5% NDF-β conditioned medium in the presence of 0, 10, or 20 μM Forskolin (Sigma). A CHO cell line expressing a soluble form of NDF-β was cultured in

DMEM/F12, 5% FCS for 7 days. Medium was collected and filter sterilized (NDF-β conditioned medium). Cells were fixed in a solution containing 35 % acetone, 35 % methanol, 5 % acetic acid and 25 % H_2O for 10 min. at roomtemperature and subsequently processed for immunochemistry or X-gal staining.

Sciatic nerve crush

Adult *Oct-6*$^{\beta\text{-}geo/+}$ and *SCE-LacZ* mice were anaesthetised with halothane and the sciatic nerve was exposed. The sciatic nerve was crushed by tight compression with flattened Biology forceps no. 5 for 10 seconds at mid-femoral level just before the point where the sciatic nerve bifurcates. The mice were sacrificed at 4, 8, 16 and 32 days after surgery, and their sciatic nerves were isolated and processed for whole mount X-gal staining, with the unlesioned contralateral nerve serving as a control for background X-gal staining.

Immunohistochemistry

Sciatic nerves were isolated from mice derived from matings between *HA/Oct-6*$^{\beta\text{-}geo/+}$ and *Oct-6*$^{\beta\text{-}geo/+}$ at the desired developmental stages and fixed overnight at 4°C in 35 % acetone/35 % methanol/ 5 % acetic acid/25 % H_2O. After dehydration and paraffin embedding 5µm sections were cut and mounted on SuperFrost*/plus glass slides (Menzel-gläser). Sections were dewaxed, rehydrated and subjected to a microwave antigen-retrieval procedure (Evers et al., 1998). All other procedures have been described (Jaegle et al., 1996; Zwart et al., 1996). Primary antibodies used were; rabbit polyclonal anti-Oct-6 serum (Zwart et al., 1996), goat anti-HA serum (Santa Cruz biotechnology), mouse monoclonal anti-P_0 (Archelos et al., 1993) and mouse monoclonal anti-HA (12CA5; Roche). Fluorochrome coupled secondary antibodies used were; goat-anti-rabbit Texas red (Molecular probes), goat-anti-mouse Oregon green (Molecular probes). Both used at 1:200 dilution. Sections were examined using epifluorescence microscopy (Leitz). Images were captured with a Sony CCD camera and directly imported into Adobe Photoshop.

Light microscopy

Animals were perfused with PBS for 3 minutes, followed by fixative (3 % paraformaldehyde (Sigma) and 1 % glutaraldehyde buffered by 100 mM sodium cacodylate at pH 7.2) for 10 minutes. Sciatic nerves were isolated, washed with cacodylate buffer, osmicated in 1 % osmium tetroxide and embedded in Epon. Semithin sections (1 µm) of Epon embedded Sciatic nerves were mounted on glass slides and stained with methylene blue.

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Chapter 6

Conclusions and future prospects

One of the functions of Schwann cells in peripheral nerve development is the elaboration of a myelin sheath around the axon. A large number of experiments have demonstrated that the axon determines whether a Schwann cell will start the myelination process [1,2]. One of the main questions in glial cell research is how axons and Schwann cells communicate to initiate myelination.

Concerning the nature of the axonal signal, there are two schools of thought: One proposes that an axonally derived factor interacts with a receptor on the Schwann cell membrane to initiate myelination. This factor could be either membrane bound or secreted with limited diffusion or half-life. Indirect evidence for this model comes from (*in vitro*) experiments showing that axonal membrane fractions induce myelin gene expression in cultured rat Schwann cells [3]. In addition, activators of adenelyl cyclase can mimic these effects [4]. It is therefore possible that the putative myelin signal receptor belongs to the family of G-protein coupled receptors.

Another group of researchers favours a more mechanical model in which a Schwann cell intrinsic mechanism monitors the curvature of the axonal surface. It is suggested that the less curved surface of larger calibre axons induce Schwann cells to single out these axons and initiate myelination. In contrast to the previous model this suggests that Schwann cells are able to myelinate independent of axons. Indeed, Ernyei et al demonstrated that Schwann cells are able to myelinate artificial fibres, such as glass, nylon or other synthetic fibres [5]. However, Field et al and other researchers were unable to repeat these experiments [6]. In addition, increasing axonal diameter of post-ganglionic neurons in the superior cervical ganglion by increasing targeted innervation size leads to myelination of these otherwise non-myelinated axons [2]. However, these experiments are by no means conclusive as it cannot be ruled out that a larger target size induces, in addition to increasing axonal diameter, a myelin-inducing factor in these neurons. Whatever the exact nature of the axonal signal, it must be relayed to the nucleus of the Schwann cell to activate a co-ordinate programme of gene expression.

Several transcription factors have been identified in Schwann cells that mediate these signals. One of those proteins is the POU domain transcription factor Oct-6. Several experiments suggest that Oct-6 plays an important role in mediating axonal signalling. First, Oct-6 is expressed in Schwann cells and its expression is dependent on axonal contact [7]. Second, in cultured rat Schwann cells, agents that elevate intracellular cAMP levels can induce Oct-6 and Oct-6 induction precedes that of major myelin genes, such as P0 and MBP [4]. These data suggest a role for Oct-6 as a nuclear regulator of myelination in response to axonal signalling.

We and others demonstrated through targeted deletion of the Oct-6 gene in mice that Oct-6 is required for Schwann cell differentiation. In particular in the absence of Oct-6 function, Schwann cells are transiently arrested at the promyelin stage [8,9]. However, Oct-6 does not appear to be essential for myelination, since myelination proceeds relatively normal after a temporal delay of approximately two weeks after birth in Oct-6 null mice (this thesis; chapter 2)[8]. These results underscore the importance of Oct-6 in Schwann cell differentiation. However many questions remain. For example, how does Oct-6 exactly exert its function or in other words: what are the target genes of Oct-6? Second, what are the factors that eventually stimulate myelination in Oct-6 deficient Schwann cells two

weeks after birth? Third, how is Oct-6 itself regulated? These and related questions will be discussed in the following sections.

