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The Protooncogene Ski Controls Schwann Cell Proliferation and Myelination

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Summary

Schwann cell proliferation and subsequent differentiation to nonmyelinating and myelinating cells are closely linked processes. Elucidating the molecular mechanisms that control these events is key to the understanding of nerve development, regeneration, nerve-sheath tumors, and neuropathies. We define the protooncogene Ski, an inhibitor of TGF-β signaling, as an essential component of the machinery that controls Schwann cell proliferation and myelination. Functional Ski overexpression inhibits TGF-β-mediated proliferation and prevents growth-arrested Schwann cells from reentering the cell cycle. Consistent with these findings, myelinating Schwann cells upregulate Ski during development and remyelination after injury. Myelination is blocked in myelin-competent cultures derived from *Ski*-deficient animals, and genes encoding myelin components are downregulated in *Ski*-deficient nerves. Conversely, overexpression of Ski in Schwann cells causes an upregulation of myelin-related genes. The myelination-regulating transcription factor Oct6 is involved in a complex modulatory relationship with Ski. We conclude that Ski is a crucial signal in Schwann cell development and myelination.

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Introduction

Schwann cells, the main glial cells of the peripheral nervous system (PNS), are responsible for the protection and support of axons and for the synthesis of myelin sheaths. During embryonic development, Schwann cells proliferate and migrate along axons, subdividing the nerve fascicle into smaller groups of nerve fibers. Eventually, Schwann cells differentiate into either nonmyelinating or myelin-forming Schwann cells in postnatal nerves (Lobsiger et al., 2002). Following injury, axons distal to the damaged site degenerate, and the myelin sheaths break down. Schwann cells dedifferentiate and proliferate. If axon regrowth is allowed, Schwann cells will stop dividing and start remyelination (Stoll and Muller, 1999). The molecular mechanisms that regulate these processes are only partially understood. The myelination program is crucially dependent on the expression of at least two transcription factors, Oct6/Scip/Tst-1 (Monuki et al., 1989) and Krox20/Egr2 (Zorick et al., 1996). Myelination is delayed in *Oct6*-deficient mice (Bermingham et al., 1996; Ghazvini et al., 2002; Jaegle et al., 1996), while in *Krox20*-deficient animals, compact myelin is never formed (Topilko et al., 1994).

Regulated exit from the cell cycle is a prerequisite for Schwann cells to achieve myelination in development and regeneration. Understanding the underlying signals may have practical implications for treatments of peripheral nerve tumors (Schwann cell hyperplasia), peripheral neuropathies secondary to diabetes, cancer chemotherapeutic agents, toxins, and autoimmune disorders (Berger and Schaumburg, 1995). Aberrant Schwann cell proliferation is also a prominent feature in inherited peripheral neuropathies (Atanasoski et al., 2002; Suter and Scherer, 2003). Thus, we screened for proteins that are involved in the regulation of the interconnection between Schwann cell proliferation and myelination (data not shown). The nuclear protein Ski (Colmenares and Stavnezer, 1989, 1990), a component of the transforming growth factor-β (TGF-β)/Smad signaling pathway (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999; Xu et al., 2000), was identified as a likely candidate.

TGF-βs have potent effects on cell proliferation, differentiation, and extracellular matrix formation in several cell types (Kulkarni et al., 2002). Schwann cells respond to TGF-β with increased proliferation (Ridley et al., 1989), and TGF-β blocks Schwann cell myelination and the expression of myelin-related proteins in vitro (Awatramani et al., 2002; Einheber et al., 1995; Guenard et al., 1995; Mews and Meyer, 1993; Morgan et al., 1994). Furthermore, TGF-β appears to be a negative Schwann cell survival signal in perinatal nerve (Parkinson et al., 2001),

and adult *TGF-β1*-deficient animals have abnormal myelin (Day et al., 2003). In most cell types, active TGF-βs bind to heteromeric complexes between type I and type II TGF-β receptors (TβR-I, TβR-II). TβR-I phosphorylates Smad2 and Smad3. The activated Smad2/3 bind to the single common Smad4 and translocate to the nucleus where they interact with other transcription factors and activate transcription of TGF-β-responsive genes (Lutz and Knaus, 2002; Moustakas et al., 2001). Ski represses TGF-β signaling through direct interactions with Smad2, -3, and -4 (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999; Wu et al., 2002; Xu et al., 2000). The mechanism of Ski-mediated repression has been attributed to the ability of Ski to recruit the nuclear corepressor (N-CoR) and the histone deacetylase complex (HDAC) to regulatory promoter sequences, thereby modulating gene transcription (Nomura et al., 1999; Tokitou et al., 1999). Ski also interferes with the binding of Smads to the transcriptional coactivator p300/CBP (Akiyoshi et al., 1999).

We show that Ski is highly and selectively expressed by myelinating Schwann cells in vivo and induces the expression of genes encoding myelin proteins. If Ski is absent, the expression of myelin-related genes is reduced and the formation of peripheral myelin is abolished. Thus, Ski is a crucial component of the signaling machinery regulating PNS myelination.

Results

Ski Is Upregulated by Growth-Arrested Schwann Cells

To start examining the potential role of Ski in Schwann cell proliferation and differentiation, we analyzed Ski expression in growing and growth-arrested Schwann cells in vitro (Figure 1). In proliferating, Ki-67-positive cultures (Figure 1Ac, green), Ski was undetectable (Figure 1Aa, red). In contrast, when Schwann cells were maintained in minimal medium (0.5% fetal calf serum [FCS]), they stopped proliferating (Figure 1Ad; no Ki-67 expression) and concomitantly upregulated Ski in their nuclei (Figure 1Ab, purple; colocalization with nuclear DAPI). Western blot analysis confirmed the expression of Ski in extracts of nonproliferating Schwann cells (Figure 1B). So, upregulation of Ski protein levels correlates with growth arrest of cultured Schwann cells.

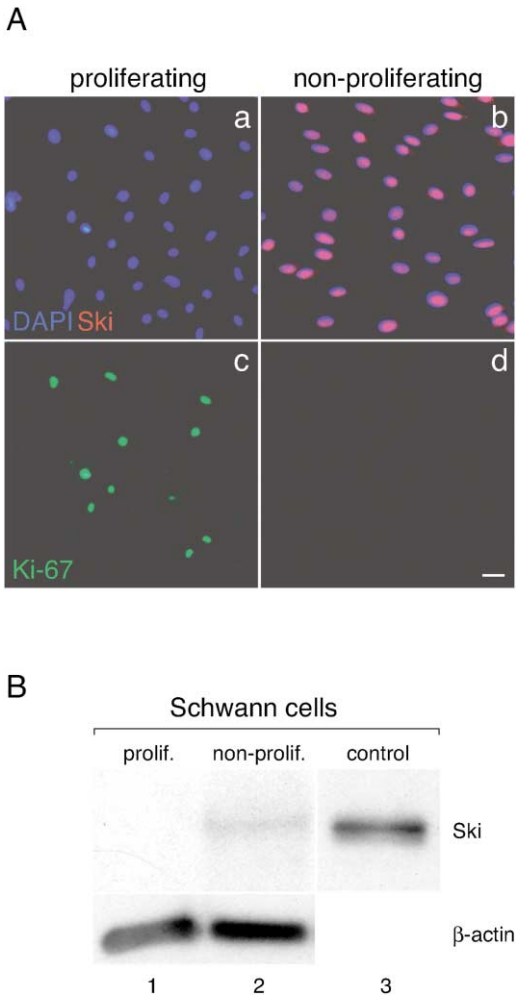

Figure 1. Ski Expression in Purified Rat Schwann Cell Cultures

(A) Triple labeling of rapidly growing (Aa and Ac) and growth-arrested (Ab and Ad) rat Schwann cells. The nuclei of Schwann cells are visualized with DAPI ([Aa and Ab], blue). Ki-67 (green) labels proliferating cells (Ac) but disappears from postmitotic cells (Ad). Ski (red) is absent in proliferating cells (Aa), whereas nonproliferating cells show significant amounts of nuclear Ski expression ([Ab], overlay with DAPI appears purple). Scale bar, 20 μm (in [Ad] for [Aa]–[Ad]).

(B) Immunoblot analysis of lysates of proliferating and nonproliferating Schwann cells. Detection with polyclonal anti-Ski antibody. Equal amounts of protein extracts controlled by β-actin detection (lower panel). Positive control: protein extract of rat Schwann cells transfected with a Ski expression plasmid (lane 3).

