Role of Oct6 in Peripheral Nerve Myelination

Noorjahan Jagalur Basheer

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Cover: 'Dancing Couple'

A couple representing axon and Schwann cell, dancing together to form a myelinated axon

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Role of Oct6 in Peripheral Nerve Myelination

Rol van Oct6 in perifere zenuw myelinatie

Thesis

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Contents

List of abbreviations

Adam22 : A Disintegrin And Metalloprotease 22
AJS : Adherens Junctions
BDNF : Brain Derived Neurotrophic factor
CAM : Cell Adhesion Molecule
CAT : Chloramphenicol Acetyl Transferase
cAMP : cyclic Adenosine Monophosphate
cDNA : complementary DNA
Cic : Capicua
Clp : Claw paw
CMT : Charcot-Marie-Tooth
CMT1 : Charcot-Marie-Tooth Neuropathy Type 1
CNS : Central Nervous System
CREBP : cAMP Response Element Binding Protein
Cx32 : Connexin32
Dhh : Desert hedgehog
DNA : Deoxyribonucleic Acid
DRG : Dorsal Root Ganglia
DSS : Dejerine-Sottas Syndrome
ECM : Extracellular Matrix
EGF : Epidermal Growth Factor
EGR2 : Early Growth Response protein 2
ERK : Extracellular signal Regulated Kinase
ERM : Ezrin-Radixin-Moesin
FAK : Focal Adhesion Kinase
FGF : Fibroblast Growth Factor
GDNF : Glial cell-line Derived Neurotrophic Factor
GGF : Glial Growth Factor
GTP : Guanosine Triphosphate
HLH : Helix Loop Helix
Ig : Immunoglobulin
ISE : Immature Schwann Cell Element
IGF : Insulin-like Growth Factor
LacZ : β-galactosidase
Lgi4 : Leucine-rich Glioma Inactivated 4
LINE : Long interspersed elements
LTR : Long terminal repeats
MAG : Myelin Associated Glycoprotein
MAGUK : Membrane-Associated Guanylate Kinase
MAPK : Mitogen Activated Protein Kinase
MBP : Myelin Basic Protein
MPZ : Myelin Protein Zero
mRNA : messenger RNA
MSE : Myelinating Schwann cell element
NC : Neural Crest
NDF : Neu differentiation factor
NFATc4 : Nuclear Factor of Activated T-cells Cytoplasmic 4
NFκB : Nuclear Factor kappa B
NGF : Nerve Growth Factor
NMSC : Non-myelinating Schwann Cell
Nrg : Neuregulin
NT3 : Neurotrophin 3
$P75^{NTR}$: p75 Neurotrophin Receptor
PDGF : Platelet Derived Growth Factor
PI3 : Phosphoinositide 3
PKA : Protein Kinase A
PMP22 : Peripheral Myelin Protein 22
PNS : Peripheral Nervous System
POU : Pit-Oct-Unc
RGNEF : Rho-A specific Guanine Nucleotide Exchange Factor
RNA : Ribonucleic Acid
ROCK : Rho Associated Protein Kinase
RT-PCR : Reverse Transcription Polymerase Chain Reaction
SC : Schwann Cell
SCE : Schwann Cell Enhancer
SCP : Schwann Cell Precursor
Shh : Sonic hedgehog
SLI : Schmidt-Lantermann Incisures
SREBP : Sterol Regulatory Element Binding Protein
TGF :Transforming Growth Factor
TF : Transcription Factor
PDGF : Platelet Derived Growth Factor
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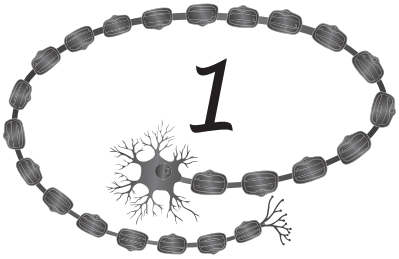
Aim and scope of this thesis

A multicellular organism is build up of widely different cell types such as blood cells, neural cells, muscle cells and skin cells. The origin of all these cells is a single cell; the fertilized egg. With very few exceptions, all cells contain the same set of genes. Then what makes them different from one another morphologically and functionally? Essentially it is not the genes that determine the fate of these cells, but it is the expression of these genes that makes one cell different from another, resulting in an eye or hand or foot.

The genetic code is transcribed into a messenger mRNA in the nucleus (transcription), which is translated into proteins (translation) by ribosomes in the cytoplasm. A range of transcription factors, arranged in regulatory circuits, modulate the transcription of genes. Like any other cell type, Schwann cells, the major glial cells in the peripheral nervous system are controlled by an array of transcription factors. They control every aspect of Schwann cell differentiation, from their origin in the neural crest to their final differentiation into either myelinating or non-myelinating Schwann cell. Several studies have identified a regulatory network consisting of Oct6, Brn2 and Sox10 that activate Krox20 and drives the transition from promyelinating to myelinating Schwann cell. *Oct6* is considered to be one of the initial transcription factors whose expression is tightly controlled. However, the regulatory machinery behind this controlled expression and functions of Oct6 other than activation of Krox20 are still unclear.

The main aim of this thesis is to identify the factor(s) that act upstream and downstream of Oct6 in Schwann cells and to identify Oct6 interacting molecules that contribute to Oct6 function in Schwann cells. **Chapter 1** of this thesis first gives an overview of the different types of cells of the peripheral nervous system, their development and their functions. In the second part of the introduction, emphasis is on the transcription factors involved in the myelination program and how they are regulated, in particular Oct6. **Chapter 2** illustrates the identification of minimal functional regulatory elements within the Schwann cell enhancer (SCE) sufficient for the activation of Oct6 in transgenic mice. Data presented in this chapter establish that the HMG domain protein Sox10 is involved in the activation of Oct6 through binding to a dimeric DNA site present within the SCE. **Chapter 3** describes Krox20 as the major transcriptional target of Oct6 in Schwann cell differentiation. In addition, p190Rgnef is identified as a novel downstream target of Oct6 activity in Schwann cells. An overview of interacting partners of Oct6 identified by mass spectrometry is presented in **Chapter 4**. Finally, **Chapter 5** presents a comprehensive outlook of the results obtained in the previous chapters, together with some future perspectives and concluding comments.



Introduction

1 - Introduction

1.1 - The vertebrate nervous system and its components

The nervous system of mammals is highly intricate and complex. It is classified into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the brain, spinal cord and optical nerve. All the other nerves are classified under PNS. A peripheral nerve consists of a bundle of axons ensheathed by glial cells and surrounded by extensive collagen networks that provide strength. The nerve further contains blood vessels and is protected by an epineurial layer that provides a highly selective barrier between the nerve and the tissue through which the nerve navigates.

1.1A - Neurons

Neurons are among the most ancient of all specialized animal cell types. Neurons or nerve cells are electrically excitable cells in the nervous system that process and transmit information from both internal and external environments. So, the basic function of a neuron is to communicate information. A generic neuron consists of four parts: dendrites, a cell body, an axon and axon terminals (Figure 1). A neuron receives stimuli through its highly branched dendrites. A nerve impulse is generated at the cell body, which is passed along the axon to the nerve endings or axon terminals. These axon terminals makes synapses with neighboring dendrites to transmit the stimuli to different cell types like a muscle cell or gland cell (1).

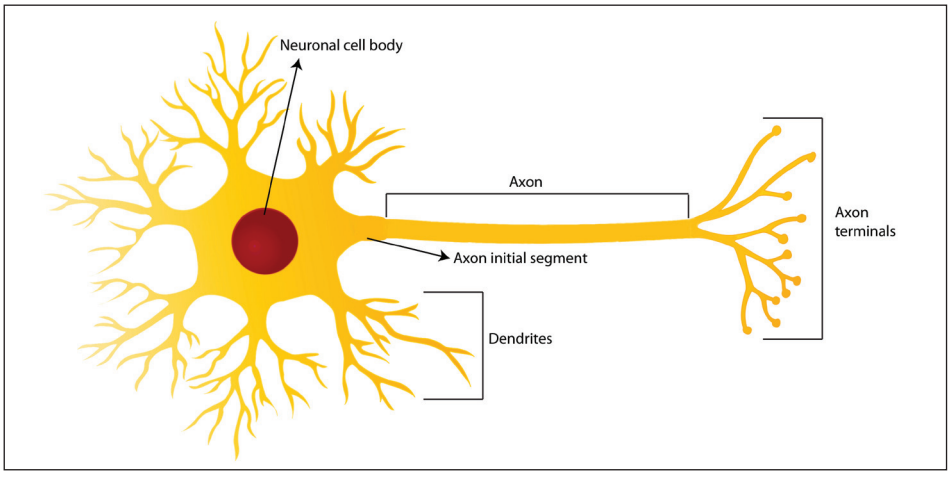


Figure 1 - Neuron
Graphic representation of a neuron and its distinct cellular domains

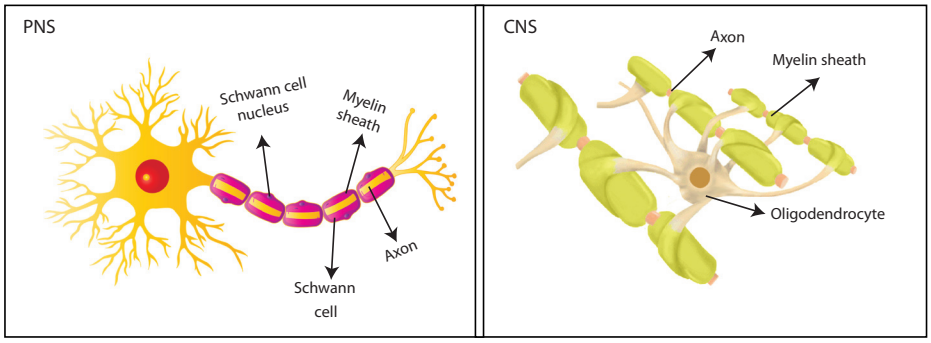


Figure 2 - Myelinated axons
Illustration of myelinated axon(s) in the peripheral nervous system (left) and the central nervous system (right).

1.1B - Glial cells of the PNS and the CNS

All the non-neuronal cells of neurectodermal origin are collectively termed glia. Glia, which is Greek for "Glue" has been traditionally viewed as the glue that holds the neurons together (2). However, there is growing evidence that glial cells are more than just glue and exist in an almost symbiotic relationship with neurons. They surround and support neurons, provide nutrition, insulate electrically, regulate neuronal survival and differentiation, help maintain homeostasis, destroy pathogens, help in neuronal repair and participate in signal transmission in the nervous system. Following injury, glial cells are the major regulators of neuronal repair and differences in glial cell type largely accounts for the difference in regenerative capacity between the CNS and the PNS (3,4).

Glial cells can be divided into microglia and macroglia. Microglia, as the name suggests, are smaller in size compared to macroglia. Microglia are specialized macrophages of the CNS, performing the function of phagocytosis and eliciting immune responses. They are the only glial cells that do not have an ectodermal origin, but are of hematopoietic, and thus of meso-endodermal, origin. Macroglia of the CNS consists of oligodendrocytes, astrocytes and ependymal cells. Oligodendrocytes enwrap axons with a myelin sheath, thereby speeding up the conduction velocity of electrical impulses. They also provide trophic support for the neurons (Figure 2). Astrocytes perform many functions including, contribution to homeostasis in the brain by providing neurons with energy and substrates, biochemical support for the endothelial cells which form the blood – brain barrier, help in repair and scarring process of the brain and spinal cord following traumatic injuries. Ependymal cells create and secrete cerebrospinal fluid which protects the nervous tissue and play a role in maintaining homeostasis.

The PNS contains a number of distinct macroglial cells. Based on their morphology, biochemical make up, and types of neurons they associate with, they can be classified either as myelinating or non-myelinating Schwann cells, satellite cells, teloglia and enteric glial cells in the gut wall. Satellite cells are present in the ganglia to regulate their homeostasis and provide nutritional support (3,5). Myelin forming Schwann cells, like oligodendrocytes in the CNS, produce the myelin sheath around the axons, provide trophic support to the neurons, perform phagocytosis and assist in nerve repair upon injury, while the non-myelin forming Schwann cells engulf the small diameter axons. Teloglial cells cover the axon terminals at the skeletal neuromuscular junctions (6). The enteric glial cells provide structural support for the enteric nervous system, modulate the homeostasis of enteric neurons, and are involved in neurotransmission (7).

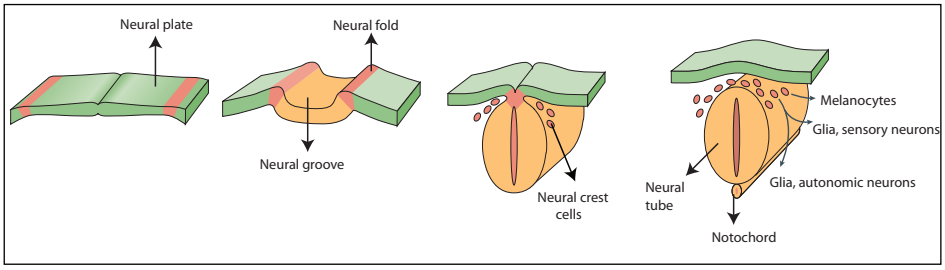


Figure 3 - Neurulation and formation of neural crest derived cells
The neural plate folds to create the neural groove. As the neural folds fuse to form the neural tube, neural crest cells delaminate and migrate away from the neural tube. Laterally migrating neural crest cells give rise to melanocytes and ventrally travelling cells give rise to glial cells, sensory neurons in DRGs and autonomic neurons. The neural tube gives rise to both the brain and the spinal cord (Figure based on (6).

1.2 - Origin and lineage specification of Schwann cells

1.2A - Origin from the neural crest

Schwann cell development occurs through a series of transitional embryonic and postnatal stages, which are tightly regulated by a number of signals. Schwann cells originate from the multipotent neural crest cells. During embryonic development the neural plate gradually folds to create the neural groove (Figure 3)(6). As the neural folds fuse to form the neural tube, the neural groove pinches out and neural crest cells delaminate and migrate along the trunk region of the neural tube to give rise to a diverse array of cells including glial cells of the PNS, melanocytes, smooth muscle cells, connective tissue, and sensory and autonomic neurons (6,8). Some of the neural crest cells retain self-renewal and multipotency properties throughout development and adulthood (9,10). Along with neural crest cells, which are the

primary source of Schwann cells, boundary cap cells also serve as secondary source for Schwann cells (11). Boundary cap cells are neural crest derivates that form clusters at the surface of the neural tube, at the entry and exit points of peripheral roots. They are multipotent stem cells capable of forming neurons and glial cells and of self -renewal (12).

1.3 - Schwann cell differentiation

The generation of Schwann cells involves two embryonic transitional stages; first is the specification of Schwann cell precursors from neural crest cells and second the maturation of those cells into immature Schwann cells (13). Schwann cell precursors (SCPs) occupy nerve trunks at embryonic day (E) 12-13 in the mouse and later differentiate into immature Schwann cells (ISCs) between E13-15 (6). These cells further differentiate to form either myelin forming or non-myelin forming cells depending on the size of the axon. The fate of each of these transitional stages is controlled by different mitogens and by differentiation signals from the axon with which these cells continuously communicate (Figure 4)(6).

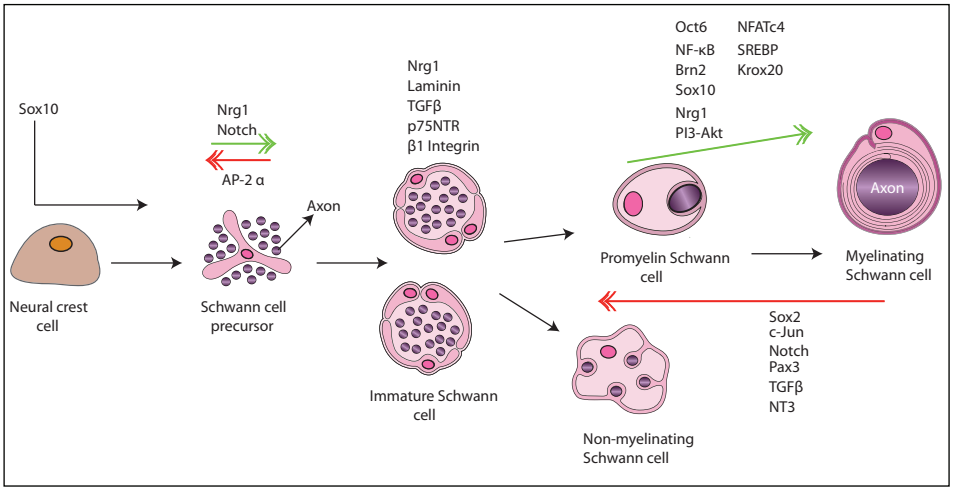


Figure 4 - Schwann cell differentiation

Schematic representation of the different Schwann cell types and the developmental transitions that connect them. Schwann cells originate from the neural crest and migrate along the growing axon (precursor cell stage). During the immature Schwann cell stage, each Schwann cell surrounds multiple small axons. One Schwann cell isolates a single large caliber axons (promyelin Schwann cell) and myelinates it (Myelinating Schwann cell). Many small diameter axons are not myelinated and are ensheathed by non-myelinating Schwann cells. Different molecules involved in these stages are shown. The red arrows indicates the irreversibility of myelination process and factors involved. The green arrows denote the molecules required for the differentiation process (Figure is based on (6).

During differentiation of Schwann cell precursor to immature Schwann cell, nerves become vascularized and a thin layer of perineurium surrounds them. Immature Schwann cells enwrap clusters of axons, forming "axon-Schwann cell families" (14). There is a high rate of cell division during the Schwann cell precursor stage, which peaks up later at the immature Schwann cell stage (15). Lack of laminins 2 and 8 or subunit laminin γ1 results in reduced proliferation and increased apoptosis of Schwann cells together with defective axonal sorting prior to myelination (15,16). Transforming growth factorβ (TGFβ) is also implicated in cell division. Inhibition of TGFβ signaling through overexpression of the oncogene Ski blocks proliferation (17-19). Neurites or neurite membrane fractions can also induce Schwann cell proliferation. Neuregulin 1 (Nrg1) appears to be the neuronal mitogenic factor responsible for this induction (20-22). Parallel to proliferation, a high rate of apoptosis also marks the immature Schwann cell stage as an appropriate number of Schwann cells is to be maintained to sort the axons (13). Two factors, nerve growth factor (NGF) acting through its receptor, $p75^{NTR}$ and TGFβ, promote apoptosis to maintain appropriate numbers of Schwann cells required to myelinate the axons (18,23,24). Thus, there should be a balance between Schwann cell proliferation and apoptosis to get the numbers right for myelination of the axons.

An important difference between Schwann cell precursors and immature Schwann cells is that, from now on, Schwann cells can survive on their own in an autocrine fashion by secreting cocktails of survival factors like Nrg1, Insulin-like Growth Factor (IGF), Neurotrophin 3 (NT3), Platelet Derived Growth Factor (PDGF-B), E-twenty six (Ets) and laminins (15,25-27). There are profound morphological changes taking place as Schwann cells differentiate into either myelinating or non-myelinating cells. Schwann cells send cytoplasmic processes into groups of axons, progressively defasciculating them. Large diameter axons become selectively ensheathed by immature Schwann cells and form a 1:1 relationship with them. This process is called radial sorting. Some of the molecular mechanisms behind radial sorting have been recently established. Laminins, the main component of the basal lamina surrounding Schwann cells interact with β1 integrins that are expressed by Schwann cells. β1 integrins regulate Rac1 and are involved in efficient radial sorting of axon bundles (28-30).

After having selected and reached a 1:1 relationship with the axon, immature Schwann cells exit the cell cycle to differentiate into promyelinating Schwann cells, a differentiation stage that is marked by high levels of the transcription factors Oct6 and Brn2. They, along with Sox10 accelerate the process of myelination by further upregulating Krox20 and inducing myelin genes. A high level of myelin gene

expression during myelination is coordinated with the production of vast quantities of lipids required for the creation of mulitiple layers of cholesterol-rich membrane. The sterol regulatory element binding proteins (SREBPs) together with Krox20 regulates genes involved in cholesterol synthesis (31,32)

1.4 - Non-myelinating Schwann cells

The small diameter axons are associated with non-myelinating Schwann cells. Each non-myelinating Schwann cell engulfs several thin axons (generally less than 1 μm in diameter) to form a Remak bundle, keeping individual axons separated by a thin layer of cytoplasm (5,33). They are called Remak Schwann cells. The other non-myelinating Schwann cells include the specialized terminal Schwann cells called teloglia at neuromuscular junctions. All these cells have the potential to myelinate if they receive the appropriate neuronal signal to do so (34). Non-myelinating Schwann cells also respond to denervation in a way similar to that of myelinating Schwann cells (35). Recent evidence indicates that non-myelinating Schwann cells are involved in the maintenance of unmyelinated axon integrity throughout life and as in the case of myelin forming Schwann cells, Nrg1-ErbB signaling is required for the survival of non-myelinating Schwann cells (36).

1.5 - Structure and molecular organization of peripheral nerves

A peripheral nerve resembles a telephone cable consisting of bundles of axons that connect a multitude of points in the body (Figure 5). Within a nerve, connective tissue called endoneurium surrounds axons and Schwann cells. Many axons are grouped together to form fascicles and each nerve fascicle is surrounded by a protective epithelial sheath called perineurium. Finally the entire nerve fiber that contains many of such fascicles is wrapped up in a connective tissue called the epineurium. Perineurium and epineurium layers also contain blood vessels and macrophages and create a barrier between nerve and tissue, making them impermeable to proteins and migratory cells. Major cellular components of these layers are fibroblasts, which along with Schwann cells produce extracellular matrix (ECM) that is also rich in collagen (37,38).

Peripheral nerves contains two types of fibers: Sensory or afferent fibers and motor or efferent fibers. Cell bodies of the sensory neurons reside within the dorsal root ganglion. They are activated by sensory input like touch and relay the information to the CNS. Cell bodies of motor neurons reside in the ventral part of the spinal cord. They relay the information from the brain to muscle tissue.

The spiral wrapping of Schwann cell plasma membrane extensions around

the axons of the PNS forms the myelin sheath (Figure 6). A myelinated axon has a unique architecture with distinct domains. These domains include the node of Ranvier, the paranodal junction, the juxtaparanodes and the internodal regions. Since the main function of the myelin sheath is to insulate axons, it is mainly composed of non-conducting macromolecules and aqueous cytosolic material is largely excluded. The innermost layer of the Schwann cell that contacts the axon is called inner mesaxon and the layer facing the basal lamina is called outer mesaxon. The basal lamina is mainly composed of laminins and collagen and act as an anchor for intracellular molecules.

While paranodes, juxtaparanodes and Schmidt Lanterman incisures (SLI) make the non-compact myelin, the internodal region (region between the nodes) constitutes the compact myelin. Non-compact myelin provides cytoplasmic continuity along the layers of the myelin sheath from the perikaryon to the nodes and adaxonal membrane. They also differ in their protein composition.

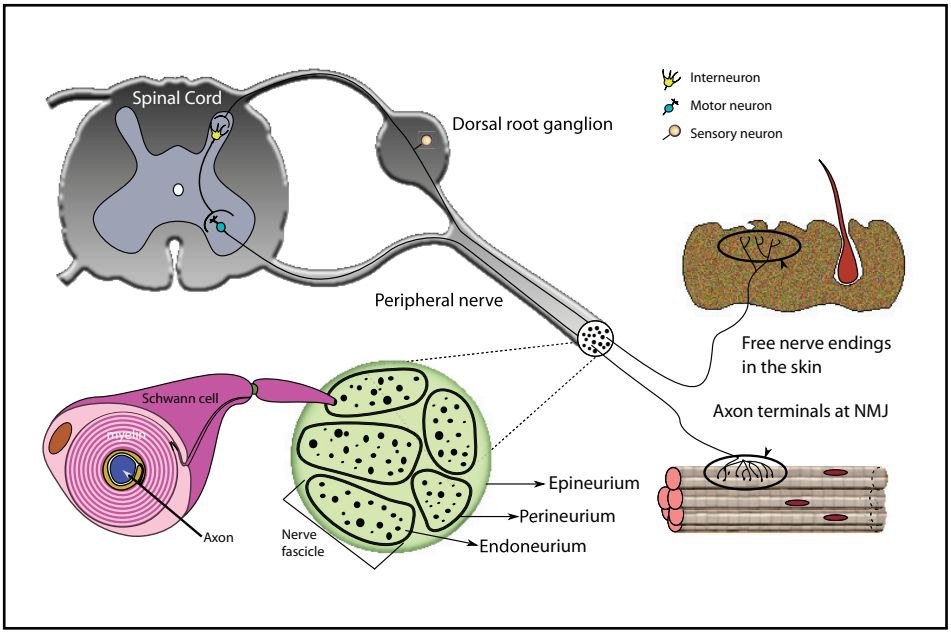


Figure 5 - Representation of peripheral nerves
The position of the cell bodies of motor neurons in the spinal cord and sensory neurons in dorsal root ganglion are shown. The diagram shows how one end of the axon of a sensory neuron innervates the skin. The other end enters the spinal cord at the dorsal side and forms synapses on interneurons that connect to motor neurons. The motor neuron innervates the muscle and forms special synapses called neuromuscular junctions. Endoneurium surrounds an axon and Schwann cell. Many axons are grouped together to form fascicles and each nerve fascicle is surrounded by a perineurium. Many such fascicles are wrapped up in an epineurial sheath.

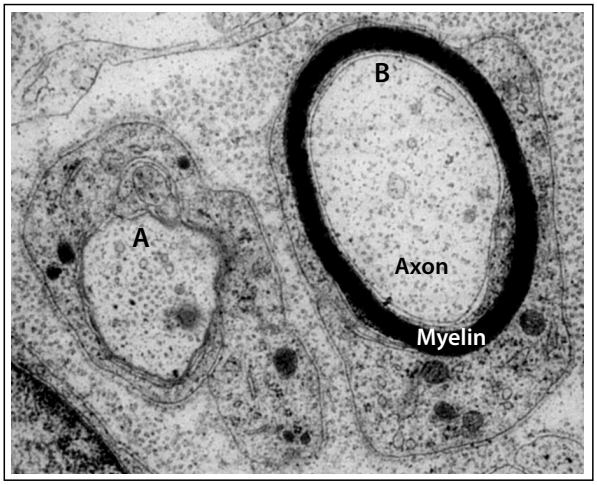


Figure 6 - Myelinated axon in the sciatic nerve
An electron micrograph of a transverse section through the sciatic nerve of a mouse. The myelin sheath is visible as dark rings around the axon. A) shows a pro-myelinating Schwann cell and B) shows a myelinating Schwann cell

Non-compact myelin membrane is rich in Myelin associated glycoprotein (MAG), E-cadherin, α6β4 integrin, Necl1, 2, 4 and connexin-32 (Cx32) and compact myelin contains proteins such as Myelin protein zero (MPZ), Myelin basic protein (MBP) and Peripheral Myelin Protein 22 (PMP22). All these proteins are up-regulated upon signals emanating from either axons or Schwann cells during myelination.

Myelinated axons are completely covered by a myelin sheath except at nodes of Ranvier, which are around 1 μm wide. They are the small gaps between consecutive Schwann cells along the myelinated fiber and form the functional epitome of myelinated axons. They are highly enriched in various voltage gated ion channels, notably sodium channels along with potassium channels (39-42). Nodes also contain cell adhesion molecules including NrCAM, Neurofascin 186 (NF186), laminins, axonal cytoskeletal protein ankyrin G and spectrin βIV (43). Nodes are contacted by interdigitating microvilli that are extended by the outer layer of Schwann cells and are covered by the basal lamina (Figure 7). They are enriched in ERMs (ezrin, radixin and moesin) which are actin binding proteins that help in node formation and function.

The paranodal region, characterized by septate-like junctions, flanks the nodes of Ranvier and is formed by cytoplasmic extensions of Schwann cells contacting the axolemma. At paranodes, the axonal membrane contains a complex of two cell adhesions molecules, contactin-associated protein (Caspr) and contactin, which are essential for the formation of axo-glial junction (44). Caspr and Contactin knockout mice are characterized by the wide gap between the axon and the paranodal loops and loss of septate like junctions (45,46) ascertaining their role in myelin sheath compaction.

Juxtaparanodes, as the name denotes, are located next to the paranodes. They are characterized by the presence of Kv1.1 and Kv1.2 channels that modulate the propagation of action potentials at nodes (44,47,48). On the axonal surface of juxtaparanodes, Caspr2 and TAG1 adhesion molecules interact with each another. They are involved in the organization of juxtaparanodal regions. Recently, Ogawa and co-workers (49) identified Adam22 (A disintegrin and metalloproteinase 22), an axonal transmembrane protein, as a component of the Kv1 channel protein complex localized at juxtaparanodes. Adam22 null mice have a severe hypomyelination phenotype (50). Analysis of these mice demonstrated the need of Adam22 for the clustering of guanylate kinases (MAGUKs) at juxtaparanodes (49).

The Internode, the largest domain of the myelinated fiber corresponds to compact myelin sheath. The majority of myelin in the PNS is in the form of compact myelin. Among the myelin proteins, MPZ is by far the most abundant protein accounting for approximately 50% of the total amount of protein in the sheath. MPZ can form tetramers and this oligomerization property of MPZ leads to dense compaction of the myelin sheath. This view is supported by the observation that MPZ null mice fail to form compact myelin (51-54) leading to hypomyelination, axonal degeneration and deregulation of some of the myelin genes. MPZ is also required for the proper formation of adherens junctions and the outer mesaxon (55,56). Normal myelination in the PNS is under strict dosage control of MPZ, as miniscule overexpression of MPZ in transgenic animals leads to defects in myelination (57). Myelin degeneration observed in the MPZ null or heterozygous mice is similar to the symptoms observed in human neuropathies like Charcot-Marie-Tooth disease (CMT) and Dejerine-Sottas syndrome (DSS) (52,58). In both the diseases, the MPZ gene is mutated which encodes a non-functional protein.

PMP22, another key molecule of PNS myelin interacts with the extracellular domain of MPZ (59,60). PMP22 is also found duplicated in the vast majority of CMT1A patients and mutated in CMT, DSS and hereditary neuropathy with liability to pressure palsies. The phenotype and severity of the disease depends on the nature of the mutation (61). Like for MPZ, PMP22 over-expression leads to myelination defects, demonstrating that the level of PMP22 is crucial (62).

Myelin basic protein (MBP) is present in both the CNS and the PNS. Its requirement for myelinogenesis is different in both the cell types. In the CNS, MBP is absolutely required (63) whereas in the PNS its null mutation results in rather normal myelination (64). MBP probably plays a role in PNS myelination too, but its absence might be compensated by MPZ (52,53). Analysis of mice lacking both MBP and MPZ demonstrated the need for these proteins in myelin compaction and

thickness in the PNS (52,53).

Compact myelin is characterized by SLIs, adherens junctions, tight junctions and gap junctions present in adjacent layers of the myelin sheath (Figure 7) (65). The SLIs run vertically along the length of compact myelin. They play a role in radial transport of molecules, myelin organization and stability (66). MAG is highly expressed during myelination and accumulates in the SLIs during myelin sheath maturation (67). It belongs to the immunoglobulin superfamily of cell adhesion molecules along with nectin-like cell (Necl) molecules. MAG mutant mice do not have any severe myelination defects (68), but show chronic myelinated axonal atrophy with reduced axonal calibers, reduced neurofilament spacing and phosphorylation (69). Schmidt-Lanterman incisures contain adherens junctions (AJs), the key component of which is calcium dependent E-cadherin that forms homophillic dimers (70). Cytoplasmic partners of E-cadherin are catenins that link E-cadherin to the actin cytoskeleton (71). Cell adhesion molecules, Necl1 and Necl2 expressed by axons and Necl4 expressed by Schwann cells, are highly enriched at SLIs and interact with each other. Inhibiting the interaction between these molecules in DRG co-cultures inhibits myelination, indicating that Necls act as mediators at axo-glial junctions during myelination (72,73).

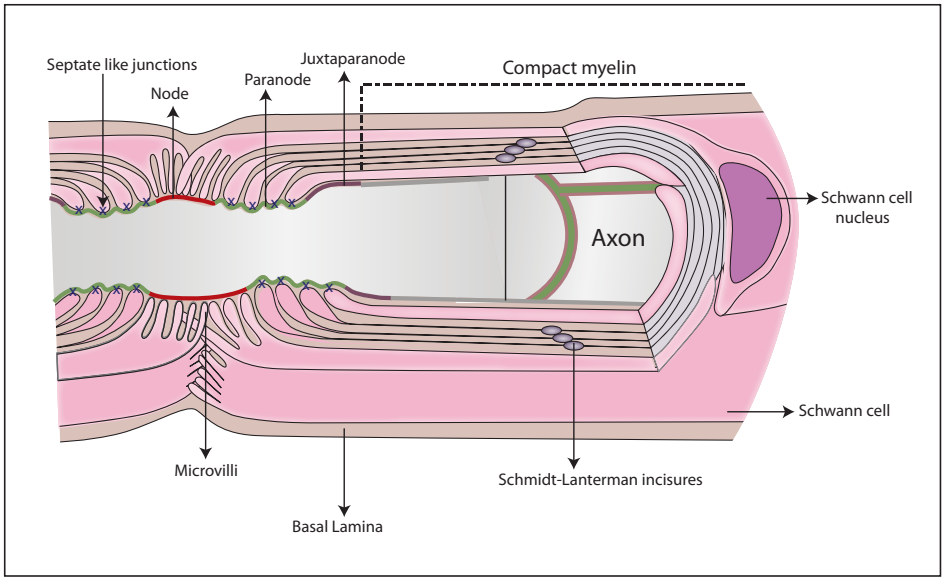


Figure 7 - Illustration of the organization of a myelinated peripheral nerve fiber
Major regions of the axonal membrane are marked. Dashed lines indicates the region of compact myelin formation (Figure is based on (66).

Connexin (Cx32) is localized at the incisures of SLIs and the paranodes of myelinating Schwann cells, where it appears to form reflexive gap junctions (74). Mutations in Cx32 are implicated in CMT disease, a hereditary demyelinating neuropathy, which results in non-functional gap junction formation (75-77). Another gap junction protein Cx29 is also expressed in Schwann cells similar to Cx32. Hence, this protein could function as a redundant gap junction protein in the PNS (78).

Taken together, these observations suggest that the composition and precise architecture of the myelin sheath serves the physiology and integrity of the axon.

1.6 - Important regulators of myelination

1.6.A - Neuregulin – a multifaceted axonal signal molecule

Intricate and intimate interactions between axons and Schwann cells, modulated by several extrinsic and intrinsic signals, steer the myelination process in the PNS. Neuregulin1 (Nrg1), also known as heregulin, Neu differentiation factor (NDF) or glial growth factor (GGF) is produced by the neurons (79,80). Nrg1 and its receptor ErbB on the Schwann cells are key mediators regulating many aspects of Schwann cell development (Figure 8)(81).