6.1 Oct-6 function - identification of target genes

As Oct-6 is a transcription factor, it is most likely that Oct-6 exerts its function by directly regulating transcription of genes essential for the myelination program to be set out. In chapter 2 we demonstrated that the major myelin genes (such as P0, PMP-22, MBP, CNP, and MAG) fail to be upregulated on schedule in Oct-6 -/- mice. POU domain binding sites have been demonstrated in the P0 promoter and Oct-6 has been demonstrated to bind these sites *in vitro* [10,11]. However in these reports Oct-6 repressed P0 expression, while *in vivo* data from Oct-6 null mice suggest the opposite. Therefore, there is as yet no convincing evidence available to suggest that Oct-6 directly regulate these genes.

The Oct-6 knockout data also contradict with the ΔSCIP transgenic data, which suggested that Oct-6 acts as a repressor of the major myelin genes [12]. Several suggestions were proposed to explain these conflicting results. For example, strong overexpression of the Oct-6 POU-domain might have non-specific effects on gene regulation by other POU proteins. This could either as a result of disturbance of the equilibria between transcription factors and co-factors which interact with them, or by the binding of the Oct-6 POU-domain to less preferred binding sites, which are not bound by wildtype Oct-6 under normal conditions. Therefore, together with the observation that Oct-6 is already expressed in Schwann cell precursors, it can be postulated that Oct-6 has two separate effects during Schwann cell development: a positive effect in early development, and later as a negative regulator of myelination [13].

Another explanation would be that *in vivo*, the Oct-6 POU-domain would be sufficient for normal Oct-6 function during Schwann cell development, and that the P0ΔSCIP transgene would have a dominant positive effect instead of dominant negative. The phenotype of the transgenic mice could then be interpreted as Oct-6 over-expression mice [14].

Recently, compelling evidence was generated supporting the latter explanation, by crossing the P0ΔSCIP transgene into the Oct-6 homozygous knockout background (Weinstein, unpublished results). Several of these mice survived after birth a demonstrated normal myelination, indicating that the Oct-6 POU-domain is able to activate the myelination process independent of wildtype Oct-6. This demonstrates that the trangene indeed functions as a dominant positive, but it does not rule out the possibility that later in Schwann cell development Oct-6 might function as a repressor.

Identification of unknown and known genes regulated by Oct-6 will be necessary to further elucidate the role of Oct-6 in this process. One approach that our group currently takes is to compare full-length cDNA pools derived from E18.5 sciatic nerves of Oct-6 knockout to those of normal mice [15,16]. This time point is chosen for the reason that wildtype and Oct-6 null nerves are indistinguishable at that stage. Furthermore, in normal nerves, E18.5 is the stage when peak Oct-6 expression levels are reached. This suggests that at this stage we have the highest chance of finding differentially expressed genes directly

regulated by Oct-6. Characterisation of cDNAs of differentially expressed genes, over- or underrepresented in the Oct-6 knockout nerves, will lead to the identification of potential direct target genes.

Furthermore, identification of DNA sequences pulled down in a chromatin immuno-precipitation assay using Oct-6 antibodies after UV or formaldehyde cross-linking will reveal direct targets of Oct-6 regulation (reviewed in [17]). However, the limited number of Schwann cells that can be obtained from neonatal rodent nerve makes this approach impractical.

A genetic approach to detect Oct-6 target genes would be to identify genes affected in mouse mutants with a similar peripheral nerve phenotype as Oct-6 null mice. In principal such an approach would identify genes that are either upstream or downstream of an Oct-6 dependent pathway or the gene functions in a parallel non-redundant pathway. The mouse autosomal recessive mutation claw paw (clp) fits these criteria [18]. Homozygous clp/clp mice produce striking limb posture abnormalities together with delayed and abnormal myelination in the peripheral nervous system but not in the central nervous system [18]. The clp defect has been mapped to mouse chromosome 7. Transplantation experiments of sciatic nerves from normal and clp/clp animals demonstrated that the clp mutation is a Schwann cell autonomous mutation (our unpublished results). We have also shown that in clp/clp mice Oct-6 is expressed. This suggests that clp acts either downstream of Oct-6 in an Oct-6 dependent pathway, or functions in a parallel non-redundant pathway. Our group is currently in the process of characterising the gene (or genes) affected in clp mice using a positional cloning approach.

Two known genes that have been identified as potential Oct-6 target genes by genetic approaches, are Krox-20 and Oct-6 itself. During Schwann cell development and nerve regeneration, Oct-6 expression precedes that of Krox-20 [19]. Furthermore, there is *in vitro* evidence that Krox-20 activates P0 expression [20]. These data are in agreement with the suggestion that Oct-6 is upstream of Krox-20 in the genetic hierarchy that governs myelination. The suggestion that Oct-6 regulates Krox-20 expression is supported by the following observations. First, Oct-6 expression is normally induced at the promyelinating stage in homozygous Krox-20 knockout animals [20]. Second, Krox-20 expression is absent in transiently arrested Schwann cells at the promyelin stage of homozygous Oct-6 knockout mice (Mandemakers and Meijer, unpublished results). Finally, the presence of two potential POU specific octamer binding sites in the Krox-20 promoter region, in agreement with direct regulation of Krox-20 by Oct-6 [21]. The relevance of these sites, however, remains to be determined. Thus, these data support a model in which Oct-6 is required for activation of the Krox-20 gene expression, which in turn activates, possibly together with Oct-6, the major myelin genes (See Fig. 1).

The suggestion that Oct-6 is involved in regulation of its own transcription came from the following observation. The β-galactosidase gene in the targeted Oct-6 transcription unit was used to determine the activity of the *Oct-6* locus in newborn and adult heterozygous and homozygous Oct-6 knockout animals. Although the *Oct-6* locus was normally activated in nerves of newborn pups of both genetic backgrounds, only the locus in the nerves of heterozygous Oct-6 knockout mice was properly down regulated in adult animals. Nevertheless, myelination had proceeded normally in adult mice of both genetic backgrounds [14]. Thus, these results suggest that Oct-6 itself is involved in the down

regulation of its own expression (directly or indirectly) as Schwann cells proceed to myelinate their axons. Potential mechanisms of how Oct-6 is involved in its own down regulation will be discussed in section 6.3.2

6.2 Oct-6 redundant factors

Another conclusion that can be drawn from our analysis of Oct-6 null mice is that Oct-6 does not appear essential for myelination per se. After a temporal delay, Oct-6 null Schwann cells eventually initiate the myelination programme [8]. One can think of several possible explanations for these observations. All of these explanations assume that a factor must exist that can partially substitute for Oct-6 function. The first possibility is that this factor binds to the same DNA recognition sequences as Oct-6 and contains a similar protein domain able to activate or repress the same target genes as Oct-6 does. This would most likely be another closely related POU-domain transcription factor. The temporal delay in myelination in Oct-6 null mice could result then either from a reduced output of target genes or a temporal difference in expression between those factors and Oct-6. Until recently, the only other POU domain factor present in Schwann cells, in addition to Oct-6, is the ubiquitously expressed Oct-1 protein [22,23]. As both factors bind to similar DNA-binding sequences, it is possible that Oct-1 partially replaces Oct-6 function. Such functional redundancy between POU factors has been demonstrated also in other systems, such as in the oligodendrocyte lineage [24]. If indeed Oct-1 does regulate normal Oct-6 targets, it probably does so inefficiently. A delayed build up of physiologically relevant levels of target proteins would then explain the delayed onset of myelination.