Ski Specifically Antagonizes TGF-β1-Mediated Schwann Cell Proliferation and Prevents Reentry into the Cell Cycle

Next, we asked whether Ski has a functional inhibitory effect on Schwann cell proliferation. Thus, we examined the consequences of forced Ski expression (Figure 2). Schwann cells were grown under different conditions promoting proliferation, including Schwann cell medium containing 10% FCS, glial growth factor (GGF), and forskolin, or in minimal media containing 0.5% FCS supplemented with the Schwann cell mitogens GGF (Lemke and Brockes, 1984) or TGF-β1 (Ridley et al., 1989), or basic fibroblast growth factor (bFGF; Davis and Stroobant, 1990). Cells were transiently transfected with a Ski-expression vector, and 48 hr later, proliferating cells were labeled with bromodeoxyuridine (BrdU) for 16 hr. The percentage of Ski/BrdU-positive cells was determined in relation to the total number of Ski-transfected cells for each culture condition (Figure 2A, gray bars). Ski-transfected cells, easily identified based on strong nuclear Ski expression, did not show significant cell death (data not shown). Parallel cultures were transfected with a control plasmid expressing enhanced green fluorescent protein (EGFP), and the percentage of EGFP/BrdU-double-positive cells in relation to the total number of EGFP-transfected cells was calculated (Figure 2A, black bars). First, this analysis revealed different proliferation rates under different growth conditions (Figure 2A, compare black bars). More importantly,

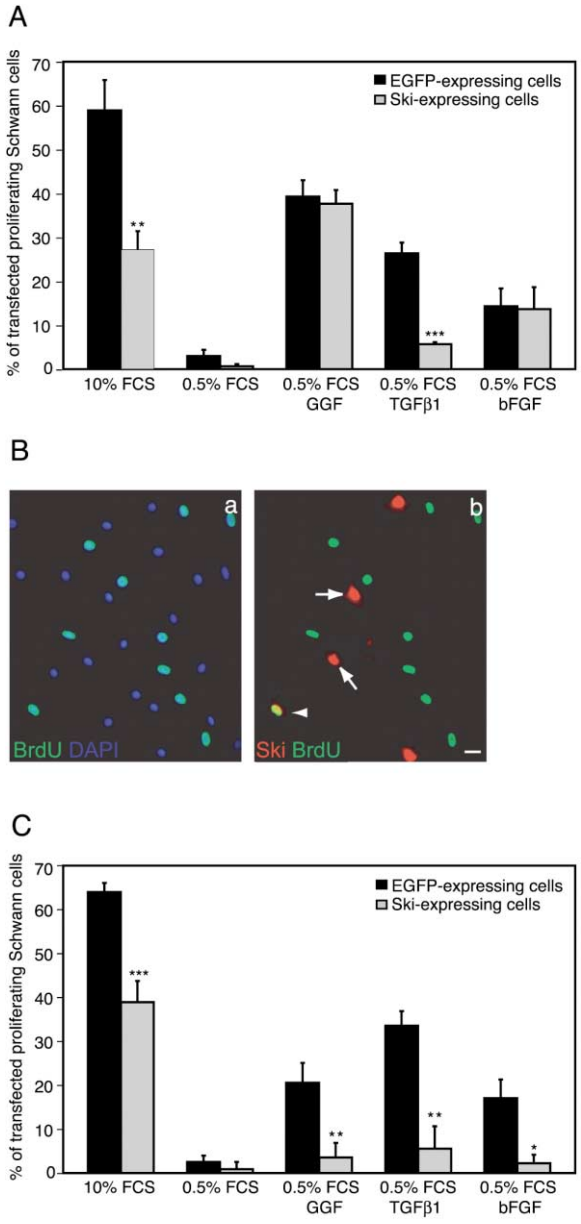

Figure 2. Ski Regulates Schwann Cell Proliferation

Ski inhibits TGF-β1-mediated Schwann cell proliferation (A and B) and prevents Schwann cells from reentering the cell cycle (C). (A) Quantitative analysis of proliferation rates of Ski-expressing and control EGFP-expressing Schwann cells grown under different conditions. Control-transfected Schwann cells (black bars) showed the highest proliferation rate when cultured in Schwann cell medium containing 10% FCS, 100 μg/ml crude GGF, and 2 μM forskolin. The proliferation rate was lowest in minimal medium containing 0.5% FCS with no additional growth factors. Addition of recombinant GGF or TGF-β1 or bFGF to minimal medium led to an increase in the percentage of BrdU-labeled nuclei. Overexpression of Ski specifically decreased the proliferation capacity of cells grown in the presence of TGF-β1 or in Schwann cell medium (gray bars). Data are mean values of three experiments ±SD. Student's t tests were applied to test for significance: $^{**}p \leq 0.01$, $^{***}p \leq 0.005$. (B) Triple immunostaining of a representative Ski-transfected culture grown in minimal medium supplemented with 0.5% FCS and TGF-β1. (Ba) DAPI, blue; DAPI/BrdU, greenish blue; (Bb) BrdU, green; Ski, red. Arrows in (Bb) indicate selected Ski-expressing cells; the arrowhead points to a cell that is double positive for Ski and BrdU (yellow in the overlay). Scale bar in Bb (for Ba and Bb), 10 μm. (C) Quantitative analysis of proliferation rates of Ski- and control EGFP-expressing Schwann cells induced to divide by different mitogens. Under all conditions tested, exogenous Ski-expression significantly decreased the capacity of Schwann cells to proliferate (gray bars), compared to control EGFP-expressing cells (black bars). Data: mean values of three experiments ±SD. Student's t test was applied to test for significance: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.005$.

transgenic expression of Ski specifically inhibited proliferation mediated by TGF-β1 (Figures 2A and 2B) but had no effect on GGF- or bFGF-mediated proliferation. In addition, Ski also significantly decreased the proliferation rate of Schwann cells in medium containing 10% FCS. These data indicate a specific antagonistic function of Ski in TGF-β1-mediated Schwann cell proliferation.

To investigate whether exogenous Ski-expressing cells are able to reenter the cell cycle after addition of growth factors, parallel cultures were transfected with a Ski or an EGFP expression vector and maintained for 3 days in minimal medium containing 0.5% FCS to achieve growth arrest (Figure 2C). Subsequently, the cells were reinduced to proliferate by adding various proliferation-promoting media, including Schwann cell medium containing 10% FCS or minimal media supplemented with GGF, TGF-β1, or bFGF. Cells were cultured for an additional 2 days, and the percentage of dividing cells was determined by BrdU incorporation for 16 hr. Regardless of the growth-promoting media tested, a significantly lower number of exogenous Ski-expressing cells reentered the cell cycle (Figure 2C, gray bars) compared to EGFP-expressing cells (Figure 2C, black bars). Thus, Ski overexpression blocks Schwann cells from reentering the cell cycle, irrespective of the growth factors used to induce proliferation.

TGF-β1 Prevents Ski Expression in Myelinating Cocultures

Our findings suggested that Ski was expressed and functional in growth-arrested rather than in proliferating Schwann cells. Given the interconnection of cell cycle exit and differentiation in Schwann cells, we tested whether Ski expression was induced by neurons and by subsequent myelination. The model of Schwann cells cocultured with dorsal root ganglion (DRG) neurons followed by the induction of myelination with ascorbic acid and progesterone provides an excellent experimental setting to examine these questions (Eldridge et al., 1987; Notterpek et al., 1999). In the absence of myelination-promoting factors, such cocultures showed very low expression of myelin-associated glycoprotein (MAG) (Figures 3Aa and 3Ad, red) and no detectable expression of Ski by immunofluorescence analysis (Figures 3Aa and 3Ad, green). Upon induction of myelination, myelinated internodes were prominent as visualized by anti-MAG antibody stainings (Figures 3Ab and 3Ae, red). Ski was highly upregulated in the nuclei of Schwann cells (Figures 3Ab and 3Ae), independent of whether Schwann cells were myelinating an axonal segment (arrowheads) or not. Interestingly, upregulation of Ski was not found in pure Schwann cell cultures treated with ascorbic acid