Nrg1 was shown to promote differentiation from rat neural crest stem cells into glial cells by inhibiting neurogenic differentiation *in vitro* (82,83). Mice with mutations in Nrg1, ErbB2 or ErbB3 have severely reduced number of early Schwann cell precursors. At the later developmental stages ErbB2 and ErbB3 mutant mice lack Schwann cells (84-87). In the presence of Nrg1, cultured Schwann cells proliferate actively (88,89). Nrg1 through its ErbB receptors activates a number of signaling pathways including Ras/MAPK, PI3 kinase and FAK which results not only in proliferation, but also in increased motility and survival (90-94). Nrg1 is an essential survival factor for Schwann cell precursors in embryonic nerves as Schwann cell precursors are still not capable of surviving on their own. Transection of sciatic nerves results in axonal degeneration and leads to Schwann cell apoptosis. This suggests that some axonal factor is necessary for the survival of Schwann cells. This factor appears to be Nrg1 as exogenously applied Nrg1 prevents Schwann cell death to some extent (25).

Nrg1 also promotes differentiation of Schwann cell precursors into immature and promyelinating Schwann cells. Experiments conducted in cell culture system provide strong evidence for this role of Nrg1. When cultured in the presence of exogenous Nrg1 for 4 days, E14 Schwann cell precursors develop a phenotype resembling that of Schwann cells which have attained 1:1 relationship with axons *in vivo* (90,95,96). These results showed that Nrg1 can mimic the axonal contact in

an *in vitro* set up. Mice carrying targeted mutations in the Nrg1 gene or its receptors ErbB2 and ErbB3 lack Schwann cells in the peripheral nerves (97).

Nrg1 signaling is essential for the interactions between axons in both the myelinating and non-myelinating Schwann cells. The continued expression of Nrg1 and ErbB2/3 into the adulthood suggests a potential role in mature Schwann cells (36,98,99). However, evidence for their requirement in myelinating mature Schwann cells is still obscure. There are a few reports emphasizing the role of Nrg1 – ErbB2/3 signaling in adult non-myelinating Schwann cells. Expression of a dominant ErbB2 in adult non-myelinating Schwann cells results in a high proliferation and death of these Schwann cells and degeneration of unmyelinated axons leads to fewer axons per Remak bundle (36). Conditional ablation of Nrg1 in unmyelinated and thinly myelinated neurons leads to formation of abnormally large Remak bundles and reduced sensitivity to heat and cold stimuli (100). Thus it can be inferred that non-myelinating Schwann cells provide trophic support for the unmyelinated axons through Nrg1.

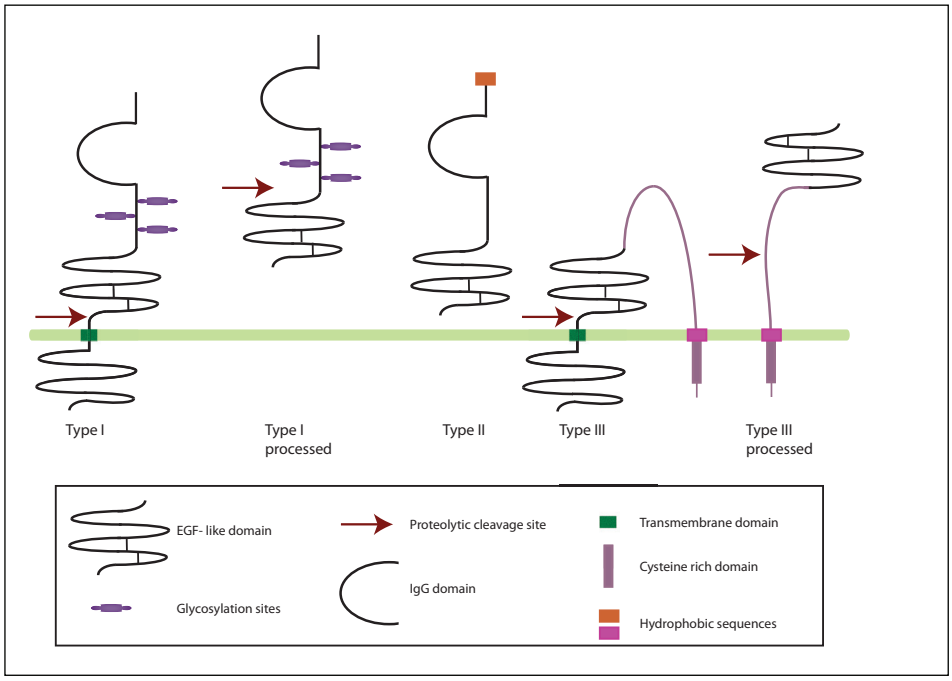


Figure 8 - Schematic display of the major Nrg1 isoforms. The three major isoforms of Nrg1 involved in myelination is illustrated (Figure based on (81)

Box 1

Neuregulin – ErbB signaling

Neuregulins (Nrgs) belong to a family of membrane associated proteins which contain an extracellular epidermal growth factor (EGF) like domain. There are four Neuregulin genes (Nrg1-4), the most prominent and well characterized being, Neuregulin-1. Three different isoforms of Neuregulins (I- III) can be generated through transcription from different promoters and differential mRNA splicing. They are classified based on their N-terminal domains. All of them have a common EGF like domain, which is sufficient to bind and activate tyrosine kinases of ErbB receptors. Other domains include an Ig-like domain (Type I and II), a domain rich in potential glycosylation sites (type I) and a Cysteine rich domain (type III). Type II is a secreted form, whereas type I and III are transmembrane isoforms. These isoforms undergo cleavage by different proteases such as Adam17 (TACE), Adam19 (Meltrin) and BACE 1 (102-105).

Nrg1 type I controls neural crest migration and development. Type II has dose dependent effects on Schwann cell myelination; at low concentration inhibiting myelination and at high concentration promoting differentiation into the promyelination phenotype (106,107). Both type I and II are shed from the cell surface upon cleavage and act as paracrine signaling molecules. Type III, however remains adhered to the cell surface after the cleavage and act as a juxtacrine signal (102,108). Nrg1 type III is critical for multiple aspects of Schwann cell development, including initiation, migration, differentiation and survival (34,101,109).

Nrg1 signals through ErbB2/3, which are tyrosine kinase receptors. Nrg1 first binds to catalytically inactive ErbB3, which further induces heterodimerization with ErbB2. ErbB2, a tyrosine kinase, initiates a cascade of signaling pathways such as RAS/MAPkinase, PI3kinase, FAK and NF-κB pathways. Nrg1 regulates the proliferation of Schwann cell precursors (SCPs), migration of SCPs along the axon, survival or apoptosis of immature Schwann cells and finally myelination through differentiation of Schwann cells.

Transgenic mice with reduced dosage of Nrg1, ErbB2 and/or ErbB3 show a thinly formed myelin sheaths (86,101) indicating that the number of lamellae produced by Schwann cells around the axon is dependent on the levels of Nrg1. Taveggia and co-workers (34) provided compelling evidence that the ensheathment fate of axons to become myelinated or remain unmyelinated depends on the dosage of Nrg1 type III (Box1). In the presence of high level of Nrg1 type III, a thick myelin sheath is produced, intermediary levels result in thinner myelin, whereas low levels direct axons to form Remak bundles (102).

Sox10 could be involved in Nrg1-ErbB signaling as it regulates the expression of ErbB3 and in the Sox10 mutant mice, ErbB3 expression is down-regulated (Britsch et al., 2001).

1.6B - RhoGTPases and myelination

RhoGTPases (Rho-family of small guanosine triphosphatases) are a subgroup of the Ras superfamily. In Schwann cells, they are required for proliferation, sorting, ensheathment, and myelination of axons (110,111). These signaling molecules shuttle between inactive GDP bound and active GTP bound state. In their active state RhoGTPases bind to a variety of effectors that regulate the actin cytoskeleton and its interaction with microtubules (110,112). They act as molecular switches to integrate the signals from extracellular matrix (ECM) proteins to modulate Schwann cell cytoskeletal rearrangement and cell proliferation (28,30,113).

Different classes of regulatory proteins GEFs, GAPs and GDIs regulate this constant shuttling. GEFs (GTPase exchange factors) promote the exchange of the bound GDP to GTP, thus activating RhoGTPases (114). GAPs (GTPase activating proteins) activate GTPases to hydrolyze the bound GTP to GDP, thus promoting inactivation of RhoGTPases (115). GDIs (Guanine nucleotide dissociation inhibitors) stabilize the GDP-bound form of the GTPase and inhibit nucleotide exchange and activation (116).

RhoGTPases are grouped into 8 subfamilies. Among all the RhoGTPases, Rac1 (Ras related C3 botulinum toxin substrate 1), Cdc42 (cell division cycle 42) and RhoA (Ras homologous member A) are studied extensively. (117). All three of them are expressed and active in both neurons and Schwann cells (113). Specific ablation of Rac1 in the Schwann cell lineage results in a delay of radial sorting and an arrest in myelination (28,29,118,119). Rac1 is regulated by β1 integrins in Schwann cells. Schwann cells lacking β1 integrin have lower active Rac1 levels and fail to extend

radial lamellipodia on axons. The defect of radial sorting observed in β1-integrin$^{-/-}$ mice can be ameliorated by the expression of active Rac1 (29). Apart from β1 integrin, Rac1 is also regulated by several growth factors and neurotrophins such as IGF-1, FAK, BDNF and Nrg1 and also by a tumor suppressor Merlin/Nf2 (120-124).

Interestingly, another RhoGTPase, Cdc42, does not affect Schwann cell process extension when specifically ablated in Schwann cells, but reduces proliferation and eventually affects myelination (28). Schwann cell division is at its peak during radial sorting and Cdc42 is critical at this stage to maintain the threshold number of Schwann cells. Cdc42 is required to promote Schwann cell cycle progression either alone or in combination with some mitogens (125-127). Cdc42 is strongly activated by Nrg1. It is possible that ErbB-FAK interactions mediate this activation. FAK is an integrin-dependent tyrosine kinase. The proliferative effect of Cdc42 is also achieved through inhibition of tumor suppressor, Schwannomin/Nf2/Merlin (124).

RhoA and its effectors, ROCK1 and 2 (Rho-associated coiled-coil containing protein kinase) are highly expressed at the onset of myelination. RhoA regulates Schwann cell morphology, cell clustering and substrate adhesion (128). After activation of RhoA, ROCK phosphorylates several substrates that regulate cytoskeletal assembly (129). Their expression drops once myelination has been established (130). Inhibition of ROCK in Schwann cell-neuron cocultures results in aberrant myelination. It causes the formation of short internodal segments containing multiple nodes and paranodes, without changes in cell proliferation or differentiation, suggesting that ROCK normally suppresses abnormal branching of the myelin sheath (130).

P190^{RhoGEF} (Rgnef) is a Rho-A specific GEF that activates RhoA in neuronal cells. P190^{RhoGEF} binds directly to FAK in neuronal cell lines. P190^{RhoGEF} is phosphorylated and activated by FAK, thereby increasing RhoA activity in cells. These events lead to the enhanced neurite outgrowth and guidance (131,132). Moreover, disruption of association of FAK with P190^{RhoGEF} results in a phenotype very similar to that of neurons lacking FAK (133). Cell specific ablation of FAK increases the number of axonal terminals and synapses formed by neurons *in vivo*. Furthermore, P190^{RhoGEF} overexpression rescues the phenotype. Autosomal recessive mutations in a GEF for Cdc42 called *Frabin/FGD4* results in CMT4H, a specific subtype of hereditary motor and sensory neuropathies ((134,135).

These data suggest that RhoGTPases and their regulators are involved in various biological processes that are important for myelination in the PNS.

1.6C - Negative regulators of myelination

Myelinating Schwann cells are highly plastic cells that are able to dedifferentiate and re-enter the cell cycle depending on the external situation. When Schwann cells are denervated, dedifferentiation of myelinating Schwann cells takes place. The cells re-enter the cell cycle and attain the phenotype of immature Schwann cells. This process is called Wallerian degeneration (136). Major negative regulators of myelination include c-Jun, Sox2, Pax3, Krox24, NT3 and Notch. c-Jun, an important component of the AP-1 complex, promotes proliferation and is downregulated by Krox20 upon initiation of myelination. c-Jun is rapidly upregulated in Schwann cells of transected nerves and contributes to dedifferentiation by antagonizing Krox20 activity (137). c-Jun acts synergistically with Sox2, a marker of immature Schwann cells in dedifferentiating cells (137,138). The role of Pax3 and Krox24 in reactive Schwann cells is less well understood.

1.7 - Transcriptional controls underlying Schwann cell differentiation and myelination

Schwann cell myelination is a remarkable example of a cell-cell interaction in which an intimate association between a Schwann cell and an axon induces radical changes in the morphology of the Schwann cell, culminating in the formation of a highly specialized cell. This multistage differentiation process is under the control of partially overlapping, serially expressed transcription factors (TFs). They direct gene expression by binding to DNA regulatory elements either alone or in co-operation with other transcription factors. Regulatory TFs are composed of usually two types of domains: a DNA binding domain, which serves to interact with its cognate DNA target sequence, and a transcription regulation domain, which serves to activate or repress transcription. The recognition sequences for these DNA binding domains vary. In general, they are shorter for eukaryotes than prokaryotes. For example, the average motif length in Drosophila is 12.5 bp compared to an average of 24.5 bp in *E.coli* (155). The shorter length of binding sequence and often loose sequence requirement in eukaryotes results in loss of specificity which is compensated by cooperative binding of multiple transcription factors. In addition TFs frequently function in a network of cross-regulatory loops, which increases the complexity of transcription program (156). Apart from gene activation, TFs can also function as repressors either by the recruitment of repressing complexes, DNA modifying enzymes or just by blocking the DNA and thereby preventing the binding of activating complexes.

Box2

Transcriptional regulation

Differential expression of genes can be achieved at multiple levels. One of the levels includes transcription initiation, elongation and termination. Transcriptional regulation is one of the most important levels of gene regulation and can be accomplished via the association of transcription factors with regulatory DNA sequences. A large proportion of the regulatory information is located at the promoter region immediately upstream of the transcription start sites of the genes. Mostly in prokaryotes, promoter sequences are sufficient to modulate the levels of transcription. However, eukaryotic genomes including yeast, contain thousands of genes, most of which are differentially expressed in time and space. How then is the diversification accomplished?

Essentially, the diverse array of gene expression is achieved by even more diverse sets of regulatory elements that are decoupled from the promoter proximal region and distributed, near and far. Such regulatory elements are termed enhancers. The enhancers can be located from a few hundred base pairs to megabases away, both upstream and downstream from the transcription start site. Enhancers usually have binding sites to which transcription factors bind to promote transcription. As a general rule, enhancers are capable of activating transcription from heterologous promoters, and in fact in the majority of gain-of-function assays reporter gene expression generally follows the pattern dictated by the enhancer, and not by the promoter.

It is still a matter of debate how enhancer-promoter contact is established. Data accumulating from several laboratories point towards a "looping" mechanism wherein certain transcription factors bind to the enhancers and bring them into close proximity of the promoters (139-141). In an alternative model of enhancer function, a "tracking" or "scanning" is proposed where the enhancer diffuses one-dimensionally along the chromatin fiber to meet the promoter (141). In addition to the above two mechanisms, another model proposed as "oozing" or "linking" model suggests that recruitment of proteins modifies the chromatin structure between enhancer and promoter. This model states that a complex is formed at the enhancer and polymerized along the chromatin fiber bi-directionally until it reaches the promoter (142). Among all the proposed models, the "looping" model has gained more prominence because of the support received from studies of nuclear architecture utilizing "chromosome conformation capture" (3C) and its derivates 4C and 5C (143-145).

Box 2 continued

Whereas the promoter of a gene can simply be identified by analyzing the sequences present 5' upstream of the gene's transcription start site, identification of enhancers is difficult as there is no simple, straightforward way to pinpoint the location of enhancers. The majority of enhancers is present within the introns of the genes that they regulate. Sometimes they are located within the introns of neighbouring genes. Enhancers are by and large conserved across species and DNA sequences that are non-coding, but still conserved, are presumed to have some regulatory function. The sequencing of various genomes has facilitated phylogenetic comparisons for identifying such regulatory elements. Furthermore, genome-wide studies have suggested certain chromatin "signatures" for enhancers, which sets them apart from promoters (146-148). For example, H3K4me1 is observed mostly at enhancers but not at transcription start sites and H3K4me3 is largely absent from enhancers and predominantly present at active promoters (149-151). The H3K4 methylation signature has been correlated with enhancer activity in gain-of- function assays, however, not precisely (146,152). Recent studies established that the p300 histone acetyltransferase binds enhancer sequences and this could therefore be used as an additional marker for enhancer elements (153,154).

TFs are grouped into families based on their predicted DNA binding domains (157). To date, more than 100 different DNA binding domains have been described (158). How can cis-regulatory elements be identified? Classical techniques such as CAT and luciferase assays *in vitro* and transgenic organisms are used to test a range of deletion and point mutation constructs to pinpoint sequences relevant for transcriptional activity. As whole genome sequences of more and more organisms are becoming available, phylogenetic footprinting has become a powerful tool to support and focus these classical approaches (159,160).

Some of the transcription factors, implicated in the regulatory network of myelination include Sox10, Oct6, Brn2, Krox20, SREBPs and NFκB (161-166)(Figure 9). Some of these factors are discussed in the following sections.

1.7A - HMG domain transcription factor Sox10

Sox10 belongs to a family of DNA binding proteins, with a highly conserved domain first found in the testis-determining gene SRY. This conserved domain is referred to as the Sry box or Sox, hence the name Sox. The Sry box is closely related to the

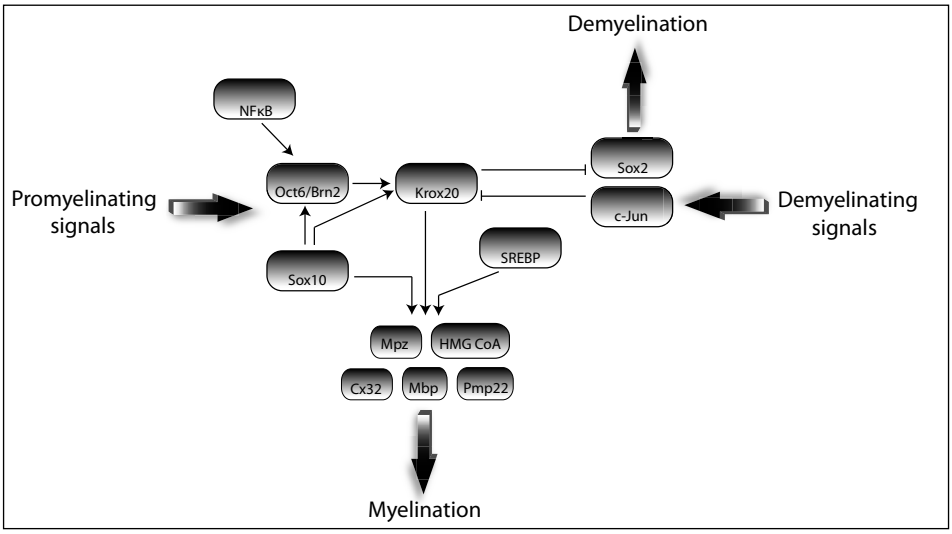


Figure 9 - Schematic representation of a gene regualtory network of myelin-associated Schwann cell differentiation.
The major transcription factors involved in myelination and demyelination are depicted. Arrows indicate activation while blunt lines indicate repression. Figure based on (250)

High Mobility Group (HMG) domain and thus Sox proteins are grouped under the HMG box superfamily (167). They are involved in a diverse array of developmental processes like germ layer formation, organ development, pluripotency and cell type specification. In mammals, there are more than 20 Sox proteins which are classified into seven sub groups (A-G) based on the conservation of the HMG domain. Members of the same subgroup usually share >80% homology at the amino acid level (168-170).

Sox10 belongs to the group of Sox E proteins together with Sox8 and Sox9. They bind to a loosely defined heptameric DNA element (A/T)(A/T) CAA (A/T) G (171). Sox10 is required for neural crest development and specification of several cell lineages including peripheral glia (172,173). Schwann cell precursors failed to develop from neural crest cells in mice harboring a targeted deletion of Sox10 (172). Intriguingly, Sox10 is expressed during all stages of Schwann cell development and continues to be present in both myelinating and non-myelinating Schwann cells of the adult PNS (174) (Figure 10). This indicates that Sox10 functions extend beyond glial specification. Finzsch and colleagues (175) generated an allele which allows deletion of Sox10 specifically in immature Schwann cells. Mutant mice died from peripheral neuropathy before the seventh postnatal week. Schwann cells failed to

develop beyond the immature Schwann cell stage and both myelinating and non-myelinating Schwann cells were absent. Heterozygous Sox10 loss-of-function mutations have been reported in patients with Waardenburg-Hirschsprung syndrome and its murine model, *Dominant megacolon* (176,177). In these mutant mice, a frame shift mutation was found to result in premature termination of Sox10. The Homozygous *Dom* mutation results in embryonic lethality. The mice show loss of neurons and glial cells in the PNS and lack the enteric nervous system. Inoue and colleagues (178) identified a dominant-negative Sox10 mutation in a patient with severe leukodystrophy and peripheral myelinopathy in conjunction with a Waardenburg syndrome type 4, which highlights the importance of Sox10 not only in Schwann cell specification, but also in the initiation of myelination both in the peripheral as well as the central nervous system. Furthermore, tissue culture analysis showed that Sox10 activates the expression of several peripheral myelin genes including MPZ, MAG and Cx32 (179-182)

MPZ expression, one of the major structural genes of myelin sheath requires Sox10 during early embryonic stage. (183). Sox10, like the other family members, Sox8 and Sox9, can bind regulatory DNA sites as a monomer and as a dimer. DNA binding results in significant bending of DNA, altering the chromatin architecture to facilitate interactions between target promoters and more distal regulatory elements (For a review see, (173). Sox10 controls MPZ expression through binding to the MPZ promoter. The MPZ promoter contains multiple Sox10 binding sites designated as site B and site C. Site B conforms to the consensus heptameric Sox binding site, but site C contains two adjacent Sox sites with single mismatches. They are referred as site C and C' or in short C/C'. Even though site C and C' do not conform to the consensus Sox site, two molecules of Sox10 bind co-operatively to this dimeric C/C' site to mediate the effect of Sox10 on the MPZ promoter (179). It could be that the dimeric sites compensate for the non-perfect consensus sequence in the regulatory elements.

The Sox10 protein is composed of a number of domains (Figure 11). The roles of these domains have been addressed in a number of recent studies (168,184,185). The HMG domain (amino acids 101-180) and a transactivation domain (amino acids 400-466) at the extreme C-terminus of Sox10 are highly homologous to a corresponding region in Sox8 and Sox9 (186). The other region with high similarity among class E proteins is the DNA dependent dimerization domain situated in the amino terminal portion of the protein (amino acids 61-101). This region is required for the cooperative binding of two Sox10 molecules on closely apposed inverted Sox10 binding sites (179,185). The mice homozygous for a Sox10 allele encoding a

Sox10 protein that does not dimerize. This mutant protein is capable to support early Schwann cell development but fails to upregulate Oct6 in late fetal life (184). This indicates that the dimerization domain is required for the immature Schwann cells to enter the promyelinating stage of Schwann cell differentiation. Another highly conserved region in Sox10 is the K2 domain, which lies between amino acids 233-306. This domain in Sox8 protein has transactivation potential (168). Interestingly, mice with a hypomorphic allele of Sox10 that lacks the K2 domain, do express Oct6 indicating that the cells have reached the promyelin stage, but fail to express Krox20 and other myelin genes (184).

1.7B - POU domain transcription factor Oct6

Oct6, also referred to as Tst-1, SCIP and POU3f1, belongs to subclass III of the POU domain family of transcription factors. The POU domain was defined following the observation that three mammalian transcription factors Pit-1, Oct-1, Oct-2 and the *C.elegans* gene UNC-86 shared a highly homologous region (187).

The POU domain, a bipartite DNA binding structure, is divided into two sub-domains. The amino terminal portion of the POU domain is ≈ 75 amino acids long and is specific for the POU class of proteins (POU_s) domain. The carboxy terminal portion is ≈ 60 amino acids long and is the POU homeo (POU_{hd}) domain. A short non-conserved linker of variable length connects these two domains (188).

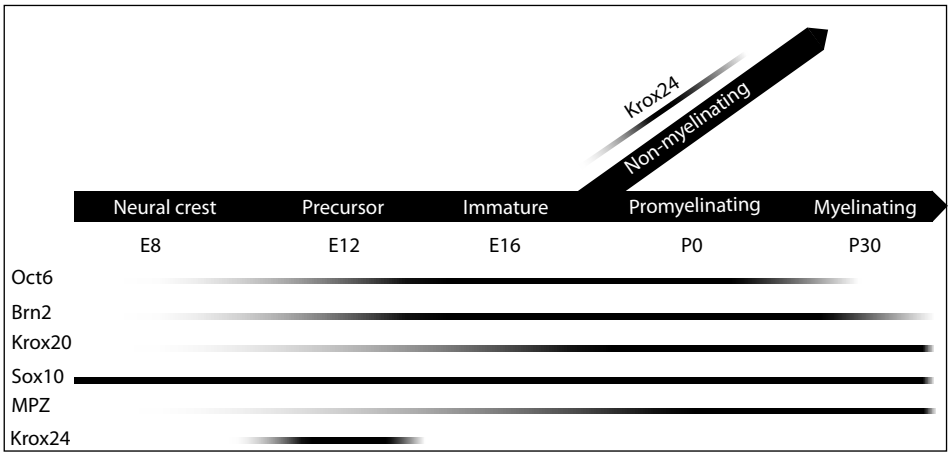


Figure 10 - Schematic view of the transcription factor expression profile in Schwann cell differentiation.
The expression of important transcription factors in developing peripheral nerve and their relation to the anatomical changes at various stages of Schwann cell differentiation is depicted. E12, E16 refers to embryonic day 12 and 16. P0 and P30 refers to postnatal day 0 and 30.

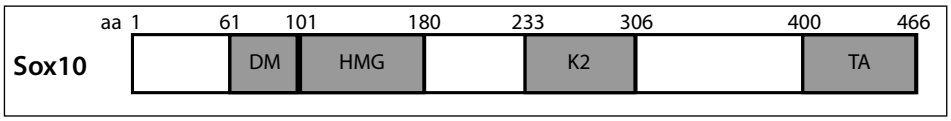


Figure 11 - Schematic representation of Sox10 protein.
The different domains with their respective amino acid position are marked. DM: dimerization motif; HMG: High mobility group DNA binding motif; K2: highly conserved potential transactivation motif; TA: C-terminal transactivation motif.

All POU domain proteins bind to a cognate octamer DNA sequence: 5'-ATGCAAAT- 3' with the POU specific domain contacting the ATGC half site on one side of the helix and the POU homeodomain contacting the AAAT half site on the opposite face of the helix (189). Thus both regions of the POU domain are involved in DNA binding. Oct6 has a classical nuclear localization signal (NLS) at the N-terminal end of the POU homeodomain, which is highly conserved in all POU proteins (190). Oct6 also contains a nuclear export signal (NES) within its POU homeodomain. Mutations in the amino acid sequence of the NES slightly reduce the DNA binding capacity of Oct6, but interfere strongly with its transactivation potential (191).

The Oct-6 gene is a single exon gene located on the distal part of chromosome 4 in mouse and on the short arm of chromosome 1 in human (192-195). All the four mammalian genes belonging to subclass III of the POU domain family (Oct-6, Brn-1, Brn-2 and Brn-4) are intronless (196). Oct6 is expressed in several cell types including Schwann cells, oligodendrocytes, skin, embryonic stem cells, testis, macrophages and central neuron sub-populations. Expression of Oct6 in the Schwann cell lineage is strictly controlled. Weak expression of Oct6 can be seen at the Schwann cell precursor stage (E12.5) (197). However, Oct6 expression can be observed at appreciable levels from E16 onwards (198)(Figure 10). Oct6 expression peaks around birth, concomitant with the onset of myelination. It is gradually downregulated as myelination proceeds and completely absent in adult nerves (198-202).

1.7B.1 – The role of Oct6 in myelination

Expression of the major myelin genes in Schwann cells is dependent on axonal contact, both for induction and for maintenance (203,204). Likewise, Oct6 is not expressed in transected nerves *in vivo* or primary Schwann cell cultures where axons are absent. However, this requirement for axonal contact can be overcome by treating Schwann cells with agents that elevate intracellular cAMP levels such as Forskolin (205). Hence Oct6 is also named SCIP (Suppressed cAMP inducible POU).

cAMP activates protein kinase A (PKA) in cultured Schwann cells which further upregulates Oct6. The induction of Oct6 kick starts the myelination program in the PNS by activating Krox20, which further induces myelin associated genes such as MBP and MPZ. Indeed, inhibition of cAMP dependent PKA activity in Schwann cells results in reduced formation of myelin segments (206).

Upregulation of genes like Krox20, MBP and MPZ after the induction of Oct6 points to a role for Oct6 as a transcriptional activator (205,207). In transient co-transfection experiments, Oct6 activates octamer-containing promoters (208-210). Oct6 knock out studies suggest that Oct6 functions as a positive regulator of Schwann cell differentiation. Homozygous Oct6 deleted mice ($Oct6^{-/-}$) show a transient arrest of Schwann cells at the promyelin stage and a delay in nerve myelination (162,163). These mice were born at normal Mendelian ratios and developed to term. However most of the $Oct6^{-/-}$ mice died soon after birth, only 2 to 4% survive for a longer period. The high rate of mortality observed among newborn pups could be attributed to the breathing defect observed in $Oct6^{-/-}$ mice (162). However, central myelination by oligodendrocytes proceed normally. These observations indicate that Oct6 could have a role in the regulation of pulmonary homeostasis, but not in CNS myelination.

Oct6 was postulated to be involved in the proliferation of embryonic Schwann cells in order to regulate the appropriate number of Schwann cells in peripheral nerves (210,211). This leads to a further hypothesis that Oct6- deficient Schwann cells would differentiate prematurely and their number would be reduced. However, Jaegle and colleagues (163) demonstrated that such abnormalities were not observed in $Oct6^{-/-}$ nerves. Microscopic examination showed the arrest of Oct6 mutant Schwann cells at the promyelin stage of differentiation. But eventually these Schwann cells further differentiated into myelinating Schwann cells. This suggests that Oct6 is not required for terminal differentiation of Schwann cells, but only for the transition from the promyelin to the myelin stage.

1.7B.2 – The regulation of Oct6

Axonal signals regulate the expression of Oct6 both during nerve development and regeneration. Previously, our laboratory has identified a cis-acting enhancer of Oct6, approximately 12 Kb downstream of the Oct6 structural gene. This enhancer termed as Schwann cell enhancer (SCE) is sufficient and required to activate temporally correct expression of Oct6 in the Schwann cell lineage of transgenic mice (198,212). This element does not contribute to Oct6 regulation in other cell types, but only in the Schwann cell lineage (212). Furthermore, a SCE/Oct6

promoter driven transgene is down regulated in myelinating cells and re-induced in regenerating Schwann cells following nerve transection. It is postulated that intracellular axonal signals converge on this element to activate Oct6 expression.

An increase in the intracellular concentration of cAMP can partially restore the expression of myelin proteins in cultured mammalian Schwann cells, suggesting that cAMP is part of the signaling pathway that drives myelin formation *in vivo*. One of the targets of cAMP is NFκB, a transcription factor involved in cell proliferation and differentiation of a variety of cells. Nickols and co-workers (165) demonstrated that in the absence of NFκB, Oct6 is not upregulated in promyelinating Schwann cells (Figure 9). Mammalian NFκB is a transcription factor formed by the homo or heterodimerization of 5 subunits (p65/RelA, p50, c-Rel, RelB, and p52). NFκB is highly expressed in pro-myelinating Schwann cells which is evident from the expression of the p65 subunit. Its expression slowly declines and is nearly absent from adult nerves (165). p65-p50 is a prototypical dimer of NFκB family. It is kept inactive in the cytoplasm by the endogenous inhibitor IκBα. Upon stimulation, the IκBα/p65-p50 complex disassociates and the p65-p50 dimer translocates to the nucleus (213). PKA phosphorylates the p65 subunit at Ser276, thereby initiating transcriptional activity of NFκB. Overexpression of mutant Ser276A of p65, blocked PKA mediated transcription resulting in reduced myelin formation in DRG co-cultures (214). However, stimulation of PKA alone was not sufficient to induce DNA binding by NFκB suggesting that an unknown signal is involved in the NFκB nuclear translocation. Recently, Limpert and colleagues (215) showed that axonal membrane-bound Nrg1 type III is the axonal signal responsible for initiating NFκB activation in Schwann cells during myelination via the ErbB2/3 receptors. It is possible that both PKA and Neuregulin signaling initiates myelination through the Oct6 SCE. (165). This leads to the speculation that Oct6 is a direct target of NFκB. However, a constitutively active form of NFκB does not activate a SCE reporter construct (Mehrnaz Ghavini, unpublished observations). cAMP targets Crebp (cAMP response element binding protein) through PKA. PKA phosphorylates Crebp upon cAMP addition to Schwann cells. But is still unclear whether Crebp activates Oct6 directly (216).

It is clearly established that elevation of intracellular cAMP induces Oct6 expression (205). But the mechanisms that regulate cAMP levels *in vivo* were unknown until recently. Using mutational analysis in zebrafish, Monk and co-workers (217) found that the G protein coupled receptor Gpr126 is required autonomously in Schwann cells for myelination. The authors showed that the Gpr126 acts upstream of Oct6. In Gpr126 mutants, Schwann cells failed to express Oct6 and Krox20 and

were arrested at the promyelinating stage (218). Treating Gpr126 mutants with forskolin, which increases the intracellular cAMP levels, bypassed the requirement for Gpr126 signaling and restored the expression of Oct6, Krox20 and MBP (217). The axonal signal activating Gpr126 has yet to be identified (219), but it functions independently of the Nrg1/ErbB signaling.

In terminally differentiated Schwann cells, Oct6 expression is extinguished. Jaegle and Meijer (220) have proposed that Oct6 is involved in its own downregulation either directly or indirectly, as the Oct6 locus is not downregulated in the nerves of adult animals in the absence of Oct6. Others have suggested that Krox20 is involved in down-regulating Oct6 expression since in Krox20$^{-/-}$ Schwann cells, Oct6 expression is sustained instead of being extinguished (221). This is very well possible as the Oct6 promoter is in a CpG island that contains multiple potential Krox20 binding sites. Together with Nab proteins, Krox20 could function as a repressor. However, our results discussed in Chapter 2 of this thesis, along with our earlier published data (198) suggest that sequences for Oct6 downregulation are present within the SCE and not in the promoter. Downregulation of Oct6 is essential for normal myelin development as constitutive overexpression of Oct6 in Schwann cells results in a permanent state of hypomyelination and axonal loss (222). This is relevant in view of the CMT1 neuropathies where hypomyelination also leads to axonal loss and subsequent limb weakness. Hence detailed knowledge of the mechanism of temporal expression of Oct6 is critical for understanding how myelination of peripheral nerves is achieved.