Recently, we have identified another DNA-binding protein in chicken sciatic nerves, which binds the same recognition sequence as Oct-6 (See chapter 3, Fig. 3). This protein is also present in mouse sciatic nerves and its expression profile correlates with the delayed onset of myelination in Oct-6 -/- mice (our unpublished results). We are currently characterising this interesting candidate Oct-6 redundant factor.

Second, it is still possible that the Oct-6 redundant factor is not a POU domain protein, but an unrelated transcription factor that binds to distinct cis-acting elements of Oct-6 target genes. This could be a factor, normally required for maintenance of transcriptional regulation of genes, initially activated or turned off by Oct-6. Expression of this unknown factor should be initiated at the time when Schwann cells of Oct-6 null mice start to myelinate (i.e. two weeks after birth).

6.3 Oct-6 regulation

As mentioned earlier, previous results suggest that axonal signalling is required for activation of Oct-6 expression in Schwann cells, while at least Oct-6 itself is required for silencing its own locus. Identification of the cis-acting elements mediating these events is an important step in decoding the complex signalling pathways between Schwann cells and axons governing myelination and Oct-6 down regulation. The

identification of such a Schwann cell specific regulatory element in the *Oct-6* locus and discussion is presented in chapter 5. In the next sections I will elaborate further on Oct-6 gene regulation and the role of the SCE.

6.3.1 Oct-6 regulation: activation

The Oct-6 Schwann cell specific enhancer (SCE) directs spatially and temporally correct expression of Oct-6 both during normal peripheral nerve development and regeneration (chapter 5). The SCE can also activate reporter gene expression in the presence of a heterologous promoter, indicating that all essential cis-acting elements that mediate Oct-6 activation reside within the SCE. The SCE contains two DNaseI hypersensitive sites (HSS) (HSS-6 and HSS-7), which is indicative for potential cis-acting regulatory elements [25]. Preliminary results have demonstrated that both HSS are also present in P4 peripheral nerves, supporting their involvement in Oct-6 activation (Mandemakers, unpublished results). Failure of these sites to individually generate Schwann cell specific expression suggests that both sites are required for full enhancer activity (see chapter 5).

Our transgenic experiments demonstrate that the SCE is sufficient to drive Schwann cell specific expression of a transgene, but does not exclude the possibility that additional elements with similar properties reside elsewhere in the *Oct-6* locus. Targeted deletion of the SCE is required to determine whether it is indeed the major Schwann cell specific element in the *Oct-6* locus or whether there are other elements within the locus that direct Schwann cell specific expression of Oct-6. We have now generated mice in which the SCE is deleted and preliminary results demonstrate that the SCE is indeed essential for Schwann cell specific expression of Oct-6 (W.Mandemakers and M.Ghazvini, unpublished results). In these mice Oct-6 expression in Schwann cells is lacking, while Oct-6 expression in other tissues appears unaffected. We are also in the process of generating null mutants of HSS6 and HSS7 individually, to determine the relevance of these sites *in vivo*.

The SCE homologous region in humans is also able to drive Schwann cell specific expression of a transgene in mice, indicating that the important sequences mediating Oct-6 activation are conserved during evolution. Sequence comparison between the mouse and human SCE demonstrated high sequence conservation in both HSS regions (Mandemakers, unpublished results). The high similarity between these two sequences does not facilitate easy identification of essential DNA-binding sites, required for Oct-6 activation. This would require the identification of and sequence comparison with the SCE homologous region of evolutionary more distant species, such as chicken and zebrafish, in which Oct-6 function in Schwann cells is conserved (chapter 3)[26].

In a parallel approach we are further narrowing down the HSS-6 and HSS-7 chromatin regions in Schwann cells using a PCR-based DNaseI HSS mapping method [27,28]. Subsequently, *in vivo* footprinting will determine the critical DNA binding sequences in the resulting smallest HSS region. The role of these binding sites in Oct-6 activation will be tested by *in vivo* mutagenesis or deletion in mice using homologous recombination in ES cells [29,30]. Both approaches will lead to the identification of DNA-

binding site of known and unknown transcription factors, essential for Oct-6 activation during Schwann cell development. The identity of this
putative regulator (X) could be revealed, by peptide sequencing of Schwann cell specific factors interacting with the corresponding DNA-binding site. One of the requirements for Oct-6 to be directly regulated by this factor X would in the first place be the loss of Oct-6 expression in X-null Schwann cells. Another requirement would be that the region to which factor X binds in the Oct-6 SCE, should be specifically pulled down in chromatin immunoprecipitation assays [17]. Although laborious and time-consuming, these approaches would provide the most compelling evidence that this factor is indeed involved in activation of Oct-6 expression.

In summary, the SCE contains all the cis-acting elements required for timely and spatially correct activation of Oct-6 expression in Schwann cells upon axonal contact. Identification of transcription factors that bind to these elements is in progress.

6.3.2 Oct-6 regulation: down regulation

Although the SCE mediates activation of Oct-6 during development, it is unclear whether it is also sufficient for down regulation of Oct-6. Experiments presented in chapter 5 demonstrate that a reporter driven by the SCE is correctly down regulated. However this construct still contains the endogenous Oct-6 promoter. It is therefore possible that the endogenous Oct-6 promoter is required for correct down regulation of Oct-6. We are currently testing this possibility by determining whether the lacZ reporter gene driven by the heterologous HSP68 promoter and SCE is properly regulated in adult transgenic animals of construct IX (see chapter 5). Presence of lacZ expression in adult mice of this line would suggest that the endogenous Oct-6 promoter is required for down regulation of the locus.