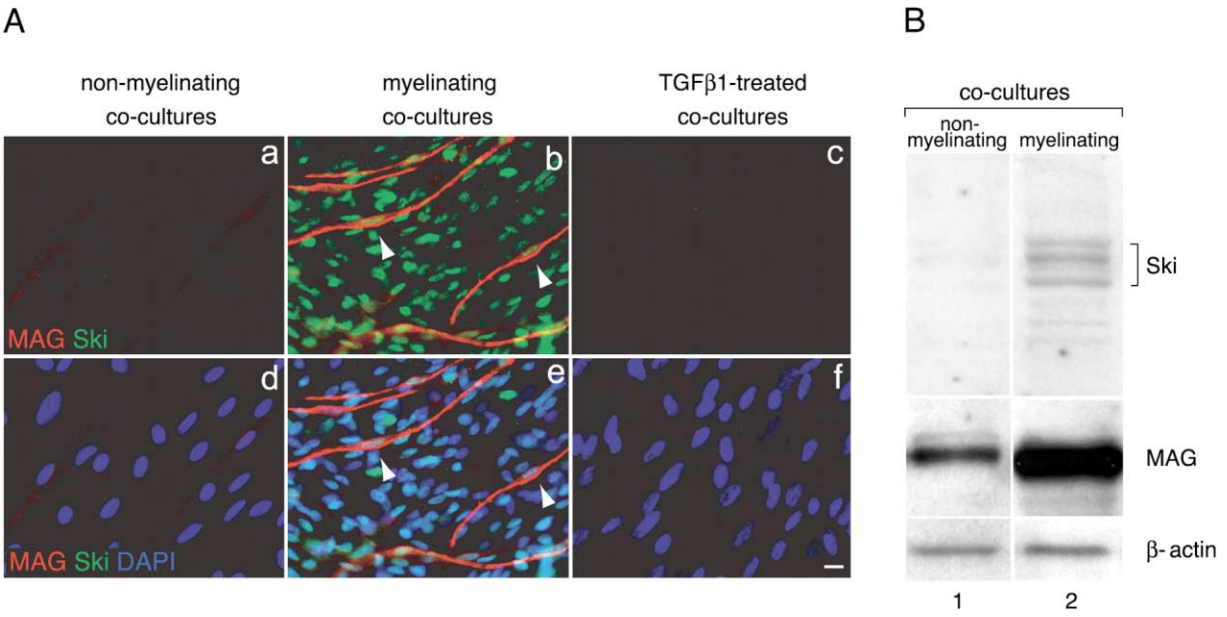

Figure 3. Ski Is Upregulated in DRG-Schwann Cell Cocultures under Myelinating Conditions

(A) Schwann cell/DRG neuron cocultures were grown for 14 days in control media that did not allow myelin formation (Aa and Ad) or in myelin-promoting media without (Ab and Ae) or with TGF-β1 (Ac and Af). Cocultures were fixed and triple stained for Ski ([Aa–Af], green), the myelin marker MAG ([Aa–Af], red), and with nuclear DAPI ([Ad–Af], blue). In nonmyelinating cocultures, Ski expression was undetectable (Aa), whereas Ski was upregulated in myelinating cocultures (Ab) and localized to the Schwann cell nuclei ([Ae], overlay with DAPI appears greenish blue). TGF-β1 prevented Ski expression (Ac). Scale bar, 10 μm (in [Af] for [Aa]–[Af]).

(B) Immunoblot analysis of lysates from nonmyelinating and myelinating cocultures. The membrane was probed with a polyclonal anti-Ski antibody. Multiple isoforms of Ski range from 90 kd to 110 kd. The myelin marker MAG is upregulated during myelination. Equal amounts of protein extract controlled by β-actin detection.

and/or progesterone (data not shown). These findings indicate that the presence of neurons, in combination with a myelination-promoting environment, is required for triggering Ski induction. Western blot analysis confirmed the upregulation of Ski under myelin-promoting conditions (Figure 3B).

TGF-β1 prevents the differentiation of Schwann cells toward the myelinating phenotype (Einheber et al., 1995). Based on our data and the described antagonistic role of Ski in TGF-β1 signaling in other cell types (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999; Xu et al., 2000), we hypothesized that Ski expression might be inhibited in TGF-β1-treated DRG-Schwann cell cocultures under myelinating conditions. Indeed, we found that TGF-β1 efficiently blocked myelination induced by ascorbic acid/progesterone (Figures 3Ac and 3Af, red) and prevented Ski expression (Figures 3Ac and 3Af, green).

Ski Is Expressed by Myelinating Schwann Cells In Vivo and Is Regulated by Axon-Schwann Cell Interactions

To further examine the role of Ski in the regulation of Schwann cell proliferation and differentiation, we investigated the expression of Ski in vivo during postnatal PNS development and before and after nerve injury (Figure 4). Using quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR), we detected no significant changes in Ski mRNA levels in the nerves of developing animals at postnatal (P) days 1.5 to 23 (Figure 4A, gray bars) or in the adult (P64, gray bar). Ski transcript levels also remained unaffected 4 days after nerve injury, when Schwann cells are highly proliferative (Figure 4B, gray bars). To assess the levels of Ski protein, we carried out Western blotting analysis using Schwann cell nuclear extracts from developing sciatic nerves at P0 (Figure 4C, lane 1) and P90 (Figure 4C, lane 2). In contrast to the mRNA levels, the Ski protein level was strongly increased in differentiated (P90) compared to immature and proliferating Schwann cells (P0). Furthermore, Ski protein levels were downregulated 4 days post nerve transection (4dpT), when Schwann cells proliferate as part of Wallerian degeneration (Figure 4C, compare lanes 2 and 3). Two months after crush injury (2mpC), when axons have regenerated and remyelinated, the expression of Ski reached similar levels as in uninjured samples (Figure 4D, compare lanes 1 and 2). In contrast, 2 months after nerve transection (2mpT) and prevention of regeneration, Ski protein levels remained low (Figure 4D, compare lanes 1 and 3).

To corroborate the biochemical studies, the subcellular localization of Ski protein on teased sciatic nerve fibers was examined (Figures 4E). In adult animals, Ski (Figure 4Ea, green) was readily detected in nuclei (Figure 4E, arrowheads in 4Ea and 4Eb) of myelinating Schwann cells as visualized by staining for myelin basic protein (MBP; Figure 4Eb, red). Four days after transection, Ski immunoreactivity was strongly reduced in the degenerating distal stump of sciatic nerves (Figures 4Ec and 4Ed; Ski in green, Schwann cell marker S100 in red). Taken together, these results indicate that Ski protein