A major target of Oct6 in Schwann cells is Krox20 (223)(Figure 9). Gene expression profiling in Oct6 mutant animals has identified additional potential Oct6 target genes (224). These genes encode fatty acid transport protein P2, LIM domain protein CRP2 and tramdorin, a protein involved in amino acid transport that is localized to paranodes and incisures. Microarray analysis of undifferentiated versus differentiated Schwann cells *in vitro* further identified p190Rgnef to be upregulated in differentiated Schwann cells. Gene expression analysis in ΔSCE/wt versus ΔSCE/Oct6$^{\beta geo}$ confirmed the microarray results (Marko Pirsoo, Thesis, 2009).

1.7C - POU domain transcription factors Brn1 and Brn2

The observation that Oct6 null Schwann cells do eventually myelinate after a transient block indicates that there are redundant factor(s) for Oct6 to take over its function. Brn2 is another member of the class III POU family. Brn2 is expressed in a developmental profile similar to that of Oct6 (Figure 10). Brn2 expression

is increased in Oct6-deficient Schwann cells compared to wild type cells and its expression is maintained beyond its normal time of expression in Oct6 null Schwann cells. Overexpression of Brn2 in Oct6 deficient Schwann cells under the control of Oct6 SCE partially rescues the developmental defects observed in Oct6 null mice (Figure 9). However, homozygous deletion of Brn2 in the Schwann cell lineage did not affect the timing or progression of peripheral nerve development (164). In contrast, deletion of both Oct6 and Brn2 resulted in further delay in the promyelinating – myelinating transition of Schwann cells relative to that observed in single Oct6 mutants (164).

Another class III POU protein, Brn1 which is normally not expressed in Schwann cells, could substitute for the loss of Oct6 in Schwann cell differentiation. In mice where Oct6 was replaced by Brn1, Krox20, the prime target of Oct6 was efficiently induced which is a pre-requisite for normal myelination (225). Thus, class III POU proteins Brn2 and Brn1 can replace Oct6 in Schwann cell development. Interestingly, all three POU factors, Oct6, Brn2 and Brn1 have identical DNA binding characteristics which could explain their redundancy in Schwann cell differentiation.

Apart from its role as a transcriptional activator, Oct6 appears to have repressive function as well. *In vitro* experiments in primary rat Schwann cells suggested that Oct6 represses the transcription of MPZ and MBP through the joint action of its POU domain and an amino terminal domain (199,210,226). Mutation of all the potential Oct6 binding sites in the MPZ promoter did not abolish the MPZ repression suggesting that Oct6 acts indirectly, possibly through titrating out an activator complex from the MPZ or MBP promoters. These results indicated that interacting factors or adapter molecules modulate Oct6 activity in a cell type and promoter context-specific manner (209,210). It is still unclear what the physiological relevance of this repressive function of Oct6 is. Oct6 null mice did not support the repressive hypothesis of Oct6 as in those mice, since, steady state levels of MBP and MPZ were reduced instead of increased (163).

1.7D - Zinc finger transcription factors Krox20 and Krox24

The major target gene of Oct6 and Brn2 is Krox20/Egr2. It belongs to the zinc finger transcription factor family that is characterized by a highly conserved DNA binding domain consisting of three zinc finger motifs. Each motif binds to three nucleotides of a nine base pair GC rich DNA element (227). Schwann cells express Krox20 only after the induction of Oct6 and expression is thereafter maintained throughout the course of myelination and adulthood (11,197,201)(Figure 10). Following

nerve crush and Wallerian degeneration, when axonal contact is lost, Schwann cells extinguish Krox20 expression. During nerve regeneration, Krox20 reappears after Oct6 expression is induced. Thus, both in development and regeneration, Schwann cell differentiation procceds following an $Oct6^{+}/Krox20^{-}$ to $Oct6^{+}/Krox20^{+}$ to $Oct6^{-}/Krox20^{+}$ expression profile. Oct6 induces the expression of Krox20 and Krox20 in turn upregulates the expression of myelin genes (161). Another zinc finger transcription factor Krox24 binds to a DNA sequence similar to that bound by Krox20 (228). During embryogenesis, both Krox20 and Krox24 are expressed in a successive manner. Krox24 is restricted to Schwann cell precursors and Krox20 to mature Schwann cells. After a short period of overlapping expression at birth, Krox20 is present only in myelinating Schwann cells while Krox24 is only found in non-myelinating Schwann cells (Figure 10). After nerve transection, when Schwann cells go back to a proliferative state, Krox24 comes up (229). This suggests that Krox20 and Krox24, although they bind to similar DNA sequences, have antagonistic roles in the Schwann cell lineage. Binding sites for Krox20/Krox24 are found in the 5' flanking region of both genes. Therefore it is possible that they repress each other at specific stages of development (230). Krox24 is essential for the expression of nerve growth factor receptor p75, which is expressed only in non-myelinating Schwann cells (231,232).

Oct6 mutant Schwann cells are transiently arrested at the promyelin stage of differentiation, while Krox20 null or hypomorphic mutations result in a permanent block in the myelination program. In Krox20 null mice Schwann cells have established a 1:1 relation with the axons, but fail to proceed with the axon spiralization process (138,161). Krox20 is not only required for myelination, but also for the maintenance of the peripheral nerve myelination. Inactivation of Krox20 in adult Schwann cells results in severe demyelination, Schwann cell de-differentiation and increased proliferation (233). Both knock out mice analysis and microarray expression analysis have supported a role for Krox20 in the expression of myelin genes such as MPZ, PMP22, MBP, MAG, Cx32 and Periaxin (138,161,181,234) (Figure 9). Together with Sox10, Krox20 transactivates most of these genes. For example, binding of Krox20 and Sox10 to the intron associated enhancers of MPZ and MAG has been shown *in vivo* (182,235,236).

A dominant Krox20 mutation reduces the binding of Sox10 to the MPZ intron element (182). Krox20 regulates the expression of Connexin 32 (Cx32), a gene associated with X-linked CMT disease through its promoter (180). Another example is the enhancer of MBP, which contains conserved binding sites for Sox10 and Krox20 proteins (237-239). In addition to myelin genes, Krox20 appears to regulate

the HMG CoA protein involved in lipid synthesis during myelination (240). Krox20 in synergy with SREBP (Sterol regulatory element binding protein) transactivates HMG CoA. SREBP plays a pivotal role in lipid and cholesterol biosynthesis during myelination (32,240).

The important role of Krox20 in PNS myelination is also supported by the fact that Krox20 is found to be associated with several human peripheral neuropathies including different forms of CMT, DSS and congenital hypomyelinating neuropathy (138,241-246). Krox20 interacts with the co-repressor complex components Nab1 and Nab2. One of the Krox20 mutations, 1268N, found in congenital hypomyelinating neuropathy (CHN) (241) affects the Krox20-Nab interaction. Loss of Nab binding results in the defective recruitment of the NuRD chromatin remodeling complex. Like Krox20 null mutants, mice lacking Nab1 and Nab2 have Schwann cells arrested at the promyelinating stage, even though Krox20 is highly expressed in these cells (247).

Genetic studies have shown that Oct6 acts upstream of Krox20 (Figure 9). Ghislain and colleagues (223) identified two cis-acting regulatory elements for Krox20. They independently act during immature and myelinating stages of cell differentiation and function in an axon dependent manner. These two elements are termed ISE (Immature Schwann cell element), active in the immature Schwann cells and MSE (Myelinating Schwann cell element), active in the myelinating Schwann cells. The MSE is a 1.3Kb fragment located 35kb downstream of the transcriptional site of Krox20. Through bioinformatics and transgenic mice analysis, four sites for Oct6 binding were identified within the MSE. Mutation of these sites results in loss of LacZ reporter gene expression in Schwann cells. Furthermore, in transient transfection experiments, it was shown that MSE mediates the effects of Oct6 and Brn2 in cooperation with Sox10 to activate Krox20 (207). Sox10 functions through multiple, but mostly monomeric, binding sites in the MSE (248). The occupancy of these sites by Sox10 was demonstrated *in vivo* both in the S16 cell line and in myelinating sciatic nerves (182). In line with the observations of Schreiner and co-workers (184), the K2 domain of Sox10 was shown to be required for the synergistic activation of Krox20. Mice carrying a $Sox10^{\Delta K2/\Delta K2}$ hypomorphic alleles did not express Krox20, resulting in failure to express the myelin genes (184,248). Krox20 is also activated by NFATc4 (nuclear factor of activated T cells) in synergy with Sox10 through the MSE. Calcineurin, acting downstream of Nrg1 dephosphorylates NFATc4 which then translocates to the nucleus to induce Krox20 expression. Mice lacking Calcineurin B1 in neural crest cells show defects in Schwann cell differentiation and myelination (166).

In the genetic hierarchy of myelination, Oct6 is the initial transcription factor to be upregulated, which sets the myelination program into motion. However, analysis of the claw paw (*clp*) mutant mouse indicates that multiple pathways for the initiation of myelination exist in Schwann cells (249). *Clp/clp* Schwann cells express reduced level of Krox20, although Oct6 is expressed highly. Hence it could be postulated that an unknown interacting partner of Oct6 is (are) necessary for the induction of Krox20 in *clp/clp* mice.

In the following chapters, I will discuss the regulation of Oct6, its downstream targets and its interacting partners during Schwann cell differentiation.

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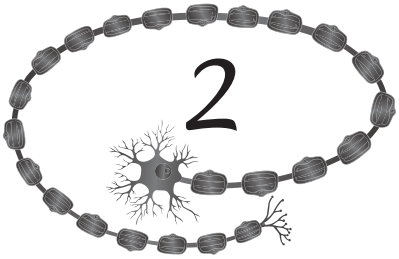

Functional dissection of the Oct6 Schwann cell enhancer reveals an essential role for dimeric Sox10 binding

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Functional dissection of the Oct6 Schwann cell enhancer reveals an essential role for dimeric Sox10 binding

Abbreviated title: Functional dissection of a Schwann cell enhancer

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Abstract

The POU domain transcription factor Pou3f1 (Oct6/Scip/Tst1) initiates the transition from ensheathing, promyelinating Schwann cells to myelinating cells. Axonal and other extra-cellular signals regulate Oct6 expression through the Oct6 Schwann cell enhancer (SCE), which is both required and sufficient to drive all aspects of Oct6 expression in Schwann cells. Thus, the Oct6 SCE is pivotal in the gene regulatory network that governs the onset of myelin formation in Schwann cells and provides a link between myelin promoting signalling and activation of a myelin related transcriptional network. In this study we define the relevant cis-acting elements within the SCE and identify the transcription factors that mediate Oct6 regulation. On the basis of phylogenetic comparisons and functional *in vivo* assays we identify a number of highly conserved core elements within the mouse SCE. We show that core element 1 is absolutely required for full enhancer function and that it contains closely-spaced inverted binding sites for Sox proteins. For the first time *in vivo*, the dimeric Sox10 binding to this element is shown to be essential for enhancer activity whereas monomeric Sox10 binding is non-functional. As Oct6 and Sox10 synergize to activate the expression of the major myelin-related transcription factor Krox20, we propose that Sox10 dependent activation of Oct6 defines a feed-forward regulatory module that serves to time and amplify the onset of myelination in the peripheral nervous system.

Introduction

Within the peripheral nervous system (PNS), Schwann cells generate and maintain a multi-lamellar insulating myelin sheath around an associated axon and impose cellular specializations that allow fast conduction of action potentials. The generation of myelinating Schwann cells from an embryonic pool of precursors and the elaboration of the myelin sheath is primarily controlled by the axons with which the cells associate (1). Axonal signals that include Neuregulin1, Notch1 ligands and neurotrophins, activate a range of signalling pathways that converge on the nucleus to initiate a program of myelin-related gene transcription (2,3).

Several Schwann cell autonomous regulators of myelination have been identified and studied over the last decades. These include the transcription factors Sox10, NfkB, Notch, Nfatc4, YY1, Srebp, Oct6, Brn2 and Krox20 (2-5). Perhaps the most intensely studied are the POU-domain transcription factor Oct6 (Pou3f1), the Sry-box protein Sox10 and the zinc finger transcription factor Krox20 (Egr2) (6-9). Whereas Sox10 is expressed at high levels at all stages of the Schwann cell lineage, both Oct6 and Krox20 are expressed dynamically in the Schwann cell lineage during development and nerve regeneration and their expression is highest in actively meylinating cells (10-13). Genetic studies have shown that deletion of Oct6 and its close relative Brn2 (a POU domain factor, also expressed in the Schwann cell lineage with kinetics similar to that of Oct6), causes severe but transient arrest at the promyelin stage of cell differentiation resulting in late onset of myelination and hypomyelinated nerves in adult animals (6,8,14). Deletion of Krox20 similarly results in a differentiation arrest at the promyelin stage but this arrest is insurmountable (9). Data showing that Oct6/Brn2 cooperate with Sox10 to upregulate Krox20 in myelinating cells through its 'myelinating Schwann cell element' (MSE) suggest a model in which Oct6/Brn2 levels serve to time the onset of myelination in response to promyelinating signals and through activation of Krox20 gene expression (2,15,16). Thus, insight into how Oct6 itself is regulated is pivotal for our understanding of regulation of myelination in the PNS.

We have previously shown that a 4,3 kb Schwann cell enhancer (SCE) sequence approximately 10 kb downstream of the Oct6 transcriptional start site is required and sufficient to drive temporally correct expression from a linked promoter in Schwann cells (17) (18). To begin to understand the nature of the signalling routes that converge on the SCE we set out to identify the functional elements within the enhancer and identify proteins that bind to these elements.

Materials and Methods

Computational analysis of the Oct6 Schwann cell enhancer

Conserved elements within the Oct6 SCE of human, mouse, rat, opossum, wallaby and zebrafish were identified using the VISTA phylogenetic alignment tool (http://pipeline.lbl.gov/cgi-bin/gateway2) (19). All sequences were aligned to the mouse sequence. The homology cut off standard is 50% but was adjusted to 90% for the rat sequence because of the close evolutionary relationship between mouse and rat. Likewise, the homology cut off was adjusted to 30 percent when mouse sequences were aligned to opossum, wallaby and zebrafish sequences. Alignments were optimized manually. Aligned sequences were further analyzed for the presence of putative transcription factor DNA binding sites using ConSite: a web-based tool for finding cis-regulatory elements in genomic sequences (20) (http://www.phylofoot.org/consite).

Cell culture, transient transfection and nuclear extract preparation

Primary Rat Schwann cell cultures were established as described previously (17,21). Rat Schwann cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza), 3% fetal calf serum (FCS; Harlan), 2μM Forskolin (Sigma), 1% Penicillin and Streptomycin (Invitrogen) and 5% NDF-β conditioned medium (22) at 37°C with 5% CO_2. Schwann cells were differentiated *in vitro* towards an early myelinating stage (23), a stage at which they express relatively high levels of Oct6. First, medium was switched to a serum free formulation of DMEM/F12 (Lonza), 1X N2 supplement (Invitrogen), 10ng/ml NGF (Harlan), 0.01%BSA (Invitrogen), 5% NDF-β conditioned medium and 1%PS for 18 hours. Cells acquire a spindle shaped form under serum free conditions. Then, differentiation was initiated by addition of 20μM Forskolin (Sigma) or 100 μM CTP-cAMP (Sigma). Cells were allowed to differentiate for 36 hours before further analysis. Differentiated cells have flattened cell morphology and express high levels of Oct6.

RT4-D6P2T rat Schwannoma cells (24,25) were obtained from ECACC and were maintained in DMEM, 10% FCS and 1% PS. For nuclear extract preparation RT4-D6P2T cells were grown in 15cm dishes to near confluence. When confluent, cells were washed once with PBS, scraped in cold PBS and nuclear extracts were prepared essentially according to Dignam and colleagues (26). Nuclei were extracted in 20 mM Hepes-KOH pH7.6, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% NP-40, 0.5 mM DTT and 1 x complete protease inhibitor mix (Roche). The salt

concentration of the extract was reduced by dialysis against a buffer containing 20 mM Hepes-KOH pH7.6, 25% glycerol, 100 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 95 µg/ml sodium metabisulfite. Precipitates were removed by centrifugation two times at 13,000 rpm for 15 minutes, at 4°C. The dialyzed nuclear extract was snap-frozen and stored in aliquots at -80°C.

HEK293T cells were maintained in DMEM, 10%FCS and 1% PS. HEK293T Cells were transfected with 20µg pCMV promoter driven full length Sox10 or MIC Sox10 (kind gift from Prof. Michael Wegner) expression cassettes in 10 cm dishes using the polyethylenimine (PEI) method. Cells were harvested 48 hours post-transfection for nuclear extract preparation (14).

Luciferase assay

Rat Schwann cells were seeded in 6 well Primaria dishes (Becton Dickinson) and grown to 70-80% confluence. Cells were transfected in triplicate with 1.125µg pGL3 luciferase reporter plasmid (Promega) containing different SCE fragments and 0.375µg pCMV-βgal reference expression plasmid in the presence of FCS, using FUGENE6 transfection reagent (Roche) at a 1:4 (DNA:FUGENE6) ratio. Cells were washed 18 hours post transfection, and medium was switched to a serum free formulation (DMEM/F12 (Lonza), 1X N2 supplement (Invitrogen), 10ng/ml NGF (Harlan), 0.01%BSA (Invitrogen), 5% NDF-β conditioned medium and 1%PS). After overnight incubation in this medium, cells were induced to differentiate through addition of CTP-cAMP to a final concentration of 100µM, and cultured for an additional 36 hours. Cells were harvested 72 hours post transfection, washed and lysed in 1X lysis buffer (Promega) and extracts were assayed for luciferase activity using Steady GLO luciferase substrate buffer (Promega) and β-galactosidase activity using 2-nitro-phenyl-galacto-pyranoside (ONPG) substrate. Luciferase activities were normalized for β-galactosidase activity. Both luciferase and β-galactosidase levels were measured on a luminometer (Perkin Elmer – Victor3). All the experiments were performed at least three times in triplicate.

DNA cloning and SCE deletion constructs

The mouse SCE was originally cloned as an HpaI-MscI DNA fragment from a mouse 129sv cosmid library (Mandemakers et al., 2000). This fragment, which corresponds to position 124,342,041-124,349,427 on mouse chromosome 4 of genome build37 (NCBIM37), was used to generate, by PCR, a series of 10 deletion constructs in which

a shifting window of approximately 500 basepairs is deleted from the entire SCE. SalI and BglII restriction sites were introduced at the 5' and 3' end respectively and these sites were used to clone the fragments into the SV40 promotor driven pGL3 luciferase reporter plasmid. Subfragments from the SCE such as HR1a and HR2 were generated by PCR using the HpaI-MscI SCE fragment as template and cloned into luciferase and LacZ reporter plasmids (Mandemakers et al., 2000). Fragment HR1a corresponds to position 124,344,894-124,345,418 and HR2 to position 124,347,550-124,348,429 on mouse chromosome 4 (NCBIM37).

Transgenic mice and whole mount β-galactosidase staining

Transgenesis and identification of transgenic embryos by PCR and southern blotting was performed as described previously (17). LacZ expression was visualized by whole-mount X-gal staining of P2-P4 pups or nerves dissected from transgenic animals at different stages of postnatal development. In brief, young mice (2-4 days of age and of either sex) were anaesthetized and sacrificed by decapitation. The body cavity was opened and the major organs were removed. All of the tissue was transferred to a solution of fixative containing 1% formaldehyde, 0.2% Glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA, 0.02% NP40 in PBS, for 1 hour at room temperature (RT). Tissues were washed in PBS/0.02% NP40 for 3 times at RT. β-galactosidase staining was carried overnight at RT in staining solution containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_20$, 2mM $MgCl_2$, 0.01% Sodium deoxycholate, 0.02% NP40 and 1mg/ml 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal). The staining was stopped by extensively washing in PBS/0.02% NP40 and post fixed in a 4% formaldehyde fixative.
All animal experiments were performed according to guidelines and protocols that had been approved by an independent committee on the ethical use of experimental animals (DEC).

Electrophoretic mobility shift assay (EMSA)

Equal amounts of nuclear extract were used in the EMSA using 50 fmole of a ^{32}P end-labeled double-strand oligonucleotide probe. Probe and nuclear extracts were incubated on ice, for 30 minutes in 10mM HEPES-KOH pH 7.9, 100 mM KCl, 5mM $MgCl_2$, 0.1% EDTA, 2mM DTT, 4μg BSA and 4% Ficoll in the presence of 2μg of competitor DNA, poly dG-dC (Sigma). Total reaction volume was 20μl. In super shift experiments, reaction mixtures were further incubated for 20 minutes with 1μg of Goat-Sox10 antibody (Santacruz). Complexed and free probe were separated on a

4% polyacrylamide gel which was pre-run for 30 minutes at 120 volts in 0.5X TBE electrophoresis buffer at RT. Gels were fixed in 10% methanol/10% acetic acid for 20 minutes, dried and exposed to a Phospho-imaging screen (Molecular dynamics). ImageJ software was used to analyze the band intensities. Probes used in the band shift assays are shown in Figure 5.

Chromatin immunoprecipitations

For Chromatin immunoprecipitation (ChIP) assays in RT4-D6P2T cells ($20x10^6$ cells/IP), dual cross-linking was performed using di(N-succimidyl)glutarate (DSG) and formaldehyde (27). Cells were washed four times with PBS, followed by incubating the cells with cross linking agent, 2mM DSG on a rotating platform at a medium speed for 45 minutes at RT. After washing the cells four times with PBS, conventional formaldehyde cross linking was performed by incubating the cells in freshly prepared buffered formaldehyde (50mM Hepes KOH pH 7.5, 100mM Nacl, 1mM EDTA, 0.5mM EGTA, 1% formaldehyde) for 10 minutes. 2.5M of Glycine was added to a final concentration of 100mM to quench the formaldehyde and cells were further incubated for 5 minutes at RT. From here on all the subsequent steps were carried at 4°C in the presence of complete EDTA free protease inhibitor (Roche). Cells were scraped in cold PBS and spun at 2000 rpm for 5 minutes. Cell pellet was resuspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0) and subsequently sonicated for 15 cycles with 15-s on and 30-s off regime. The sheared chromatin was cleared of insoluble debris by centrifugation at 13000 rpm for 10 minutes. The supernatant containing the sheared chromatin was pre-cleared with protein A Agarose/Salmon sperm DNA (Millipore) for one hour at 4°C. 1% chromatin was set aside to serve as a $1/100^{th}$ of total chromatin input control. After centrifugation, the supernatant was incubated with 10 µg of anti-Sox10 antibody (Abcam) or 10 µg of normal rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology) control antibody at 4°C on a rotating wheel overnight. Immune complexes were incubated with 60 µl of protein A Agarose/Salmon sperm DNA slurry for one hour at 4°C. After centrifugation, the immune complexes were washed once with 1 ml of low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Hcl pH 8.0, 150mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Hcl pH 8.0, 500mM Nacl), once with Lithium chloride wash buffer (0.25M LiCl, 1% NP-40, 1% deoxycholic acid(sodium salt), 1mM EDTA, 10mM Tris pH 8.0), and twice with TE buffer (10mM Tris pH8.0, 1mM EDTA) (all the wash buffers contain protease inhibitor). Immunoprecipitated chromatin was eluted twice each time

with 250 µl of elution buffer (1% SDS, 0.1M $NaHCO_3$). The elutions were pooled and 5M NaCl was added to a final concentration of 300mM. Further, cross-links were reversed by incubating the sample at 65°C for 5 hours. The solution was adjusted to 10mM EDTA and 10mM Tris-Hcl pH 6.5. ProteinaseK (10mg/ml) was added to 40mg/ml and the solution was incubated for one hour at 45°C. DNA was purified with phenol:chlorform:isoamyl alcohol, precipitated with EtOH and dissolved in water. The same reverse cross-linking and purification procedure was followed for the input sample. Comparative Ct values (28) were used to calculate the enrichment of a DNA segment relative to the total input. These values were used to calculate the relative enrichment of Sox10 antibody over rabbit IgG precipitated chromatin for the c1, -13kb and IgG2a promoter regions. Primer sets used in quantitative PCR for ChIP are as follows: C1 of SCE: 5'-GCC CTG AGG ACT CTA GCT CT-3'and 5'-GCA GGA CAA TAG CTG CAT TC-3', -13kb: 5'-AAT CAC GTC CTC ACC AAA CC-3' and 5'-TCC GGT TAG CTC GAA TCC TA-3', IgG2aP: 5'-GAA ATT CTG CCC TGC ACT TCC-3' and 5'-GCT TTG CAT TGA GGG AGG ATC-3'

In vivo formaldehyde assisted identification of regulatory elements (FAIRE)

Freshly isolated P14 rat sciatic nerves were minced in phosphate buffered saline (PBS) with 1% formaldehyde for 5 minute at RT. The nerves were washed with cold PBS and frozen at -80°C. Following thawing, the nerves were analyzed by Faire as previously described (29,30). A custom microarray (Nimblegen Roche) consisting of 50kb Oct6 rat gene locus was used for identification of regions enriched by FAIRE. Labeling of the FAIRE with Cy5 and total input with Cy3 followed by micro-array hybridization was performed as described (31) by Nimblegen. The enrichment ratio of Cy5 to Cy3 was plotted on a Log_2 scale and further processed to display a moving average using a window size of 5 probes. All raw data sets for the custom tiled array are available from the NCBI Gene Expression Omnibus website: accession number GSE23648.

Results

The Oct6 SCE contains two functionally interdependent conserved modules

Previously, DNaseI hypersensitivity mapping and alignment of the genomes of human, mouse and rat using the VISTA alignment tool presented here, localized several possible Oct6 regulatory regions including the SCE (Figure 1A). Among the

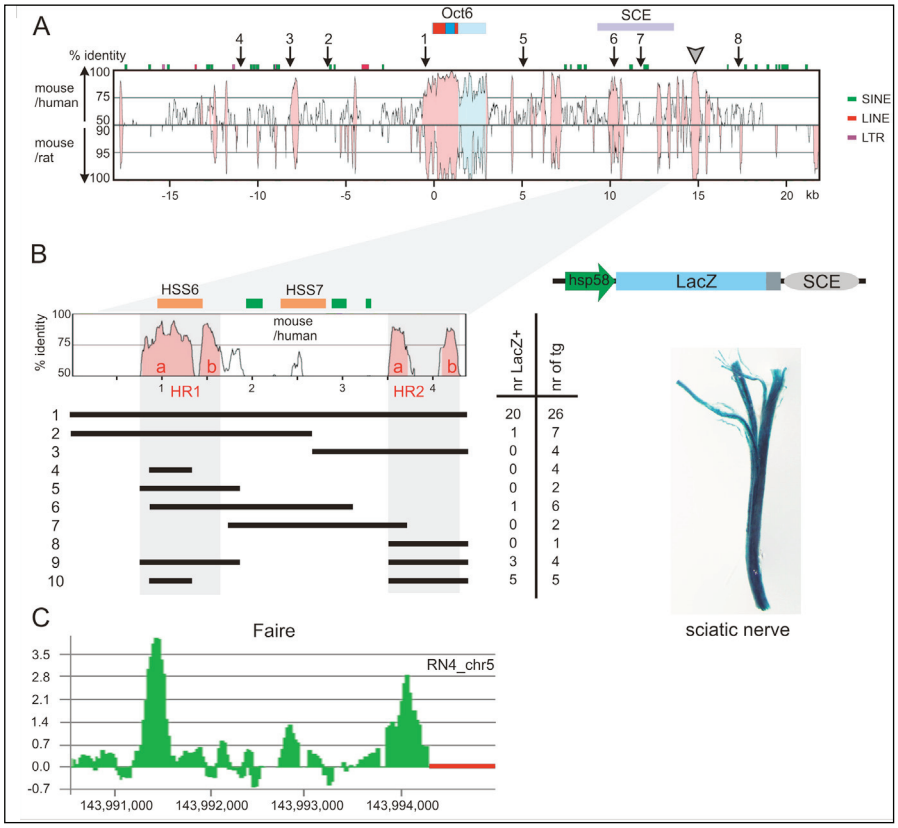

Figure 1 - Comparative genomic and functional analysis of the mouse Oct6 locus.
(*A*) Vista alignment of the mouse, rat and human Oct6 locus spanning approximately 50kb. The position of the Oct6 transcription unit and the SCE are indicated above the plot. The Oct6 open reading frame is in red with the POU domain in dark blue and the 3' untranslated region in light blue. The grey arrowhead marks the position of the ultra-conserved sequence and numbered arrows the position of the previously mapped DNaseI hypersensitive sites (17). Repeat sequences present in the mouse genome are indicated. Note that the homology threshold has been adjusted to 90% for the rat sequence.
(*B*) The enlarged section of the SCE region shows the four regions of strong homology HR1a and b and HR2a and b between mouse and human sequences. The approximate position of HSS6 and 7 as well as the short repeat sequences (in green) are indicated. The black lines represent the segments of the SCE that were cloned behind the hsp68LacZ reporter construct. Individual constructs were tested for activity in transgenic mice and the number of transgenic founders (nr of tg) and those that express the hsp68LacZ in the peripheral nerves (nr LacZ$^+$) are indicated. A dissected sciatic nerve of a P2 mouse transgenic for construct 9 is shown as a typical example.
(*C*) The FAIRE plot of the rat Oct6 SCE is shown at the same scale as the Vista plot in *B* to allow a direct visual comparison of the two plots. The coordinates of the rat genome sequence (RN4) are indicated. The red bar indicates sequences not represented on the custom made micro array used in this experiment.

strongly conserved regions within the Oct6 locus, only three regions (at –8, –0.5 and +10.5kb) correspond with previously identified DNaseI hypersensitive sites (HSS: Figure 1A; numbered arrows). The conserved peak at -4.8kb corresponds with an estradiol responsive enhancer characterized by Renner and colleagues (32). The large conserved region containing the Oct6 gene contains the promoter proximal region at -0.5kb which confers Interferon-b responsiveness and is important for the regulated expression of Oct6 in macrophages (33). An ultraconserved element flanks the 3' end of the locus (Figure 1A; arrowhead) at approximately +15kb (34) and is downstream of the SCE. All of these elements are dispensable for Oct6 expression *in vivo* in the Schwann cell lineage. The relevance of the other conserved elements, outside the boundaries of the SCE, for the regulated expression of Oct6 in cells other than Schwann cells is not known.

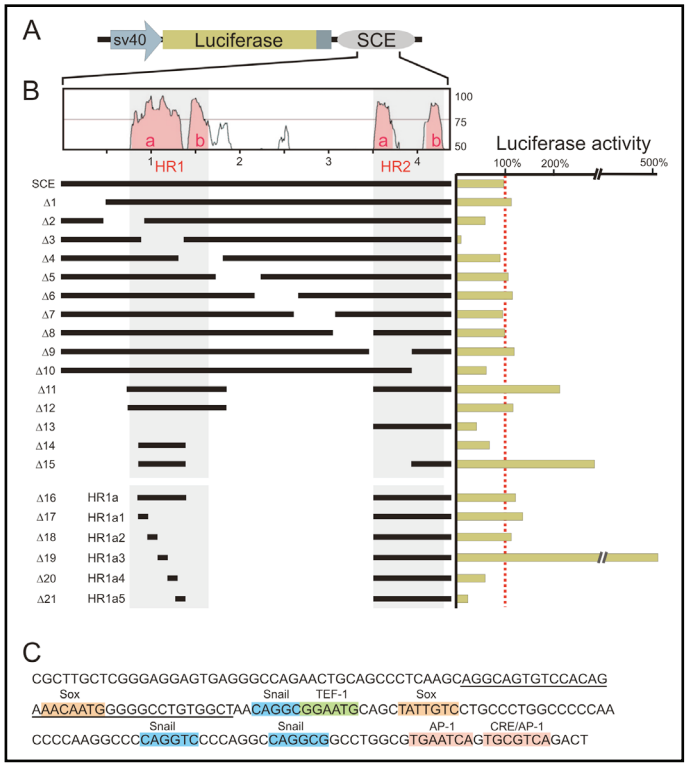

Figure 2 - Dissection of Oct6 SCE functional elements in differentiating Schwann cell cultures. (*A*) SCE constructs were cloned behind the minimal SV40 promoter-driven Luciferase cassette (pGL3) and transfected in rat Schwann cells. (*B*) The SCE constructs tested are represented with a black line and aligned with the Vista plot. Luciferase activity of each construct is expressed as a percentage of the activity of the full SCE in these experiments. All transfections were performed in triplicate. (*C*) The sequence of HR1a3, which has strong enhancer activity in the luciferase assay, shows potential binding sites for transcription factors known to be involved in Schwann cell biology. These potential binding sequences were identified using the phylogenetic footprinting program ConSite. The sequence of the c2 element, containing the consensus Sox binding site, is underlined.

Within the defined boundaries of the SCE there are two pairs of conserved elements, which we termed HR1a and HR1b (at +10 to 10.8kb) and HR2a and HR2b (at +13 to13.7kb). The HR1 region corresponds to DNaseI hypersensitive site 6, whereas HSS7 maps to a marginally conserved region between HR1 and HR2. To test the relative contributions of conserved elements within the enhancer to Schwann cell specific expression in transgenic mice we generated a number of constructs that were linked to the generic minimal promoter of the Hsp68 gene driving expression of the LacZ gene (Figure 1B). These constructs were analysed for Schwann cell specific expression of the LacZ reporter in the sciatic nerve of F_0 transgenic animals at postnatal day 2 to 4 by whole mount X-gal staining. The blue sciatic nerve in Figure 1B is a typical example of such analysis. Confirming our previous results (17), the full 4.3 kb SCE construct confers Schwann cell specific expression in transgenic animals with high frequency (construct 1 Figure 1B; 20 out 26 transgenic animals), whereas the 5' region (construct 2; 1 out of 7) and 3' region (construct 3; 0 out of 4) alone did not drive expression in Schwann cells at an appreciable frequency. However, these constructs abutted in the HSS7 region leaving open the possibility that in both these constructs essential genetic information was disrupted. To examine this possibility 5 constructs (construct 4 to 8) spanning the width of the SCE were generated. Only one (construct 6) of these constructs conferred Schwann cell expression on the hsp68 promoter in transgenic mice although again at a low frequency (1 out of 6 transgenic animals). We then combined DNA fragments covering HR1 or HR1a and HR2 in one construct (construct 9 and 10 respectively) and found that both fragments are required to confer high frequency of reporter gene expression in Schwann cells of transgenic mice. Furthermore, these data show that HR1 can be reduced to HR1a without loss of activity.