As mentioned before, Oct-6 itself is required for the proper down regulation of its own locus (see section 6.1). Auto-regulatory loops have been described for other POU domain proteins, however those are all positive regulatory loops [31-35]. Negative feedback loops have been described for many proteins such as P53, TIM, and goosecoid [36-38]. For almost as many genes that are subjected to auto-repression, a similar number of mechanisms have been proposed to bring about repression of an active locus (reviewed in [39,40]). The mechanism involved in Oct-6 auto-inhibition remains to be determined.

Another factor potentially involved in down regulation of Oct-6 is Krox-20. In the occasional Krox-20 knockout animals surviving postnatally, Oct-6 expression remains at high levels, while in normal littermates Oct-6 expression is down regulated after two weeks [20]. This observation suggests that continuous expression of Krox-20 is another requirement for Oct-6 silencing to occur. However, an equally plausible explanation is that high levels of Oct-6 expression observed in postnatal stages of Krox-20 knockout nerves is attributable to the block at the promyelinating stage of Schwann cell development [20]. Furthermore, although late, Krox-20 expression is apparently normal in the nerves of few surviving adult Oct-6 homozygous knockout animals, in which myelination has proceeded normally (Mandemakers and Meijer, unpublished results) [14]. Together with the fact that the *Oct-6* locus is not down regulated in these mice, these data argue against a direct role for Krox-20 in extinguishing Oct-6 expression [14].

Alternatively, it is possible that Oct-6 is down regulated during a narrow window of normal postnatal development, possibly by a factor that is only transiently present. Hence, either by itself or via interaction with either Oct-6 or Krox-20, this factor could bind to Oct-6 cis-acting regulatory elements and repress transcription. This could explain the lack of Oct-6 down regulation in both Oct-6 and Krox-20 knockouts. In the case of the Oct-6 knockout, either the lack of Oct-6 or too late expression of Krox-20 would disable the formation of the hypothetical Oct-6 repressive complex, resulting in constitutive activation of the Oct-6 locus. In the Krox-20 knockout, the lack of Krox-20 could interfere with the formation of this complex, which leads to the absence of Oct-6 down regulation. The presence of such a developmentally restricted factor might also explain why, in case of nerve regeneration, Oct-6 is not down regulated for at least 60 days after nerve damage [7].

The existence of such a temporally restricted factor could be tested in clp/clp animals, since they display a similar delay in myelination as in Oct-6 null mice. High levels of Oct-6 expression in adult clp/clp mice would give more support for the presence of such a factor. We are currently investigating this possibility.

In addition, the transcription factor Sox10 has been demonstrated to interact with both Oct-6 and Krox-20. However, Sox10 is continuously expressed during Schwann cell development, ruling out the possibility that it is the transient cofactor [41]. Two other interesting candidates would be the cofactors NAB1 and NAB2, which repress transcriptional activation by Krox-20 [42,43]. Preliminary results from our group demonstrated that both NAB1 and NAB2 are expressed in mouse sciatic nerves. Their expression pattern in Schwann cell differentiation remains to be determined (W.Mandemakers, unpublished results).

At present it is unresolved whether the Oct-6 SCE or its promoter is sufficient for the down regulation of Oct-6 in myelinating Schwann cells. Although various mechanisms can be proposed, it is certain that expression of Oct-6 and progression into myelination is required for shutting off Oct-6 expression.

6.4 Model and final considerations

From the data presented and discussed in the previous sections the following model emerges (Fig. 1). Following acquisition of a one-to-one relationship with an axon the Schwann cell is signalled to initiate the myelination program. One of the first targets of this signaling is upregulation of Oct-6 gene expression, an event that is mediated through the Oct-6 Schwann cell enhancer (SCE). The transacting factors binding to this regulatory element have not been identified as yet. Subsequently, Oct-6 regulates expression of a set of downstream genes, which possibly includes Krox-20, clp and the major myelin genes, driving the differentiation of these cells. Recently we have obtained additional evidence to suggest that Oct-6 act as a forward drive for myelination. As myelination proceeds, Oct-6 expression is extinguished in myelinating Schwann cells. The exact mechanism by which down regulation of Oct-6 is achieved, and whether it acts on the SCE or the Oct-6 promoter, remains to be established. However, it is clear from our experiments that at least the expression of Oct-6 itself, and possibly Krox-20, is required to silence the Oct-6 locus. The fact that myelination does proceed in the absence of Oct-6, albeit with considerable delay, suggests the existence of an Oct-6-like or redundant factor. The identity of the redundant factor is as yet unknown although we have recently identified a strong candidate.

Our results, described in this thesis, firmly establish the Oct-6 POU domain protein as an important intracellular regulator of myelination and identify the SCE as a major target of axonal signalling. The specific properties of the SCE make it an attractive target for therapeutic intervention in a clinical setting. This suggestion is based on a number of observations. Nerve regeneration following damage, takes months before connections with target organs are restored. A plethora of studies has been performed to identify neurotrophic factors that increase the speed of regeneration (reviewed in 31). Factors involved in Oct-6 activation might also improve nerve regeneration. For example, it has been shown that premature and over-expression of the POU domain of Oct-6 (ΔSCIP) in Schwann cells results in accelerated nerve regeneration following crush damage [44]. Thus, therapeutic agents that modulate Oct-6 gene expression might prove beneficial in stimulating nerve regeneration. Also, the SCE might prove a valuable tool in gene therapy approaches in myelin related diseases. For instance, viral vectors in which the SCE directs the expression of nerve regeneration promoting factors might have important advantages over more conventional vectors. It is anticipated that after regeneration is completed transgene expression is extinguished while in vectors that use constitutive promoter expression is sustained (chapter 5) [45,46].

