

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

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

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

Introduction

Over the years, a considerable research effort has focused on how the myelination programme in Schwann cells is regulated. This cellular differentiation programme is characterized by dramatic metabolic and morphological changes, including polarization of the cell by deposition of a basal lamina, the production of massive amounts of cell membrane, incorporating myelin-specific lipids and proteins, and the spiralling of these lamellae around the axon (reviewed in Mezei, 1993; Garbay *et al.*, 2000). The myelin organelle further matures into structurally and functionally distinct domains of compact and non-compact myelin such as the Schmidt–Lantermann incisures and

paranodal loops (Arroyo and Scherer, 2000; Peles and Salzer, 2000; Pedraza *et al.*, 2001). The synthesis and maintenance of myelin is an exquisitely sensitive process, as demonstrated by the many, inherited or acquired, demyelinating and dysmyelinating diseases such as Guillain-Barré Syndrome and the hereditary motor and sensory neuropathies. If we are to understand the interactions between glial cells and neurones that shape and maintain the functionally mature histo-architecture of the nerve or lead to pathogenesis, it is important to elucidate the molecular basis of the myelination programme.

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

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

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

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

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

Results

Deletion of the *Oct-6* SCE through gene targeting

To delete the 4.3 kb SCE, a gene targeting vector was constructed in which the SCE was replaced with a *puromycin* selection cassette (Figure 1A). A negative selection cassette was introduced (*Py-TK*) flanking the 5' homologous region, allowing counterselection of randomly integrated targeting constructs. The *puromycin* selection cassette was flanked by LoxP sites, which

allowed deletion of the *puromycin* gene and its regulatory sequences from the targeted allele using Cre recombinase (Le and Sauer, 2001). Of the 141 embryonic stem (ES) cell clones that were puromycin resistant and ganciclovir insensitive, four were found to contain a homologous recombination event, as judged by Southern blot analysis (Figure 1B). Of those four, three had additional random integrations of the targeting cassette and were discarded. The one correctly targeted ES cell clone had a correct number of chromosomes and was used to generate chimeric mice. Chimeric animals were mated to *Zp3-Cre* transgenic female animals (D.Drabek). *Zp3-Cre* transgenic animals express high levels of the Cre recombinase in the oocyte, resulting in the removal of the puromycin cassette on the paternal chromosome in the zygote. Offspring in which the puromycin cassette was removed and the *Zp3-Cre* transgene was absent were identified using Southern blot analysis. These mice were used for further analysis.