Active enhancer elements are associated with a relatively open chromatin configuration, devoid of nucleosomes, and these can be probed using FAIRE (formaldehyde assisted isolation of regulatory elements (35). Using this assay on chromatin derived from two week old rat sciatic nerve, strong peaks at both HR1a and HR2 were found, in line with our functional transgenic expression results. Thus, both regions are associated with a relatively open chromatin structure and are interdependently required for formation of an active enhancer in Schwann cells.

Deletion analysis of the SCE in cultured Schwann cells

To more finely delineate the essential elements within the Schwann cell enhancer an

in vitro rat Schwann cell culture system was used, since it is rapid and inexpensive compared to transgenic mouse experimentation. Primary rat Schwann cells can be cultured indefinitely (21,36) and, when exposed to agents that elevate intracellular cAMP levels, can be induced to differentiate into a cell that resembles an early myelinating Schwann cell. Under these conditions endogenous Oct6 expression is induced within three hours reaching maximum levels after 24 hours (37). We generated a series of deletion constructs (Δ1 to Δ10) covering the entire SCE (and cloned these in a SV40 promoter driven luciferase vector; Figure 2A and 2B). These constructs were co-transfected with a CMV promoter driven β-galactosidase reference reporter into rat Schwann cells. Cells were cultured for 18 hours in serum-free medium, switched to differentiation medium for another 36 hrs and measured for luciferase and β-galactosidase activity. All luciferase values were normalized for β-galactosidase activity. The activity of the entire enhancer was set to 100% (Figure 2B) and activity of the various SCE deletions is reported as percentage of the full SCE activity. From this deletion screen it is evident that a major enhancing activity is associated with the region covered by Δ3 and, to lesser extent, Δ2 and Δ10. These regions coincide with the HR1 and HR2 conserved regions.

Next we tested whether the HR1 and HR2 regions, alone or in combination, would enhance gene expression in transfected Schwann cells (Figure 2B; constructs Δ11, Δ12 and Δ13). In contrast with our transgenic data, we found that HR1 (Δ12) is as active as the full enhancer although this activity is further enhanced by the presence of HR2 (as in Δ11). HR2 by itself is less active than the full enhancer. Further restriction of HR1 to HR1a as in construct Δ14 and Δ15 suggested that the major enhancer activity of HR1 is located within HR1a, but that this activity is boosted by the presence of HR2 sequences.

To identify the major functional elements within the HR1a region we tested a series of smaller overlapping fragments HR1a1 through HR1a5 (Figure 2B; constructs Δ16 to Δ21). Whereas Δ17 and Δ18 gave full enhancer activity as compared to control Δ16 (HR1a), Δ19 further enhanced activity by 5-fold. Constructs Δ20 and Δ21 showed reduced activity. Interestingly, the HR1a3 sequence associated with very high enhancer activity contains several consensus binding sites for transcription factors known to be involved in Schwann or neural crest cell biology (Figure 2C). These include binding sites for Sox, Snail and Creb/AP-1 proteins, indicating a likely important role for these proteins in mediating the enhancer effect in differentiating Schwann cells.

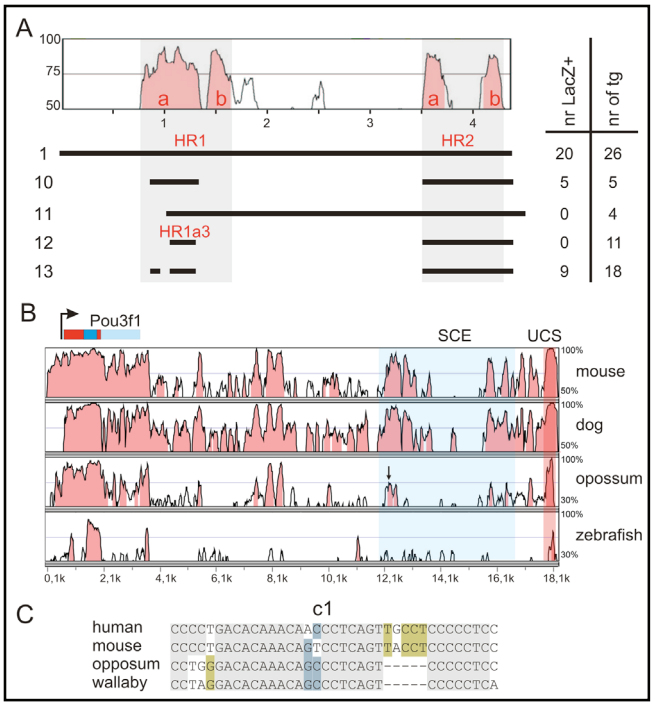

Figure 3 - Functional analysis of SCE elements in transgenic mice and comparative genomics.

(A) Schematic depiction of the SCE constructs tested in transgenic mice. The SCE constructs are represented by black lines aligned with the Vista plot of the mouse SCE to visualize the presence or absence of conserved sequences in the various constructs. The total number of transgenic animals (nr of tg) and the number of animals that express the transgene in neonatal peripheral nerves (nr LacZ$^+$) is indicated. All animals were analysed as founders. (*B*) Vista alignment of human SCE sequence with mouse, dog (*Canis familiaris*), opossum (*Monodelphis domestica*) and zebrafish (*Danio rerio*). Note that the lower homology threshold has been set to 30% for opossum and zebrafish to allow for the visualization of more diverged homologous regions. The position of core element c1 in the Vista plot of opossum is indicated by an arrow. (*C*) Alignment of core element c1 in the SCE of human, mouse, opossum and wallaby (*Macropus eugenii*). Hundred percent homologous nucleotides are boxed in black, 75% homology in light blue and 50% homology in ochre.

A 35 bp core element is essential for Oct6 activation in Schwann cells in vivo.

The activity of HR1a3 in combination with HR2 was tested in transgenic mice. Surprisingly, we found that HR1a3 has no activity *in vivo* (Figure 3A; construct 12). No Schwann cells were found to express LacZ in any of the 11 transgenics tested. Moreover, an additional construct that covers all of HR1a3 and extends to HR2 and 3' beyond the boundaries of HR2 (construct 11) also did not show activity *in vivo*. These results are in stark contrast with the activity of construct 10, which contains 175bp of extra sequence 5' to HR1a3 (see Figure 1B), and demonstrate that an essential element is missing in constructs 11 and 12.

To identify the missing element(s) we extended our phylogenetic analysis of the SCE to include the genomes of the Opossum (a non-placental mammal) and the Zebrafish.

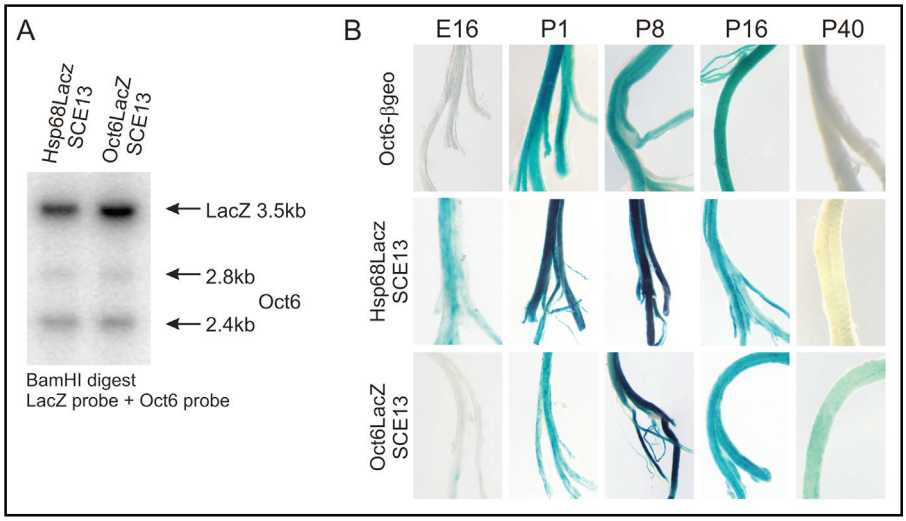

Figure 4 - Developmental control of Schwann cell specific expression through SCE13. (A) Southern blot of genomic DNA of the two transgenic lines analysed here shows that they carry a comparable number of transgenes. The transgene is detected with a LacZ probe that hybridizes to a 3.5kb BamHI restriction fragment derived from the transgenic construct. The Oct6 locus is detected with a probe that hybridizes with two fragments of 2.8 and 2.4kb in length. The equal intensity of these endogenous band shows that equal amounts of DNA were loaded in both lanes. (B) Transgenic animals carrying a LacZ reporter gene driven by either the Oct6 promoter or the Hsp68 promoter and controlled by SCE construct 13, Oct6LacZSCE13 and Hsp68LacZSCE13 respectively, were stained for β-galactosidase activity in Schwann cells of the sciatic nerve at different stages of pre- and postnatal development. Developmental expression of β-galactosidase activity in nerves of the two transgenic lines was compared to the β-galactosidase activity in Oct6 β-galactosidase-neomycin (Oct6-bgeo) heterozygous knock in animals (8,17).

As is evident in Figure 3B, homology among the orthologous sequences rapidly breaks down when more distantly related species are included. Note that the threshold for the genomes of Opossum and Zebrafish has been lowered to 30%. However, when we manually aligned the sequences abutted by the 5' border of construct 10 and 11, one short element of approximately 35 basepairs was found strongly conserved in mammals and marsupials (Figure 3C). We refer to this short conserved element as core 1 or c1.

To test whether this c1 sequence represents an essential element of the SCE, we cloned the sequence in front of HR1a3 of construct 12 to generate construct 13. Indeed, this construct drives Schwann cell specific expression of the reporter in 9 out of 18 transgenic mice (Figure 3A), demonstrating that this short element contains genetic information that is essential for Schwann cell specific enhancer function *in vivo*.

Temporal control of gene expression through the SCE is promoter independent

Previously, our experiments showing that the Oct6 SCE drives temporally correct expression of a reporter gene in the Schwann cell lineage, including its downregulation in fully differentiated cells, were performed with the Oct6 promoter driving reporter gene expression (17). The use of the Oct6 promoter leaves open the possibility that the observed down regulation of the reporter gene was mediated through Oct6 promoter sequences and/or the SCE. Now, to test whether SCE construct 13 contains all the information to drive regulated expression and does so irrespective of the linked promoter, transgenic lines (two lines for each construct) were generated in which the LacZ reporter is driven by the Oct6 promoter or the Hsp68 promoter. We choose one line for each construct with comparable copy-number for further analysis (Figure 4A). LacZ reporter gene expression in the sciatic nerve of transgenic mice was monitored at different stages of development and compared with LacZ expression in nerves of mice carrying a LacZ-neo fusion ($^{\beta geo}$) gene knocked in the Oct6 locus (8). As shown in Figure 4B, SCE construct 13 drives temporal expression from both the Oct6 and Hsp68 promoter in a pattern that largely follows that of the endogenous Oct6 gene (Oct6$^{\beta geo}$). Both lines show upregulation of reporter gene expression at later stages of embryonic development (E16 to first day of postnatal life) and expression is downregulated after the first week of postnatal development and extinguished at later stages. However, expression of Hsp68LacZSCE13 is initiated slightly ahead of Oct6$^{\beta geo}$, but is extinguished correctly, whereas Oct6LacZSCE13 expression is initiated correctly but is extinguished with slower kinetics. As we analysed only one line for each construct we do not know whether these marginal deviations in expression are caused by chromosomal integration position effects or whether they reflect differences between the Oct6 and Hsp68 promoter. Nevertheless, it is clear that SCE construct 13 contains all elements to respond to the transcription factor environment of differentiating Schwann cells and the signalling pathways that regulates their activities and that no Oct6 promoter specific sequences are required for downregulating Oct6 expression in mature myelin forming cells.

Core element 1 contains an atypical dimeric binding site for Sox10

How does core element 1 (c1) contribute to Schwann cell specific enhancer function of the SCE? Using online bioinformatic tools we identified potential binding sites within this 35bp sequence for several classes of transcription factors including Sox

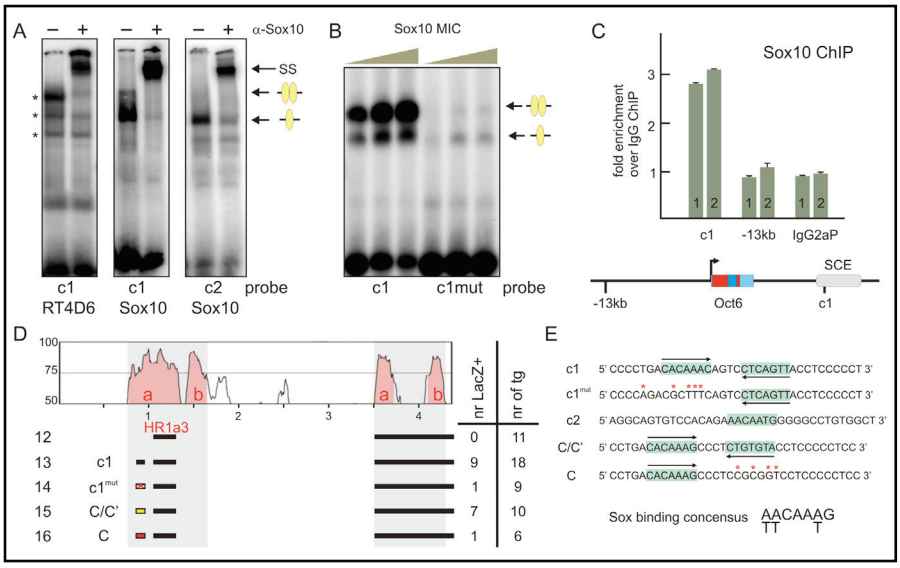

Figure 5 - Biochemical and functional analysis of core element c1.
(*A*) EMSA with radiolabelled doublestranded oligonucleotides c1 and c2. The sequence of these probes is depicted in panel E. Oligonucleotide probes were incubated with nuclear extracts from RT4-D6P2T cells or from HEK293 cells over-expressing Sox10 (labelled Sox10), in the presence (+) or absence (-) of Sox10 antibodies, as indicated. The three major DNA-protein complexes formed on c1 with RT4-D6P2T nuclear proteins are marked with an asterisk. The Sox10 monomeric and dimeric complexes are indicated with arrows. (*B*) EMSA with radiolabelled doublestranded oligonucleotide c1 and c1^{mut}. Oligonucleotide probes were incubated with increasing amounts of nuclear extracts from HEK293 cells expressing Sox10 MIC. (*C*) ChIP of Oct6 SCE sequences using Sox10 antibodies. Chromatin of RT4-D6P2T cells was precipitated with Sox10 or rabbit IgG antibodies. The non-specific rabbit IgG antibodies served as a control. Quantitative PCR on immune-precipitated and purified DNA using primers for the c1 region (c1), a region 13kb upstream of the Oct6 promoter (-13kb) and a region associated with the IgG2a promoter (IgG2aP) was performed in triplo and was used to determine the relative enrichment for these loci by Sox10 over IgG immune-precipitation. The experiment was performed twice. Data from both experiments are presented (1 and 2). (*D*) Functional analysis of Sox binding sites in element c1. The *in vivo* activity of the various SCE constructs was assessed in new born founder animals. The number of transgenic animals (nr of tg) and the number of animals that express LacZ in the peripheral nerves (nr LacZ^{+}) is indicated. Constructs 14, 15 and 16 were created from construct 13 by replacing the c1 sequence for that of c1^{mut}, C/C', and C respectively. (*E*) Sequence of c1 and related elements and its various mutant derivatives. Red asterisks indicate the nucleotides mutated in c1^{mut}. The position and orientation of the Sox10 binding sites is indicated by green boxes and arrows. The C/C' and C sequence is derived from the *Mpz* promoter (55). Differences between C/C' and C are indicated with red asterisks. The consensus Sox DNA binding motif was derived by Harley and colleagues (38).

proteins. Sox10, Sox2, Sox13 and Sox4 (10); and data not shown) are expressed in immature Schwann cells. Whereas all of these proteins bind to the consensus Sox binding site (A/T)(A/T)CAA(A/T)G, as found in element HR1a3, only Sox10 and Sox13 (not shown) bind to the c1 element in electrophoretic mobility shift assays (EMSA) (Figure 5A; (38). We examined the formation of protein-DNA complexes on the c1 probe using rat Schwannoma RT4-D6P2T nuclear extracts and nuclear extracts of HEK293 cells transfected with a Sox10 expression cassette (Sox10 in Figure 5A). RT4-D6P2T cells resemble an early myelinating cell as it expresses considerable amounts of Oct6, Krox20, Sox10 and low amounts of Mpz (39,40); and unpublished observations) and thus provide an excellent model system to study promyelin-related transcription. Three major complexes were observed (indicated with asterisks in Figure 5A) of which the slowest migrating complex contains Sox10 as evidenced by the further decrease in electrophoretic mobility of this complex after addition of Sox10 specific antibodies (Figure 5A). Interestingly, the Sox10 containing complex on the c1 probe has a lower mobility than the Sox10 complex formed on the c2 probe (Figure 5A) suggesting that Sox10 binds to c1 as a homo- or heterodimer. The sequence of the c2 probe (Figure 5E) is derived from fragment HR1a3 and contains a consensus Sox binding site (underlined in Figure 2C). Homodimeric binding of Sox10 has been described before and depends on the presence of two closely spaced heptameric Sox binding sites in an inverted orientation (41). Examination of the c1 sequence revealed the presence of a degenerate Sox binding site (AACTGAG) in an opposite orientation and 4 basepair spacing with the degenerate CACAAAC Sox binding site (Figure 5E). To confirm that Sox10 binds to c1 as a dimer we performed EMSA (Figure 5B) with nuclear extracts of HEK293 cells transfected with an expression construct encoding a truncated form of Sox10 (Sox10 MIC; aminoacid 1-189 including the HMG-box). It has been shown before that this shorter form of Sox10 binds predominantly as a dimer on closely spaced inverted Sox binding sites such as present in the Mpz promoter (C/C' Figure 5E; see (41). Indeed, Sox10MIC binds as a dimer to c1 and this binding is abolished when the 5' Sox site is mutated ($c1^{mut}$). Thus, the two degenerate Sox binding sites bind Sox10 strongly as a dimer whereas the individual 3' Sox site has very little affinity for Sox10.

Next we examined whether indeed Sox10 binds to the c1 SCE motif in chromatin of rat Schwannoma RT4-D6P2T cells. We performed chromatin immune-precipitation assay (CHIP) using an antibody against Sox10 and control rabbit IgGs. We found c1 SCE sequences precipitated with three-fold higher efficiency with Sox10 antibodies versus control IgG antibodies. Two control sequences, a -13kb sequence upstream of Oct6 and the IgG2a promoter showed no enrichment for Sox10 and

were precipitated with equal efficiency by Sox10 and IgG antibodies (Figure 5C). Thus, Sox10 occupies c1 SCE chromatin in RT4-D6P2T cells.

To test whether this dimeric Sox10 binding contributes to SCE function *in vivo* we generated a number of reporter constructs and tested their activity in transgenic animals. All these constructs are based on construct 12 and include HR1a3 and HR2. First we tested whether mutation of the 5' degenerate Sox site had any effect on the contribution of c1 to SCE activity. Mutating 5 nucleotides, including the particularly important central CAA motif, as in $c1^{mut}$ (Figure 5E) results in loss of Sox10 binding (Figure 5B). Replacing the c1 sequence in construct 13 for the $c1^{mut}$ sequence, to generate construct 14, results in a strong reduction of the number of transgenic mice expressing the LacZ reporter in Schwann cells of the sciatic nerve, from 9 out of 18 to 1 out of 9 (Figure 5D). Thus, Sox10 binding to c1 is an essential component of the fully active SCE.

As $c1^{mut}$ completely abolishes Sox10 binding we could not determine whether dimeric Sox10 binding per se, or perhaps monomeric Sox10 binding, would suffice for SCE function. To answer these questions we tested two additional constructs. In the first construct tested, construct 15, we replaced the c1 sequence with that of the well documented C/C' dimeric Sox10 binding site from the Mpz promoter (41). In the second construct tested, construct 16, we replaced the c1 sequence for the C/C' derivative C, to which Sox10 binds exclusively as a monomer (sequence is shown in Figure 5E). As is evident from the numbers presented in Figure 5D, the Mpz promoter derived C/C' Sox binding site is as effective as the original c1 sequence in supporting Schwann cell specific expression (construct 15; 7 out of 10). However, the monomeric Sox site C is not an effective substitute for c1 (Figure 5D construct 16; 1 out of 6). Thus, these experiments show that it is dimeric binding of Sox10 to the c1 sequence and not just Sox10 binding per se that provides an essential function in Schwann cell specific regulation through the Oct6 SCE.

Discussion

We have combined comparative genomics and transgenic analysis to identify regulatory regions within the Oct6 SCE that are required to drive Schwann cell specific expression in transgenic mice. We demonstrate that a 35 bp fragment called c1 is an essential element of the SCE and contains two degenerate Sox protein binding sites in an inverted orientation. Sox10 protein binds to this element as a dimer and we demonstrate that its *in vivo* activity critically depends on dimeric binding of Sox10, as enhancer activity is lost in DNA mutants that bind Sox10 as a

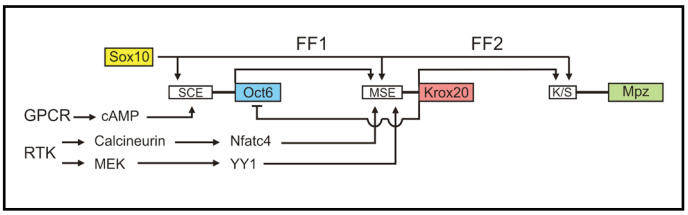

Figure 6 - The figure provides a schematic representation of the gene regulatory network that governs the transition from a premyelinating Schwann cell to a myelinating cell and incorporates findings described in this study.
Different signalling pathways converge on the regulatory elements in this network and cooperate with Sox10 to control Oct6, Krox20 and myelin gene expression (Svaren and Meijer, 2008). The Sox10 dependent activation of Oct6 and subsequent synergistic activation of Krox20 defines a feed-forward (FF) regulatory module. Sox10 then cooperates with Krox20 to control expression of myelin related genes, through closely spaced Krox/Sox (K/S) binding elements, defining a second feed-forward loop. These FF loops serve to time and amplify the onset of myelination in the PNS. For further details see text.

monomer. Thus, dimeric Sox10 binding provides a crucial function within the larger context of the Oct6 SCE including HR1a and HR2, allowing the formation of a productive enhanceosome in response to developmental cues.

Phylogenetic comparison of genome sequences is a powerful tool to identify putative gene regulatory sequences. We initially analyzed human, mouse and rat sequences of approximately 50kb surrounding the Oct6 gene and identified several conserved DNA elements surrounding the Oct6 structural gene (Figure 1A). Within the boundaries of the SCE two pairs of highly conserved blocks could be identified. Inclusion of non-placental mammals and fish in our analysis however shows that the high degree of homology is lost over larger evolutionary distances (Figure 3). This rapid decline in overall sequence conservation down the phylogenetic ladder is remarkable and suggests that the core elements of the enhancer are relatively insensitive to the composition and sequence of the DNA in which they are embedded. In line with this observation we found that the 35bp c1 could be lifted from its natural sequence context and placed directly in front of HR1a3 and HR2 to generate a functional enhancer (Figure 3A).

HSS1 is associated with the promoter region of Oct6 and is embedded in a CpG island. Promoter proximal elements mediate interferon and dsRNA responses in fibroblasts, macrophages and possibly Schwann cells through binding of the transcription factor Stat1 (33). These elements fall within the limits of HSS1. Our analysis of transgenic founder animals carrying different SCE constructs did not reveal a role for promoter proximal sequences in directing Schwann cell specific

expression. However, it did not rule out a possible contribution of these sequences to the temporal control of Oct6 gene expression, in particular downregulation in fully myelinating cells. Oct6 downregulation in myelinating Schwann cells is important as forced expression of Oct6 beyond the initial stages of myelination results in myelin degeneration and neuropathy (42). It has been suggested that Oct6 gene expression is extinguished through an auto-inhibitory mechanism and/or through the action of Krox20 (see Figure 6) possibly through binding to the many potential Krox20 binding sites in the Oct6 promoter (2,43,44). Our results with SCE13 containing constructs, shown in Figure 4, demonstrate that the Oct6 promoter can be replaced by the minimal Hsp68 promoter. The downregulation of these constructs in mature nerves suggests that downregulation is mediated through the SCE and does not depend on Oct6 promoter specific sequences. Whether Oct6 downregulation involves active repression by Krox20 or other factors, or loss of activation is an open question. Further insight into the developmental downregulation of Oct6 will depend on the future identification of the relevant sequences within the SCE.

Our detailed mapping of HR1a using a transient reporter Luciferase assay defines a 177bp fragment HR1a3 that is highly active in differentiating rat Schwann cells. This element contains binding sites for a number of transcription factors that are expressed in Schwann cells including Sox, Snail, cAMP-response-element-binding (CREB) and fos/jun proteins (Figure 2). Of particular interest here is the CRE/AP1 binding site TGCGTCA which has been shown to mediate cAMP induced expression of dopamine β-hydroxylase (45). Increased intracellular cAMP concentrations activate PKA which in turn can activate a variety of proteins including CRE-binding protein (CREB) and the p65 subunit of NfkB (46,47). A role for cAMP/PKA in Oct6 expression in Schwann cells was long suspected but compelling evidence for such a role was obtained only recently. It was shown by Monk and colleagues that activation of Oct6 in zebrafish Schwann cells depends on the G-protein coupled receptor Gpr126, a receptor hypothesized to signal through adenylyl cyclase (48). Indeed, pharmacological activation of adenylyl cyclase in Gpr126 mutant fish larvae activated Oct6 expression in Schwann cells and rescued the myelination defect in these mutant animals. Phosphorylation of CREB was also shown to result from Nrg1 signalling through the ErbB2 and ErbB3 heterodimeric receptor type tyrosine kinases, suggesting that both Gpr126 and ErbB2/ErbB3 signals contribute to regulated Oct6 gene transcription (see Figure 6) possibly through the CRE/AP1 element present in HR1a3.

However, whereas Luciferase reporter constructs carrying the HR1a3 and HR2 fragment were highly active in transient transfected rat Schwann cells, a LacZ

reporter construct carrying the same enhancer fragments was inactive in transgenic mice (Figures 2 and 3). This discrepancy could reflect differences in Oct6 regulation in Schwann cells of different species. Indeed, it has recently been shown that mouse and rat Schwann cells exhibit different sensitivities to differentiation cues *in vitro* (49). Alternatively, the observed discrepancy reflects the fundamentally different nature of the two assays employed here. In transfection assays, the DNA construct remains episomal, doesn't incorporate epigenetic marks and is relatively open to the transcription factor environment of the nucleus. In contrast, a transgenic reporter construct is integrated in the chromosome, acquires epigenetic marks and is subject to chromosomal position effects. Clearly, inclusion of the conserved 35bp fragment c1 (Figure 3 and 4) containing two inversely oriented heptameric Sox10 binding site restored *in vivo* enhancer activity to the HR1a3 and HR2 DNA fragment and thus provides information that allows productive enhancer function in a chromatin context. Interestingly, two monomeric Sox10 sites as in construct 16 (C and the c2 sequence embedded in HR1a3; Figure 5D and E) are no substitute for these inversely oriented sequences in c1 to which Sox10 binds as a dimer. Sequence dependent dimerization is a property shared by all group E Sox proteins and depends on a short peptide sequence in the aminoterminal portion of the proteins (41). The relevance of dimeric SoxE binding first became apparent when it was found that mutations in Sox9 that abolish dimeric binding but not monomeric binding, cause a form of Campomelic dysplasia in humans (50). Likewise, mutation of the homologous sequence in mouse Sox10 ($Sox10^{aa1/aa1}$) results in loss of dimeric DNA binding, causing specific defects in neural crest derived cell lineages. These include Schwann cells which are normally generated but fail to enter the promyelinating phase of differentiation and fail to activate Oct6 expression (51) (52). Our demonstration here that the c1 element is a target for dimeric sox10 binding and is essential for Oct6 activation provides a clear explanation for that observation.

How could dimeric Sox10 binding contribute to regulated expression of Oct6? A dimeric Sox10 protein binds DNA with higher affinity and induces stronger bending of the DNA helix than a monomeric Sox10 protein (41). Productive enhancer formation by dimeric Sox10 could result from the higher DNA binding affinity or, alternatively, DNA tethered Sox10 dimers assemble protein complexes different from those assembled by Sox10 on monomeric sites. It is in principle possible that the differences in DNA topology induced by monomeric and dimeric Sox binding contribute to the latter. It is of note that in $Sox10^{aa1/aa1}$ mice Schwann cells are generated in normal numbers whereas in the Sox10 null mice Schwann cells are not generated at all (7,52). As $Sox10^{aa1}$ proteins can still bind as a monomer

to Sox binding sites, this suggests that activity of a subset of Sox10 target genes in the Schwann cell lineage depends on monomeric Sox10 binding, whereas another subset of genes including Oct6 depends on dimeric Sox10 binding. Furthermore, our transgenic data suggest that dimeric Sox10 creates an open chromatin configuration in Schwann cells, allowing productive interactions of transcription factors with the Oct6 SCE. Irrespective of whether this results from qualitative or quantitative differences between the two modes of Sox10 binding, the functional implications are profound.

As Sox10 is expressed at high levels at all stages of Schwann cell differentiation, the dependency of the Oct6 SCE on Sox10 does not provide a ready explanation for the dynamic expression of Oct6 during Schwann cell differentiation. Most likely this information is encoded in HR1a3 or HR2. However, we cannot rule out the possibility that the activity of Sox10 on the Oct6 SCE is regulated through modifications such as sumoylation (53,54). Indeed, it has been shown that sumoylation of Sox10 represses its capacity to activate specific promoters such as those of the Mitf and Gjb genes (53).

It is expected that future identification of additional transcription factors interacting with regulatory elements within HR1a3 and HR2 will provide further insight into the temporal regulation of Oct6 gene expression and myelin related gene expression.

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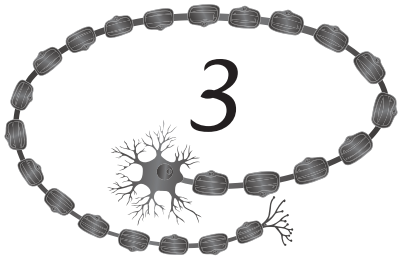

Krox20 is the major transcriptional target of the POU domain transcription factor Oct6 in Schwann cell differentiation

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Krox20 is the major transcriptional target of the POU domain transcription factor Oct6 in Schwann cell differentiation

Abbreviated title: Oct6 in Schwann cell differentiation

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Abstract

The differentiation of Schwann cells to a myelinating phenotype is controlled by axon-associated signals. These signals drive expression of Oct6 and Krox20, two transcription factors crucial for the regulation of a myelin related Schwann cell differentiation. Many aspects of Schwann cell differentiation can be mimicked *in vitro* by elevation of intracellular cAMP. To gain a better understanding of the molecular changes that occur during Schwann cell differentiation, we have analyzed changes in gene expression profiles using differentiating primary rat Schwann cells. We have identified a number of genes previously unknown to be modulated upon Schwann cell differentiation. Furthermore, we have identified mRNAs that are differentially expressed in peripheral nerves of Oct6 null versus wildtype mice. We confirmed that some of these genes are target genes of Oct6, while others are target genes of Krox20. Additionally, we show that Krox20 is the major direct transcriptional target of Oct6. Thus, Oct6 serves to integrate cellular information and to initiate myelination through activation of Krox20 expression.

Introduction

Schwann cells are embryonically derived from the neural crest and produce the myelin sheath in the peripheral nervous system (PNS). Neural crest cells associate with embryonic nerves to give rise to precursors that proliferate and migrate along the nerve fibers and differentiate into bi-potent immature Schwann cells. Around birth these cells will start to exit the cell cycle and finally differentiate to myelinating and non-myelinating Schwann cells (1).

Oct6 (POU3F1) is a POU-homeodomain containing transcription factor (2) that is transiently expressed in Schwann cells. In mouse, low levels of Oct6 are observed in immature Schwann cells as early as embryonic day 14 (E14) but Oct6 expression levels increases dramatically at late fetal life and remains high during early postnatal development, coinciding with the promyelinating stage of Schwann cells (3-5). In the PNS, Oct6 expression is restricted to the Schwann cell lineage and depends on maintained axon-Schwann cell interactions as isolation and culture of neonatal Schwann cells will result in rapid downregulation of Oct6 (6). During postnatal development, Oct6 expression is gradually downregulated in differentiating myelin-forming Schwann cells and is completely extinguished in myelinating Schwann cells of adult animals (4,6,7). The importance of Oct6 downregulation in myelinated cells is illustrated by mice in which Oct6 expression is forced in myelinated cells. These mice exhibit severe demyelination and axonal degeneration in the PNS (8).

Deletion of the Oct6 gene in mice leads to a transient arrest at the promyelinating stage of cell differentiation resulting in delayed myelination of the PNS (9,10). Gene deletion experiments with the zinc finger transcription factor Egr2 (Krox20) and its interacting proteins Nab1 and Nab2 revealed an arrest of Schwann cell differentiation at the promyelin stage. In contrast with the transient developmental arrest in Oct6 deficient mice, Krox20$^{-/-}$ and Nab1$^{-/-}$/Nab2$^{-/-}$ Schwann cells are permanently arrested at the promyelin stage (11,12). Genetic studies have shown that Oct6 acts upstream of Krox20 (13). Moreover, the Krox20 myelinating Schwann cell enhancer (MSE) contains several Oct6 binding sites that are essential for enhancer function (11). These data suggest that Oct6 directly regulates Krox20 expression in promyelinating SCs. Gene expression profiling in Oct6 mutant animals has identified an additional six potential Oct6 target genes (14).

Schwann cells withdrawn from axonal contact through nerve dissection followed by *in vitro* culturing, adopt an immature embryonic Schwann cell phenotype. Agents elevating intracellular cAMP levels can mimic the *in vivo* requirement of

myelinating Schwann cells for axonal contact (15). Schwann cells isolated from perinatal rat sciatic nerves can be propagated *in vitro* in the presence of neuregulin1 (Nrg1) (16). In a number of aspects these cells resemble embryonic Schwann cells as their survival depends on Nrg1 signaling. Upon treatment with high concentrations of forskolin their morphology changes and they start to express high levels of Oct6 and myelin protein Pzero (Mpz) (15,17,18). Thus, this culture system provides a convenient system to study molecular changes in differentiation of immature SCs and to identify Oct6 target genes.

In this study we have used a modified *in vitro* SC culture model to identify genes whose expression is regulated during differentiation (19). Using microarray approach combined with molecular indexing analysis, we identified multiple targets of Oct6 that were distinct from those of Krox20. In addition, we present data on the comparison of the gene expression between postnatal day 1 Oct6 $^{-/-}$ and wild type sciatic nerves. Furthermore, we demonstrate that Oct6 binds directly to the Krox20 Schwann cell enhancer in the chromatin of a Schwann cell line. Finally, we show that Krox20 is the major target of Oct6 regulation as a Krox20 transgene under the control of the Oct6 SCE rescues the myelination defect in Oct6 mutant mice.