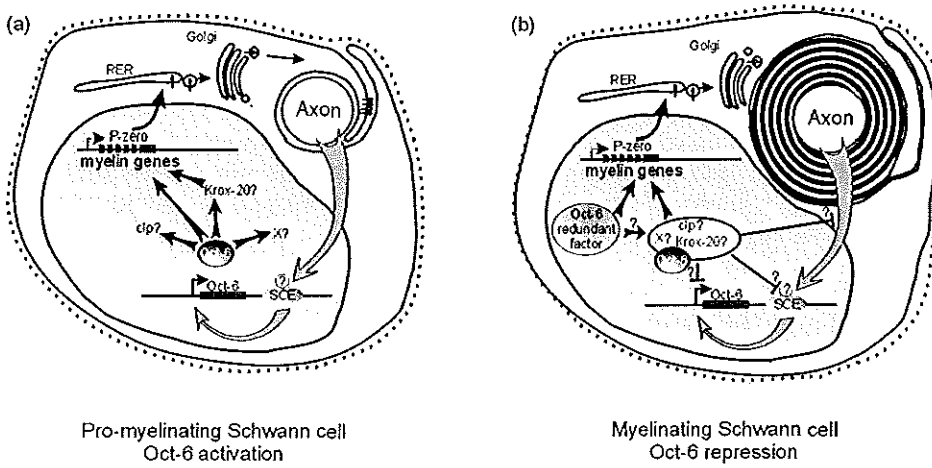

Figure 1: Activation (a) and repression (b) of Oct-6 during different stages of Schwann cell development and the involvement of the SCE in this process. For details see text. Abbreviations: SCE, Schwann cell enhancer; X?, unknown factor;?, unknown factor; clp?, claw paw gene(s); RER, rough endoplasmatic reticulum.

6.5 References

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Summary

The developmental and proper function of peripheral nerves in the vertebrate nervous system depends on the intimate interactions and continuous signalling between the Schwann cell and its associated nerve fiber(s). One important function of the Schwann cell is the elaboration and maintenance of the myelin sheath, a highly specialised laminar membranous organelle. A major consequence of the myelin sheath is that axonal depolarisation is restricted to the nodes of Ranvier, resulting in a saltatory mode of electric impulse propagation that is approximately a hundred times faster than the conduction velocity of a non-myelinated nerve fiber with the same diameter. Myelin is one of nature's most recent inventions, and clearly played an essential role in the evolution of compact nervous systems in larger animals. The myelin sheath is of vital importance to the organism, as the destruction or instability of the sheath leads to impaired nerve function and severe neurological problems. Prime examples of such neurological diseases are multiple sclerosis, the inflammatory neuropathy Guillain-Barre syndrome or the genetic diseases Charcot-Marie-Tooth, Dejerine-Sottas and Pelizaeus-Merzbacher.

Pioneering work by Aguayo and collegues have indicated that the myelination program of Schwann cells is under control of the associated axon. The axonal 'instruction' has to be relayed to the Schwann cell nucleus where a genetic program is deployed that results in the elaboration of the myelin sheath.

In this thesis, experiments are described that identifies the POU domain transcription factor Oct-6 as a key regulator of this genetic program. Activation of Oct-6 is under the control of an axonal signal, which converges on a remote cis-acting element within the Oct-6 locus. This element is sufficient to activate the Oct-6 gene in a temporally correct fashion, both during nerve development and regeneration. In addition, we show that Oct-6 gene expression is under control of a negative feedback loop that attenuates the level of Oct-6 expression. The importance of this negative feedback loop is underscored by the observation that even mild overexpression of the gene in myelinating cells results in hypermyelination and abnormal nerve function. Furthermore, we show that transcriptional silencing of the Oct-6 gene in myelinating Schwann cells is dependent on both Oct-6 itself and myelination as such.

It is anticipated that our identification of Oct-6 as a key regulator of peripheral nerve myelination and our description of the major Oct-6 regulatory sequences will open up new avenues for the development of therapeutic strategies to ameliorate the devastating effects of impaired peripheral nerve function and enhance the rate and extend of nerve regeneration following trauma.

Populaire samenvatting

Signalen uit de omgeving van gewervelde dieren, zoals de mens, worden door zintuigen (horen, zien, voelen, ruiken, proeven) via zenuwbanen doorgegeven aan het centrale zenuwstelsel. Het centrale zenuwstelsel verwerkt deze informatie en stuurt signalen bijvoorbeeld naar bepaalde spiergroepen om een gepaste reactie teweeg te brengen. De meest basale vorm van deze interactie tussen het organisme en zijn omgeving wordt een reflexboog genoemd en vindt bijvoorbeeld plaats als men in een punaise trapt en men onwillekeurig het been optrekt. De reactie op een stimulus kan echter ook beïnvloed worden door bepaalde hersengebieden die bijvoorbeeld betrokken zijn bij emotionele processen. Dit vindt bijvoorbeeld plaats als men eten wil serveren in een dure schaal die ontzettend heet blijkt te zijn als men hem oppakt. In een reflex zou men de dure schaal los laten en kapot laten vallen, maar door emotionele signalen uit de hersenen wordt de reflexboog onderdrukt en probeert men een gulden midden weg te vinden om de handen niet te verbranden en de dure schaal te sparen. Deze reacties tonen onder andere aan dat verschillende gebieden van het centrale zenuwstelsel op een nauwkeurige manier met elkaar verbonden zijn.

Het zenuwstelsel van gewervelde dieren bestaat uit een aaneenschakeling van honderden miljoenen cellen, neuronen. Neuronen zijn met elkaar, met zintuigen, met spieren of met de organen verbonden via lange uitlopers die axonen worden genoemd. Buiten de beschermende omhulling van de schedel en wervelkolom zijn axonen gebundeld in een beschermende bindweefsel laag. Deze structuur wordt een zenuwbaan genoemd en kan zijn opgebouwd uit honderden axonen. In het menselijk lichaam kunnen axonen meer dan een meter lang zijn, zoals bepaalde axonen die in zenuwbanen van het ruggenmerg tot in de tenen lopen. Tevens maken neuronen ook contact met elkaar via kortere uitlopers, dendrieten. Het zenuwstelsel is grofweg op te splitsen in het centrale zenuwstelsel (de hersenenen en het ruggenmerg) en het perifere zenuwstelsel, wat alle neuronen en zenuwenbanen omvat die buiten het centrale zenuwstelsel vallen.