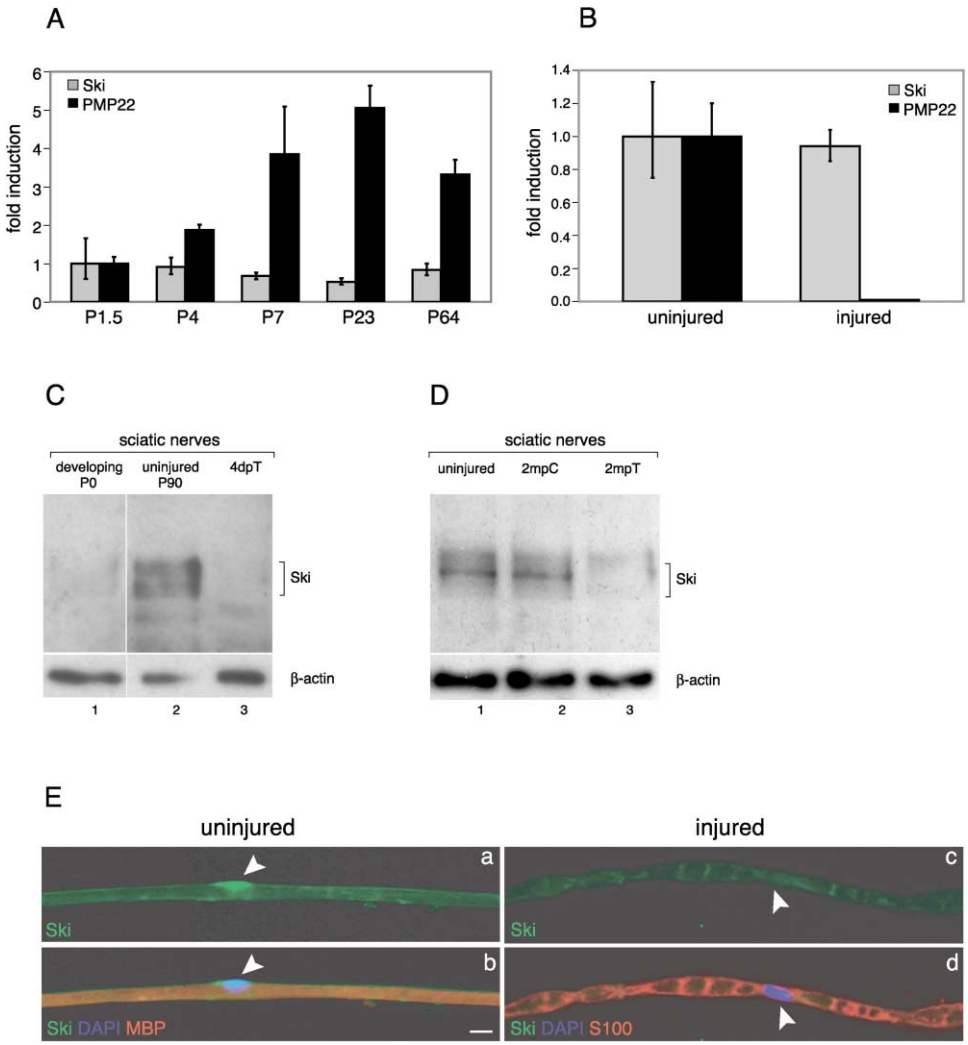

Figure 4. Ski Expression in Schwann Cells In Vivo

(A) qRT-PCR was performed to determine Ski mRNA levels in sciatic nerves during postnatal development (P1.5–P64; gray bars). As internal control for the integrity of the samples, mRNA levels of PMP22 were measured (black bars). cDNA from nerves of at least three animals was generated per time point, except for p64, where two animals were used. Each bar represents the mean value of three independent PCR experiments ±SD. Student's t test was applied to test for significance. Results are presented as ratios of Ski or PMP22 to 18S rRNA and normalized to the levels at P1.5. No significant changes in Ski expression were observed, whereas the levels of PMP22 were upregulated during the myelination process as described (Suter et al., 1994).

(B) qRT-PCR was performed to study the abundance of Ski mRNA in adult uninjured and injured sciatic nerves (gray bars). PMP22 served as internal control (black bars). cDNA from nerves of three animals per paradigm were generated. Each bar represents the mean value of three PCR experiments ±SD. Student's t test was applied to test for significance. Results are shown as ratios of Ski or PMP22 to 18S rRNA. No significant changes in the levels of Ski transcripts were observed, whereas the levels of PMP22 were downregulated after nerve injury as previously shown (Suter et al., 1994).

(C) Western blot analysis using nuclear extracts of developing (P0; lane 1), adult uninjured (P90; lane 2), and adult injured sciatic nerves 4 days posttransection (4dpT; lane 3). Membranes were incubated with a monoclonal anti-Ski antibody. Comparable loading of protein extracts was controlled by β-actin detection. Note the increase in Ski protein levels in adult versus developing nerves. Ski expression decreased drastically 4dpT as Schwann cells proliferated (lane 3).

(D) Western blot analysis using sciatic nerve nuclear extracts of adult uninjured (lane 1), 2 months post-crush lesion (2mpC; lane 2), and 2 months posttransection (2mpT; lane 3) samples. Membranes were incubated with a monoclonal anti-Ski antibody. Ski levels return to normal after a crush injury when axons are allowed to regenerate, but not when regeneration is inhibited by nerve transection. Equal loading of protein extracts controlled by β-actin detection.

(E) Triple labeling of a teased, uninjured, myelinated nerve fiber (Ea and Eb). Ski (green), DAPI (blue), and MBP (red). Arrowheads point to nuclear staining of Ski. Triple labeling of a teased nerve fiber 4 days after nerve injury (Ec and Ed). Ski (green), DAPI (blue), and Schwann cell marker S100 (red). Note the lack of Ski in Schwann cell nuclei after nerve injury (arrowheads). Scale bar, 40 μm (in [Eb] for [Ea]–[Ed]).

levels are low in proliferating Schwann cells but high in myelinating cells. This regulation involves posttranscriptional mechanisms and neuron-Schwann cell interactions.

Ski Is Not Expressed by Schwann Cells of Demyelinated Peripheral Nerves

Our results described so far show that Ski is undetectable in proliferating Schwann cells in vitro and in vivo,

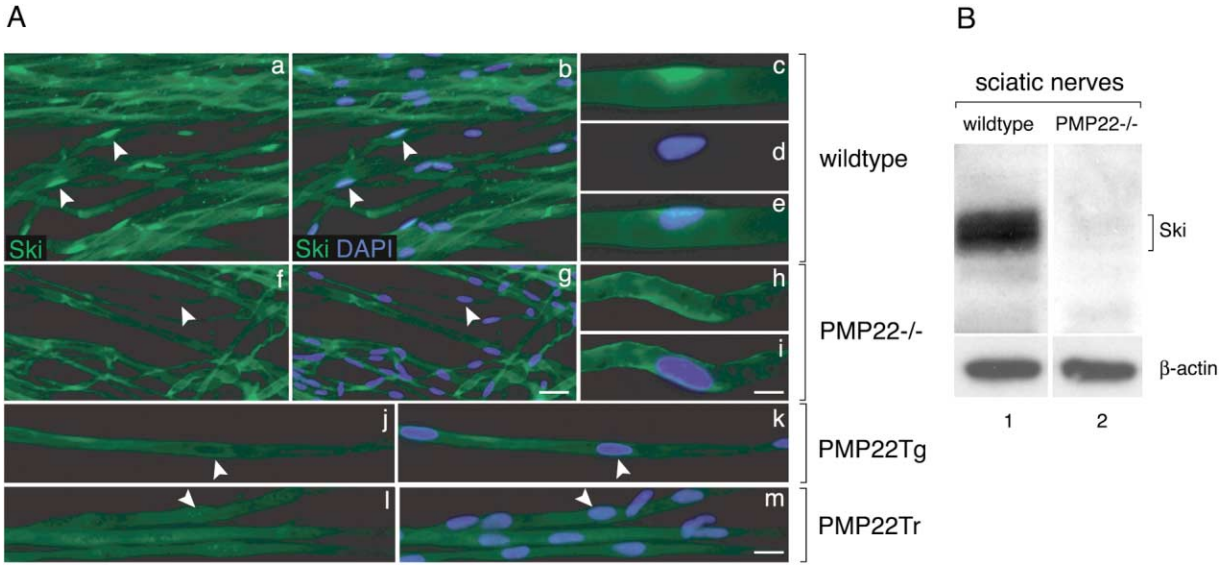

Figure 5. Ski Expression in Animal Models of Demyelinating Neuropathies

(A) Double labeling of teased sciatic nerves for Ski (green) and DAPI (blue) in wt (Aa–Ae), *PMP22*-deficient (*PMP22*$^{-/-}$; [Af–Ai]), *PMP22*-transgenic (PMP22Tg; [Aj and Ak]), and PMP22 *Trembler* (PMP22Tr; [Al and Am]) mice. In wt animals, Ski was highly expressed (Aa) and localized to the nuclei (Ab) (arrowheads in [Aa] and [Ab]). Note that the double-positive nuclei appear greenish blue (Ab). Magnification of a wt nucleus visualizing the nuclear Ski staining ([Ac], Ski; [Ad], DAPI; [Ae], overlay). In *PMP22*-deficient nerves, Ski was mainly absent from the nuclei (arrowheads in [Af] and [Ag]) ([Af], Ski; [Ag], Ski/DAPI). Magnification of a nucleus from *PMP22*-deficient nerves demonstrating low levels of nuclear Ski staining ([Ah], Ski; [Ai], Ski/DAPI). Ski is also absent in Schwann cell nuclei of *PMP22*-transgenic mice (arrowheads in [Aj] and [Ak]) ([Aj], Ski; [Ak], Ski/DAPI) and *Trembler* animals (arrowheads in [Al] and [Am]) ([Al], Ski; [Am], Ski/DAPI). Scale bar, 40 μm (in [Ag] for [Aa], [Ab], [Af], and [Ag]), 10 μm (in [Ai] for [Ac]–[Ae], [Ah], and [Ai]), and 20 μm (in [Am] for [Aj]–[Am]).