Results

Modeling early SC differentiation in a serum free cell culture system

In order to identify and characterize molecular events of Schwann cell differentiation, we modified the previously established Schwann cell culture system. First, we employed serum-free conditions, to omit gene expression changes due to cell cycle exit upon serum presence. In addition, in order to diminish the effects of DMSO (forskolin was dissolved in DMSO), we treated the control cells with volumes of DMSO that were equal to the volumes of forskolin used to induce differentiation in parallel experiments. 80% confluent Schwann cell cultures grown for 36 hours in defined medium supplemented with Nrg1 and DMSO contained multipolar cells with a relatively small soma and long processes (Figure 1A; DM). Upon stimulation with 20μM forskolin the cells flattened out, indicating induction of an active extracellular matrix synthesis, and adopted mostly a bipolar shape (Figure 1A; DM+F).

Oct6 was initially cloned as a POU-homeodomain containing gene upregulated during cAMP induced differentiation of Schwann cells *in vitro* (18). However, they used 10% FCS in their medium. Here we show that in culture conditions where serum was omitted, Oct6 protein expression was induced in differentiated Schwann cells, but not in proliferating or DMSO treated cells (Figure

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

Upon differentiation, the majority of Schwann cells adopted the flattened bipolar morphology, while rare cells with small soma and multiple processes, resembling non-differentiated Schwann cells were also observed. Next, we asked what percentage of cells expressed Oct6 upon differentiation. Using an Oct6 antibody and immunofluorescence microscopy we found that all of the differentiated Schwann cells expressed Oct6 protein (Figure 1C). In addition, some differentiated cells expressed high levels of periaxin (Figure 1C; green) whereas all cells expressed MPZ (not shown).

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

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

To analyze changes in gene expression during Schwann cell differentiation, we used the rat GEM-4 microarray chip of Incyte Pharmaceuticals, Palo Alto, CA. containing 8144 brain - enriched cDNAs as described in Materials and Methods. The experiment was performed in duplicate by swapping the dyes. The resulting hybridization

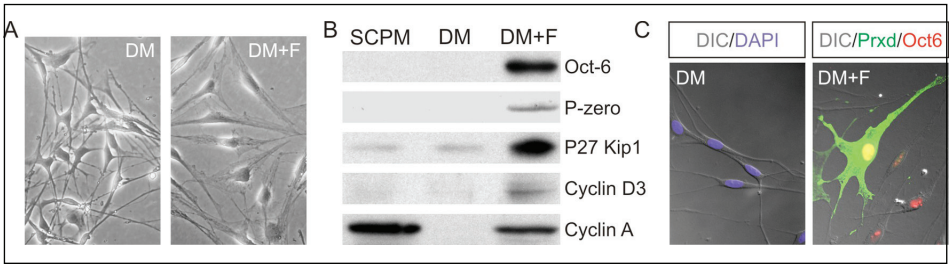

Figure 1 - Primary rat Schwann cells differentiate synchronously in culture with changing cell morphology.
(A) Phase contrast image of non-differentiated and differentiated rat Schwann cells grown in defined medium. **(B)** Expression of Oct6, MPZ, p27 Kip1 and cyclin D3 in proliferating (column 1),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

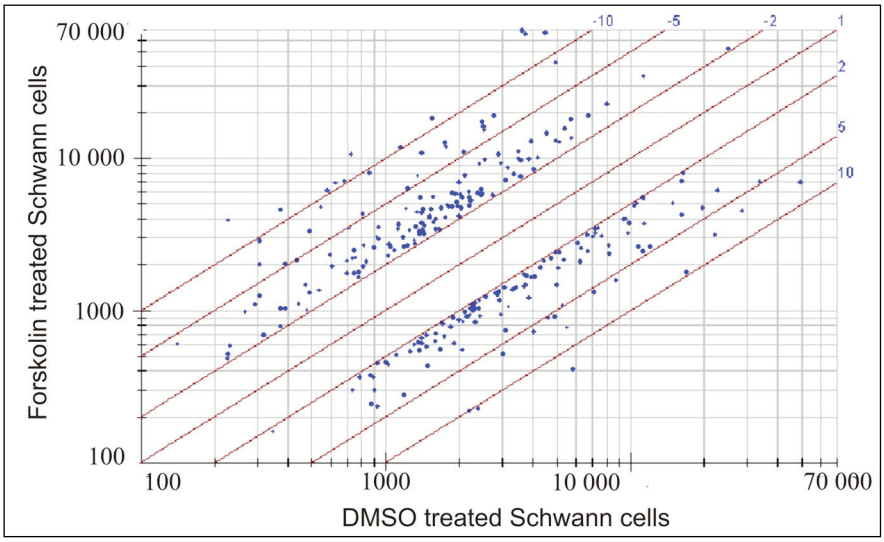

Figure 2 - Microarray analysis of differentiating Schwann cells versus proliferating Schwann cells

profiles are depicted in Figure 2 and Table 1. Signals from the differentiated (forskolin-treated) cells were plotted on the Y-axis and signals from the control (DMSO-treated) cells on the X-axis. For the sake of clarity, all datapoints that showed less than 2-fold difference in expression levels were omitted from the figure. We found a strong correlation between replicates within the same samples. Therefore, we concluded that these microarray hybridizations were meaningful and likely to provide reliable data for further analysis.

Quantification of the results of the microarray experiment shows the following: Out of 8144 data points, 315 were differentially regulated upon differentiation by at least 2-fold. Among 315, 61 different annotated cDNAs were upregulated and 62 different annotated cDNAs were downregulated upon Schwann cell differentiation. 53 differentially expressed datapoints gave similarities to ESTs. All known genes and their accession numbers found to be differentially expressed during forskolin-dependent Schwann cell differentiation are listed in Table 1. As expected, myelin-associated mRNAs (MPZ, MAG, PMP22, PLP, MBP) were all upregulated during Schwann cell differentiation. Also a number of genes encoding extracellular matrix (ECM) components were upregulated upon differentiation, indicating an active ECM synthesis. These data were consistent with the results from *in vivo* experiments, where it has been shown that Schwann cells synthesize extensive basal lamina during myelination (20). We also found that, consistent with

active basal lamina synthesis, a number of proteases and protease inhibitors were differentially regulated upon Schwann cell differentiation *in vitro* including Igfbp5, Timp-2, Timp-3, Serpine2 (GDN), serine protease inhibitor and others. In good agreement with earlier findings (14,21), Neurofilament proteins, Nfl and Nfm mRNAs were also upregulated upon differentiation. In addition, several other nervous system-specific cytoskeletal components were found to be induced upon Schwann cell differentiation. Examples are erythrocyte protein band 4.1-like 4b (Ehm-2), erythrocyte protein band 4.1-like 3 (T-II brain 4.1 isoform) and Myristoylated alanine-rich C-kinase substrate (MARCKS). Among the transcription factors, Oct6, CoupTFII and Id2 were induced. In addition, the levels of the transcription factors CP2 and Tle3 mRNAs are upregulated upon differentiation, whereas Myocyte enhancer factor 2C (MEF2c), Atf3, Zfand5 and MEF2-interacting transcription repressor Mitr (Hdac9) were downregulated during differentiation of Schwann cells.

As a complement and alternative to the microarray analysis we used modification of a differential display method called molecular indexing [Mahadeva et al., 1998] to compare cDNA populations derived from non-differentiated and differentiated Schwann cells. The results of molecular indexing are shown in Table II. We identified a total of 50 cDNAs differentially expressed in non-differentiated versus differentiated Schwann cells. Eight of them (Mpz, Nfl, Col1a1, Igfbp5, gamma filamin, Tnc, Catenin delta1 and Serpine2) were also identified by microarray analysis (see Table 1). As for the rest we found that mRNAs encoding 3 different protein kinases (p90 S6 kinase, PTEN induced kinase and brain creatine kinase) were induced during Schwann cell differentiation. We also identified a number of additional cDNAs encoding cytoskeletal proteins that were modulated during Schwann cell differentiation, out of which utrophin mRNA level was induced and talin and vimentin levels downregulated, indicating broad rearrangements in the cyoskeletal compartment of Schwann cells that take place during differentiation. Interestingly, although both cell populations analyzed were in G0/G1 phase of the cell cycle (data not shown), the expression of Cyclin D3 mRNA was upregulated during Schwann cell differentiation. The induction of Cyclin D3 was further confirmed by Western blot analysis (Figure 1B).

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

Previous studies have shown that inactivation of Oct6 in mice results in a transient arrest of Schwann cell differentiation at the promyelination stage (9,10). Therefore

it was of interest to examine whether the genes for which differential expression was established *in vitro* were also differently regulated in the perinatal nerves of Oct6 deficient mice as compared to peripheral nerves of wt animals.

Most Oct6 deficient mice die postnatally. To obtain nerves in quantities sufficient for RNA analysis, we used a mouse in which one allele of the Oct6 locus was inactivated by insertion of LacZβgeo reporter gene into the coding region of Oct6 and the other inactivated by deletion of the Schwann cell specific enhancer of Oct6 (10,22). These $Oct6^{\Delta SCE/\beta geo}$ mice have all the characteristics of the Schwann cell phenotype observed in Oct6 null animals but live well into adulthood due to the absence of the CNS phenotype and therefore present an excellent genetic system to address questions related to the transcriptional targets of Oct6 in Schwann cells.

We performed semi-quantitative RT-PCR analysis on 14 transcripts that showed differential expression during Schwann cell differentiation *in vitro*. As shown in Figure 3, four transcripts, Id2 and CoupTFII, myelin proteins Cd9 and Fibulin5, identified as upregulated upon Schwann cell differentiation *in vitro*, displayed similar patterns of expression during Schwann cell differentiation in $Oct6^{\Delta SCE/wt}$ and $Oct6^{\Delta SCE/\beta geo}$ mice. The levels of Krox20 and Rgnef were strongly reduced in the nerves of the mutant animal as compared to the wildtype (Figure 3).

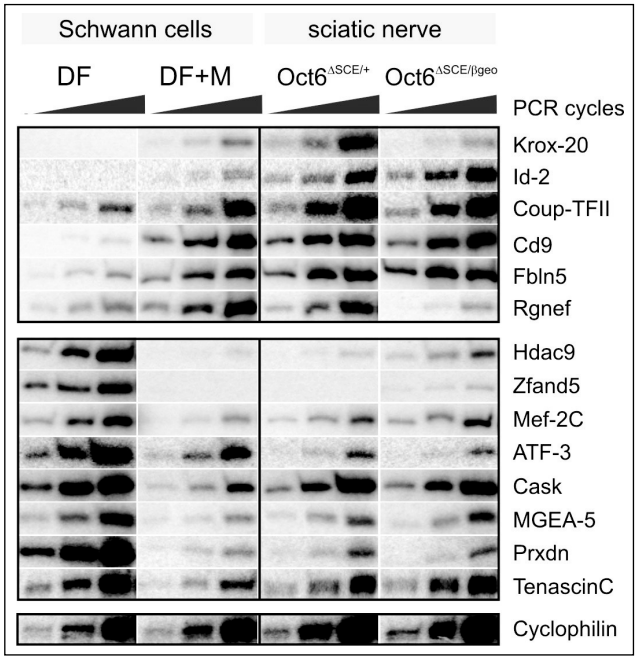

Figure 3 - Gene expression analysis of potential Oct6 target genes in undifferentiated versus differentiated Schwann cells and in P1 sciatic nerves of Oct6 ΔSCE/+ versus Oct6 ΔSCE /βgeo animals using semi-quantitative RT-PCR. Cyclophillin mRNA served as a control.

Semi-quantitative RT-PCR analysis of the RNAs found to be downregulated during Schwann cell differentiation *in vitro* revealed that the levels of two transcription factors – Zfand5 and Mitr/Hdac9 – were regulated accordingly *in vivo*, since the levels of Zfand5 and Mitr/Hdac9 in P1 mutant nerves were significantly higher as compared to the levels in nerves of $Oct6^{\Delta SCE/\beta geo}$ mice (Figure 3). However, levels of other mRNAs tested, including mRNAs encoding transcription factors Mef2C and Atf3, were reduced upon Schwann cell differentiation in culture, but remained the same in nerves of both mutant and heterozygous animals (Figure 3).

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

Krox20, Rgnef and Fibulin5 are targets of Oct6 activity.

We and others have shown that both Oct6 and Krox20 mRNAs are induced during Schwann cell differentiation. *In vivo* experiments have provided clear evidence that Krox20 functions downstream of Oct6 in the genetic hierarchy of Schwann cell differentiation, compatible with the possibility that Krox20 is a direct target gene of Oct6 (11,13). We tested this possibility by transient transfection of Oct6 in undifferentiated rat Schwann cells. As shown in Figure 4, overexpression of Oct6 significantly induces expression of endogenous Krox20, whereas over-expression of Krox20 had no effect on Oct6 mRNA levels (data not shown).

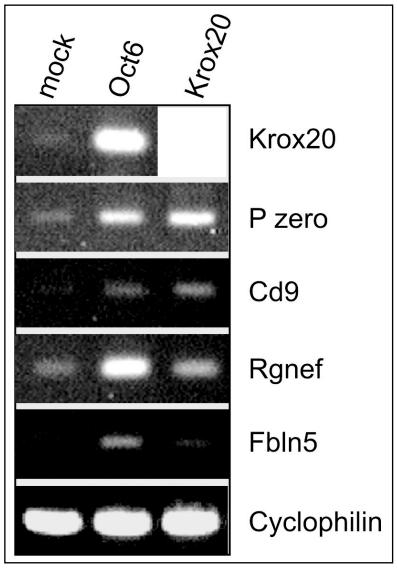

Figure 4 - RT-PCR analysis of the induction of endogenous Krox20, MPZ, Cd9, Rgnef and Fibulin5 mRNAs after transfection of empty vector (column1), Oct6 expression vector (column 2) and Krox20 expression vector (column 3) into undifferentiated Schwann cells.

Next, we asked whether other differentially regulated genes that we identified in previous experiments were also direct targets of Oct6 or indirect targets through regulation by Krox20. As illustrated in Figure 4, MPZ and Cd9 mRNA levels in undifferentiated Schwann

cells were upregulated by overexpressed Krox20. These results indicate that these myelin genes are targets of Krox20. In contrast, Rgnef and Fibulin5 expression is upregulated through overexpression of Oct6 but not Krox20 (Figure 4) suggesting they are downstream of Oct6 and potentially could be direct transcriptional targets of Oct6.

Knockdown of P190RhoGef in rat Schwann cells

The P190RhoGef (Rgnef) protein is a Rho-GTPase nucleotide exchange factor that is highly enriched in neural tissue and was found to associate with focal adhesion kinase (FAK) and microtubules. It regulates cell motility and focal adhesion formation (23-25). To test the role of Rgnef in Schwann cell biology we used lentiviral delivery of shRNA against Rgnef to knockdown expression of the gene in Schwann cells. Two lentiviral constructs were selected from the mouse shRNA Mission library as their RNA targets were identical in the rat Rgnef mRNA. As controls we used a scrambled shRNA clone and a shRNA clone directed against the rat Oct6 mRNA. We transduced rat Schwann cells with the lentiviral vectors and counter-selected the non-transduced cells using puromycin. First we tested the level of knock down of Rgnef and Oct6 in these populations of Schwann cell by quantative RT-PCR and plotted the expression levels as percentage of the expression level in non-transduced Schwann cells. As shown in Figure 5A, Oct6 knockdown to approximately 40%, results in a reduction of Krox20, Rgnef and MPZ expression in differentiating Schwann cells. Of the two Rgnef shRNA constructs, Rgnef#1 resulted in a reduction of Rgnef mRNA to 40% of wildtype levels whereas Rgnef#2 reduced Rgnef expression to 80%. However, expression of Oct6, Krox20 and MPZ was not affected by knockdown of Rgnef (Figure 5A).

Next, we tested the consequence of knockdown of Rgnef in Schwann cells in co-culture with mouse primary sensory neurons. Neuronal cultures were established from E13 mouse embryos. These neuronal cultures were seeded with rat Schwann cells and myelination was initiated a week later by addition of ascorbic acid. Myelination was monitored by staining of the cultures with MBP antibodies. Wildtype and control shRNA Schwann cells readily form myelin around mouse axons (Figure 5B). Also, Rgnef shRNA Schwann cells form myelin in these co-cultures, suggesting that knockdown of Rgnef levels to around 40 or 80% does not affect the ability of these cells to elaborate myelin. The Oct6 shRNA control Schwann cells also formed myelin but at a reduced frequency. Westernblot analysis of these control cultures confirmed this as expression of Oct6 and Pzero was significantly reduced

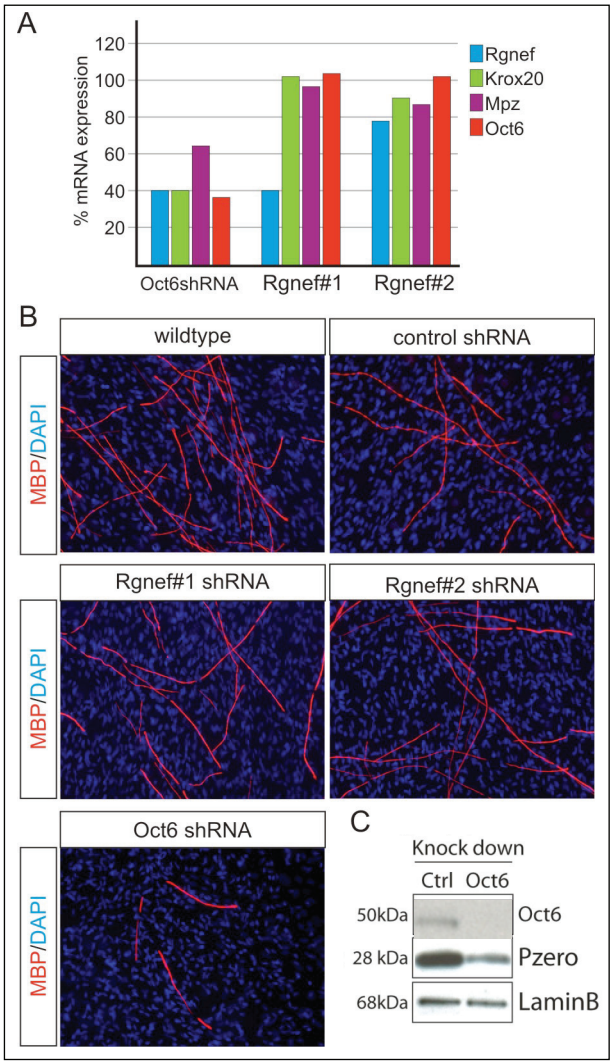

Figure 5 - Rgnef knock down in rat Schwann cells does not affect myelination. **(A)** Quantitative RT-PCR analysis of differentiated Schwann cells after knock down of Oct6 and Rgnef. Gene expression is shown in terms of % of mRNA expression after knock down. Knock down with a non-targeting shRNA is used for the normalization. **(B)** Sensory neurons are normally myelinated with wildtype rat Schwann cells or Schwann cells knocked down with control shRNA and Rgnef shRNA. Very few myelin segments are observed in Oct6 knock down Schwann cells seeded on wild type neurons. The level of myelination was evaluated by immunohistological staining with an MBP antibody (red). Schwann cell nuclei were stained with DAPI (blue). **(C)** Western blot showing the expression of Oct6 and MPZ in differentiated Schwann cells knocked down with either control shRNA or Oct6 shRNA. Lamin B is used as an internal control. The molecular weight of the respective proteins is indicated.

(Figure 5C). Thus at this point, it is not possible to conclusively deduce the role of Rgnef in Schwann cell myelination.

Krox20 is a major target of Oct6 regulation

Previous work has indicated that Oct6 directly regulates Krox20 in differentiating Schwann cells *in vivo* (11,13). In line with these studies we found that overexpression

of Oct6 in cultured rat Schwann cells results in Krox20 activation. To further corroborate the idea that Oct6 directly regulates Krox20 we performed Oct6 immunoprecipitation assays on chromatin of RT4D6-P2T rat Schwannoma cells (26,27). These cells express Oct6, Krox20, Sox10 and a number of myelin related genes such as MPZ and provide a suitable model for early myelinating cells. Oct6 antibodies recover the Krox20 MSE with higher frequency than rabbit IgG antibodies (Figure 6). Such an enrichment is not seen on an unrelated silent promoter such as that of the IgG2a gene. Thus Oct6 occupies the Krox20 MSE in RT4D6-P2T Schwannoma cells.

Given the central role of Krox20 in the transcriptional network of myelination we asked whether Krox20 is the major target of Oct6 regulation. To address this question we designed an expression construct that brings HA-tagged Krox20 under the control of the Oct6 regulatory sequences (Figure 7A). We generated a transgenic line with this construct and crossed the transgene into the $Oct6^{\Delta SCE/+}$ and $Oct6^{\Delta SCE/\beta geo}$ genetic backgrounds. We analysed the expression of Krox20, Oct6 and P-zero in sciatic nerves of these animals at 4 days after birth. At this stage both Oct6 and Krox20 are highly expressed (Figure 7B; $Oct6^{\Delta SCE/+}$) and the vast majority of myelin competent Schwann cells initiate myelination around this time of postnatal development. Krox20 and Oct6 are expressed at very low level in nerves of $Oct6^{\Delta SCE/\beta geo}$ animals (Figure 7B). However, in nerves from $Oct6^{\Delta SCE/\beta geo}$ animals that express the Krox20 transgene, expression of Pzero is restored to wildtype levels. Interestingly, in addition to the HA-tagged Krox20 encoded by the transgene, the endogenous Krox20 is also expressed. As these cells do not express Oct6, this suggests that endogenous Krox20 is activated through a Krox20 autoregulatory feedback loop.

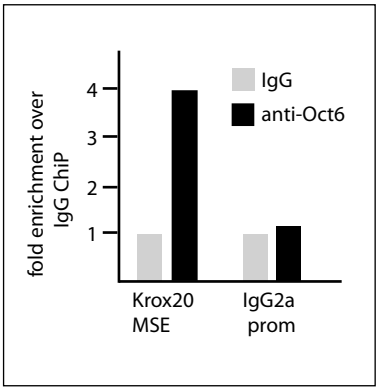

Figure 6 - ChIP of Krox20 MSE sequences using Oct6 antibodies.
ChIP of Krox20 MSE sequences using Oct6 antibodies. Chromatin of RT4D6-P2T cells was precipitated with an Oct6 antibody or control rabbit IgG. Quantitative PCR on immunoprecipitated and purified DNA was performed for the MSE region in triplicate and was used to determine the relative enrichment of these loci by Oct6 over IgG immunoprecipitation. The experiment was performed twice and data is a representation of one of the experiments.

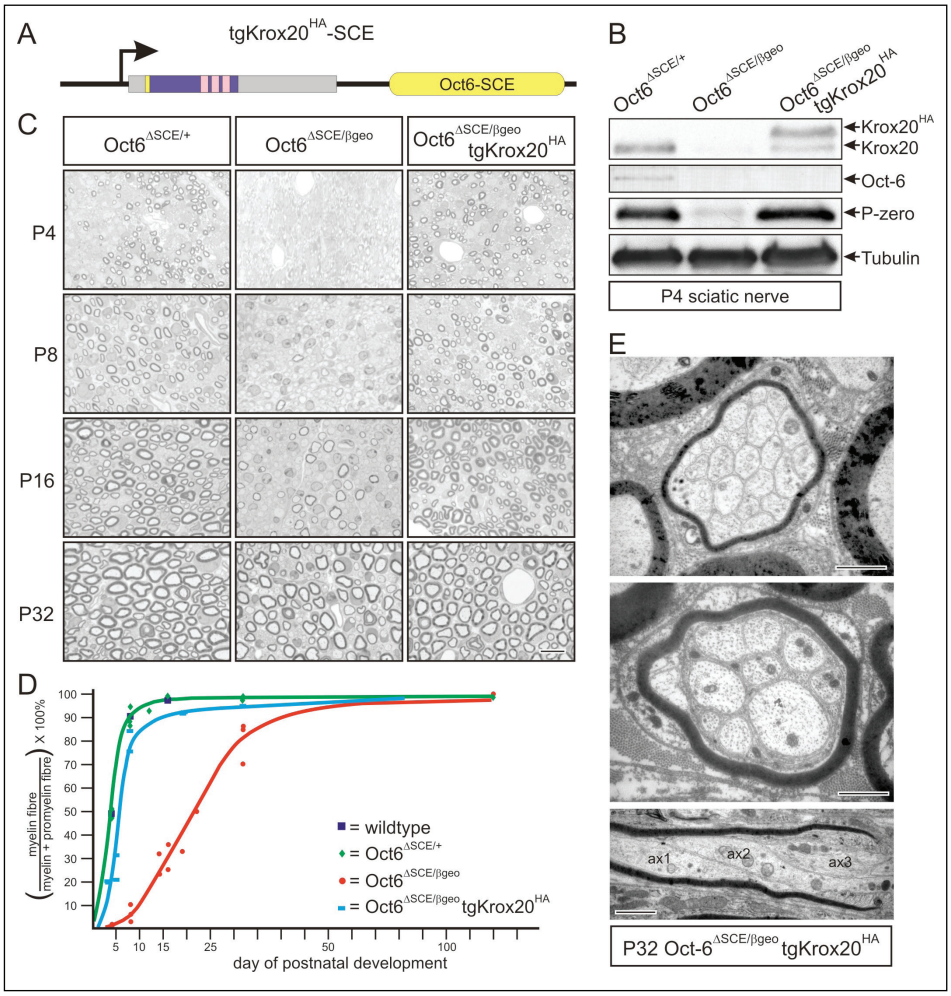

Figure 7 - Krox20 expression in an Oct6 mutant background rescues the congenital hypomyelination phenotype in Oct6 mutant mice. **(A)** Schematic representation of the tgKrox20HA-SCE construct used to generate transgenic mice. Krox20HA is under the control of Oct6 SCE. **(B)** Expression of Krox20, Oct6 and MPZ was examined in P4 sciatic nerves of Oct6ΔSCE/+ (column 1), Oct6ΔSCE/β-geo (column 2) and Oct6ΔSCE/β-geo x tgKrox20HA (column 3). Note that Krox20HA exogenous protein runs higher than the endogenous Krox20 protein due to the presence of the HA tag. Amounts of nerve extract loaded were normalized for acetylated tubulin. **(C)** TgKrox20HA under the control of Oct6 SCE rescues the developmental defect observed in Oct6 mutant mice. PPD (p-phenylenediamine) stained representative sections of sciatic nerves are shown at four developmental time points (P4, P8, P16 and P32). In Oct6SCE/+ mice, most myelin competent axons are actively being myelinated by P16. By P32, these nerves are fully matured. This pattern contrasts with that observed in Oct6ΔSCE /β-geo animals, which exhibit a strong delay in the differentiation of Schwann

cells. At P16, the majority of Schwann cells are arrested at the promyelin stage, with only few larger myelinated axons. However, by P32, many axons are myelinated in Oct6ΔSCE/β-geo animals. Introduction of tgKrox20HA in the Oct6-deficient background rescues the phenotype observed in the Oct6 mutant mice. Myelinated axons are present at the P4 stage similar to the observations in Oct6 heterozygote mice. **(D)** Quantification of the promyelin-myelinating transition in percentages. The transition of the promyelin Schwann cell to myelinating Schwann cell is shown in wildtypes, Oct6SCE/+, Oct6 ΔSCE /β-geo and Oct6 ΔSCE/β-geo :: tgKrox20HA mice during postnatal development. **(E)** Representative electron micrographs of the rare myelin abnormalities observed in Oct6 ΔSCE/β-geo :: tgKrox20HA mice. The top photo shows a myelinated bundle of low caliber axons. These fibers are normally segregated into Remak fibers. The middle picture shows a bundle of larger axons segregated by Schwann cell cytoplasmic extensions and surrounded by a myelinating Schwann cell. Note that in this configuration the myelinating Schwann cell does not contact an axon but the membrane of another Schwann cell. The lower panel shows a longitudinal section of multiple axons surrounded by one myelin sheath. The paranodal loops on the right touch down on the axonal membrane of at least 3 axons (ax1, ax2 and ax3). The spacebar represents 1 μm.

Next, we examined the morphology of the sciatic nerve in $Oct6^{\Delta SCE/+}$, $Oct6^{\Delta SCE/\beta geo}$ and $tgKrox20^{HA}/Oct6^{\Delta SCE/\beta geo}$ animals at several stages of postnatal development (Figure 7C). As previously described, $Oct6^{\Delta SCE/\beta geo}$ nerves exhibit congenital hypomyelination with delayed onset of myelination (10,22). In contrast, $tgKrox20^{HA}/Oct6^{\Delta SCE/\beta geo}$ nerves are myelinated with the same kinetics as of the nerves of $Oct6^{\Delta SCE/+}$ animals (Figure 7C). Quantification of the transition from a promyelinating to a myelinating cell corroborates this observation. Indeed, Schwann cells transit to the myelinating stage with near normal kinetics (Figure 7D). Thus, expression of Krox20 under control of the Oct6 SCE restores the congenital hypomyelination defect in $Oct6^{\Delta SCE/\beta geo}$ mice. Closer examination of transverse sections of nerves under higher magnification revealed a number of intriguing abnormalities (Figure 7E). These include polyaxonal myelin figures and myelinated Remak fiber bundles. Although rare, such abnormalities are found in nerves of all $tgKrox20^{HA}$ mice. Therefore, these abnormalities do not result from deregulated expression of other Oct6 target genes but from expression of $tgKrox20^{HA}$.

Discussion

To identify novel genes whose expression is modulated during Schwann cell differentiation, we compared the transcriptome of undifferentiated and differentiated Schwann cells *in vitro* using microarray hybridization. We have identified 84

different transcripts that are upregulated and 83 transcripts that are downregulated during forskolin mediated Schwann cell differentiation *in vitro*. We further analyzed whether some of the identified cDNAs were differentially expressed in the nerves of Oct6 mutant mice and compared expression of a selected set of genes by RT-PCR in $Oct6^{\Delta SCE/+}$ and $Oct6^{\Delta SCE/\beta geo}$ sciatic nerves. We found that some of the identified genes were potential direct transcriptional targets of Oct6, whereas others are targets of Krox20. Finally we demonstrate that Krox20 is the major target of Oct6 regulation in myelinating Schwann cells.

Myelin gene expression was found to be activated during forskolin mediated Schwann cell differentiation. In accordance with previous reports (14,17) we found that MPZ, PMP22, Cd9, MBP, PLP, and MAG mRNA expression was induced. Also, the expression of a number of proteases and protease inhibitors (Igfbp5, CarboxypeptidaseE, C1r, ITI-like protease, Timp2 and Timp3) was increased, while levels of other protease and protease inhibitors were decreased (Adamts1, Lgl1, Serpine2, Calpastatin, Furin, Serpinb6A). Among the latter, Serpine2 has been shown previously to be upregulated in response to Krox20 in Schwann cells [Nagarajan et al., 2001]. Our finding that Serpine2 was downregulated during Schwann cell differentiation is therefore unexpected, since levels of Krox20 increase during differentiation. However, it has been shown that Serpine2 is downregulated upon forskolin and not Nrg1 treatment of Schwann cells (21). The modulation of the levels of proteases and protease inhibitors is expected, since there is an active ECM synthesis and reorganization during myelination (20). This assumption is further corroborated by the observation that a number of laminin and collagen genes were upregulated during Schwann cell differentiation, while others were downregulated (Table 1). Downregulation of Tnc has been shown before and it was found to be dependent only on forskolin and not on Nrg1 (21).

In addition to Nfl and Nfm upregulation and gamma filamin downregulation that has been shown previously (14), we found the levels of a number of other cytoskeletal proteins to be modulated. Also, we found that there is a change in Cl^- channel repertoire during Schwann cell differentiation. Voltage-gated CLCN3 was downregulated, while calcium activated CLCA1 was upregulated (Table 1). We have shown here for the first time the expression of two types of Cl^- channels in Schwann cells (28).

A number of transcription factors in addition to Oct6 were found upregulated during Schwann cell differentiation. These include Id2, CoupTFII, CP2 and the groucho-related corepressor Tle3. Induction of Id2 has been observed before in dbcAMP (a membrane permeable cAMP analogue) induced primary Schwann cells.

Also, the level of Id2 mRNA is elevated in postnatal day 0 sciatic nerve as compared to E19 sciatic nerve (29). Tle3 is particularly interesting, since members of this family interact and regulate a variety of transcription regulatory systems such as Notch and Wnt which are known to regulate Schwann cell development (30) and therefore Tle-3 might be one of the global regulators of Schwann cell differentiation.

Hdac9 and Zfand5 (Znf216) were found to be downregulated during Schwann cell differentiation and the levels of these mRNAs were higher in the perinatal nerves of Oct6$^{\Delta SCE/\beta geo}$ animals than those of Oct6$^{\Delta SCE/+}$ animals. These genes might be negatively regulated by Oct6. Hdac9 is a class-IIa histone deacetylase known to interact with many transcription factors including Mef2 (31). Interestingly we also found Mef-2 to be downregulated during Schwann cell differentiation, but Mef2 was expressed at normal level in Oct6 mutant nerves. It is possible that compensatory factors retain Mef-2 expression in Oct6 mutant nerves, but that Hdac9 is regulated negatively by Oct6. Zfand5 is particularly interesting, since it is a potent inhibitor of Nf- κB (32). Nf-κB activity is important for myelination in the PNS (33). Zfand5 interacts with the IκB kinase non-enzymatic regulatory subunit, IKKγ and prevents phosphorylation of Nf-κB (32). It is possible that Oct6 is required to reduce the levels of Zfand5, thereby relieving the inhibitory action of IκB kinase.

Rgnef is a RhoA interacting protein that specifically activates RhoA and interacts with microtubules and FAK (24). Rgnef was upregulated during Schwann cell differentiation and was also found to be expressed at lower levels in Oct6$^{\Delta SCE/\beta geo}$ perinatal nerves as compared to Oct6$^{\Delta SCE/+}$ perinatal nerves. Therefore it might be activated by Oct6. Furthermore, Rgnef colocalizes with microtubules and might provide a link between microtubule dynamics and RhoA signalling. Our knockdown experiments on Rgnef in Schwann cell neuron co-cultures failed to reveal a role for Rgnef in myelination. It is possible that our knockdown levels are not low enough to resort an effect or, alternatively, Rgnef redundant factors (other RhoGef proteins) compensate for the lower P190RhoGef levels. P190RhoGef is known to interact with FAK (25). FAK is associated with integrin signalling in Schwann cells and deletion of FAK results in a failure to sort axons (35).

Cyclins D3 and the Cdk inhibitor, p27kip1 are both induced during forskolin-mediated Schwann cell differentiation. Cyclin D3 is upregulated upon treatment of Schwann cell culture with a combination of Nrg1 and forskolin (21). We also found elevated levels of Cdk inhibitors p27kip1 and p57kip2 in wildtype nerves, as compared to the Oct6 deficient nerves. This indicates a role for these proteins in the differentiation process rather than in the proliferation of Schwann cells since there is no proliferation defect in Oct6 mutant mice (10).