De aaneengeschakelde neuronen geven impulsen door via een elektrochemisch proces. Bijvoorbeeld, het licht van een computer beeldscherm activeert lichtreceptoren die achter in het oog liggen. Deze lichtreceptoren zijn verbonden met axonen van neuronen (sensorische neuronen) in de hersenen. Stimulering van lichtreceptoren veroorzaakt een verandering van de verdeling van geladen deeltjes binnen en buiten de celmembraan van het axon. Deze verandering, depolarisatie, zorgt dat een elektrische stroom ontstaat wat zich via een domino-effect over de hele lengte van het axon van het oog naar het cellichaam van het sensorische neuron in de hersenen verplaatst. Aan het andere uiteinde van het neuron bevinden zich dendrieten, die contact maken met andere neuronen. Op de plaatsen waar het sensorische neuron contact maakt met het volgende neuron (synapsen) scheidt het sensorische neuron, in reactie op de depolarisatie, biologisch actieve stoffen uit die weer een depolarisatie kunnen veroorzaken in het volgende neuron. Op deze manier worden impulsen van receptor tot neuron en van neuron tot neuron doorgegeven. Uiteindelijk zal het resulteren in activatie van neuronen (motoneuronen) die de impulsen doorgeven aan spieren in de vingertoppen, die er voor zorgen dat bepaalde toetsen op het toetsenbord worden ingedrukt.

Van het moment van activatie van lichtreceptoren in het oog tot het moment dat de vingertoppen het toetsenbord raakt, duurt ongeveer een duizendste van een seconde. Deze hoge snelheid van impulsoverdracht zorgt dat gewervelde dieren snel kunnen reageren in bijvoorbeeld levensbedreigende situaties. Een aantal aanpassingen in het zenuwstelsel zorgt

voor een hogere snelheid van impulsoverdracht in axonen. Ten eerste zorgt een grotere diameter van het axon voor een hogere snelheid. De grotere diameter zorgt voor een lagere weerstand, waardoor de impuls zich sneller langs de lengte van het axon kan verplaatsen. Deze aanpassing is vooral duidelijk in ongewervelde dieren zoals inktvissen, waar axonen een diameter van ongeveer 1 mm kunnen bereiken. In vergelijking zijn de dikste axonen in het menselijk lichaam 20 µm (50 maal zo klein!). Desalniettemin, is de snelheid van impulsoverdracht in axonen met dezelfde diameter in gewervelde dieren 100 maal zo hoog als in ongewervelde dieren. Dit verschil wordt veroorzaakt door een andere aanpassing van het zenuwstelsel, wat alleen in hogere klassen van de gewervelde dieren, zoals de mens, heeft plaatsgevonden.

Deze aanpassing bestaat uit het aanbrengen van een eiwit- en vetrijke isolatielaag rond de axonen, welke myeline wordt genoemd. Er zijn twee typen cellen geassocieerd met axonen die deze isolatielaag aanmaken. In het centrale zenuwstelsel zijn dat de oligodendrocyten en in het perifere zenuwstelsel zijn dat de Schwann cellen. Omdat dit proefschrift over Schwann cellen handelt, zullen in het vervolg de oligodendrocyten slechts zijdelings ter sprake komen. Schwann cellen wikkelen zich meerdere malen rond een axon, vergelijkbaar met het aanleggen van een bandage rond een arm of been. Het aantal lagen wat gevormd wordt door de Schwann cel membraan en de lengte daarvan hangt samen met de dikte van het axon. Hoe dikker het axon, hoe meer lagen een Schwann cel vormt en hoe langer het omwikkelde segment. In tegenstelling tot oligodendrocyten, maakt een Schwann cel contact met maar één axon. Er zitten echter meerdere Schwann cellen dicht aaneengesloten langs een axon, waardoor ze een bijna ononderbroken isolatielaag rond het axon vormen. De enige plaatsen waar het axon niet bedekt is door de isolatielaag, is tussen twee Schwann cellen in. Deze openingen in de myeline laag zijn vernoemd naar hun ontdekker en worden knopen van Ranvier genoemd. Depolarisatie van een gemyelineerd axon kan allen bij deze knopen van Ranvier plaats vinden, waardoor een impuls zich 'sprongsgewijs' langs het axon verplaatst. Deze sprongsgewijze impuls geleiding gaat vele malen sneller dan geleiding volgens een domino-effect zoals bij een niet gemyelineerd axon. Echter beschadiging of instabiliteit van de myeline laag leidt tot een verslechterde geleiding van signalen langs deze zenuwbanen, hetgeen resulteert in ernstige neurologische problemen. Bekende voorbeelden van ziektes waarbij de myeline laag is aangedaan zijn onder andere de autoimmuun ziektes multiple sclerose (MS) en Guillain-Barre of de genetische afwijkingen Charcot-Marie-Tooth (CMT), Dejerine-Sottas en Perlizaeus-Merzbacher.

Naast het aanleggen van de myelinelaag zijn Schwann cellen betrokken bij processen die bijvoorbeeld leiden tot de formatie van perifere zenuwen tijdens de embryonale ontwikkeling, maar ook tijdens de regeneratie van perifere zenuwen na een beschadiging. Het laatst genoemde feit is uniek voor perifere zenuwen. In het centrale zenuwstelsel van volwassen dieren herstellen zenuwbanen maar zeer beperkt of helemaal niet na een beschadiging, zoals bijvoorbeeld na een dwarslaesie in het ruggenmerg. Er zijn enkele factoren die hierbij een rol spelen waar nu niet verder op ingegaan wordt. Echter, in het perifere zenuwstelsel zijn axonen wel in staat om na beschadiging terug te groeien naar hun oorspronkelijke contactplaats, wat kan leiden tot volledig herstel van de verloren functies. Schwann cellen spelen een belangrijke rol in dit proces, onder andere doordat ze groeifactoren voor neuronen uitscheiden waardoor axonen kunnen terug groeien naar hun doel. Dit aspect van Schwann cellen heeft veel aandacht van klinische onderzoeksgroepen, omdat factoren die het herstel van perifere zenuwen versnellen misschien ook kunnen

worden toegepast om herstel van schade in het centrale zenuwstelsel te bevorderen. Het is daarom van belang om de rol van Schwann cellen in processen zoals zenuwherstel en het aanmaken van een myeline laag tot op moleculair niveau te onderzoeken.

Een eerste vereiste voor een Schwann cel om een myeline laag aan te leggen is contact met een axon. Een tweede vereiste is dat de diameter van het axon groot genoeg moet zijn. Als de diameter van het axon kleiner is dan 1 µm, dan wordt het axon niet gemyelineerd. Naast deze vereisten is er nog weinig bekend over hoe het axon een Schwann cel instrueert om een myeline laag te vormen. Dit proefschrift toont aan dat de Schwann cel intrinsieke factor Oct-6 een belangrijke rol speelt in het aanleggen van de Myeline laag.