(B) Western blot analysis of nuclear extracts from adult wt (lane 1) and PMP22-deficient (lane 2) sciatic nerves. The membrane was incubated with a monoclonal anti-Ski antibody. Both lanes contain equal amounts of protein extract as controlled by β-actin detection. Note the strong downregulation of Ski in mutant animals.

i.e., during development or after nerve injury. Hence, Ski appears to play an important role in regulating cell cycle exit, Schwann cell differentiation, and, possibly, myelination. We therefore examined Ski expression in another clinically important and mechanistically different paradigm in which Schwann cell proliferation and myelination are affected in vivo. In hereditary demyelinating diseases such as Charcot-Marie-Tooth type 1 (CMT1), both Schwann cell proliferation and myelination are disturbed (Atanasoski et al., 2002; Dyck et al., 1993; Sancho et al., 1999, 2001; Suter and Scherer, 2003). In affected nerves, repeated cycles of demyelination and remyelination are associated with aberrant Schwann cell proliferation. In contrast to nerve lesions, however, there is no major acute damage to the axons, although axonal atrophy and loss are slowly developing features in CMT1 (Suter and Scherer, 2003). We examined sciatic nerves of two authentic mouse models for CMT1A, the *Trembler* and the *PMP22*-transgenic mouse (Figure 5). The *Trembler* mouse carries a point mutation in the peripheral myelin protein 22 (*PMP22*; Suter et al., 1992), and *PMP22*-transgenic mice carry additional copies of the *PMP22* gene (Magyar et al., 1996). In both mouse strains, axons are largely devoid of myelin. We detected no Ski expression in Schwann cell nuclei (Figure 5A, arrowheads in 5Aj–5Am) of adult mutant animals in contrast to wild-type (wt) mice (Figures 5Aa–5Ae, arrowheads in 5Aa and 5Ab). Similarly, Ski was mostly not detectable in adult *PMP22*-deficient mouse nerves (Figures 5Af–5Ai, arrowheads in 5Af and 5Ag), which are largely demyelinated (Adlkofer et al., 1995). Immunoblot analysis confirmed that Ski levels were below the detection limit in nerves of *PMP22*-deficient animals (Figure 5B). Thus, Ski is not expressed by demyelinating Schwann cells.

Ski Is Required for Myelination of Peripheral Nerves and Regulates Myelin Gene Expression

Since Ski expression is closely associated with myelination, we asked whether Ski is essential for Schwann cells to produce myelin. To this end, we employed a genetic approach using *Ski*-deficient mice. As these animals die perinatally (Berk et al., 1997), preventing us from studying the postnatal event of PNS myelination in vivo, we established DRG explant cultures under myelination-permissive conditions from mutant and wt animals (Carenini et al., 1998). Myelination (Figure 6A, MBP, red) was readily apparent in DRG cultures originating from wt (*Ski*$^{+/+}$) animals (Figure 6Aa) but absent in *Ski*$^{-/-}$ samples (Figure 6Ab). As underscored by the statistical analysis (Figure 6B), there is an absolute requirement for Ski in the formation of PNS myelin.

Ski-mediated regulation of myelin-related genes is a potential mechanism by which Ski may affect myelination. Thus, we performed in situ hybridization experiments on transverse sections from E19.5 embryos of wt and *Ski*-deficient mice (Figure 7). *Ski*$^{+/+}$ mice express the glial marker Sox10 (Figure 7A) and the myelin-related genes *MBP* (Figure 7C), *PMP22* (Figure 7E), and *MPZ/P0* (Figure 7G) in DRGs (arrows in Figures 7A, 7C, 7E, and 7G) and peripheral nerves (arrowheads in Figures

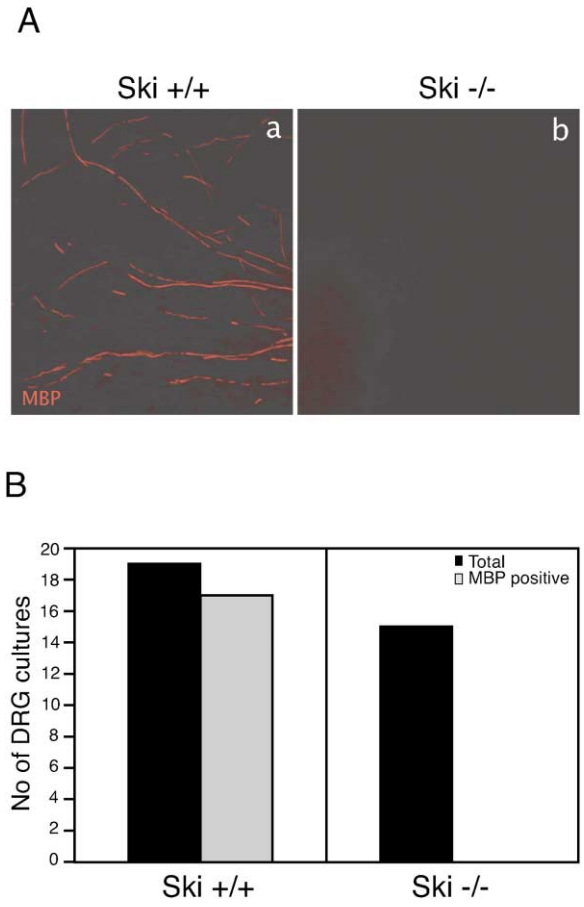

Figure 6. Absence of Myelin Formation in DRG Explants from *Ski*-Deficient Animals

(A) MBP immunofluorescence in control ([Aa], $Ski^{+/+}$) and mutant ([Ab], $Ski^{-/-}$) DRG explant cultures grown in myelination-promoting media. Note the complete absence of myelin in *Ski*-deficient samples.

(B) Numbers of analyzed DRG cultures of $Ski^{+/+}$ (19) and $Ski^{-/-}$ (15) animals (black bars). Seventeen control $Ski^{+/+}$ samples showed MBP-positive internodes, whereas no MPB staining was detected in $Ski^{-/-}$ DRG cultures (gray bars).

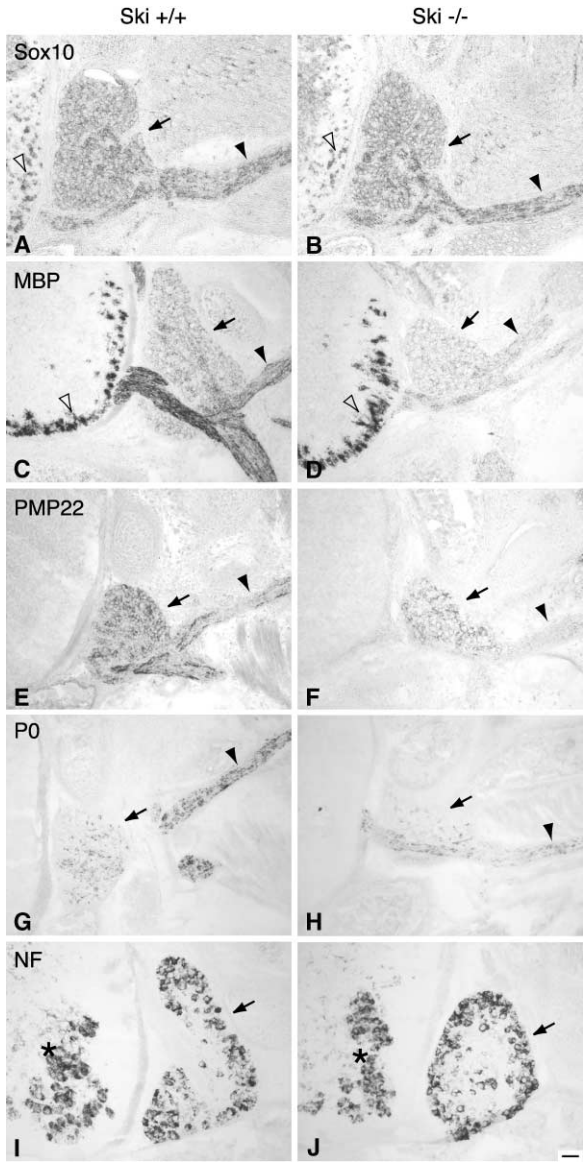

Figure 7. In Situ Hybridization Analysis of Glial and Neuronal Markers on Transverse Sections of E19.5 Embryos

The glial marker Sox10 (A and B) showed no difference in expression in mutant ([B], $Ski^{-/-}$) versus wt ([A], $Ski^{+/+}$) embryos. Expression of MBP (C and D), PMP22 (E and F), and MPZ/P0 (G and H) in wt and *Ski*-deficient embryos revealed that all myelin gene-related markers tested were strongly downregulated in the DRG (arrows) and peripheral nerves (arrowheads) of the mutant (D, F, and H) compared to control embryos (C, E, and G). Open arrowheads point to oligodendrocytes in control and mutant mice, which show no difference in Sox10 (A and B) and MBP expression (C and D). Similarly, the neural marker neurofilament (NF; [I and J]) showed no difference in expression in mutant (J) versus wt (I) embryos. Arrows (I and J) point to the DRG, and the asterisk (I and J) marks neurons in the CNS. Scale bar, 40 μm (in [J] for [A]–[J]).