All of the cDNAs identified by our analysis might in principle be divided into 3 categories. First, they might be target genes of Oct6, second they might be target genes of Krox20. Third, they might be induced by the sharp activation of the PKA pathway. To distinguish between these possibilities we transfected undifferentiated Schwann cells with either Oct6 or Krox20 expression vectors and examined the induction of endogenous MPZ, Cd9, Rgnef and Fibulin5. We found that in accordance with the work of Nagarajan and colleagues (34) myelin gene expression is activated by Krox20, whereas Rgnef and Fibulin5 are activated by Oct6.

Importantly, we found that Krox20 itself is directly activated by Oct6. Moreover, our transgenic experiments show that Krox20 is the major target of Oct6 as expression of Krox20 in an Oct6 mutant background is sufficient to restore myelination. However, expression of Krox20 under control of the Oct6 SCE also resulted in rare myelin abnormalities that include polyaxonal myelination and myelination of Remak fibers. Most likely these abnormalities result from premature myelin differentiation. As Krox20 is now controlled by the Oct6 SCE, expression of Krox20 sets in too early in these cells resulting in myelination of, for example, unsorted bundles of smaller axons. These abnormalities are not caused by a defective Oct6 gene regulation as they are also observed in mice that have the $Krox20^{HA}$ transgene on a wildtype or $Oct6^{\Delta SCE/+}$ background.

Materials and Methods

Primary Schwann cell (SC) culture and transient transfection assays.

All cell culture reagents were obtained from Invitrogen (USA), unless indicated otherwise. Rat SC primary culture was established as previously described (16) with slight modifications. In brief, sciatic nerves of P3 rat pups were dissected aseptically and collected into L-15 medium, nerves were teased with fine forceps and tissue was further disrupted by enzymatic digestion using 1 mg/ml collagenase (Roche,USA) for 30 min at 37°C in L15 medium. Cells were washed once with L15/10% fetal calf serum (FCS), plated onto uncoated Primaria tissue culture dishes (Becton Dickinson, USA) in DMEM/10% FCS medium and incubated overnight at 37°C and 5%CO_2. The following day, medium was replaced with fresh medium containing 10 μM cytosine arabinoside (Ara-C; Sigma) to remove fibroblasts. Cultures were treated this way for 3 days, refreshing the Ara-C containing medium daily. Pure SC cultures were maintained and passaged in DMEM, 3%FCS, 2μM forskolin (Sigma) and 5% NDF-beta conditioned medium on Primaria tissue culture dishes (36). In the differentiation experiments, medium was replaced by a defined medium (DMEM/F12, 1xN2

supplement and 5% NDF-β conditioned medium) and incubated overnight. The following day, medium was replaced by a defined differentiation medium containing 20μM forskolin to induce the differentiation of Schwann cells. The control Schwann cell cultures were subjected to defined medium containing 0.2%DMSO. 36 hours later, the cells were used for subsequent analysis.
SCs in defined medium were transfected with pCDNA3 (Invitrogen,USA), pSCTKrox20 (kind gift from Walter Schaffner) or pEVOct6 (37) expression vectors using Fugene6 (Roche, USA) according to manufacturer's instructions. 1 μg of DNA was used per 60 mm cell culture dish using Fugene6 /DNA ratio 3:1. 12 hours post-transfection the medium was replaced with fresh defined medium and cells were harvested after 36 hours.

Western blotting and immuno-fluorescence analysis.

Equal amounts of cell extracts prepared from proliferating, DMSO-treated and forskolin-treated SC cultures were resolved on a 12% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). Membranes were blocked with 3%BSA, 0.05% Tween20 in PBS for 1 hour at room temperature. After incubation with respective antibodies for 2 hours, the filters were washed 5 times with 0.5% Tween20 in PBS and incubated with secondary antibodies for 1 hour in blocking buffer. Following 5 washes with 0.5% Tween20 in PBS the antigens were visualized by luminol.
For immunofluorescence analysis, the cells were fixed with 4% PFA 20 min at RT followed by 3 washes with 1x PBS and subsequent blocking with 1%BSA, 0.05% Tween20 in 1x PBS solution for about 1 hour. Cells were incubated with respective antibodies over night at 4^0C. Next day, the cells were incubated with respective secondary antibodies for one hour at room temperature. Cell nuclei were visualized with DNA-specific fluorophore DAPI.

Microarray analysis

RNA from undifferentiated and differentiated SC cultures were prepared by using Trizol (Invitrogen, USA) and polyA+ RNA was purified with Oligo dT linked Dynabeads (Dynal Biotech) according to manufacturers' instructions. cDNA, hybridization of rat GEM2 microarray (Incyte Genomics) and data collection were performed in Incyte Genomics (USA). Briefly, random primed cDNA was generated by reverse transcriptase in the presence of Cy3 or Cy5 labeled nucleotide precursors and competitively hybridized onto the rat GEM2microarray. Primary data was normalized, and defective hybridization spots removed using Incyte Gemtools

software. A difference greater than 2-fold was considered as significant. Hybridization signals were determined by laser scanning fluorography and corrected via internal controls. The experiment was performed in duplicate.

Molecular Indexing

Molecular indexing was performed as described in Mahadeva et al., 1998. In brief, 20 ugs of total RNA from undifferentiated and differentiated rat SC cultures was prepared using Trisol and reverse transcribed into double strand cDNA using oligo(dT) as the first strand primer by standard techniques. cDNAs were digested with a type II restriction enzyme Bbv I (New England Biolabs) at 37°C overnight. Digested cDNAs were divided into 120 pools and a combination of 2 adaptors were ligated onto each pool in a reaction containing 3.3 nM of each adaptor pool. The volume of the ligation reaction was 30 ml. Adaptor sequences were as described in Mahadeva et al., 1998. Ligation mixtures were heated at 65°C for 5 minutes and cooled to 37°C prior to adding the T4 DNA Ligase (Promega, USA). After ligation the unincorporated adaptors were removed by using Qiaquick PCR purification colums (Qiagen). Adaptored cDNA fragments were amplified by using primer common to all the adaptor combinations in a 10 ml reaction in the presence of [^{32}P]dATP and 0.5 mM primer with PlatinumTaq (Invitrogen). PCR conditions used for amplification were 94°C 5 minutes, (94°C 30 seconds, 60°C 30 seconds, 72°C 2 minutes and 30 seconds) x 28, 72°C 10 minutes. Amplified PCR reactions were resolved on 6% denaturing polyacrylamide gels, and analyzed by autoradiography. Differentially expressed PCR fragments were eluted from gel by boiling gel-slices in TE for 5 minutes. 1/50th of the eluted PCR fragment was used for re-amplification in a 25 ml reaction. Re-amplified PCR products were cloned into T vector (Promega) and sequenced.

Semi-quantitative RT-PCR analysis.

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

different cDNAs.

Chromatin immunoprecipitations

The ChiP assays were performed on dual-cross linked chromatin, as previously described in Chapter 2. Comparative Ct values (38) were used to calculate the enrichment of a DNA segment relative to the total input. These values were used to calculate the relative enrichment of Oct6 antibody over rabbit IgG precipitated chromatin for the Krox20 MSE and IgG2a promoter regions. Primer sets used in quantitative PCR for ChIP are as follows: MSE **:** 5'- AGC TGA AAT CCT GCA GTG TG -3'and 5'- GCA GCT ATT TCC CAA GCA TT-3', IgG2aP: 5'-GAA ATT CTG CCC TGC ACT TCC-3' and 5'-GCT TTG CAT TGA GGG AGG ATC-3'

Lentivirus production

Clones from the Sigma Mission shRNA library were used for the knockdown experiments in rat Schwann cells, including control non-targeting shRNA (SHC002). Lentivirus particles were produced by transfections of 293T cells according to standard protocols (39). Rat Schwann cells were transduced with concentrated virus particles and further selected with 0.4µg/ml puromycin. Rat Schwann cells were harvested after 36 hours of differentiation. The expression levels of Oct6, Rgnef, MPZ and Krox20 were determined by RT-PCR. Total RNA was isolated from differentiated rat Schwann cells using RNA-Bee. 500 ng of RNA was used to synthesize first strand cDNA using SuperScript II (Invitrogen) according to manufacturer's instructions. Q-PCRs were carried out in triplicate with Platinum Taq polymerase (Invitrogen). HPRT gene transcription was used as a reference for normalization. Primers used are listed below:
HPRT: 5' TCA GTC AAC GGG GGA CAT AAA 3' (for), 5' GGG GCT GTA CTG CTT AAC CAG 3' (rev), Oct6: 5' ATG TGC AAG CTC AAG CCG CT 3' (for), 5' CGA GTC GGT CTC CTC CAG C 3' (rev), MPZ: 5' CTG GTC CAG TGA ATG GGT CT 3' (for), 5' CAT GTG AAA GTG CCG TTG TC 3' (rev), Krox20: 5' GAA CGG AGT GGC TGG AGA TG 3'(For), 5' ACT GAG GGT CAA TGG AGA ATT TGC 3' (rev), Rgnef: 5' ATG TCC TCC AAT CTA GCA TTC CA 3(For)', 5' GTC CAC GTA GGT CAC TGA GC 3' (rev)

Primary sensory neuron cultures

Dorsal root ganglia (DRGs) were isolated aseptically from E13.5 mouse embryos. After dissociation by trypsination and trituration, the cells were plated onto 18mm poly-L-Lysine (Sigma) and collagen-coated coverslips (Thermo Scientific),

and maintained in MEM (Invitrogen), 3% FCS, 100 ng/ml NGF, 1% Penicillin and Streptomycin (referred as M1 medium). The medium was refreshed every other day. To obtain neuron-only cultures, the cells were treated with 10µm FUDR (Sigma) in M1 medium for 2 days. Two cycles of FUDR treatment (48 hours with FUDR, 48 hours without FUDR) was usually sufficient to obtain neuron–only cultures. The puromycin selected, knock down rat Schwann cells were starved of neuregulin in DMEM, 10% FCS, PS for 3 days before seeding 2 x 10^5 cells in M1 medium onto neuron-only cultures. The Schwann cells were allowed to associate with the neurons for an additional 5 days, and then myelination was induced by addition of ascorbic acid to a final concentration of 50 µg/ml (Sigma). Cultures were maintained in M1 medium containing 50 µg/ml ascorbic acid and 10% FCS for 2 weeks and subsequently analyzed for the presence of myelin using MBP antibody using immunofluorescence.

Antibodies

Primary antibodies were from the following sources: Rabbit-anti-Oct6 (Jaegle et al., 2003) (Western blotting (WB), 1:500), mouse-anti-P0 (Archelos et al., 1993) (WB, 1:1000), goat-anti-LaminB (Santacruz; C-20 and M-20, WB, 1:2000), anti-cyclin D3 (Santacruz; WB, 1:2000), anti-p27 (Santacruz; WB, 1:2000), rat-anti-myelin basic protein (Millipore; Immunofluorescience (IF) IF, 1:300), mouse-anti-Oct6 (IF: 1:400), rabbit-anti-Periaxin (1:200). The following secondary antibodies were used. DyLight594-conjugated donkey-anti-rat (Jackson Immuno Research, IF, 1: 300), goat-anti-rabbit Alexa488 (IF:1:300), chicken-anti-mouse Alexa594 (IF:1:300), HRP-goat-anti –rabbit (Dako; WB, 1:5000), HRP-goat-anti-mouse (Dako; WB, 1:5000), HRP-donkey-anti-goat (Santacruz; WB, 1:5000)

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Table 1

cDNAs induced during forskolin dependent Schwann cell differentiation identified with microarray analysis

Fold - Δ	Gene	Accession no.
Myelin genes		
-16,9	Schwann cell peripheral myelin protein (P-0)	K03242
-9	PMP-22	X62431
-4,1	CD9 mRNA for cell surface glycoprotein.	X76489
-3,9	Myelin basic protein	M25889
-3	Myelin proteolipid protein (PLP)	M25888
-2,9	Myelin-associated glycoprotein (MAG)	M22357
Proteases and protease inhibitors		
-7,2	Insulin-like growth factor binding protein 5 protease	AF179370
-2,2	Complement C1r	AAA51851
-2,3	Carboxypeptidase E	M31602
-6,4	Tissue inhibitor of metalloprotease 3 (TIMP-3)	U27201
-5,2	Inter-alpha (globulin) inhibitor H5-like	CAA18605
-2,8	Matrix metalloprotease inhibitor (TIMP-2)	L31884
Cell surface receptors		
-6,7	Nonselective-type endothelin receptor	S65355
-2,3	Pre-PDGF receptor mRNA	X04367
Extracellular matrix proteins		
-3,9	Type VII collagen	AAB66593
-3,9	Thrombospondin 2	L06421
-3,6	Laminin B1	M15525
-3	Laminin gamma 1	X94551
-2,7	Laminin-2 alpha2 chain	U12147
-2,4	Collagen alpha1 type I	Z78279
-2,3	Laminin alpha 4	Y09827
-2,2	Collagen alpha-1 type-III	M18933
-2,1	Collagen type IX alpha 3	AF237721
Cytoskeletal proteins		
-10,9	Light molecular-weight neurofilament (NF-L)	AF031880
-4,8	Neurofilament protein middle (NF-M) mRNA	Z12152
-3,5	Type II brain 4.1 minor isoform	AB032828
-3,4	Myristoylated alanine-rich C-kinase substrate (MARCKS)	M60474
-2,8	Ehm2 mRNA	AB032366
-2,6	Anillin	BAA91711
-2,6	Protein 4.1G mRNA	AF044312
-2,4	Gamma-adducin	U35775
-2	Non-muscle alpha-actinin 1	AF115386

Transporters		
-17,1	Ca(2+)-sensitive chloride channel 2 (Cacc)	AF108501
-4,1	ATP-binding cassette protein (Abca8) mRNA	AF213393
-3,8	mRNA for sodium channel.	Y09164
-2	Sodium channel I mRNA	M22253
-2	Sodium channel II mRNA.	M22254
-2,2	(Na+, K+)-ATPase-beta-2 subunit mRNA	J04629
-2	Sodium-hydrogen exchanger 6 (NHE-6)	AF030409
Transcription factors		
-3,7	CoupTFII	X76653
-3,1	Oct6	M72711

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-7,2	Insulin-like growth factor binding protein 5 protease	AF179370
-2,2	Complement C1r	AAA51851
-2,3	Carboxypeptidase E	M31602
-6,4	Tissue inhibitor of metalloprotease 3 (TIMP-3)	U27201
-5,2	Inter-alpha (globulin) inhibitor H5-like	CAA18605
-2,8	Matrix metalloprotease inhibitor (TIMP-2)	L31884
Cell surface receptors		
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-2,3	Pre-PDGF receptor mRNA	X04367
Extracellular matrix proteins		
-3,9	Type VII collagen	AAB66593
-3,9	Thrombospondin 2	L06421
-3,6	Laminin B1	M15525
-3	Laminin gamma 1	X94551
-2,7	Laminin-2 alpha2 chain	U12147
-2,4	Collagen alpha1 type I	Z78279
-2,3	Laminin alpha 4	Y09827
-2,2	Collagen alpha-1 type-III	M18933

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

Fold - Δ	Gene	Accession no.
Proteases and protease inhibitors		
10,6	ADAMTS-1	AF149118
5,9	Late gestation lung protein 1 (Lgl1)	AF109674
4,9	Glia-derived nexin (GDN)	M17784
3,4	Calpastatin, clone RNCAST104	Y13588
2,1	Furin.	X55660
2	Serine protease inhibitor (SPI3)	U25844
Cell surface receptors		
4	Glypican	L34067
3,3	p120 (catenin delta1)	Z17804
3,2	ST7 protein	AAD44360
2,5	Thymocyte mRNA for cell surface protein (MRC OX-2)	X01785
2,4	Cadherin-10	BAA87417
2,3	NC1 protein	AJ250730
2,1	VPS10 domain receptor protein SORCS 2	NM_030889
Extracellular matrix proteins		
6,3	Tenascin C	D90343
4,7	collagen, type XX, alpha 1	BAA96034
2,1	Collagen XII alpha 1	U57362
Cytoskeletal proteins		
4,6	Beta-centracetin	CAA57691
3,4	Myosin X (myo 10 gene).	AJ249706
3,1	Gamma-filamin	AAF67190
3	Kinesin heavy chain (Khcs)	L27153
2,8	T-plastin.	X70706
2,6	Moesin	AF004811
2,5	E-septin long form mRNA	AF180525
2,5	MID2	Y18881
2,1	RLC-A gene for myosin regulatory light chain	X54617
2	Erythrocyte tropomodulin (E-Tmod)	AF287746
Transporters		
7	Vesicular monoamine transporter	L00603
3	Ion channel homolog RIC mRNA	U72680
2,2	ATPase, H+ transporting, lysosomal accessory protein 2	CAB43210
2,2	Multidrug resistance protein 1a (Pgy1)	AF257746
2,2	Dihydropyridine-sesitive L-type calcium channel alpha-2 subunit	M86621
2	CLCN3	X78520

Transcription factors		
3,6	MEF2-interacting transcription repressor (Mitr)	NM_024124
2,6	Myocyte enhancer factor 2C (Mef2c)	NM_025282
2,4	Basic transcription factor 2, 35 kD subunit	CAA82909
2	PEBP2 beta	AF087437
Others		
10,75	Mouse ectropic viral integration site 2 (Evi-2) ORF	M34896
8,3	GAP-43	L21191
5,6	Developmentally-regulated cardiac factor (DRCF-5)	U95001
4,7	Cytochrome c oxidase subunit Vb	D10951
4,2	CGI-78 protein	AAD34072
4,1	Beta-galactoside-alpha 2,6-sialyltransferase	M83143
3,7	Phytanoyl-CoA hydroxylase interacting protein	BAA13402
3,4	Smooth muscle cell LIM protein (SmLIM) mRNA	U44948
3	Plasma glutathione peroxidase precursor	D00680
3,2	Platelet-derived growth factor A-chain (PDGF A)	L06894
3,1	M-Sema F	S79463
2,7	ARFGEF1	BAA91912
2,7	Peripheral plasma membrane protein CASK	U47110
2,6	Ubiquitin carboxyl-terminal hydrolase PGP9.5	D10699
2,5	Ri1	X76454
2,4	Protein phosphatase-2A catalytic subunit	X14159
2,4	Galanin	J03624
2,4	Diphosphoinositol polyphosphate phosphohydrolase type 2 (NUDT4)	AF191654
2,3	Beta-1,3-N-acetylglucosaminyltransferase	AF092050
2,3	PE31/TALLA	D26483
2,3	Phosphofurin acidic cluster sorting protein 2	BAA25528
2,3	Stress activated protein kinase alpha II	L27112
2,2	Guanine nucleotide binding protein gamma subunit 11	AF257110
2,2	Cctq-cytosolic chaperone containing TCP-1, theta subunit	Z37164
2,2	Evectin-2 (Evt2)	AF189817
2,1	Vascular protein tyrosine phosphatase-1 rDEP-1	U40790

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

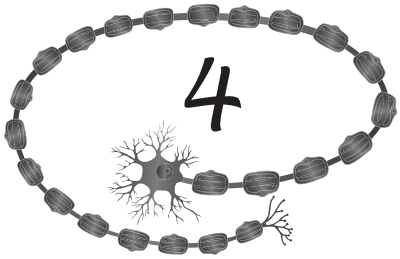
Table 2
cDNAs induced during forskolin dependent Schwann cell differentiation identified with molecular indexing

Gene	Accession no.
Myelin protein zero (Mpz)	NM_017027
Neurofilament, light polypeptide (Nefl)	NM_031783
Lamin A (Lmna)	NM_021755
Utrophin (Utrn)	NM_013070
Alpha-spectrin 2 (Spna2)	X90845
Collagen, type V, alpha 3 (Col5a3)	NM_021760
Collagen, type VI, alpha 1 (Col6a1)	XM_001079629
Collagen, type I, alpha 1 (Col1a1)	Z78279
Plexin B3 (Plxnb3)	NM_001135878
Transforming growth factor, beta receptor III (Tgfbr3)	NM_017256
Fibulin 5 (Fbln5)	AF137350
Sperm associated antigen 9 (Spag9)	NM_0275691
Insulin-like growth factor binding protein 5 (Igfbp5)	AF139830
Ferritin, heavy polypeptide 1 (Fth1)	NM_012848
Rho-guanine nucleotide exchange factor (Rgnef)	NM_001108542
Fibroblast growth factor (acidic) intracellular binding protein (Fibp)	NM_172334
Sequestosome 1 (Sqstm1)	Y08355
Ribosomal protein S6 kinase polypeptide 2 (Rps6ka2)	D83013
Brain creatine kinase (Ckb)	NM_012529
PTEN induced putative kinase 1 (Pink1)	XM_216565
Cyclin D3 (Ccnd3)	NM_012766
Transcription factor CP2 (Tcfcp2)	NM_001134714
Transducin-like enhancer of split 3 (Tle3)	NM_053400
Ribosomal protein S4 (Rps4x)	X14210
Ribosomal protein L5 (Rpl5)	NM_031099
Inscuteable homolog (Insc)	NM_001106285

cDNAs down-regulated during forskolin dependent Schwann cell differentiation identified by molecular indexing

Gene	Accession no.
Gamma C filamin (Flnc)	XM_342653
Talin 1 (Tln1)	NM_001039025
Vimentin (Vim)	NM_031140
Serine (or cysteine) peptidase inhibitor, clade B, member 5 (Serpinb5)	U58857
Non-erythrocyte beta-spectrin (Sptbn1)	AF337905
Dystonin (Dst)	NM_001108208
Peroxidasin homolog (Pxdn)	XM_001053103
Tenascin C (Tnc)	NM_053861
Catenin (cadherin associated protein), delta 1 (Ctnnd1)	NM_001107740
Sialic acid binding Ig-like lectin E (RGD1563073)	XR_009371
Transforming growth factor, beta induced (Tgfbi)	AF305713
Guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1 (Gnb2l1)	U03390
Serine (or cysteine) peptidase inhibitor, clade E, member 2 (Serpine2)	M17784
Guanine nucleotide-binding protein G-s alpha subunit (Gnas)	NM_019132
Meningioma expressed antigen 5 (Mgea5)	NM_131904
Procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1 (Plod1)	L25331
Suppressor of Ty 5 homolog (Supt5h)	U885391
Activating transcription factor-3 (Atf3)	NM_012912
Zinc finger, AN1-type domain 5 (Zfand5)	NM_001106356
Heterogeneous nuclear ribonucleoprotein K (Hnrnpk)	NM_057141
Ribosomal protein L10 (Rpl10)	NM_031100
Ribosomal protein L13 (Rpl13)	NM_031101
Acidic ribosomal protein P0 (Arbp)	NM_022402
Tetratricopeptide repeat domain 1 (Ttc1)	NM_001005529

[1] refers to mouse cDNA



The interactome of Oct6: Searching for partners of Oct6 in Rat Schwann cells

Noorjahan B. Jagalur, Ali Shahmoradi, Karel Bezstarosti,
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(Work in progress)

The interactome of Oct6: Searching for partners of Oct6 in Rat Schwann cells

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Abstract

Oct6/POU3f1/SCIP is a POU domain transcription factor that plays a pivotal role as an intracellular regulator of Schwann cell differentiation. Oct6 deficient mice show congenital hypomyelination with delayed onset of myelination. The mechanism through which Oct6 exerts its function in myelination is still unclear. On one hand Oct6 induces expression of Krox20, but on the other hand it is involved in repressing promoters of myelin specific genes such as myelin basic protein (MBP) and myelin protein zero (MPZ). It is therefore likely that the activating and repressive functions of Oct6 result from participation of different interacting proteins and/or post-translational modifications. Apart from a direct interaction between the HMG domain of Sox proteins and the POU domain of Oct6, no other interaction partners of Oct6 are known in Schwann cells. To define the complete set of Oct6 interacting proteins in Schwann cells, we immuno-purified Oct6 from Schwannoma cell nuclear extracts and identified co-purifying proteins by mass spectrometry. Our analysis revealed a range of Oct6 interacting proteins, including transcription factors, chromatin remodeling complexes, factors involved in protein transport and mRNA splicing. This list provides an important source for future experiments that aim to establish the exact mechanism of Oct6 function in Schwann cell development.

Introduction

Schwann cells are the myelin forming cells in the peripheral nervous system. They originate from neural crest cells, undergo a transition from Schwann cell precursors to promyelinating cells to finally differentiate as either myelinating or non-myelinating Schwann cells (1,2). During the last two decades a number of transcription factors have been identified that play important roles in Schwann cell differentiation and the execution of the myelin-related program of gene expression. Key factors in this myelination program include Oct6, Brn2, Nfatc4, YY1, c-Jun, Sox2, NF-kB, Srebp and Sox10 (2-7). These transcription factors appear to regulate the expression and activity of the transcription factor Krox20, which is the key activator of myelin related gene expression.

Expression of Oct6 in the Schwann cell lineage is strictly controlled. Oct6 expression is initiated at around embryonic day (E)12.5, peaks around birth at the onset of myelination and is gradually downregulated as myelination proceeds and is completely extinguished in adult nerves (8-10). Homozygous Oct6 deleted mice ($Oct6^{bgeo/bgeo}$) show a transient arrest of Schwann cells at the promyelin stage of cell differentiation, resulting in late onset of myelination and hypomyelinated nerves in adult animals (5,11,12). Having established that Oct6 plays a critical role in the myelination program (previous chapters), we decided to purify Oct6 in order to identify its interaction partners. It is generally assumed that tissue-specific transcription factors interact with coactivators and corepressors in order to modulate gene expression.

To understand the exact mechanism of Oct6 function in Schwann cell differentiation and, in particular, Krox20 gene regulation requires a complete catalogue of Oct6 interacting proteins. Toward this end we undertook an unbiased proteomic approach and identified potential Oct6 interacting proteins through mass spectrometry analysis of Oct6 immunoaffinity purified protein complexes .

Results and discussion

Mass spectrometry analysis of Oct6 interacting proteins

Using a polyclonal rabbit antibody raised against the amino-terminal portion of Oct6, we immunopurified Oct6 from RT4-D6P2T nuclear extracts. RT4-D6P2T is a rat Schwannoma cell line that resembles an early myelinating Schwann cell. It expresses considerable amounts of Oct6, Krox20, Sox10, PMP22 and MPZ ((13-15) and unpublished observations). The RT4-D6PT2 cell line was established from the

RT4 tumor of a sciatic nerve derived from N-ethyl-N-nitrosourea treated rats (16,17). Oct6 immunoprecipitation from RT4D6-P2T nuclear extracts was carried out twice without and once with benzonase treatment. The inclusion of benzonase treatment reduces the co-purification of proteins through DNA binding (18). After extensive washing and elution of bound proteins with low pH, Oct6 and associated proteins were resolved on SDS-PAGE followed by Coomassie staining. Oct6 is identified as a prominent band in the Oct6 sample (Figure 1). The immunoprecipitated proteins were subjected to mass spectrometry analysis. The analysis identified Oct6 and several potential interacting proteins (Table1 and Table 2). This list of putative interacting proteins included 19 transcription factors of which four were basal transcription factors. We also identified a number of chromatin modifying complexes including subunits of the SWI/SNF complex and the NURD complex. Oct6 protein interaction network in RT4D6-P2T cells is illustrated (Figure 2).

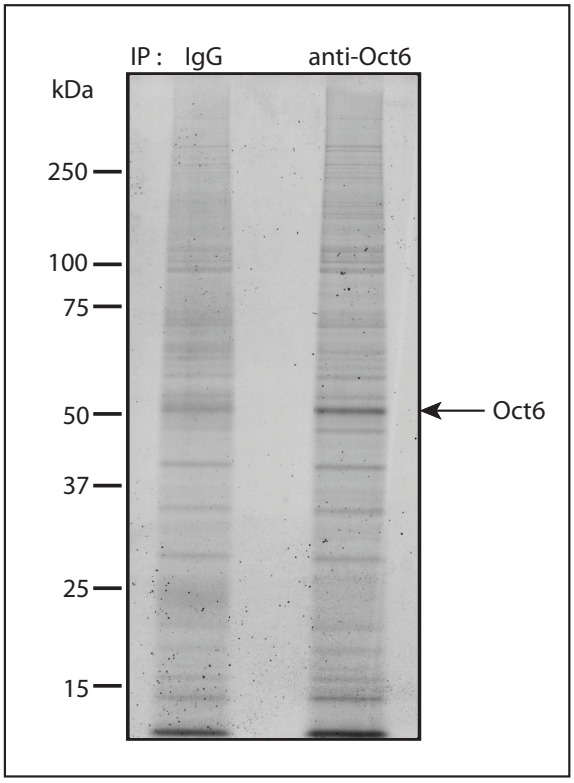

Figure 1 - Purification of Oct6 and its interacting partners.
Colloidal Coomassie-stained SDS polyacrylamide gel of immuno-precipitated endogenous Oct6 and a control immuno-precipitation using IgG. The Oct6 band is indicated by an arrow.

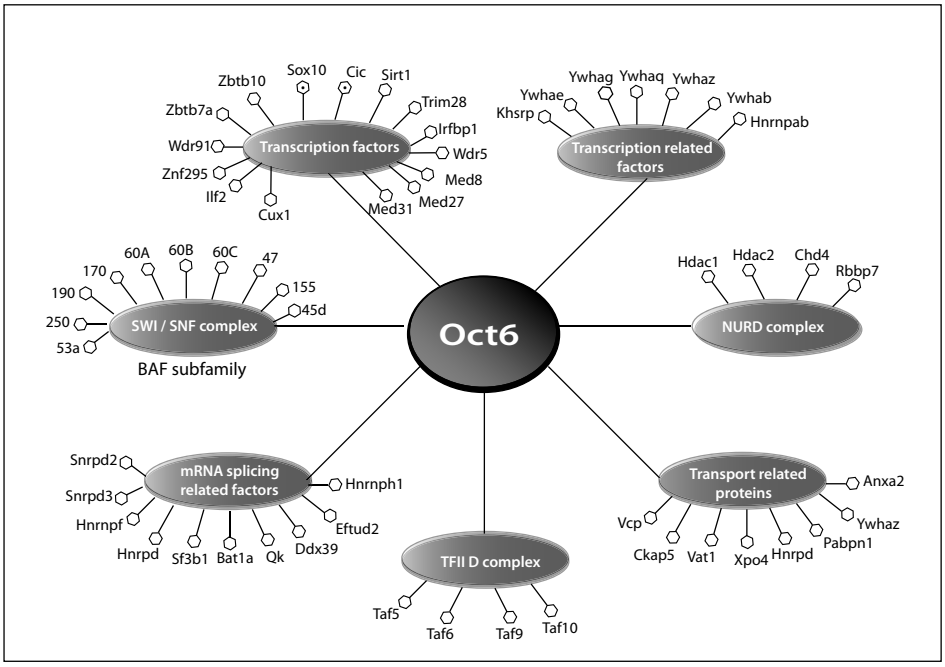

Figure 2 - Interactome of Oct6 protein in rat Schwannoma cells.
Oct6 protein interaction network in RT4D6-P2T cells, including Oct6 associated transcription factors, transcription related factors, chromatin remodeling complexes, mRNA splicing related factors, TFIID factors and transport related factors. Black dots in polygonal shapes indicate the experimentally validated interacting proteins in our laboratory.

Oct6 interacts with several transcription factors

Among the Oct6 associated proteins identified by mass spectrometry the HMG box protein Sox10 topped the list (Table 1). The interaction between Oct6 and Sox10 was confirmed by co-immunoprecipitation. Indeed, Oct6 antibody efficiently co-immunoprecipitated Sox10 from RT4D6-P2T nuclear extract (Figure 3A). Treating the nuclear extract with benzonase ruled out the possibility that Oct6 and Sox10 interaction is only mediated through nucleic acids. Mass spectrometric analysis revealed the presence of only Sox10 while none of the other Sox factors was detected, suggesting that they did not co-purify with Oct6. Together with Sox8 and Sox9, Sox10 belongs to the E class of Sox proteins (19,20). In transfection experiments, all three Sox proteins are able to synergize with Oct6 in the activation of the myelinating Schwann cell enhancer of Krox20 (MSE) (21). Moreover, it is known that they are expressed in RT4D6-P2T cells (13). Sox E proteins are highly conserved in vertebrates, exhibit overlapping expression patterns in mouse and play important

roles in different developmental processes (19,22-24). Krox20, c-Jun, Dlxf5 and C/EBPα are efficiently co-immunoprecipitated by Sox8 as well as by Sox10 (25). The observation that only Sox10, co-precipitates with Oct6 suggests that the interaction between Oct6 and Sox10 is very specific.

Sox10-Oct6 interaction is K2 domain independent

The Sox10 protein has several distinct domains that are shown to play different functional roles in Schwann cell biology (26). These domains are the HMG domain, a dimerization domain, the K2 domain and a transactivation domain (Figure 3B). The HMG domain is necessary for DNA binding. Recently, Reiprich and colleagues (21) showed that a mutation in the HMG-box of Sox10 abolishing DNA binding destroys MSE activation and synergism with Oct6 *in vitro*. In contrast, mutational inactivation of the dimerization motif of Sox10 (Sox10aa1) neither interfered with MSE activation nor with the synergistic interaction with Oct6. The other conserved domain, the K2 domain, is highly conserved in class E Sox proteins (27). Mice in which the K2 domain of Sox10 is deleted fail to activate myelin genes such as MBP and MPZ (26). However, these mice do express Oct6 implying that the promyelin stage is unaffected. To activate myelin genes, Krox20 is necessary and it is not expressed in K2 mutant mice (21). We therefore reasoned that the K2 domain of Sox10 might be involved in MSE activation. Both DK2Sox10 and wildtype Sox10 were equally active in activating the luciferase reporter gene expression through the MSE (Figure 3C). However, synergistic activation between Oct6 and Sox10 was lost upon deletion of the K2 domain (Figure 3C). This indicates that the K2 domain plays an essential role in mediating the synergism between Oct6 and Sox10 in line with the observations made by Reiprich and co-workers (21).

To establish whether the observed synergism between Oct6 and Sox10 involves direct protein-protein interactions, possibly through the K2 domain, we performed Oct6 immunoprecipitation experiments using extracts from HEK 293T cells transfected with either wildtype or mutant (DK2) Sox10. HEK 293T cells express endogenous Oct6. We found that Oct6 antibodies co-immunoprecipitated DK2Sox10 protein as efficiently as wildtype Sox10 protein (Figure 3D). Previously it has been shown that the K2 domain by itself is incapable of precipitating Oct6 (21). However, the aminoterminal portion of Sox10, including the HMG domain, did precipitate Oct6. Previous reports suggested that the HMG domain of Sox10 interacts with the DNA binding domain of Oct proteins (25). Hence, although the K2 domain is essential for synergism, it is unlikely that it is exerting this role through direct protein–protein interactions with Oct6.