Zoals alle andere cellen in het lichaam van gewervelde dieren bestaat een Schwann cel uit verschillende compartimenten, zoals bijvoorbeeld het endoplasmatisch reticulum of de celkern. De celkern bevat een groot aantal lange strengen moleculen die DNA worden genoemd. DNA is opgebouwd uit 4 bouwstenen (basen) welke in een specifieke volgorde met elkaar verbonden zijn. In deze specifieke volgorde van basen ligt de genetische informatie van het organisme opgeslagen. In bepaalde delen van het DNA codeert een specifieke volgorde van basen, een gen, voor een bepaald eiwit. Een gen kan worden herkend door specifieke eiwitten (algemene transcriptiefactoren) die er voor zorgen dat de code van het gen wordt 'overgeschreven' (transcriptie) waardoor een boodschapper molecuul (mRNA) ontstaat wat weer door andere eiwitten kan worden vertaald (translatie) in het eiwit waarvoor het gen codeert. Het vertaalde eiwit kan bijvoorbeeld een functie hebben in alle cellen van het organisme, zoals DNA herstel eiwitten. Echter, het kan ook een eiwit zijn wat alleen aanwezig behoort te zijn in Schwann cellen, zoals myeline eiwitten, of in rode bloed cellen, zoals de eiwitten die hemoglobine vormen. Om te voorkomen dat bijvoorbeeld hemoglobine eiwitten in Schwann cellen aanwezig zijn en myeline genen in bloedcellen, hebben cellen een aantal regulatie mechanismen ontwikkeld. Een belangrijke rol in deze is weggelegd voor de zogenaamde weefsel specifieke transcriptiefactoren. Deze factoren zijn aanwezig in celtypes waar ze alleen die genen reguleren die een DNA code hebben die door deze transcriptiefactoren kunnen worden herkend. Een voorbeeld van zo'n weefselspecifieke transcriptie factor in Schwann cellen is het POU domein eiwit Oct-6. Het POU domein van Oct-6 is een deel van het eiwit wat een specifieke DNA code kan herkennen en daaraan binden om vervolgens met andere delen van het Oct-6 eiwit transcriptie van een gen te reguleren (bijvoorbeeld: aan of uit zetten). De aanwezigheid (expressie) van Oct-6 zelf in Schwann cellen is ook strikt gereguleerd. Tijdens de embryonale ontwikkeling is Oct-6 alleen aanwezig in cellen die op het punt staan om een myeline laag om het axon te leggen. Zodra de myeline laag wordt aangelegd wordt Oct-6 als het ware weer uitgeschakeld. Een vergelijkbaar proces vindt plaats in Schwann cellen na beschadiging van perifere zenuwen in volwassen dieren.

Na beschadiging van een perifere zenuw vindt een ingenieus proces van regeneratie plaats. Een neuron verliest het axon tot voorbij de schade, maar behoud het axon wat nog verbonden is met het cellichaam van het neuron tot vlak voor de plaats van de beschadiging. De Schwann cellen die in contact waren met het verloren stuk axon verliezen hun myeline laag en gaan weer op embryonale Schwann cellen lijken. Deze Schwann cellen scheiden factoren uit die het teruggroeien van het axon bevorderen. Zodra het axon weer in contact komt met die Schwann cellen, wordt Oct-6 in die cel aangezet en uiteindelijk zal die cel een myeline laag om het axon aanbrengen. Zoals eerder vermeldt zal dit alleen plaatsvinden als het axon dik genoeg is.

Het lijkt er dus op dat Oct-6 expressie in een Schwann cel belangrijk is om een axon te myelineren. Tevens is uit eerder onderzoek in ratten en muizen gebleken dat, tot nu toe, Oct-6 het eerste eiwit is dat wordt aangeschakeld in een Schwann cel die uiteindelijk zal gaan myelineren. Deze observaties brengen onder andere de volgende vragen ter berde. Ten eerste, wat is de functie van Oct-6 in een Schwann cel? Is het betrokken bij de aanleg van myeline of misschien bij een ander aspect van Schwann cel functie. Ten tweede, wat zijn de signalen die er voor zorgen dat Oct-6 wordt aangeschakeld in Schwann cellen die gaan myelineren en wordt uitgeschakeld in cellen die een myeline laag hebben gevormd? De experimenten die in dit proefschrift beschreven staan hebben tot doel een antwoord te vinden op deze vragen.

In hoofdstuk 2 is met een bepaalde techniek, homologe recombinatie, het Oct-6 gen verwijderd uit het DNA van alle cellen van de muis en vervangen door een inactief gen. Deze muizen kunnen geen Oct-6 eiwit meer aanmaken. Uit ons onderzoek is gebleken dat de Schwann cellen in die muizen niet goed meer functioneren. De myeline laag wordt wel aangemaakt, maar met een vertraging van ongeveer twee weken in verhouding tot even oude muizen die het Oct-6 eiwit nog wel kunnen aanmaken. Verder, zij er waarschijnlijk andere factoren die op Oct-6 lijken en die de functie van Oct-6 in afwezigheid van Oct-6 overnemen, waardoor Schwann cellen in afwezigheid van Oct-6 uiteindelijk alsnog gaan myelineren.

Experimenten beschreven in hoofdstuk 3 tonen aan dat Oct-6 ook in Schwann cellen voorkomt in dieren die evolutionair gezien ver van de muis afstaan, zoals de kip en de zebravis. Ten eerste duidt deze conservering van Oct-6 expressie er op dat ook de functie van Oct-6 bewaard is gebleven. Ten tweede is het mogelijk dat ook de signalen die ervoor zorgen dat Oct-6 specifiek in Schwann cellen wordt aan- en uitgeschakeld tijdens de evolutie zijn geconserveerd. Experimenten in hoofdstuk 4 en 5 hebben tot doel om duidelijkheid te verschaffen over hoe Oct-6 specifiek in Schwann cellen wordt aan- en uitgeschakeld.