7A, 7C, 7E, and 7G). In addition, Sox10 and MBP are also detected in oligodendrocytes (open arrowheads in Figures 7A–7D). Mutant mice ($Ski^{-/-}$) show a strong reduction in the expression of the myelin-related genes (Figures 7D, 7F, and 7H), both in peripheral nerves (arrowheads) and DRGs (arrows). This lower expression is not due to reduced glia cell numbers, since Sox10 levels are unchanged in the mutants (Figure 7, compare 7A and 7B). In contrast to the PNS, MBP was not reduced in the CNS of mutant animals (Figure 7D, open arrowhead). Expression of the neuronal marker neurofilament (NF; Figures 7I and 7J) was also unaffected both in DRGs (arrows) and the spinal cord (asterisk) of mutant mice. We conclude that the absence of Ski specifically affects the expression of myelin-related genes in the PNS, but not in the CNS.

To directly assess the influence of Ski on myelin gene regulation, we infected primary Schwann cells with an EGFP- or a Ski-expressing adenovirus. Cells were collected 3 days postinfection and processed for further analysis. Compared to control EGFP-infected cells (Figure 8A, gray bars), qRT-PCR experiments revealed an ~3-fold induction of the myelin gene transcripts PMP22, MPZ/PO, and periaxin in Ski-infected Schwann cell cultures (Figure 8A, black bars). This induction was also

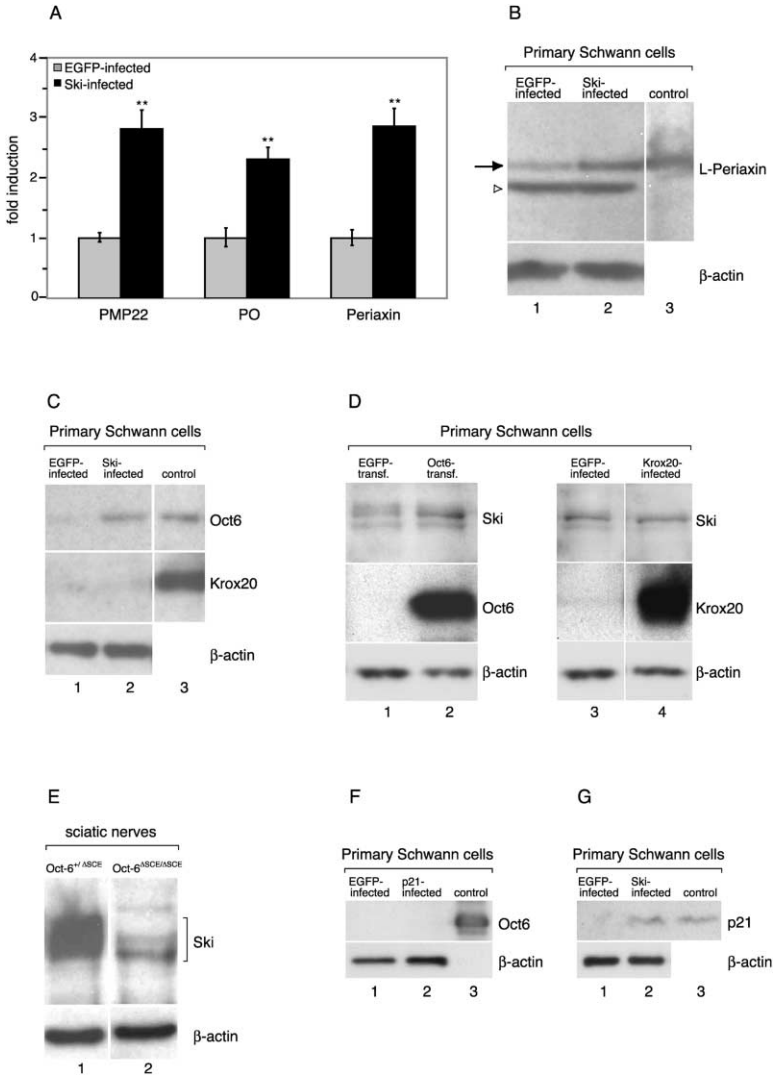

Figure 8. Ski Regulates Genes Related to Myelination

(A) qRT-PCR was performed to study the abundance of various myelin-related markers (PMP22, MPZ/P0, and periaxin) in control EGFP (gray bars) versus Ski-infected (black bars) primary rat Schwann cells. cDNA from three different cultures per paradigm were generated. Each bar represents the mean value of three independent PCR experiments ±SD. Student's t test was applied to test for significance: $^{**}p \leq 0.01$. Values of control-infected samples arbitrarily set to 1. All tested myelin-related molecules were significantly upregulated by Ski overexpression.

(B) Western blot analysis of extracts from control EGFP (lane 1) versus Ski-infected (lane 2) primary Schwann cells showed a 3-fold induction in periaxin levels upon Ski overexpression (arrow). Extracts of myelinating DRG explant cultures were used as a positive control (lane 3). Both lanes contain similar amounts of protein extract as verified by β-actin detection. Open arrowhead indicates unspecific bands.

(C) Western blot analysis of Oct6 and Krox20 expression in control EGFP (lane 1) and Ski-infected (lane 2) primary rat Schwann cells. Comparable loading of protein extract controlled by β-actin detection. Schwann cells overexpressing Oct6 or Krox20 were used as positive controls (lane 3). Note the upregulation of Oct6 upon Ski infection.

(D) Western blot analysis of Ski expression in control EGFP (lane 1) and Oct6-transfected (lane 2) primary rat Schwann cells. Membranes were incubated with polyclonal antibodies against Ski or Oct6, showing an ~2-fold upregulation of Ski as a result of Oct6 overexpression (lane 2). In contrast, Ski protein levels remained the same in control EGFP (lane 3) and Krox20-infected (lane 4) primary rat Schwann cells. Membranes were incubated with polyclonal antibodies against Ski or Krox20. Similar loading controlled by β-actin detection.

(E) Western blot analysis of sciatic nerve extracts of control ($Oct6^{+/\Delta SCE}$; lane 1) and mutant animals carrying Schwann cell-specific *Oct6* hypomorphic alleles ($Oct6^{\Delta SCE/\Delta SCE}$; lane 2). The membrane was incubated with monoclonal antibodies against Ski. Comparable protein loading confirmed by β-actin detection. Note the ~10-fold reduction of Ski expression in mutant versus control animals.

(F) Western blot analysis of Oct6 expression in control (lane 1) and p21-infected (lane 2) primary rat Schwann cells. Membranes were incubated with polyclonal antibodies against Oct6. Schwann cells overexpressing Oct6 were used as positive control (lane 3). Comparable loading of protein extract controlled by β-actin detection.

(G) Western blot analysis of p21 expression in control (lane 1) and Ski-infected (lane 2) primary rat Schwann cells. Membranes were incubated with polyclonal antibodies against p21, showing upregulation of p21 as a result of Ski overexpression (lane 2). Schwann cells overexpressing p21 were used as positive control (lane 3). Similar loading controlled by β-actin detection.

reflected at the protein level, as Western blot analysis revealed an ~3-fold induction in periaxin protein upon Ski overexpression (Figure 8B, lanes 1 and 2).

Ski Modulates the Expression of Oct6, and Schwann Cells Deficient in Oct6 Show Decreased Levels of Ski

We hypothesized that the observed functional role of Ski in Schwann cells might be related to the regulation of Oct6 and Krox20 since these transcription factors are crucially involved in the control of Schwann cell differentiation. To approach this question, cultured Schwann cell were infected with EGFP- or Ski-expressing adenovirus, and cells were collected 3 days later. Western blot analysis revealed that Oct6 was upregulated in Schwann cells overexpressing Ski (Figure 8C, lane 2, upper panel) compared to control EGFP-infected cells (Figure 8C, lane 1, upper panel). In contrast, Krox20 was barely detectable in EGFP-infected and in Ski-overexpressing Schwann cells (Figure 8C, lanes 1 and 2, middle panel). Next, we examined whether Oct6 and Krox20 influence the expression of Ski. Primary Schwann cells transfected with an Oct6 expression plasmid showed an ~2-fold increase in Ski protein levels (Figure 8D, lane 2, upper panel) compared to control EGFP-transfected cells (Figure 8D, lane 1, upper panel). Krox20-overexpressing Schwann cells showed no difference in Ski expression levels (Figure 8D, lane 4, upper

panel) in comparison to EGFP-infected cells (Figure 8D, lane 3, upper panel). In summary, Ski and Oct6 appear to mutually regulate each other, while Krox20 is not affected by Ski overexpression or vice versa.