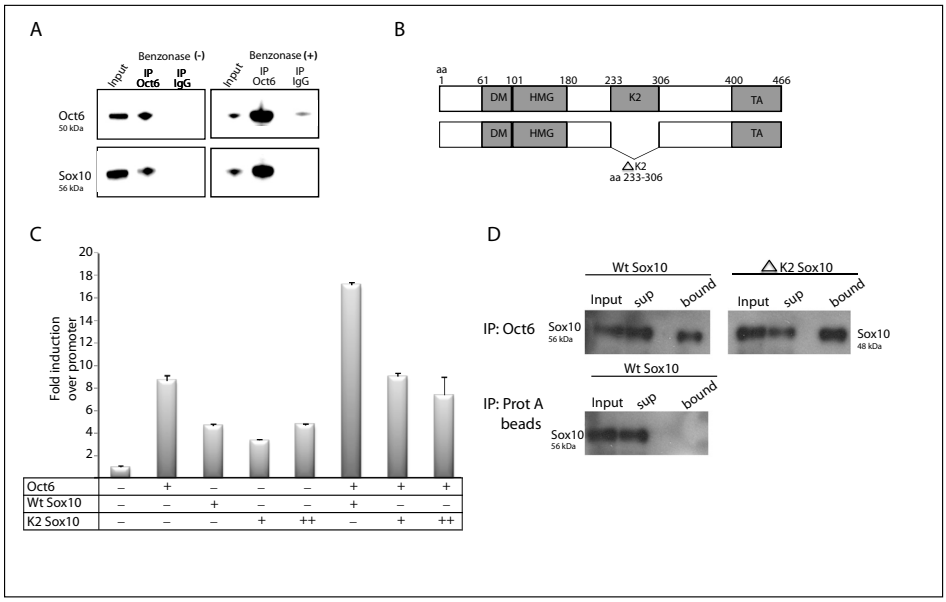

Figure 3 - Oct6 interaction with Sox10 is K2 domain independent.
A) Western analysis shows the co-immunoprecipitation of Sox10 with Oct6 antibody. The molecular sizes of both Oct6 and Sox10 are indicated. The IgG immunoprecipitation serves as a negative control. Benzonase was added where indicated (+).
B) Schematic representation of Sox10 protein. Different domains with their respective amino acid position are marked. DM: dimerization motif; HMG: High mobility group DNA binding motif: K2: highly conserved potential transactivation motif; TA: C-terminus transactivation motif. Δ K2 refers to the deletion of the K2 domain.
C) 1.3 kb Krox20 MSE luciferase reporter plasmid was transfected alone or in the presence of Oct6, wt Sox10 and ΔK2 sox10 expression vectors in various combinations. The luciferase activity with the promoter alone was arbitrarily set to 1 and fold activation with effectors was calculated. Note that ΔK2 sox10 reduces the transactivation of MSE. Error bars represent the standard deviation between the replicates. The experiment was performed at least three times and the results are reproducible.
D) Oct6 efficiently co-immunoprecipitates both wild type (wt) and mutant Sox10 (ΔK2 Sox10) as shown in the western blot using the Sox10 antibody. HEK 293T cells were transfected with either wt or mutant Sox10 expressing equal amount of protein. Bound fraction with Prot A beads (negative control) does not show any Sox10 specific band. Sup: unbound fraction; bound: precipitated fractions.

Oct6 interacts with Capicua

Another interesting protein found in our mass spectrometry analysis is a protein called Capicua (Cic). Cic is a transcriptional repressor and contains an HMG domain similar to that of Sox proteins. The human homologue of Cic is implicated in CNS development (28). Cic is a direct nuclear target of the ErbB signaling pathway in flies

(29,30). ErbB receptors are essential for Schwann cell development and myelination (7,31). Therefore, Cic is an interesting candidate as an Oct6 co-regulator involved in myelin related gene expression.

In RT4D6-P2T cells, there are two isoforms of Cic: Cic-L (large isoform) and Cic-S (small isoform) both of which are expressed in RT4D6-P2T cells (Figure 4A). Cic antibodies co-immunoprecipitated Oct6 from RT4D6-PT2 nuclear extracts, further validating the mass spectrometry results (Figure 4B). Cic is associated with the polyglutamine protein Ataxin1 (Atxn1) in humans and *Drosophila* and this interaction is necessary for modulating the repressive activity of Cic (32). Expansion of a polyglutamine tract in Atxn1 protein reduces the association of mutant Atxn1 with Cic *in vivo*, resulting in the neuropathological disorder spinocerebellar ataxia type1 (SCA1) (32). Interestingly, we have also found Atxn1 and another protein similar to Atxn1 in the mass spectrometry results (Table 1). It is tempting to speculate that this protein complex plays a role in mediating Oct6 regulated gene expression by determination of activation versus repressive functions of Oct6. Future experiments in which Cic complex components are knocked down or overexpressed may lend further credence to this hypothesis.

Chromatin remodeling complexes as co-players in Schwann cell differentiation and Oct6 function

The regulation of temporal and spatial gene expression is crucial for normal development and differentiation in higher eukaryotes. This is ultimately achieved through delicate cross-talk between the chromatin and the transcriptional apparatus. Reorganization of chromatin structure facilitates or inhibits the access of transcription factors to their target genes, leading to transcriptional activation or repression. Chromatin remodelers modify chromatin structure or serve as a bridge between transcription factors and basal transcription machinery. Several groups of chromatin-remodeling complexes are responsible for this process (33,34). One such group is the SWI/SNF enzyme complex that utilizes the energy of ATP hydrolysis to slide or peel away histone octamers around which nucleosomal DNA is wrapped. This process exposes the DNA and facilitates the binding of sequence specific transcription factors (35).

A number of subunits of the SWI/SNF complex are prominently present in our list of Oct6 interacting proteins (Table 1). SWI/SNF complexes are evolutionarily conserved from yeast to human, and have been implicated in many biological processes, such as transcriptional regulation, tumorigenesis, development, and differentiation (36). They can be grouped into two major complexes: BRG1 or hbrm-

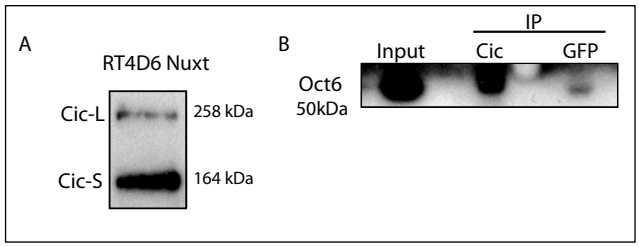

Figure 4 - Capicua co-immunoprecipitates Oct6 in RT4D6 cells.
A) Capicua (Cic), an HMG box protein is expressed in RT4D6-P2T cells. Both short (Cic-S) and long (Cic-L) isoforms are indicated with their respective molecular size.
B) Western blot analysis shows a much higher amount of Oct6 in the Cic IP than in the control IP with a GFP antibody.

associated factor (BAF) and Polybromo-associated BAF (PBAF) (37-41). Both BAF and PBAF complexes share eight identical subunits; they are distinguished by three unique subunits. BAF180 (also called Polybromo) and BAF200 are specific subunits of PBAF and BAF250 (also called Arid1a, Smarcf1) is specific for BAF (37,41,42).

Because both complexes share similar subunits, it can be assumed that they perform similar functions. However, Lemon and colleagues (43) showed that PBAF, but not BAF, is necessary for ligand-dependent transcriptional activation by nuclear hormone receptors *in vitro*. These two complexes are also involved in selective regulation of different interferon alpha inducible genes. IFITM1 is dependent on BAF200 and IFITM3 is dependent on BAF250 for their optimal expression (42). It is interesting to note that in the mass spectrometry analysis, we only found the specific subunit of the BAF complex, BAF250, but not either BAF180 or BAF200. Hence, a hypothesis can be contemplated in which Oct6 specifically recruits the BAF complex to regulate the expression of its downstream targets or alternatively this complex first remodels the target sequences of Oct6 so that it can gain access and stably bind to the DNA.

It has been shown that a specific subunit switch in the SWI/SNF complex regulates neuronal cell fate (44). Western blot analysis of mouse brain nuclear extracts from different developmental stages showed a switch in the expression of BAF45 and BAF53 subunits during neurogenesis. BAF45a and BAF53a expressed around E10.5-11.5 gives way to BAF45b/c and BAF53b, respectively during later development (E13.5 onwards). E12-13 is the time point when neural stem cells in the developing brain cease to proliferate and start to differentiate into neurons. During neuronal differentiation, BAF45a and BAF53a (neural progenitor specific BAF – npBAF complex) are exchanged with BAF45b/c and BAF53b (postmitotic neuron

specific BAF - nBAF complex). Thus exchanges of subunits within BAF complexes accompany the transitions from multipotent stem cells to neuroblasts and finally to post mitotic neurons (44-47). We find BAF53a in our immunoprecipitation. However, it would be premature to draw conclusions from the presence of BAF53a in the list of Oct6 interacting proteins. To address the question if npBAF and nBAF complexes play a role in the differentiation of Schwann cells, functional assays need to be carried out in primary Schwann cells.

Some of the other components of the SWI/SNF complex found in the analysis are BAF190, BAF155, BAF170 and BAF60a/b/c. BAF190 (Brg1) is the catalytic subunit of SWI/SNF complex. Previous studies indicate an important role for Brg1 in neural crest induction and neurogenesis (48). Brg1-deficient zebrafish showed reduced expression of Krox20 and Engrailed2 genes in brain (48). Studies conducted in *Xenopus* demonstrated that Brg1 regulates neural differentiation by mediating the transcriptional activities of the proneural bHLH Neurogenin or NeuroD proteins (49). Matsumoto and colleagues (50) showed that Brg1 is required to repress neuronal differentiation in neural stem cells as a means of permitting glial cell differentiation in response to gliogenic signals. This suggests a potential role of Brg1 in regulating the switch from neurogenesis to gliogenesis. Considering the importance of chromatin remodelers in neural development, it is tempting to speculate that Oct6 recruits these chromatin remodelers to the Krox20 MSE (3).

We also identified HDAC1/2, Rbbp7 and Chd4, components of NuRD complex as potential Oct6 interactors (Table1). Hyperacetylated histones are associated with gene activation and hypoacetylated histones mark gene repression. Histone deacetylases (HDACs) play a major role in achieving the balance between gene activation and gene repression (51-53). Several studies investigated the role of HDACs and HDAC inhibitors in oligodendryocyte differentiation and neural regeneration upon injury (54-56). Recently, HDAC1 and HDAC2 were found to be essential for Schwann cell survival and myelination ((57,58). HDAC1 regulates Schwann cell survival, while HDAC2, regulates myeliantion through interactions with NF-κB and activation of the Sox10 promoter. The Co-immunoprecipitation further revealed that HDACs interacts with Sox10, but they could not detect Oct6. However, in the double knock out HDAC1/2 mice, Oct6 mRNA levels were reduced (58). Previous reports have suggested that Oct6 represses the transcription of MPZ and MBP through quenching of an activator complex (59). It can be speculated that Oct6 forms a complex with HDAC1/2 to repress MPZ expression.

The activation or repressive function of Oct6 could be achieved by associating with different proteins. In principle we find that Oct6 associates with repressive and

activating chromatin remodelers suggesting that different Oct6 complexes in the cell exist and that the specific recruitment of the different complexes result in either repressive or activating activity. We predict that activating complexes cooperate with Oct6 to regulate the Krox20 MSE.

Transport related factors

We found exportin 4 (Xpo4) as an interacting partner of Oct6 in RT4D6-PT2 cells. It has been shown previously that Oct6 is a nucleo-cytoplasmic shuttling protein and a nuclear export signal (NES) has been defined in its DNA binding POU domain (60). The nuclear export signal of Oct6 is leucine-rich and like most other leucine-rich NES containing proteins, nuclear export of Oct6 is dependent on CRM1/Exp1 and RanGTP (60,61). However, we found only Xpo4 in the Oct6 immunoprecipitations where RanGTP was likely present. It is possible that the amount of RanGTP was not sufficient to have Exp1 in the nuclear extract. Recently it was shown that Xpo4, in addition to its established function in nuclear export, also acts as nuclear import receptor for Sox2 and SRY (62). Thus, Xpo4 might be involved in bringing Oct6 into the nucleus.

Concluding remarks

Taking a proteomics approach, we show that the POU domain factor Oct6 interacts with many nuclear proteins. Among these nuclear proteins there are proteins involved in regulating different aspects of chromatin organization, mediating ErbB signaling and nuclear-cytoplasmic transport. We validate the results obtained in the mass spectrometry by immunoblotting for two proteins, Sox10 and Cic. The question is whether all these proteins form one large complex. More likely, Oct6 exerts its function as a transcriptional activator or repressor in Schwann cell differentiation by forming different complexes with the proteins identified in this screen.

Binding of Oct6 and Sox10 on the MSE activates Krox20 expression. However, this DNA binding is not solely responsible for the induction of Krox20 as *claw paw* (*clp*) mice fail to upregulate Krox20 despite the presence of both Oct6 and Sox10 (63). *Clp* mutation is a result of 225bp insertion in the Lgi4 gene (64). The similarities in delayed myelination observed in Oct6 and in *clp* mice suggest an interaction between the two genes. It is also possible that Oct6 and Sox10 are recruiting chromatin remodelers to modify the chromatin architecture, paving the way for the induction of Krox20. Additionally, association of Oct6 and/or Sox10 with novel protein(s) might be required to stabilize DNA binding of Oct6 at target

promoters and enhancers. Dissecting the Oct6 and Sox10 protein network might provide clues to the absence of Krox20 expression in *clp* mice. Finally the interaction network of Oct6 identified by mass spectrometry contains many novel proteins that were not previously linked to myelination. Future research will address their potential role in this process.

Experimental procedures

Cell culture and nuclear extract preparation

RT4D6-P2T Schwannoma cell line were grown in 15 cms cell culture dishes containing DMEM/10%FCS/1%PS. Twenty such dishes were washed once with PBS, the cells were scraped off and nuclear extracts were prepared essentially as described in Chapter 2.

Transfection and luciferase assay

Hela cells were seeded in 6 well plates and grown to 90% confluence. Cells were transfected with 250 ng of reporter plasmid containing Krox20 MSE, 200 ng of Oct6, 200 ng of wild type and 30 ng or 50 ng ΔK2 Sox10 (effector plasmids) using Lipofectamine (Invitrogen) in the presence of fetal calf serum. The concentration of wt Sox10 and ΔK2 Sox10 was selected such that both the constructs express similar amount of Sox10 protein. Cells were washed next day and refreshed with 10% fetal calf serum and 1%Penicillin/Streptomycin containing DMEM medium. 48 hours after transfection, cells were washed with PBS and lysed in Reporter lysis buffer (promega). β-galactosidase levels were measured in an activity assay using 2-nitro-phenyl-galacto-pyranoside (ONPG) substrate. Luciferase was assayed using the steady GLO luciferase assay substrate from Promega.

Immunoprecipitation

Equal amounts of anti-Oct6 rabbit serum and pre-immune rabbit serum containing approximately 10mg/ml total IgG was precipitated using 4M ammonium sulphate pH 7.0 and subsequently resuspended the pellet in Tris borate buffer. Equal amounts of anti-Oct6 or control IgGs were crosslinked to 50 µl protein A Sepharose beads (Repligen). Antibody beads, equilibrated in C-100* buffer (20mM HEPES pH 7.6, 10% glycerol, 100mM KCl, 1.5 mM MgCl2, 0.2mM EDTA, 0.02% NP 40 and 1X complete EDTA-free protease inhibitor (Roche)) were blocked with 0.1mg/ml Insulin (Sigma), 0.2mg/ml Chicken egg albumin (Sigma), 1% cold-water fish skin gelatin (Sigma) for 1 hour at 4^0C . Subsequently, they were incubated with 1 ml of nuclear extracts

made from RT4D6-P2T cells containing benzonase for 3 hours at 4^0C in no stick microcentrifuge tubes. After washing 5 times for 5 minutes with C-100* buffer at 4^0C, either the beads were boiled in SDS-loading dye (Table 1: IP#2) or eluted with 100mM Glycine pH 2.5 (Table 1: IP#1). The eluted samples were neutralized with required amount of 2M Tris base, concentrated using 100% Trichloro acetic acid, 0.1% sodium deoxycholic acid , washed with acetone and finally resuspended in SDS-loading dye. Finally the fractions were separated on a 4-12% pre-casted SDS-PAGE (Invitrogen) and stained by colloidal Coomassie-staining (Invitrogen). Gel lanes were cut, processed and analysed by mass spectrometry .

Mass spectrometric analysis

1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described by Wilm and co-workers (65). Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) both operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the IPI database (IPI_rat_20110124.fasta). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 50. Individual peptide MS/MS spectra with Mascot scores below 30 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table.

Protein interaction network criteria

To be included in Table 1, proteins had to be present in at least one of the independent purifications with a minimum Mascot score of 50 and at least 2.5 fold higher than the score in the corresponding control (IgG) purification. Classification of proteins into different molecular functions was based on http://www.uniprot.org/uniprot.

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Table 1: Oct6 interacting partners as identified by Mass spectrometry Analysis by Oct6 immuno precipitation

			IP #1		IP #2			
			Without Benzonase		Without Benzonase		With Benzonase	
Protein	Accession		Mascot [a]	Pept. [b]	Mascot [a]	Pept. [b]	Mascot [a]	Pept. [b]
Oct6 (Pou3f1)	gi|20301972	POU class 3 homeobox 1]	736	8	842(350)	11(4)	815(279)	11(4)
Transcription factors								
Sox10	gi|9507131	SRY-box containing gene 10	638	9	867(109)	12(3)	939(35)	12(1)
Cic	gi|158711712	Capicua homolog	2233	29	633	10	539	8
Wdr91	gi|187469149	WD repeat domain 91 protein	877	12	234	4	222	3
Wdr5	gi|16554627	WD repeat domain 5 protein	399	5	164	2	-	-
Sirt1	gi|149043900	Silent mating type information regulation 2 homolog -1	1178	17	491	8	859	13
Trim28	gi|209870077	Trpartite motif-containing 28	1210(177)	16(2)	273(102)	4(1)	148	2
Zbtb10	gi|149048469	zinc finger and BTB domain containing 10	809	11	327	5	295	4
Zbtb7a	gi|149034453	zinc finger and BTB domain containing 7a	972	12	119	2	78	1
Znf295	gi|149060268	zinc finger protein 295	615	9	273	3	170	2
Ilf2	gi|13385872	Interleukin enhancer binding factor 2	490	6	145	2	99	2
Irf2bp1	gi|157819153	Interferon regulatory factor 2 binding protein 1	1239	14	199	3	204	4
Med31	gi|209447013	Mediator of RNA polymerase II transcription subunit 31	128	1	-	-	-	-
Med27	gi|157818903	Mediator of RNA polymerase II transcription subunit 27	195	3	-	-	-	-
Med8	gi|149035494	Mediator of RNA polymerase II transcription subunit 8	177	2	-	-	-	-
Cux1	gi|109495543	Similar to homeobox protein Cut like 1	294	5	82	2	151	4
Transcription related factors								
Hnrnpab	gi|4378711	Nucleic acid binding factor pRM10	387(102)	6(1)	106	1	155	2
Khsrp	gi|19424312	KH-type splicing regulatory protein	1220	15	481	9	515	8
RGD1308009	gi|198386347	similar to KIAA1007 protein	4227	61	704	12	-	-
Ywhab	gi|9507243	14-3-3 beta/alpha	483	5	368	7	-	-
Ywhaz	gi|1051270	14-3-3 zeta	782	8	738(179)	10(3)	-	-
Ywhaq	gi|6756039	14-3-3 theta	693	7	514	8	-	-
Ywhag	gi|9507245	14-3-3 gamma	336	4	402	6	-	-
YWHAE	gi|5803225	14-3-3 epsilon	1358(111)	16(2)	764(252)	13(4)	349(58)	6(1)
TFIID complex								
Taf5	gi|157822291	TATA box binding protein (TBP) - associated factor 5	576	10	-	-	126	3
Taf6	gi|3024673	TATA box binding protein (TBP) - associated factor 6	320	6	230	4	266	5
Taf9	gi|68299787	TATA box binding protein (TBP) - associated factor 9	625	7	405	7	80	2
Taf10	gi|197927135	TATA box binding protein (TBP) - associated factor 10	185	2	107	1	-	-

NURD complex								
Chd4	gi|62648161	chromodomain helicase DNA binding protein 4	103	2	337	6	-	-
Hdac1	gi|70794768	Histone deacetylase 1	543(78)	8(1)	174	3	168	2
Hdac2	gi|171846760	Histone deacetylase 2	449(78)	8(1)	156(64)	3(2)	135(78)	2(2)
Rbbp7	gi|149035823	Retinoblastoma binding protein 7	791(112)	11(3)	144(77)	3(2)	325	6
SWI/SNF complex								
Smarcf1 (Arid1a,BAF250)	gi|157817412	AT rich interactive domain 1A (SWI-like)	2865	39	2397(311)	34(5)	2007(332)	30(7)
Smarca4 (BAF190, Brg1)	gi|22095171	Brahma-related protein 1	1204	19	2264(325)	32(5)	1610(793)	26(13)
Smarcc2 (BAF170)	gi|109480098	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, c2	2229	27	1341	21	1449	20
Smarcd1 (BAF60A)	gi|157824218	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, d1	1686	23	764	12	817	14
Smarcd2 (BAF60B)	gi|2723484	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, d2	969	14	447	7	477	8
Smarcd3 (BAF60C)	gi|58865508	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, d3	1200	17	379	5	457	9
Smarcb1 (BAF47)	gi|6755578	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, b1	702	9	489(298)	9(6)	379(174)	7(3)
Smarcc1 (BAF 155)	gi|109485390	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, c1	2567	35	1919(891)	31(15)	2240(749)	37(12)
BAF45d (Dpf2)	gi|157817959	BRG1-associated factor 45D, D4, zinc and double PHD fingers family 2	464	6	433(142)	7(2)	437	6
Actl6a (BAF53A)	gi|84781662	BRG1-associated factor 53A	0(150)	0(3)	534(379)	9(6)	673(119)	8(2)
Transport								
Xpo4	gi|157819617	Exportin 4	2222	29	515	9	636	10
Pabpn1	gi|149063930	poly(A) binding protein, nuclear 1	247	4	112	2	149	3
Anxa2	gi|9845234	Annexin a2	1228	15	1038(140)	14(3)	861	12
Ckap5	gi|109468432	Spliceosome RNA helicase Bat1	291	5	196	4	218(46)	5(1)
Ywhaz	gi|1051270	14-3-3 zeta	782	8	738(179)	10(3)	-	-
Vcp	gi|17865351	valosin-containing protein	2259	29	364	8	459	8
Vat1	gi|76096306	vesicle amine transport protein 1 homolog	1081	13	773	11	984	14
mRNA splicing								
Ddx39	gi|3132829	Nuclear RNA helicase, DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	-	-	120	3	113	2
Bat1a	gi|9790069	HLA-B-associated transcript 1A	-	-	120	3	113	2
Hnrpd	gi|9588098	Heterogeneous nuclear ribonucleoprotein D-like	552	8	199	2	279(46)	3(1)
Hnrnph1	gi|10946928	heterogeneous nuclear ribonucleoprotein H1	447(117)	6(1)	-	-	787(211)	11(3)
Hnrnpf	gi|19527048	heterogeneous nuclear ribonucleoprotein F	448	6	-	-	563	8
Hnrpm	gi|56789218	heterogeneous nuclear ribonucleoprotein M	1013(35)	13(1)	-	-	557(98)	9(2)
SF3B1	gi|54112117	splicing factor 3b, subunit 1 isoform 1	239	3	176	3	250	3
Eftud2	gi|62657153	elongation factor Tu GTP binding domain containing 2	287	5	-	-	209(67)	3(1)
Qk	gi|11527388	quaking protein [Mus musculus]	611(70)	9(1)	118	2	-	-
SNRPD2	gi|4759158	small nuclear ribonucleoprotein polypeptide D2	370	5	-	-	230	3
SNRPD3	gi|4759160	small nuclear ribonucleoprotein polypeptide D3	111	1	-	-	-	-

Others								
Nap1l4	gi|58865912	nucleosome assembly protein 1-like 4	1163	14	720	9	902	11
EIF4A1	gi|4503529	eukaryotic translation initiation factor 4A isoform 1 [Homo sapiens]	473	7	-	-	276	4
Pkn3	gi|114145515	protein kinase N3	303	4	370	5	201	3
Tnks1bp1	gi|109468388	tankyrase 1 binding protein 1	2264	28	361	6	389	7
Ddx3x	gi|157823027	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	420	6	225	4	-	-
Nap1l1	gi|7657357	nucleosome assembly protein 1-like 4	710	9	163	3	335	6
Sorbs3	gi|54114997	sorbin and SH3 domain containing 3	642	9	119	3	-	-
Serbp1	gi|52783155	Serpine1 mRNA binding protein 1	-	-	117	1	116	1
EIF3E	gi|4503521	eukaryotic translation initiation factor 3, subunit 6 48kDa	666(35)	12(1)	-	-	-	-
similar to ataxin-1	gi|109508128	PREDICTED: similar to spinocerebellar ataxia 1 homolog	445	8	-	-	212	3
Pafah1b2	gi|11693154	platelet-activating factor acetylhydrolase alpha 2 subunit [Rattus norvegicus]	556	7	445(80)	8(2)	127	3
Csnk2a1	gi|16758674	casein kinase 2, alpha 1 polypeptide [Rattus norvegicus]	1161	15	594(160)	10(3)	389	6
Kif4	gi|62667042	PREDICTED: similar to Chromosome-associated kinesin KIF4A (Chromokinesin)	269	4	-	-	181	4
Dynll1	gi|4505813	dynein light chain 1 [Homo sapiens]	233	4	-	-	147	2
Actn4	gi|6636119	Actinin alpha 4	1301	19	268(60)	5(1)	145	3
Atxn1	gi|6981504	spinocerebellar ataxia 1	130	3	-	-	-	-
Clip2	gi|149063106	cytoplasmic linker 2 [Rattus norvegicus]	1978	31	775(70)	15(1)	966(72)	17(1)
Capza1	gi|149030414	F-actin capping protein alpha-1 subunit [Rattus norvegicus]	651	8	134	2	57	1
Fhod1	gi|109507992	PREDICTED: Formin homology 2 domain-containing protein 1	1770	23	440	5	552	7
GABARAP	gi|6005764	GABA(A) receptor-associated protein	141	4	-	-	-	-
Pcbp4	gi|62654235	PREDICTED: similar to Poly(rC)-binding protein 4 (Alpha-CP4)	-	-	141	3	-	-
RGD1308009	gi|198386347	similar to KIAA1007 protein	4227	61	704	12	-	-

Thresholds for inclusion of the identified proteins into Table1 are in Experimental Procedures

[a] Mascot score for the specified protein in the Oct6 sample, purified by Oct6 - immunoprecipitation, Mascot score for the specified protein in the corresponding control purification, if present, is between brackets.

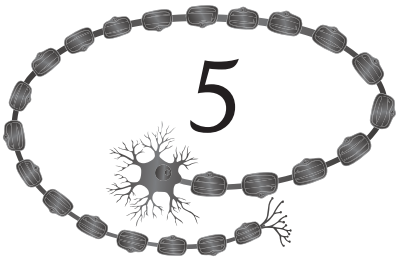
[b] Number of identified unique-non-redudant peptides for the specified protein in the Oct6 sample. Number of identified unique peptides in the control purification is between brackets.

Table 2: Oct6 interacting partners: emPAI scores

Protein	Accession	IP #1 Without Benzonase emPAI[a] value	IP #2 Without Benzonase emPAI[a] value	With Benzonase emPAI[a] value	average emPAI
Oct6 (Pou3f1)	gi|20301972	4.05	3.47(0.45)	1.13(0.25)	2.88
Transcription factors					
Sox10	gi|9507131	1.55	5.46(0.14)	0.55	2.52
Cic	gi|158711712	0.86	0.19	0.21	0.42
Wdr91	gi|187469149	0.56	0.19	-	0.38
Wdr5	gi|16554627	0.43	0.21	-	0.32
Sirt1	gi|149043900	1.91	0.67	2.37	1.65
Trim28	gi|209870077	-	-	0.09	0.09
Zbtb10	gi|149048469	0.45	0.22	-	0.34
Zbtb7a	gi|149034453	1.55	0.13	-	0.84
Znf295	gi|149060268	0.31	0.12	-	0.22
Ilf2	gi|13385872	0.59	0.18	0.6	0.46
Irf2bp1	gi|157819153	1.49	0.19	0.1	0.59
Med31	gi|209447013	0.23	-	-	0.23
Med27	gi|157818903	0.33	-	-	0.33
Med8	gi|149035494	0.41	-	-	0.41
Cux1	gi|109495543	0.44	-	0.04	0.24
Transcription related factors					
Hnrnpab	gi|4378711	-	-	0.2	0.20
Khsrp	gi|19424312	1.71	0.41	0.51	0.88
RGD1308009	gi|198386347	1.45	0.16	1.09	0.90
Ywhab	gi|9507243	1.57	1.42	-	1.50
Ywhaz	gi|1051270	2.7	2.56(0.43)	0.43	1.88
Ywhaq	gi|6756039	1.29	1.75	-	1.52
Ywhag	gi|9507245	0.8	1.12	-	0.96
YWHAE	gi|5803225	14.32(0.26)	3.3(0.58)	0.99(0.12)	6.20
TFIID complex					
Taf5	gi|157822291	0.36	-	0.3	0.33
Taf6	gi|3024673	0.32	0.22	1.49	0.68
Taf9	gi|68299787	1.26	1.39	0.27	0.97
Taf10	gi|197927135	0.16	0.38		0.27
NURD complex					
Chd4	gi|62648161	0.03	0.1	0.19	0.11
Hdac1	gi|70794768	0.72(0.06)	0.21	0.13	0.35
Hdac2	gi|171846760	0.54	0.19(0.12)	0.12(0.12)	0.28
Rbbp7	gi|149035823	1.31(0.15)	0.16(0.14)	1.61	1.03
SWI/SNF complex					
Smarcf1 (Arid1a,BAF250)	gi|157817412	1.22	1.01(0.09)		1.12
Smarca4 (BAF190, Brg1)	gi|22095171	0.43	0.94	5.24(0.28)	2.20
Smarcb1 (BAF47)	gi|6755578	1.17	1.29(0.48)	0.84(0.26)	1.10
Smarcc1 (BAF 155)	gi|109485390	2.55	1.73(0.6)	2.13(0.51)	2.14
Smarcc2 (BAF170)	gi|109480098	1.87	0.84	0.83	1.18
Smarcd1 (BAF60A)	gi|157824218	4.01	1.37	-	2.69
Smarcd2 (BAF60B)	gi|2723484	1.9	0.6	0.77	1.09
Smarcd3 (BAF60C)	gi|58865508	2.28	0.6	0.3	1.06
BAF45d (Dpf2)	gi|157817959	0.56	0.89(0.16)	-	0.73
Actl6a (BAF53A)	gi|84781662		1.28(0.53)	0.92(0.15)	1.10
Apoptosis/cell death					
Pabpn1	gi|149063930	0.67	0.4	0.55	0.54
Taf9	gi|68299787	1.26	1.39	0.27	0.97
Actn4	gi|6636119	0.78	0.19	0.85	0.61
Hdac1	gi|70794768	0.72	0.21	-	0.47
Sirt1	gi|149043900	1.91	0.67	-	1.29
Vcp	gi|17865351	3.66	0.27	-	1.97
Taf10	gi|197927135	0.16	0.38	-	0.27
14-3-3 epsilon(YWHAE)	gi|5803225	14.3	3.3	0.99	6.20

Cytoskeletal organization					
Clip2	gi|149063106	1.47	0.55	2.23	1.42
Capza1	gi|149030414	2.04	0.3	0.29	0.88
Fhod1	gi|109507992	1	0.15	-	0.58
Transport					
Xpo4	gi|157819617	1.61	0.25	0.17	0.68
Pabpn1	gi|149063930	0.67	0.4	-	0.54
Anxa2	gi|9845234	6.87	2.63	-	4.75
Ckap5	gi|109468432	0.07	0.08	0.06(0.02)	0.07
Ywhaz	gi|1051270	-	2.56(0.43)	0.43	1.50
Vcp	gi|17865351	3.66	-	-	3.66
Vat1	gi|76096306	4.09	1.71	-	2.90
mRNA splicing					
Ddx39	gi|3132829	-	-	0.16	0.16
Bat1a	gi|9790069	-	-	0.11	0.11
Hnrpd	gi|9588098	1.52	-	0.5(0.1)	1.01
Hnrnph1	gi|10946928	0.4(0.07)	0.34	4.13(0.23)	2.24
Hnrnpf	gi|19527048	0.55	0.87	-	0.71
Hnrpm	gi|56789218	-	0.21	-	0.21
SF3B1	gi|54112117	1.44	0.08	-	0.76
Eftud2	gi|62657153	0.13	0.07	0.17(0.07)	0.12
Qk	gi|11527388	2.04(0.1)	-	-	2.04
SNRPD2	gi|4759158	3.18	0.29	-	1.74
SNRPD3	gi|4759160	0.59	0.28	0.32	0.40
Others					
Nap1l4	gi|58865912	5.71	1.66	1.72	3.03
EIF4A1	gi|4503529	1.06	0.59	-	0.83
Pkn3	gi|114145515	-	-	-	
Tnks1bp1	gi|109468388	0.72	0.11	-	0.42
Ddx3x	gi|157823027	0.63	0.52	-	0.58
Nap1l1	gi|7657357	2.02	0.27	1.04	1.11
Sorbs3	gi|54114997	0.45	-	-	0.45
Serbp1	gi|52783155	-	-	0.08	0.08
EIF3E	gi|4503521	1.45(0.07)	0.15	-	0.80
similar to ataxin-1	gi|109508128	0.43	0.38	-	0.41
Pafah1b2	gi|11693154	2.63	1.62(0.3)	0.22	1.49
Csnk2a1	gi|16758674	4.13	1.81(0.25)	0.26	2.07
Kif4	gi|62667042	0.07	-	0.08	0.08
Dynll1	gi|4505813	3.59	-	1.86	2.73
Actn4	gi|6636119	0.78	0.19(0.03)	0.85	0.61
Atxn1	gi|6981504	0.08	0.25	-	0.17
Clip2	gi|149063106	1.47	0.55(0.03)	2.23(0.03)	1.42
Capza1	gi|149030414	2.04	0.3	0.29	0.88
Fhod1	gi|109507992	1	0.15	-	0.58
GABARAP	gi|6005764	1.56	0.65	-	1.11
Pcbp4	gi|62654235	-	0.19	-	0.19
RGD1308009	gi|198386347	1.45	0.16	1.09	0.90

[a] emPAI score for the specified protein in the Oct6 sample. emPAI score for the specified protein in the corresponding control purification, if present, is between brackets.