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

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

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

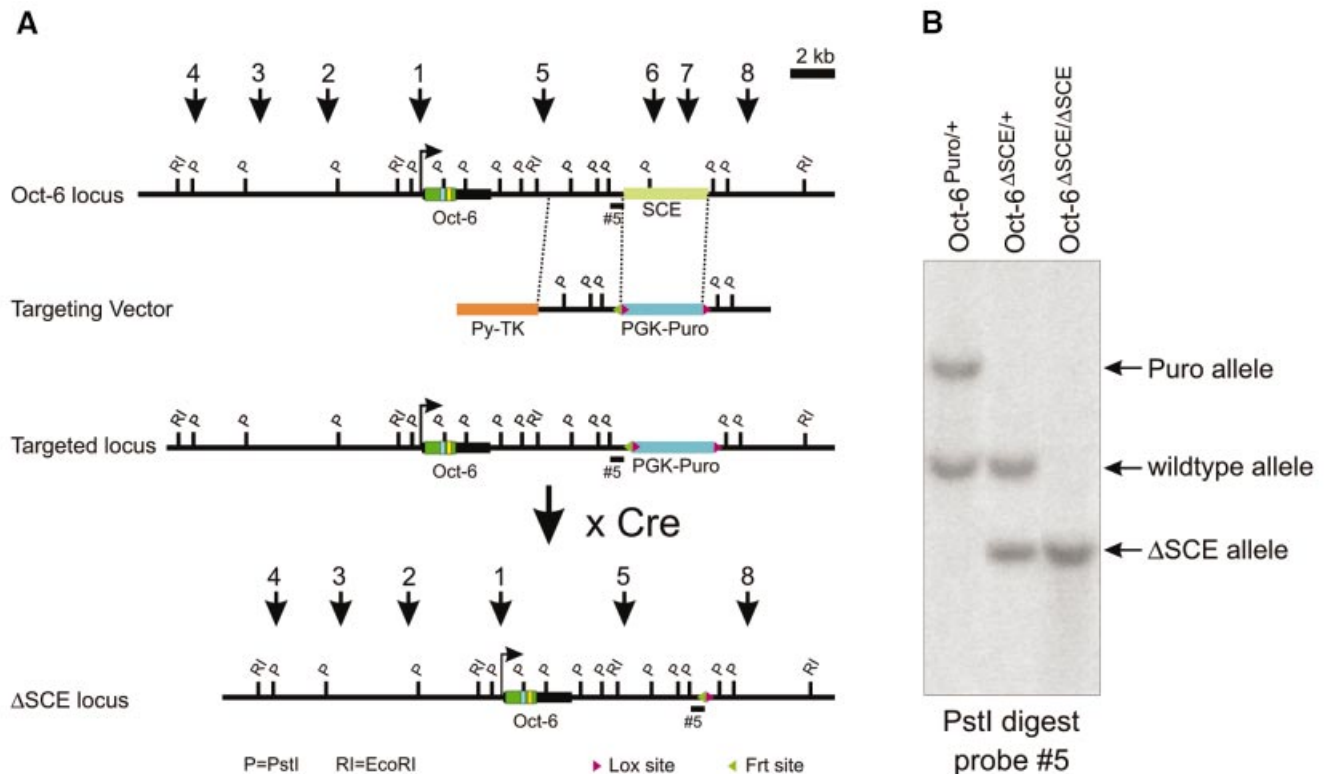


Fig. 1. Deletion of the 4.3 kb SCE from the *Oct-6* locus. (A) Gene targeting scheme for the *Oct-6* SCE. The SCE is indicated with a light green bar and is located ~12 kb downstream of the *Oct-6* gene CAP site. The intronless *Oct-6* transcription unit is indicated with a thick black line and the open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

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

Developmental delay in peripheral nerve development

Mice homozygous for complete loss-of-function alleles show delayed peripheral nerve development with

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

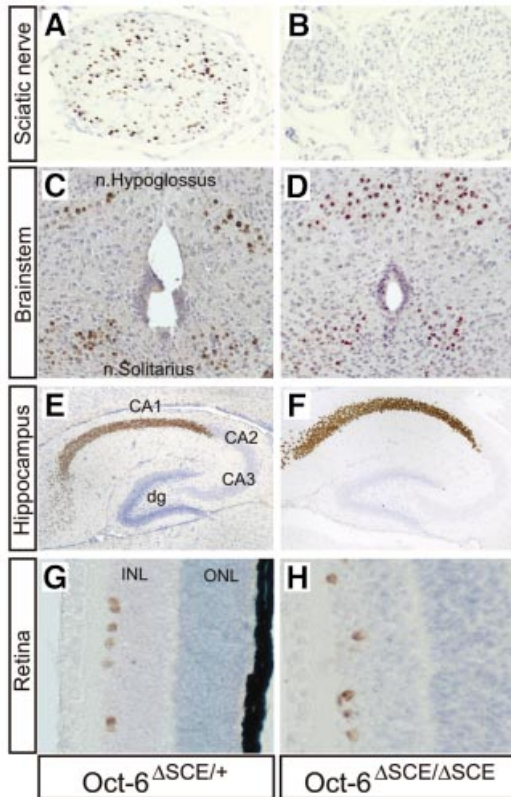


Fig. 2. The Δ SCE allele affects Oct-6 expression in the Schwann cell lineage only. (A) A brown precipitate identifies Oct-6 protein-expressing Schwann cell nuclei in transverse sections of sciatic nerve of *Oct-6 Δ SCE/+* mice at P8. (B) None of the Schwann cells in the sciatic nerve of *Oct-6 Δ SCE/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

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

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

The developmental delay in peripheral nerves of mice homozygous for the Δ SCE allele appears slightly milder