Experimenten gedaan in reageerbuizen (*in vitro*) en in muizen (*in vivo*) in hoofdstuk 4 tonen aan dat codes in het DNA vlak voor het Oct-6 gen (promotor) waar transcriptiefactoren aan kunnen binden, betrokken zijn bij de activatie van het Oct-6 gen, maar niet voldoende zijn om Oct-6 specifiek in Schwann cellen aan te schakelen. Deze observatie komt vooral naar voren in de *in vivo* experimenten. Hierbij is met behulp van moleculair biologische technieken een gen uit een bacterie, lacZ genaamd, aan het Oct-6 gen gevoegd. Stukken DNA van verschillend grootte die dit 'fusie-gen' bevatten zijn vervolgens doormiddel van microinjectie ingebracht in bevruchte eicellen van de muis. De muizen die uit deze eicellen geboren worden en het fusie-gen in zich hebben worden transgene muizen genoemd. Als het stuk DNA waarop het fusie-gen ligt codes bevat voor transcriptiefactoren die nodig zijn voor specifieke expressie in Schwann cellen, dan kan het fusie-gen in Schwann cellen tot expressie komen. Dit kan dan eenvoudig bepaald worden door gebruik te maken van de activiteit van het lacZ gen. Dit gen is instaat om een kleurloos substraat om te zetten in een onoplosbare blauwe neerslag wat eenvoudig te detecteren is. Omdat bacterieel lacZ normaal niet in de muis aanwezig is, wordt de blauwe neerslag alleen gevormd in de weefsels waar het transgen actief is. Muizen die een transgen bevatten wat alleen bestaat uit de Oct-6 promotor en het fusie-gen op een stuk DNA wat is opgebouwd uit ongeveer 7000 basen, vertonen geen blauwe kleuring in de Schwann cellen. Echter, in muizen die transgeen zijn voor een fusie-gen wat op een stuk DNA ligt van 32000 basen kleuren de Schwann cellen wel blauw. Tevens verdwijnt de blauwe kleur weer in myelineerende Schwann cellen van volwassen transgene muizen. Hieruit valt op te

maken dat, op een stuk DNA waar het Oct-6 gen ongeveer in het midden ligt, de DNA codes (sequenties) waaraan transcriptiefactoren kunnen binden die zorgen dat Oct-6 specifiek in Schwann cellen wordt aan- en uitgeschakeld zich buiten de 7000 basen, maar binnen die 32000 basen moeten bevinden. De locaties van die sequenties zijn nauwkeuriger bepaald in hoofdstuk 5.

Door stap voor stap het stuk DNA van 32000 basen kleiner te maken, hebben we in hoofdstuk 5 een regio van ongeveer 4000 basen kunnen identificeren, wat voldoende is om, net als normale Oct-6 gen, het transgen specifiek in Schwann cellen aan- en uit te schakelen zowel tijdens de embryonale ontwikkeling als na zenuw beschadiging. Dit fragment hebben we de Schwann cel enhancer (SCE) gedoopt. De SCE ligt op enige afstand achter het Oct-6 gen. Het vergelijkbare stuk DNA uit het genoom van de mens is eveneens in staat om het transgen specifiek in muizen Schwann cellen tot expressie te brengen. Deze resultaten duiden er op dat in de SCE de DNA sequenties waaraan transcriptiefactoren kunnen binden tijdens de evolutie geconserveerd zijn. Een aanwijzing voor de aanwezigheid van dergelijke sequenties in de SCE komt uit experimenten waarin we het genoom van muizen cellen hebben behandeld met een enzym, DNAse, wat het DNA specifiek op plaatsen knipt waar normaal transcriptie factoren binden. Uit deze experimenten komt naar voren dat er twee DNAse gevoelige regio's in het DNA van de SCE aanwezig zijn. Deze DNAse gevoelige regio's zijn potentieel de sequenties waaraan de transcriptiefactoren kunnen binden die er voor zorgen dat Oct-6 specifiek in Schwann cellen tot expressie komt. Muizen die een transgen dragen met slechts een beide DNAse gevoelige regio's van de SCE vertonen geen blauw kleuring in Schwann cellen, wat er op duidt dat beide regio's tezamen nodig zijn om Schwann cel specifieke expressie te bewerkstelligen.

In hoofdstuk 6 trekken we de conclusies uit de resultaten van het onderzoek beschreven in dit proefschrift en doen we een aantal suggesties over hoe we dit onderzoek kunnen voortzetten. Ten eerste kan geconcludeerd worden dat Oct-6 een belangrijke rol speelt tijdens de Schwann cel ontwikkeling door het myelinatie proces tijdig te laten beginnen. Waarschijnlijk reguleert Oct-6 genen die het myelinatie proces ten uitvoer moeten brengen. Ons huidig onderzoek is er op gericht om deze genen te identificeren. Vervolgens blijkt dat DNA sequenties in de SCE van belang zijn om het Oct-6 gen zelf tijdig aan- en uit te schakelen. Toekomstig onderzoek is er onder andere op gericht om de DNA sequenties in de SCE waaraan transcriptie factoren binden nauwkeurig te bepalen. Vervolgens zullen die transcriptiefactoren worden gekarakteriseerd die Oct-6 specifiek in Schwann cellen activeren en ook weer uit schakelen. We hopen dat ons onderzoek uiteindelijk zal leiden tot beter begrip over de functie van Schwann cellen en tot de ontwikkeling van therapieën die de desastreuze effecten van de eerder genoemde ziektes kunnen tegen gaan en het herstel van zenuwen in het perifere en centrale zenuwstelsel na beschadiging kunnen bevorderen.

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Curriculum Vitae

Naam:	Wilhelm Johannes Mandemakers
Geboortedatum:	13 maart 1970
Geboorteplaats:	Waalwijk
1983-1988	V.W.O. Atheneum aan het Willem van Oranje College te Waalwijk.
1988-1993	Doctoraal opleiding Medische Biologie aan de Rijksuniversiteit Utrecht.
1991-1992	Stage bij de afdeling Immunologie aan de Rijksuniversiteit Utrecht onder supervisie van Dr. M.E.M. Schutte en Dr. T. Logtenberg. Onderwerp: 'Cloning and analysis of immunoglobulin variable heavy chain genes of bovine origin'.
1992-1993	Stage bij het Nederlands Instituut voor Ontwikkelingsbiologie te Utrecht onder supervisie van Dr. F. Meijlink, Dr. R. Vogels en Dr. F. Zwartkruis. Onderwerp: 'Regulation of the mouse Hox-B7 gene' & 'Overexpression of K-2a/MHOX in 10.5 days p.c. mouse embryos'.
1994-2000	Assistent in opleiding aan de afdeling genetica van de Erasmus Universiteit Rotterdam onder supervisie van Prof. Dr. D. Bootsma, Prof. Dr. F.G. Grosveld en Dr. D. Meijer. Onderwerp: 'On the role of Oct-6 in Schwann cell differentiation'.

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WM Wim Bimo