Conclusions and future prospects

Conclusions and future prospects

The invention of myelin was a defining moment in the evolutionary history of vertebrates. As myelin enabled axons to propagate electrical impulses at unprecedented speed with relatively low energetic costs, it facilitated the evolution of highly complex nervous systems. Oligodendrocytes and Schwann cells are the myelin forming macroglia of the vertebrate nervous system and myelinate axons in the central nervous system and the peripheral nervous system respectively. Schwann cells are derived from the neural crest, a transient, migratory embryonic population of stem cells that originate from the dorsal margin of the neural tube. Although little is known about the early stages of Schwann cell development, it is assumed that migratory crest cells that contact the outgrowing axons are triggered to become Schwann cell precursors. This highly proliferative and migratory precursor population invades the embryonic nerves and segregates axon bundles in a process called radial sorting. At the same time, precursors differentiate into immature Schwann cells, cells that can be distinguished from precursors on the basis of growth factor dependency and marker expression (1). Myelination promoting signals from axons trigger the transition of immature, promyelinating cells to myelinating cells. Depending on the size of the axons they are associated with, they differentiate into either a myelinating or a non-myelinating Schwann cell. During myelination, Schwann cells repeatedly wrap and tightly compact large sheets of plasma membranes around axons. This process involves synthesis of considerable amounts of lipids and myelin proteins like MBP, MPZ and PMP22. Activation of these myelin genes is largely controlled by the transcription factors.

A significant amount of work has been done to define the transcription factors in Schwann cells, their regulatory networks, and the intracellular pathways that modulate their activity. The backbone of the promyelinating regulatory network is formed by the POU domain transcription factor Oct6 which together with Brn2 and Sox10 regulates Krox20 expression (See chapter 1) (2,3).

Oct6 mutant Schwann cells are transiently arrested at the promyelin stage of Schwann cell differentiation and adult animals have hypomyelinated axons (4-6). We have previously identified a Schwann cell enhancer element (SCE) approximately 10kb downstream of the transcription start site of Oct6 that controls Oct6 expression both in time and space (7). Homozygous deletion of the SCE from the Oct6 locus (DSCE allele) results in the selective loss of Oct6 gene expression in Schwann cells, but expression in the brain is unaffected (8). Accordingly, peripheral

nerves of Oct6$^{\Delta SCE/\Delta SCE}$ mice exhibit delayed myelination similar to that observed in Oct6 null mice.

The SCE assembles a range of transcription factors into a multi protein/DNA complex referred to as an enhanceosome. Typically, enhanceosomes contain architectural proteins which bend DNA to facilitate interaction among the factors binding to the enhancer (9,10). Accordingly, the SCE contains binding sites for several dimeric and monomeric Sox proteins, which have been shown to be important in activating Oct6 (chapter 2), Krox20 (3) and several myelin genes (11-15)

In this thesis, we have identified functional cis-acting elements within the SCE and the transcription factor(s) that mediate Oct6 regulation in Schwann cells. Combination of these cis-acting elements into a fragment with reduced size is referred to as the mini-SCE. The mini SCE contains all the information for both the upregulation and downregulation of Oct6 (chapter 2). An essential component within this mini-SCE is a short fragment named core1 or c1. This element is absolutely required for the full enhancer function in peripheral nerves of transgenic animals. However, c1 alone is not sufficient to activate the reporter gene in transgenic mice (chapter2 and unpublished observations). The other elements necessary for the enhancer activity are HR1a3 and HR2 (chapter 2).

We have identified the SRY-box transcription factor Sox10 as an activator of Oct6 and this activation is mediated by dimeric binding of Sox10 to the c1 element of the SCE (Figure 1). We have demonstrated through transgenic analysis that two monomeric sites in lieu of c1 do not compensate for the normal dimeric Sox DNA binding motif of c1 (Chapter 2). However, Sox10 is expressed at a high level at all stages of Schwann cell differentiation. Thus, the dependence of Oct6 activation on Sox10 does not provide an obvious explanation for the regulated expression of Oct6. How could Sox10 contribute to the temporal control of Oct6 gene expression? It is possible that dimeric binding of Sox10 to the SCE induces strong DNA bending thereby facilitating the binding of other proteins and the formation of a functional enhanceosome. In this scenario, temporal control is exerted through additional factors that bind to other elements within the mini SCE. Future work should aim to identify these factors binding to HR1a3 and HR2 to establish a link between developmental signals and Oct6 gene activation. Alternatively, it is possible that temporal control is exerted through for example post-translational modifications of Sox10. Sumoylation of Sox proteins has been described to modulate the function of these proteins in Xenopus neural crest and inner ear development (Taylor and Labonne 2005).

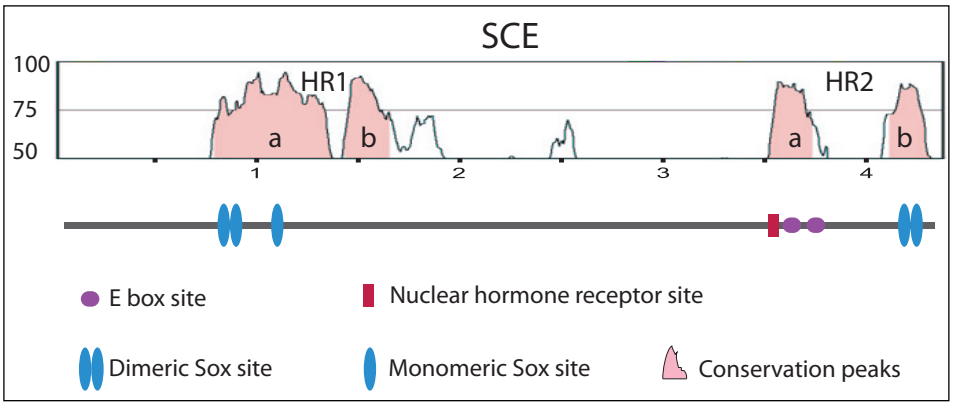


Figure 1 - Schematic representation of potential binding factors of HR1 and HR2 region of SCE

We have further extended the phylogenetic analysis of HR1a3 and HR2 to include the opossum and wallaby genomes and have preliminary data suggesting the minimal functional elements (300bp) required for the functioning of SCE (Jagalur et al., unpublished observations). We are at present extensively studying small DNA elements within HR2 that are highly conserved between placental and non-placental mammals. Of particular interest are two conserved regions in HR2 (Figure 1). Conserved sequences match consensus-binding sites for nuclear hormone receptors and basic-helix-loop-helix (bHLH) proteins (E-box binding proteins). These classes of transcription factors are most likely involved in the regulation of Oct6 expression. How these proteins might play a role in the Oct6 regulation is discussed below.

The nuclear hormone receptor superfamily includes transcription factors such as chicken ovalbumin upstream promoter-transcription factors (COUP-TFs), Retinoic acid receptors (RARs), Retinoid X receptors (RXRs) and Peroxisome proliferator-activated receptors (PPARs). Members of the nuclear hormone receptor family are involved in development, homeostasis and differentiation of different cell types. The ligand binding to a nuclear receptor results in a conformational change in the receptor, which, in turn, activates the receptor. The receptor, homo- or heterodimerizes with other nuclear receptors and binds to specific DNA sequences of target genes to up or down-regulate their expression. The HR2 region contains one consensus half site spaced by one degenerate site for nuclear receptor proteins (Consensus site: 5' AGGTCA 3').

Many of the RXRs play substantial roles in nerve development and nerve repair both in the PNS and CNS (16,17). The ability of RXR to heterodimerize with

a number of nuclear receptors suggests that RXR can modulate the expression of different genes, depending on when and with which receptor it heterodimerizes. Retinoic acid (RA) is a potent inhibitor of myelin formation in the PNS. RA acts through its binding to Retinoid X receptors (RXR) and Retinoic acid receptors (RAR) (18). RA activates RXR, which, in turn, up-regulates Krox20 expression. The Krox20 expression leads to over-expression of MBP and MPZ causing the blockage of myelin formation. However, Retinoid addition increased Oct6 expression marginally, only in Schwann cells, but not in myelinating co-cultures. On the other hand Brn2 and Sox10 expression were unaffected (18). In a recent study Latasa and co-workers (19) showed that RXR-γ is up-regulated upon axonal contact and axonal mimickers, forskolin and BPE, can replace the need for the physical contact of axons to induce RXR-γ in Schwann cells. Furthermore, RXR- γ was found to be differentially expressed during spontaneous remyelination in the CNS (17). They showed that RXR agonists or retinoids stimulate oligodendrocyte differentiation and remyelination in the injured CNS. It would be interesting to know what genes are transcribed in response to RXR- γ activation in the CNS as well as in the PNS.

Another nuclear receptor protein, PPARγ modulates the transcription of target genes by heterodimerization with Retinoid x receptors (RxR)(20). PPAR proteins respond to various lipophilic substances and activate key genes involved in lipid metabolism. During the myelination process, myelinating Schwann cells have to produce massive amount of fatty acids and lipids to meet the demand. SREBP-1c, another key molecule in lipid metabolism, has been suggested to be involved in adipogenesis by activating PPAR-γ (21). As discussed earlier, RXRs heterodimerize with an array of different nuclear receptors to regulate expression of different target genes. Therefore it is possible that different cross-regulatory networks are present to activate the myelin genes and the lipid synthesis genes. Lipids to be incorporated in the myelin sheath are mostly produced in the myelinating glial cell itself as the blood barrier inhibits uptake of lipids from the blood circulation (22). The coordination of myelin gene induction with high level of lipid synthesis is required to produce the multilayered, cholesterol-rich myelin membrane.

COUP-TFs are the other prominent nuclear receptors. They have two homologs, COUP -TFI (NR2F1) and COUP-TFII (NR2F2), in the mouse (23-25). The protein sequences are highly homologous across species, suggesting functional conservation (26). COUP-TFI null mutant mice exhibit delayed myelination and dysmyelination in the central nervous system (27). The researchers demonstrated that COUP-TF1 null mice exhibit lower expression of Oct6 in the neuronal cells

and oligodendrocytes. They further showed that reporter construct of Oct6 gene promoter can be upregulated by COUP-TFI in transient transfection assay, suggesting that COUP-TFI is the upstream regulator of Oct6 in the CNS. However, Oct6 expression in the PNS of COUP-TFI null mice was not affected. It is possible that COUP-TFII compensates for the function of COUP-TFI in the mutant mice. Our preliminary data shows that COUP-TFII protein is differentially upregulated in differentiated Schwann cells compared to proliferating Schwann cells (Thesis of Marko Pirsoo). Both COUP-TFI and COUP-TFII are expressed in nerves of mice at 2 days of age (Jagalur et al., unpublished observations). Further expression analysis, mutagenesis and chromatin immunoprecipitation experiments could address the relevance of COUP-TF binding sites in the SCE.

Together with binding sites for nuclear receptors, HR2 also harbors signatures for E box binding proteins. E boxes are short hexameric (CANNTG) DNA core sequences, to which transcription factors from the basic helix-loop-helix (bHLH) family of proteins bind (28,29). Schwann cells express a number of HLH proteins that could potentially interact with this E-box. Preliminary data from our lab show that in an EMSA one prominent complex is formed on these E-box elements. E box proteins such as Olig 1/2 and Id proteins play an important role in myelination in the CNS but the importance of HLH proteins in Schwann cells have not been established as yet (30-32). In oligodendrocytes, Olig1 and Sox10 act synergistically to drive MBP transcription (30). Moreover, Olig1 is important for differentiation of oligodendrocyte precursors into myelin-forming oligodendrocytes during development and is thought to play a crucial role in remyelination during multiple sclerosis (33). However Olig genes are not expressed in Schwann cells (34).

Sterol regulatory element binding proteins (SREBPs) consisting of SREBP-1a, SREBP-1c and SREBP-2, belong to the family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors that regulate lipid metabolism. SREBPs are highly expressed in Schwann cells (35,36) and are required for proper myelination (35). SREBP has dual DNA binding specificity binding to both Sterol regulatory elements (SREs)(5' ATCACCCCAC3') and E-boxes (5'ATCACGTGA3')(37). SREBP activation protein (SCAP) is an activator of SREBPs and deletion of SCAP in Schwann cells leads to a loss of SREBP-mediated gene expression involving cholesterol and fatty acid synthesis (38). In SCAP mutant Schwann cells, Oct6 expression is prolonged in adult nerves (P56), but eventually decreased with ageing (P210). Hence it is tempting to speculate that SREBPs are required for the timely downregulation of Oct6 in Schwann cells. However, all the three SREBPs (SREBP-1a, SREBP -1c and SREBP -2)

failed to activate or repress a SCE reporter construct in a transient transfection assay (unpublished observations).

Apart from the known binding sites that can be identified by bioinformatical approaches, it is also possible that HR2 contains binding sites for transcription factors whose target sites are not yet characterized. Detection of such proteins could be achieved by purifying the DNA binding proteins by DNA affinity chromatography and later identifying the bound nuclear proteins by mass spectrometry analysis. This unbiased approach helps to identify the proteins, which have not yet been characterized and as such, would be overlooked when scrutinized for putative transcription factor binding sites. Additionally, if the target site is bound by a transcription factor composed of various subunit combinations such as AP1, the mass spectrometry analysis will identify the exact subunit composition. Their expression pattern and western blotting can further ascertain the relevance of an identified potential factor. Proteomic analysis of nuclear factors binding to regulatory elements has been successfully achieved previously (39,40).

Downregulation of Oct6

In chapter 2 we show that a reporter construct containing the mini SCE (SCE 13: chapter 2) is temporally correct regulated in Schwann cells of transgenic mice irrespective of whether the reporter is driven by a hsp68 or Oct6 promoter. Hence, the mini SCE contains all the sequences necessary for down-regulating Oct6 expression in mature myelin forming cells (Figure 2). Ryu and co-workers (41) generated mice that constitutively expresses Oct6 in the peripheral glia. The mice exhibited persistent hypomyelination and eventual axonal loss. This data supports a dual role for Oct6 in peripheral nerve development (4,42,43). Oct6 facilitates the transition of promyelinating Schwann cell to a myelinating Schwann cell by inducing Krox20, thereby acting as an activator, while simultaneously withholding the cells from premature full differentiation by repressing MPZ and MBP. However, the continued presence of Oct6 would result in continued repression of myelin proteins. Hence, down regulation of Oct6 is important in Schwann cells as the forced expression of Oct6 beyond its normal expression window results in myelin degeneration and neuropathy (41). Therefore, it is important to understand the molecular machinery behind the timely down regulation of Oct6.

Our laboratory results have shown that Oct6 locus is not down-regulated in

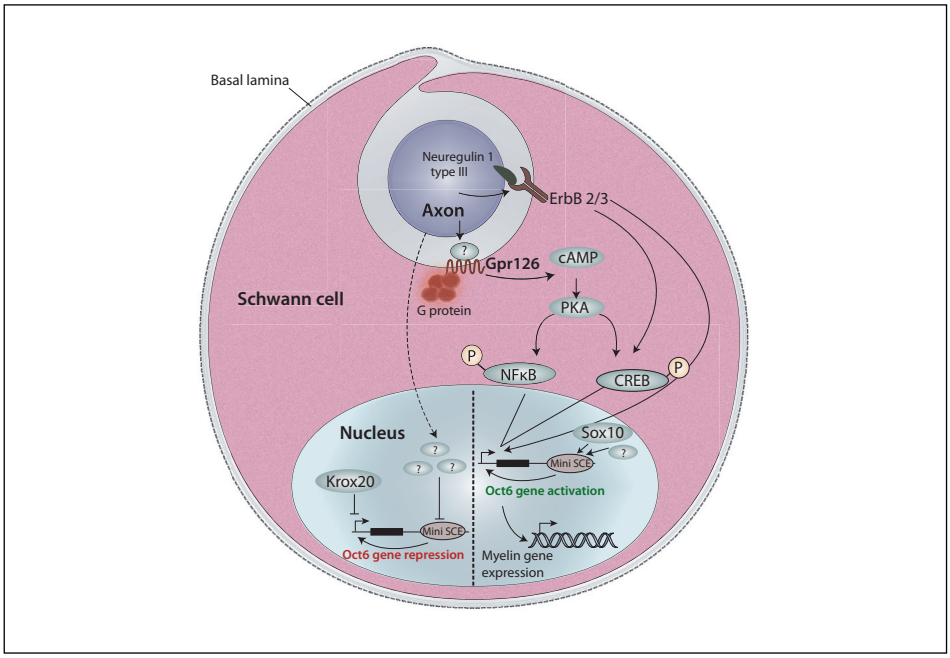


Figure 2 - Upregulation and downregulation of Oct6 in Schwann cells

the nerves of adult animals in the absence of Oct6, but faithfully recapitulated in the nerves of adult heterozygote gene targeted animals (44). The simplest interpretation would be that Oct6 itself is required for the downregulation of its own gene either directly or indirectly. These kinds of autoregulatory loops have been described for the other POU genes such as pit-1 and ventral veinless (45,46). It is possible that Oct6 activates a repressor which represses the active locus of Oct6 during the later phase of Schwann cell differentiation. Alternatively, it is possible that Oct6 is downregulated during a narrow window of normal postnatal development, by factor(s) transiently expressed. Hence, in the Oct6 null mice, where the myelination process is initiated at a later time, the putative repressive factor is not induced and hence Oct6 is not downregulated. This could explain why, during nerve transection and regeneration, Oct6 is not downregulated for at least two months after injury (47).

Another factor potentially involved in Oct6 downregulation is Krox20. In the Krox20 knockout animals, Oct6 expression is switched from being transient to sustained expression (48). This observation suggests that Krox20 could be involved in extinguishing the Oct6 expression either alone or together with Oct6 or other

factor(s). This is very well possible as the Oct6 promoter region is in a CpG island and harbors many Krox20 binding sites. However, our transgenic lines expressing mini SCE driven by either hsp68 or Oct6 promoter argues for promoter independency in the downregulation of Oct6. Deleting the conserved elements within SCE and analyzing the adult nerves, where normally Oct6 expression is extinguished could give us a clue as to the exact sequences required for the downregulation.

Additionally, gene expression is a result of genetic and epigenetic processes. Histone modifications have become an important epigenetic process determining gene expression. The fact that Oct6 is very precisely regulated by the SCE, one could speculate about how much of a role is played by different histone modifications in determining the optimum expression of Oct6. Key histone modifications are H3K4me3, H3 and H4 acetylation that are marks for gene activation and H3K9me3, H3K27me3 and H4K20me3, which are markers for gene repression. A Histone H3K27ac mark on the enhancers distinguishes active enhancers from inactive/ poised enhancer elements which contain the H3K4me1 mark (49). Furthermore, large sets of inactive developmental genes in hematopoietic stem cells are associated with H3K4me1-enriched distal enhancers (50). Sikorska and co-workers (51) showed that epigenetic silencing is observed for the Sox2 locus in neurons. Sox2 contains two enhancers SRR1 and SRR2, out of which the SRR2 enhancer contains bipartite binding sites for Sox2-Oct4 proteins and is essential for Sox2 expression in embryonic stem cells (52-54). They examined DNA methylation and histone H3 acetylation at both enhancers and demonstrated that both enhancers were differentially epigenetically marked to generate neuron and astrocyte specific epigenomes from a common progenitor cell. DNA Methylation of the SRR2 enhancer was specific for neurons and coincided with gene silencing. Hence, it is possible that downregulation of Oct6 could be orchestrated due to epigenetic marking like DNA methylation on the SCE. The presence of one or a few of the above marks will shed light on the role of enzymes involved in putting these modifications in regulation of Oct6. Such kind of epigenetic silencing is observed for the Sox2 locus in neurons (51).

Interestingly, genome-wide studies on Polycomb binding proteins have revealed that the Oct6 promoter is bound by the Human PRC2 (55). Polycomb is an important complex regulating silencing of differentiation specific genes and its components like EZh2 and SUZ12 are upregulated in several cancers (56). Could Polycomb have a role in SCE regulation?

Target genes of Oct6

As a transcription factor, Oct6 regulates genes required for Schwann cell differentiation and myelination. Several lines of evidence suggest that the zinc finger transcription factor Krox20 is a major target of Oct6 in Schwann cells. In Oct6 null mice, Krox20 expression is severely reduced (4). The myelinating Schwann cell enhancer (MSE) of Krox20 harbors many binding sites for Oct6/Brn2 and mutation of the binding sites results in loss of expression of linked reporter gene in transgenic mice (3). Oct6 and/or Brn2, synergistically activate the MSE together with Sox10 in co-transfection experiments.

We have demonstrated that transgenic Krox20 driven by Oct6-SCE can rescue the phenotype observed in $Oct6^{\Delta SCE/\beta geo}$ mice (Chapter 3). This clearly indicates that whatever may be the signals emanating from axons, they must converge on the SCE to activate Krox20 expression. This proves the earlier hypothesis that, even though Oct6 is expressed in the early myelinating cells, Oct6 is not required for the execution of the transcriptional program of myelination. When Krox20 is expressed at a stage where Oct6 is actively expressed (P4) as in the case of $Oct6^{\Delta SCE/\beta geo}$::$tgKrox20^{HA}$ – SCE mice, Krox20 takes care of the myelination program.

Apart from Krox20, RhoA specifc GTP exchange factor (Rgnef) was induced during Schwann cell differentiation *in vitro* and also severely reduced in the nerves of the Oct6 deficient mice as compared to the wild type (wt) (chapter 3). However, knock down of Rgnef did not directly affect myelination in DRG co-cultures (Chapter 3). So what could be the role of Rgnef in Schwann cells? During myelination two different cell types (axons and Schwann cells) come together to produce the myelin sheath. Different classes of proteins orchestrate this process and RhoGTPases are one among them. RhoGTPases such as, RhoA, Rac1 and cdc42 coordinate the signals from extracellular matrix (ECM) proteins to transform Schwann cell proliferation and more importantly Schwann cell cytoskeletal re-arrangement (57-59). Rgnefs promote the exchange of bound GDP to GTP, thus activating RhoGTPases (60). Our knockdown experiments on Rgnef in Schwann cell neuron co-cultures failed to reveal a role for Rgnef in myelination (Chapter 3). Alternatively, it is possible that redundant RhoGEF factors compensate for the lower p190RhoGef levels. Only either achieving sufficient knock down of Rgnef either by ShRNA or SiRNAs can draw any conclusive data. Rgnef colocalizes with microtubules and might provide a link between microtubule dynamics and RhoA signalling. Though we did not study the morphology of Rgnef knock down cells in detail, there were no obvious alterations in the phenotype of myelinating co-cultures with knock down Schwann cells and wild type neurons.

Interacting proteins of Oct6

Identifying the interaction partners of a protein sheds more light on the function of the protein. In an attempt to identify the interacting proteins of Oct6, we carried out Oct6 immunoprecipitation in RT4D6-P2T cells. The type of material (cells or tissues) we use plays a substantial role in the identification of relevant proteins. Because of the difficulties associated with availability of primary Schwann cells or nerves from young animals, we used a Schwannoma cell line. We have identified several novel partners of Oct6 which may have a role in peripheral nerve myelination. However, the observed interaction with Oct6 is not always sufficient to accurately predict the biological relevance of the proteins(s). The functional significance of their interaction with Oct6 needs to be established. Out of the proteins we have identified through mass spectrometry analysis, Capicua (Cic) is the most interesting one. It is a transcriptional repressor and is a direct target of ErbB receptors in flies (61,62). As discussed in chapter 1, ErbB receptors are essential for normal Schwann cell development and myelination (63-65)

We are currently setting up experiments to knock down Capicua in rat Schwann cells. Further inducing them for differentiation or culturing the knock down cells with neurons could provide us with clues about the function of Cic in the peripheral nervous system.

Final remarks

Enhancers were first characterized using transient reporter gene assays in cultured cell lines. These assays measured the activation of transcription irrespective of the regulatory elements' location or orientation relative to the promoter within a plasmid construct. Later, when the genome era started and more and more genomes were sequenced, identification of enhancers through phylogenetic footprinting became possible. Though a powerful tool, phylogenetic analysis has some drawbacks. Identification of enhancers particularly becomes difficult when they are situated megabase pairs away from the gene of interest. The classical example is the Sonic hedgehog (Shh) gene enhancer, which is located within the intron of another gene more than 1Mb from the Shh gene promoter (66,67).

The advent of chromatin immunoprecipitation coupled with high throughput sequencing has facilitated the identification of a large number of tissue specific enhancers in mouse embryo. A recent study made use of the fact that p300 histone acetyltransferase binds enhancer sequences and could be used as a marker for enhancer elements (65). This study highlights the fact that there are a large number of enhancers, probably acting in concert with the promoters in determining

the precise expression of the developmental specific gene. Identification and characterization of all these enhancers will lead to a better understanding of their contribution to developmental processes and human disease.

Finally, the identification of small conserved sequences that play indispensable roles in Oct6 activation has been the key feature of this project. Furthermore, this project has opened up fresh possibilities to understand the regulation and mechanism of Oct6 function. It would be interesting and important to know how Oct6 is downregulated in a timely fashion.

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Summary

Summary

The high conduction velocity of nerve fibers in higher vertebrates owes to the myelin sheath elaborated around the axons. During development, specialized glial cells repeatedly wrap and tightly compact large sheets of plasma membranes around the axons. Myelin is, arguably, one of nature's most fascinating creations that undoubtedly has played an essential role in the evolution of compact nervous systems in larger animals. Continuous molecular and cellular interactions between axons and myelinating glia are important during development and maintenance of the myelin sheath. The damage or dysfunction of the myelin sheath invariably leads to diseases such as multiple sclerosis and hereditary leukodystrophies in the CNS and Guillain Barré syndrome, Charcot-Marie-Tooth disease in the PNS.

Schwann cells, the myelinating glia cells of the PNS, initiate myelin formation in response to axon-associated signals that include Neuregulin1. A network of transcription factors that include Oct6, Krox20, Brn2 and Sox10, governs the onset of myelination in the PNS. Oct6 facilitates the transition of a promyelinating Schwann cell to a myelinating Schwann cell through activation of downstream targets. The expression of Oct6 is under control of a downstream enhancer called Schwann cell enhancer (SCE), which is both required and sufficient to drive all aspects of Oct6 expression in Schwann cells. Thus, the Oct6 SCE plays an important role in the gene regulatory network that governs the onset of myelin formation in Schwann cells and provides a link between myelin promoting signaling and activation of a myelin related transcriptional network.

In the studies presented in this thesis we combined different approaches, to gain further insight into the cis-acting regulatory elements within the SCE and the factor(s) controlling Oct6 expression in Schwann cells. We identified several conserved sequences within the SCE and of them, core element 1 (C1), was found to be absolutely essential for enhancer activity. Additionally, we demonstrated that a dimeric Sox motif present in C1 is essential for its function.

Further, we established that the zinc-finger transcription factor Krox20 is the major transcriptional target of Oct6 in peripheral nerve development. We also show that Krox20 under the control of Schwann cell enhancer of Oct6 can rescue the myelination defects of Oct6 mutant mice identifying Krox20 as the major target of Oct6 regulation.

In an attempt to gain a deeper understanding of Oct6 function in Schwann cells, we immune-purified Oct6 protein complexes from a Schwannoma cell line and identified the Oct6 co-purifying proteins by mass spectrometry. Our analysis revealed a range of Oct6 interacting proteins, including transcription factors, chromatin remodeling complexes, factors involved in protein transport and mRNA splicing. This list of proteins provides an important source for future experiments that aim to establish the exact mechanism of Oct6 function in Schwann cell development and myelination.

The studies presented in this thesis highlight the importance of regulatory elements in Schwann cell specific Oct6 gene regulation. A deeper understanding of Oct6 protein function, its regulatory targets and interacting partners in the peripheral nervous system might eventually help in developing combinatorial therapies for demyelinating diseases.

Nederlandse Samenvatting

De hoge geleidingssnelheid van zenuwvezels in gewervelde dieren is te danken aan de isolerende myelineschede die zich om de axonen bevindt. Gedurende de ontwikkeling vormen gespecialiseerde glia cellen de myelinschede door het herhaaldelijk wikkelen van grote hoeveelheden plasmamembraan rondom het axon. De myelineschede is een van de meest fascinerende creaties in de levende natuur die zonder twijfel de evolutie van complexe zenuwstelsels in grotere organismen heeft mogelijk gemaakt. Complexe cellulaire en moleculaire interacties tussen de myelinevormende glia cel en het axon liggen ten grondslag aan de aanleg en onderhoud van de myelineschede. Schade aan de myelineschede leidt zonder uitzondering tot ziektes zoals multiple sclerose en de erfelijke leukodystrofiën in het centraal zenuwstelsel en Guillain-Barré syndroom en de ziekte van Charcot-Marie en Tooth in het perifeer zenuwstelsel.

Schwann cellen, de myeline vormende cellen van het perifeer zenuwstelsel, initiëren myeline vorming onder invloed van axon geassocieerde signalen zoals het membraan gebonden eiwit neureguline1. Een netwerk van transcriptie factoren, zoals Oct6, Krox20, Brn2 en Sox10, drijft het myelinatie proces in het perifeer zenuwstelsel. Oct6 is nodig voor de overgang van een pro-myelinerende cel naar een volledig myelinerende Schwann cel door regulatie van een aantal andere genen. De expressie van Oct6 zelf staat onder controle van een enhancer die we de 'Schwann Cell specific Enhancer' (SCE) hebben genoemd. De SCE is voldoende en noodzakelijk voor de gereguleerde expressie van Oct6 in Schwann cellen. De SCE speelt dus een centrale rol in het regulatie netwerk van myeline vorming en vormt een directe schakel tussen de axon geassocieerde signalen en het transcriptioneel netwerk van celdifferentiatie.

In het werk beschreven in dit proefschrift hebben we aantal experimentele strategieën gecombineerd om meer inzicht te verkrijgen in de architectuur van regulerende elementen binnen de SCE en de factoren die aan deze elementen binden en expressie van Oct6 in Schwann cellen aansturen. We hebben een aantal van zulke elementen genetisch geïdentificeerd en aangetoond dat zij onmisbaar zijn voor functie. Een element, c1 genaamd, hebben wij verder onderzocht en vonden dat het twee geinverteerde Sox bindende elementen bevat. De transcriptie factor Sox10 bind hier als een dimeer en dit is essentieel voor SCE functie.

Verder konden we laten zien dat de transcriptie factor Krox20 een belangrijk target

van Oct6 regulatie is in Schwann cellen. We hebben laten zien dat wanneer we Krox20 onder controle brengen van de Oct6 SCE als een transgeen in muizen, dit Krox20-SCE transgeen in staat is om het myelinatie defect in Oct6 deficiënte muizen te herstellen. Dit experiment toont onomstotelijk aan dat in myelinerende Schwann cellen, Krox20 de belangrijkste target van Oct6 regulatie is.

In een volgende serie experimenten hebben we geprobeerd een beter begrip te krijgen van de functie van Oct6 in Schwann cellen door middel van zuivering van Oct6 geassocieerde eiwitten uit een Schwannoma cellijn. We hebben deze eiwitten geïdentificeerd met behulp van massa spectrometrie. Onder de geïdentificeerde eiwitten bevinden zich transcriptie factoren zoals Sox10, chromatine modificerende eiwitten en eiwitten betrokken bij eiwit transport over het nucleaire membraan en mRNA splicing. Deze lijst van eiwitten is een belangrijke bron voor verder onderzoek naar het mechanisme van Oct6 functie in Schwann ontwikkeling en myeline vorming.

De studies beschreven in dit proefschrift onderstrepen het cruciale belang van cis-acting regulerende elementen in de genregulerende netwerken die cel differentiatie en proliferatie aansturen. Een verder verdieping van onze kennis betreffende het mechanisme van Oct6 functie in myelinatie en zenuwregeneratie zal hopelijk in de toekomst bijdragen aan de ontwikkeling van gecombineerde therapieën die zenuwschade in eerste instantie beperken maar mogelijk ook het herstel van schade bevorderen.

Curriculum Vitae

Name : Noorjahan Jagalur Basheer
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Education

2005-2010 PhD student
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2003-2005 Assistant Director of Agriculture, Government of Karnataka, India

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Publications

1. **Jagalur NB**., Ghazvini, M., Mandemakers W., Jaegle M., Driegen S., Svaren J., Meijer D., *Functional dissection of the Oct6 Schwann cell enhancer reveals an essential role for dimeric Sox10 binding* (In press: Journal of Neuroscience)

2. Mul JD., Nadra K., **Jagalur NB**., Nijman IJ., Toonen P.W., Medard JJ., Gres S., de Bruin A., Han GS., Carman GM., Saulnier-Blache JS., Meijer D., Chrast R and Cuppen E. *A hypomorphic mutation in Lpin1 induces progressively improving neuropathy and lipodystrophy in the rat* (Submitted: Journal of Biological Chemistry).

3. Pirsoo M., **Jagalur NB**., Driegen S ., Van der Spek P., Grosveld F., Jaegle M., Meijer D. *Krox20 is the major transcriptional target of the POU domain transcription factor Oct6 in Schwann cell differentiation* (In preparation)

ERASMUS UNIVERSITEIT ROTTERDAM

PhD Portfolio

Department of Cell Biology and Genetics Research School: Medisch-Genetisch Centrum	PhD period: June 2005- September 2010 Promoter: Prof.dr. Dies Meijer

PhD Training	Year
General academic skills	
- Biomedical English Writing and Communication	2008
- Workshop Browsing Genes and Genomes with Ensembl IV	2009
- Adobe Photoshop and Illustrator Workshop	2010
- Adobe Indesign	2011
Research skills	
- Radiation safety course	2005
- Laboratory Animal Science	2006
- Safe laboratory techniques	2007
In-depth courses (e.g. Research school, Medical Training)	
- Experimental Approach to Molecular and Cell Biology	2006
- Yeast to Man	2006
- *In Vivo* imaging: From Molecule to Organism	2007
- From Development to Disease (Embryos, Genes and Developmental Defects)	2008
- Signal Transduction Pathways Regulating Aging and Disease	2009
- Epigenetic Regulation (Epigenetic Mechanisms in Health and Disease)	2008
- Technology Facilities (Proteomics, Genomics and Transcriptomics)	2009
Presentations	
Winterschool Kleinwalsertal	2007-2008
15th MGC PhD Workshop	2008
International conferences and Workshops	
8th European Meeting on Glial Cells in Health and Disease (poster) UK	2007
Summer School on Chromatin and Transcription (poster), Greece	2008
Eutracc, Transcription Networks Symposium (poster), Germany	2011
Seminars and Workshops	
MGC PhD workshop	2006
1st and 2nd SCDD symposium BSIK, Amsterdam	2007, 2008
Neuroscience meeting, Utrecht	2010
Teaching Activities	
Supervision of MSc (Molecular Medicine) students	2009, 2011

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Think where mans glory most begins and ends, and say my glory was I had such friends (William Butler Yeats).

What seemed to be a mammoth task is finally over and I am penning the last words of this thesis. The thesis you are holding now owes its existence to the help, support and inspiration of many people.

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- Noorie