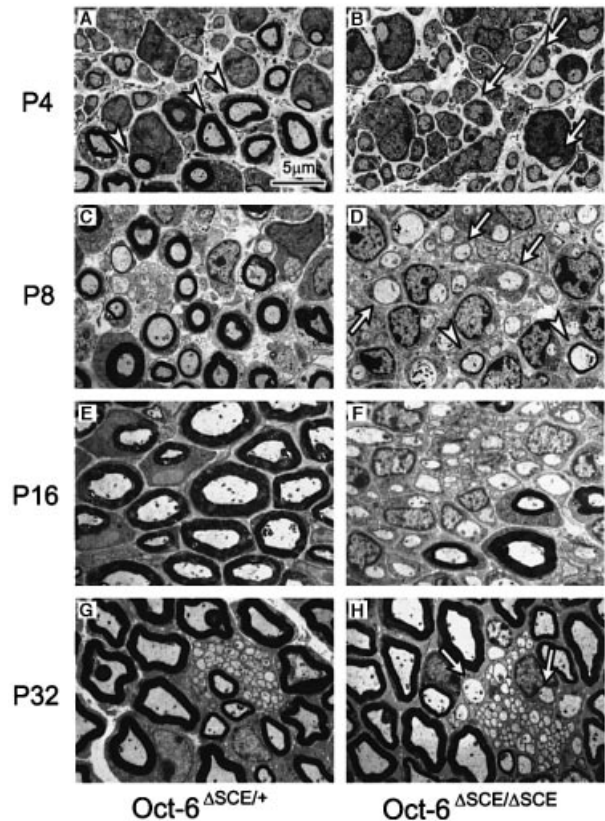


Fig. 3. Schwann cell differentiation is delayed at the promyelin stage in developing nerves of *Oct-6 Δ SCE/ Δ SCE* mice relative to heterozygous mice. (A–H) Representative electron micrographs of transverse sections through the sciatic nerve of homozygous and heterozygous animals at different postnatal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

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

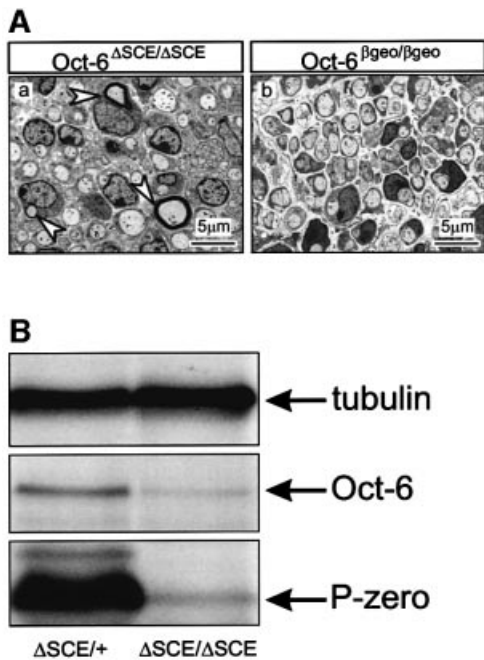


Fig. 4. The Δ SCE allele is a hypomorphic allele of *Oct-6*. (A) Comparison of sciatic nerve morphology in *Oct-6* $^{\Delta$ SCE/ Δ SCE (a) and *Oct-6* $^{\beta$ geo/ β geo (b) mice at P8 reveals that a minority of Schwann cells in *Oct-6* $^{\Delta$ SCE/ Δ SCE mice have progressed to form compact myelin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

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

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

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

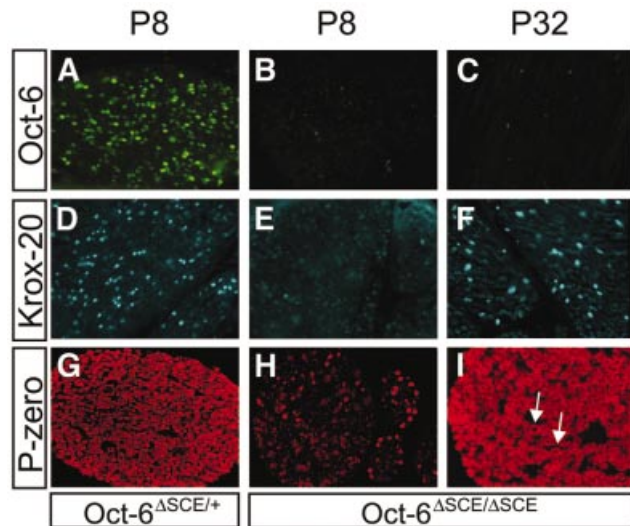


Fig. 5. Oct-6 expression is lost in Schwann cells of mice homozygous for the Δ SCE allele. (A) Homozygous deletion of the SCE results in loss of Oct-6 expression in Schwann cells of the developing nerve and delayed appearance of Krox-20 and the major myelin protein P-zero. Transverse sections of paraffin-embedded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