Based on the observed functional interplay between Ski and Oct6 in gain-of-function experiments, we further tested this mechanism in a loss-of-function paradigm in Schwann cells lacking Oct6. Western blot analysis of nerve extracts from adult animals carrying Schwann cell-specific *Oct6* hypomorphic alleles (*Oct6*$^{\Delta SCE/\Delta SCE}$; Ghazvini et al., 2002) and controls demonstrated an ~10-fold reduction of Ski expression in the absence of Oct6 (Figure 8E, compare lanes 1 and 2). These results corroborate our findings of a functional interplay between Ski and Oct6 and suggest that Schwann cell differentiation involves Oct6-mediated activation of Ski.

Ski Links Growth Arrest and Differentiation in Schwann Cells

Growth arrest and differentiation are tightly coupled during postnatal Schwann cell development. Based on our data, we therefore asked whether Ski was triggering the differentiation program as a default consequence of inhibiting proliferation. To test this possibility, the cell cycle inhibitor p21 (Sherr, 1995) was overexpressed in Schwann cells, causing efficient growth arrest (data not shown). Oct6 was not induced by exogenous p21 (Figure 8F, lanes 1 and 2), similar to the myelin-related genes *PMP22*, *MPZ/P0*, and *periaxin* (data not shown). Conversely, to determine whether Ski-mediated growth arrest involves p21, Schwann cell cultures were infected with EGFP or Ski-expressing adenovirus and analyzed by immunoblots (Figure 8G). p21 was upregulated in Schwann cells overexpressing Ski. We conclude that Ski does not induce differentiation merely by stopping Schwann cell proliferation but plays an active role in both the regulation of Schwann cell growth arrest and myelination.

Discussion

In this report, we show that the protooncogene Ski plays a crucial role in the control of Schwann cell proliferation and peripheral nerve myelination. This conclusion is based on multiple lines of in vitro and in vivo evidence. First, Ski expression is inversely correlated with Schwann cell proliferation both in vitro and in vivo. Second, Ski overexpression causes growth arrest in Schwann cells that are cultured in the presence of TGF-β1. Third, Ski protein expression is upregulated in myelinating Schwann cells, and organotypic DRG cultures derived from *Ski*-deficient mice are unable to form myelin. Fourth, Ski regulates genes encoding myelin proteins and Oct6, a major regulator of PNS myelination. Finally, Ski links the two biologically interconnected events of cell cycle exit and differentiation in Schwann cells.

Ski exerts its functions in concert with various intracellular partners, including the Smad proteins (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999; Wu et al., 2002; Xu et al., 2000) that are mediators of the TGF-β receptor signal transduction (Shi et al., 1997, 1998). Binding of Ski to Smads represses their ability to activate target genes of TGF-β family signaling. In this manner, Ski is likely to be involved in the regulation of a wide range of cellular functions and developmental processes. Consistent with an antagonistic role of Ski in the TGF-β pathway, Ski overexpression in our study repressed specifically TGF-β1-mediated Schwann cell proliferation but had no effect on GGF and bFGF-mediated cell division. In further support of this hypothesis, Ski and Smad2/3 can be coimmunoprecipitated from confluent Schwann cells cultured with TGF-β1 (data not shown). In contrast, when Ski was overexpressed in growth-arrested Schwann cells, GGF and bFGF, like TGF-β1, were unable to trigger the reentry into the cell cycle. Thus, the effect of Ski appears to be dependent on the extracellular environment as well as on the growth and differentiation status of the cell (Engert et al., 1995; Ichikawa et al., 1997). A similar differential behavior has been observed in quail embryo fibroblasts where Ski can induce both oncogenic transformation and terminal muscle differentiation, depending on the growth conditions (Colmenares and Stavnezer, 1989). The results also suggest that Ski probably not only acts through the TGF-β pathway and Smads in Schwann cells—interactions with other Ski partners, including the retinoic acid receptor (RAR), Gli3, retinoblastoma protein, Skip, C184M, and NFI may also be involved (Dahl et al., 1998a, 1998b; Dai et al., 2002; Kokura et al., 2003; Tarapore et al., 1997; Tokitou et al., 1999).

As expected for an antagonistic role of Ski in TGF-β signaling, Ski protein was not expressed in myelin-competent cocultures of sensory neurons and Schwann cells in the presence of TGF-β1, conditions that inhibit myelination. In the absence of TGF-β1, however, Ski protein levels were high in Schwann cells in cocultures, regardless of whether the Schwann cells were myelinating or not. Intriguingly, this upregulation was not observed in pure Schwann cell cultures in the same medium. Thus, yet unidentified factors produced by neurons under these myelin-inducing conditions regulate Ski protein expression in Schwann cells.

Germline inactivation of *Ski* in mice causes perinatal lethality and decreased precursor cell numbers in the neuroepithelial and skeletal muscle lineages, in addition to other abnormalities (Berk et al., 1997). We found that loss of Ski prevents myelination in organotypic DRG cultures, indicating that Ski expression is required for peripheral nerve myelination. Since Schwann cell proliferation and myelination are dependent on axon-Schwann cell interactions, this raises the question of which cell type is mainly dependent on Ski expression. In this regard, we found that overexpression of Ski in cultured Schwann cells causes upregulation of myelin protein genes and of the regulator Oct6. Conversely, transcript levels of myelin protein genes were reduced in *Ski*-deficient peripheral nerves in late embryonic development. Together, these findings indicate a major regulatory function for Ski in myelin protein gene regulation and Schwann cell differentiation. However, Ski is also expressed by motor and sensory neurons (Lyons et al., 1994; data not shown). *Ski*-deficient DRG cultures showed some transient delay in initial neurite outgrowth (data not shown) that may also indicate some role of Ski in neurons. To examine for early developmental defects with respect to axonal outgrowth, we analyzed E19.5

Ski-mutant peripheral nerves by electron microscopy but found no gross abnormalities (data not shown).

Ski induces Oct6 expression in cultured Schwann cells, and Oct6 also upregulates Ski. In addition, *Oct6*-deficient Schwann cells express Ski at much lower levels than wt Schwann cells. These findings indicate that Ski and Oct6 act in concert to control the Schwann cell differentiation program. The corresponding mechanisms, however, remain to be clarified. One might speculate, for example, that the delay in myelination observed in *Oct6*-deficient mice is related to this interplay. Experiments including overexpression of Ski in *Oct6*-deficient Schwann cells to potentially rescue the developmental delay phenotype will shed more light on this interesting issue.

Induction of Schwann cell growth arrest by Ski overexpression is accompanied by the upregulation of myelin-related genes. This might be interpreted so that Schwann cell differentiation is caused directly as a consequence of the effect of Ski on growth arrest. However, we found that overexpression of the cell cycle inhibitor p21 (Sherr, 1995) induced only growth arrest but no upregulation of myelin-related genes. Since Ski can also induce growth arrest through p21, it is likely that Ski is independently required for both growth arrest and differentiation. We anticipate that identifying additional target genes of Ski will elucidate the mechanisms by which Ski is coupling the biologically interconnected events of proliferation and differentiation in postnatal Schwann cell development.

In summary, our data show that Ski is necessary for peripheral nerve myelination and is a major regulator of Schwann cell proliferation and differentiation. Future conditional and cell-type specific *Ski* knockout experiments will further define the specific roles of Ski in Schwann cells and, potentially, neurons.

Experimental Procedures

Animals

Animal experiments (Wistar rats and C57bl/6 mice; Elevage Janvier, France) were approved by the veterinary office of the Canton of Zurich. Mutant mice: Schwann cell-specific *Oct6* null mice (Ghazvini et al., 2002), *PMP22*-deficient animals (Adlkofer et al., 1995), *Trembler* mice (Suter et al., 1992), *PMP22*-transgenic mice (Magyar et al., 1996), and mice deficient in *Ski* (Berk et al., 1997; Colmenares et al., 2002).

Sciatic Nerve Injuries and Preparation of Teased Fibers and Cryosections

Injuries and further processing were carried out as described (Atanasoski et al., 2001).