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

Nerve regeneration

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

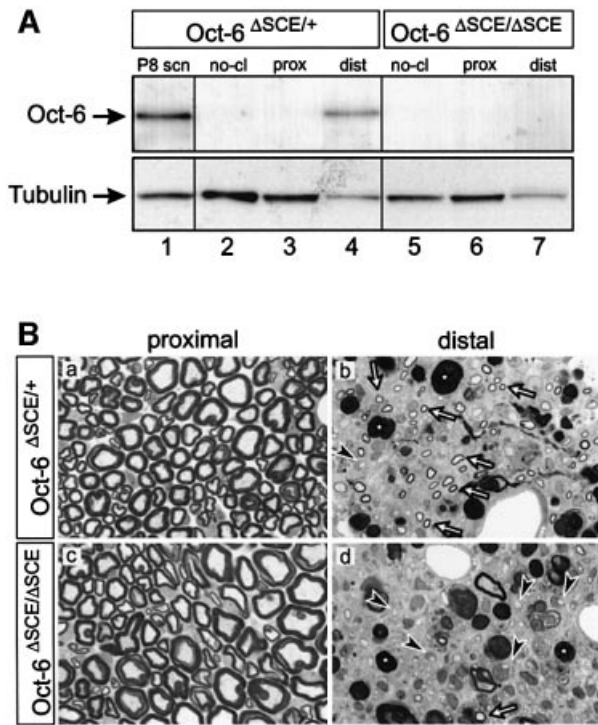


Fig. 6. Oct-6 regulation and function in the regenerating nerve. (A) Reactivation of Oct-6 expression is mediated through the SCE. Western blot analysis of sciatic nerves 12 days after crush lesion from adult *Oct-6 Δ SCE/+* (lanes 2–4) and *Oct-6 Δ SCE/ Δ SCE* mice (lanes 5–7). Nerves were divided in a proximal part (prox, lanes 3 and 6) and a distal part (dist, lanes 4 and 7) of equal length. Controls included are the undamaged nerves, contralateral to those operated on (no-cl, lanes 2 and 5), and developing sciatic nerve at P8 (P8 scn, lane 1). Oct-6 is not expressed in Schwann cells of the adult nerve, while at P8, Schwann cells express high levels of Oct-6. Tubulin served as loading control. However, tubulin expression is reduced in the distal part of the regenerating nerve because of incomplete regeneration. (B) Schwann cell myelination is delayed in regenerating nerves of *Oct-6 Δ SCE/ Δ SCE* mice. Regenerating nerves, 12 days post-operation, were examined by light microscopy. Nerves were embedded in plastic and semi-thin sections were cut at 5 mm proximal (a and c) and 3 mm distal (b and d) to the lesion site. Sections were stained with ppd. In both genotypes, much myelin debris is still present (asterisk in b and d). Many regenerating fibres in the *Oct-6 Δ SCE/ Δ SCE* mouse are ensheathed by Schwann cells that have not elaborated compact myelin yet (arrowheads in b and d). In contrast, many compact myelin figures are found surrounding the regenerating fibres in *Oct-6 Δ SCE/+* mice (arrows in b and d).

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

We next examined how nerve regeneration at the morphological level was affected in the absence of *Oct-6* reactivation. The sciatic nerves of *Oct-6 Δ SCE/+* or *Oct-6 Δ SCE/ Δ SCE* animals were crush lesioned at mid-femoral level. The extent of regeneration was assessed by serial sectioning and microscopic analysis of the regenerating nerves. At 12 days post-transection, many regenerating axon fibres were seen at 3 mm distal of the lesion. In addition, we observed many myelin ovoids that had not been cleared yet by macrophages or had not been autophagocytosed (arrowheads in Figure 6B, b and d). The degree of degeneration and axonal ingrowth of the

distal nerve stumps was similar in *Oct-6 Δ SCE/+* and *Oct-6 Δ SCE/ Δ SCE* nerves. Many regenerating nerve fibres are being actively myelinated in the *Oct-6 Δ SCE/+* nerves, as demonstrated by the many thin compact myelin figures (arrow in Figure 6B, b). In contrast, myelination of regenerating fibres in *Oct-6 Δ SCE/ Δ SCE* nerves was much less advanced at this stage. Many fibres that had not yet progressed beyond the promyelin stage of ensheathment and those that were myelinated had thinner myelin sheaths. These observations indicate that, as in development, myelination is delayed in the absence of *Oct-6* gene function.

Discussion

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

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

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

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

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

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

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

Function of the *Oct-6* Schwann cell enhancer

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

The reduced levels of *Oct-6* expressed in Schwann cells of *Oct-6* ^{$\Delta SCE/\Delta SCE$} mice result in a slightly less severe peripheral nerve phenotype than that observed in *Oct-6* ^{β_{geo}/β_{geo}} mice, which do not express *Oct-6* at all. In particular, we found that the number of Schwann cells that have entered the myelinating phase of differentiation at P8 is lower in *Oct-6* ^{β_{geo}/β_{geo}} mice than in *Oct-6* ^{$\Delta SCE/\Delta SCE$} mice. These observations suggest that the level of *Oct-6* determines the rate at which a Schwann cell progresses through the promyelin stage of differentiation. This suggests that increased levels of *Oct-6* might result in an increased rate of differentiation of Schwann cells, potentially resulting in early onset of myelination and hypermyelination. Weinstein *et al.* (1995) have previously shown that expression of a mutant form of the *Oct-6* protein ($\Delta SCIP$) under the control of the P-zero promoter in Schwann cells of transgenic animals results in early onset of myelination and hypermyelination. Although

these results were initially interpreted differently, involving a dominant-negative action of the $\Delta SCIP$ protein, more recent interpretation suggests that the protein acts as a dominant positive (Wu *et al.*, 2001). This reinterpretation strongly suggests that the levels of *Oct-6* are rate limiting in Schwann cell differentiation. Such transcription factor dose-dependent differentiation has also been demonstrated for a number of other systems, including the haematopoietic system (McDevitt *et al.*, 1997; Vivian *et al.*, 1999). For example, it has been demonstrated that 80% reduction in *Gata-1* expression levels results in a decreased rate or efficiency of red blood cell maturation.

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

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

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

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

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

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

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

Materials and methods

Targeting of the *Oct-6* SCE

A genomic clone encompassing the 4.3 kb *HpaI*-*MscI* Schwann cell enhancer fragment was subcloned from cosmid clone pTBE 6Cos. From this clone, a 3.2 kb *MscI*-*HpaI* fragment, containing homologous genomic sequences 5' of the SCE, was cloned behind the negative selection cassette py-TK. This selection cassette consists of the herpes simplex virus (HSV) *thymidine kinase* gene including its own promoter and a variant polyoma virus enhancer. A second clone was generated containing the *puromycin* resistance gene driven by the *phosphoglycerate kinase-1* (PGK) promoter. This clone was flanked on both sites by *LoxP* sites that have the same orientation. An FRT site was introduced

immediately 5' of the 5' *LoxP* site. This also introduces a unique *SwaI* site at the 5' end of this clone. Downstream of the 3' *LoxP* site, a unique *SnaBI* site was used to introduce a 2.8 kb *MscI* fragment containing the 3' genomic homologous region. The entire fragment, encompassing the FRT-*LoxP*-PGK-*puromycin*-*LoxP*-3' 2.8 kb *MscI*, was excised as a *SwaI*-*NotI* fragment and cloned in the *HpaI*-*NotI*-linearized Py-TK plasmid. This resulted in the targeting vector, as depicted in Figure 1A. The targeting vector was first linearized using *NotI* before electroporation into E14 ES cells. Electroporation and selection of cells in which homologous recombination had occurred were carried out as described. G418-resistant and ganciclovir-insensitive ES cell clones were screened for homologous recombination by Southern blotting of genomic DNA digested with *PstI* using a ³²P-labelled DNA probe derived from the 5' homologous region (see Figure 1A). Chimeric mice were generated by injection of ES cells from the correctly targeted clone into C57BL/6 blastocyst embryos. Chimeric males were crossed with FVB females and offspring were genotyped by Southern blotting of tail DNA digested with *PstI* using the probe described above. Offspring carrying the chromosome with the targeted SCE allele were identified (see Figure 1B) and subsequently crossed to mice carrying the *Zp3-Cre* transgene to obtain offspring in which the *puromycin* cassette was removed. These mice were then inter-crossed to obtain mice homozygous for the deleted SCE allele (see Figure 1B).

Immunohistochemistry and western blotting

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

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

Electron microscope analysis

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

Animal surgery

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

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

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