Cell Culture

Rat Schwann cells were grown in Schwann cell medium (Dulbecco's modified Eagle's medium [DMEM], GIBCO BRL), containing 10% FCS, 50 μg/ml gentamicin (Sigma), 100 μg/ml crude GGF (BioReba Biotechnology Inc.), and 2 μM forskolin (Sigma) as described (Atanasoski et al., 2001). For growth arrest, cells were incubated for 3 days in minimal medium plus 0.5% FCS. Minimal medium: DMEM/F12 (GIBCO), human apo-transferrin (100 μg/ml), progesterone (60 ng/ml), insulin (5 μg/ml), putrescine (16 μg/ml), L-thyroxin (400 ng/ml), selenium (160 ng/ml), triiodothyronine (10 ng/ml), and 300 μg/ml BSA (Fluka). Supplements were from Sigma, unless stated otherwise. Schwann cell growth factors: human recombinant GGF (20 ng/ml), TGF-β1 (10 ng/ml; R&D Systems), and bFGF (10 ng/ml; PeproTech).

For DRG explant cultures, DRGs were excised (E19.5) and plated on glass coverslips (Carenini et al., 1998). Cultures were maintained in myelination-promoting medium for up to 3 weeks.

DRG-Schwann cell cocultures were established as described (Notterpek et al., 1999). Where indicated, TGF-β1 was added to the myelination-promoting medium to a final concentration of 10 ng/ml.

Ski Adenovirus Generation and Production

To construct a Ski adenovirus, an internal ribosome entry site (IRES) along with the GFPq (Quantum Biotechnology) gene was subcloned in the pShuttle-CMV plasmid (Mosser et al., 1997). The human Ski open reading frame was then inserted between the CMV promoter and the IRES leading to a CMV-driven cassette expressing both Ski and GFP, whereas the control vector expresses GFP alone. Recombinant adenoviruses were generated with the AdEasy system (Stratagene). The resulting adenoviral vector was amplified in HEK293 cells, purified (BD Adeno-X Virus Purification Kit [Clontech]), and stored in elution buffer.

Transient Transfection and Infection of Schwann Cells with Recombinant Adenovirus Vectors

Rat Schwann cells were grown at a density of 8000 cells/cm^2 on laminin-2-coated dishes in Schwann cell medium. Fugene 6 (Roche)-mediated transfection of human Ski (Xu et al., 2000) or EGFP expression plasmids (kind gift from B. Amati, ISREC, Lausanne) was performed at a ratio of 1.5 μl Fugene/μg plasmid DNA. BrdU labeling and detection was carried out according to the manufacturer's instructions (Roche).

Primary rat Schwann cells were isolated and maintained in Schwann cell medium. The following day, cells were either transfected with expression plasmids using Fugene 6 or infected with the indicated adenovirus. Fugene 6-mediated transfection of Oct6 expression plasmid (Meijer et al., 1992) and infection with Krox20/Egr2 (Ehrengruber et al., 2000) or ADV-p21 (Yang et al., 1996) adenovirus were performed as described. Adenovirus vectors were used at m.o.i. of 1000. Twenty-four hours later, the medium was changed and the cells maintained in Schwann cell medium for another 2 days before analysis. Transfection and infection experiments were repeated at least twice to insure the reproducibility of the results.

Immunofluorescence Microscopy

Longitudinal nerve sections and teased fibers were blocked for 1 hr in PBS containing 10% goat serum, 1% BSA, and 0.3% Triton X-100. Incubation with primary antibodies was carried out for 16 hr at 4°C. Rat monoclonal antibodies against MBP (1:50 dilution; Serotec) and Ki-67 (1:20; DAKO), mouse monoclonal antibodies against Ski (G8, 1:20; Colmenares et al., 1991), and rabbit polyclonal antibodies against S100 (1:300; DAKO) were used. For MBP staining, nerve fibers were permeabilized with 100% Triton X-100 for 40 min and organotypic DRG cultures with 5% Triton X-100 for 15 min at room temperature prior to the blocking step. After incubation with the primary antibody, the tissue samples or fixed cells were washed and exposed to the appropriate donkey anti-rat Cy3 (1:200), goat anti-rat Texas red (1:200), goat anti-mouse FITC (1:100), or goat anti-rabbit Cy3 (1:200) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Appropriate controls including secondary antibodies showed no significant stainings. Note that anti-mouse IgG antibodies showed a diffuse overall background staining on mouse tissue. Finally, specimens were washed and mounted in AF1 (Citifluor, Canterbury, UK) supplemented with DAPI (1:1000; Roche). Immunoreactivity was visualized by fluorescence microscopy and documented using a Hamamatsu color chilled 3CCD camera. Pictures were processed with Adobe Photoshop 7.0 for Macintosh.

Rat Schwann cell cultures and Schwann cell/DRG cocultures were fixed in 3.7% formaldehyde for 10 min at room temperature. Cocultures were additionally postfixed and permeabilized in 100% methanol at −20°C for 5 min. Unspecific binding was blocked for at least 30 min in PBS containing 10% goat serum, 1% BSA, and 0.3% Triton X-100. Primary antibodies against Ski (1:100; Upstate Biotechnology), Ki-67 (1:10; DAKO), MBP (1:500; Roche), MAG (1:1000; gift of M.B.Tropak, S.Lunenfeld Research Institute, Toronto, Canada), and p75 (1:300; Chemicon) were incubated in blocking buffer overnight at 4°C, followed by incubation with secondary antibodies in blocking buffer for 1 hr at room temperature. BrdU labeling and

detection was carried out according to the manufacturer's instructions (Roche). After washing in PBS, cells were mounted in AF1 supplemented with DAPI (1:1000; Roche).

Western Blotting

Sciatic nerve tissue was dissected, frozen in liquid nitrogen, pulverized with a chilled mortar and pestle, and nuclear extracts were prepared (Atanasoski et al., 1997). Alternatively, cells were harvested and lysed in SDS gel sample buffer (Atanasoski et al., 2001). Proteins were electophoresed on 7.5% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membrane, and immunoblotted with antibodies against Ski (Upstate Biotechnology; 1:100), MAG (1:1000), Periaxin (gift of P. Brophy, University of Edinburgh, UK; 1:100), Krox20 (BabCO; 1:100), Oct6 (Ghazvini et al., 2002; 1:100), p21 (Santa Cruz Biotechnology, Inc.; 1:1000), and β-actin (Sigma; 1:1000). After incubating with goat anti-rabbit horseradish peroxidase- (Santa Cruz Biotechnology, Inc.) or goat anti-mouse κ chain alkaline phosphatase-conjugated secondary antibodies (Southern Biotechnology Associates), immunoreactive bands were visualized by Western Lightning (PerkinElmer Life Sciences, Inc.) or CDP-Star (Roche). Cell lysates of Ski-transfected primary rat Schwann cells served as positive controls. The blots were quantified using ImageJ software (http://rsb.info.nih.gov/ij/), normalizing the individual lanes to the β-actin signal. Note that several isoforms of Ski have been found on Western blots. Although the molecular basis for this phenomenon remains to be elucidated, all bands represent genuine forms of Ski, since they are detected by two different polyclonal anti-Ski sera and a monoclonal anti-Ski antibody, all of which identify different epitopes (unpublished data).

RNA In Situ Hybridization

Antisense riboprobes were labeled with digoxigenin according to the manufacturer's instruction (Roche Diagnostics) and were used as desribed (Paratore et al., 1999). Riboprobes: Rat P0 (Lemke and Axel, 1985); rat PMP22 (Welcher et al., 1991); mouse MBP (de Ferra et al., 1985); human Sox10 (gift from M. Wegner, University of Erlangen-Nurnberg, Germany), and mouse NF (gift from M. Oblinger, Chicago Medical School, North Chicago).

RNA Isolation from Schwann Cell Cultures and Sciatic Nerve Tissue

Sciatic nerve tissue of C57bl/6 mice was dissected, flash frozen in liquid nitrogen, and processed with a Polytron PT 1200 homogenizer. Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions (GIBCO). Alternatively, Schwann cells were harvested, and total RNA was extracted (RNeasy Mini Kit; Qiagen) and quantified.

Reverse Transcription and Real-Time PCR

Total RNA (200 ng) was reverse transcribed with Superscript II according to the manufacturer's instructions (Invitrogen). Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System cycler (Applied Biosystems). Reactions were done in triplicate. Primers were as follows. Ski (detected by SYBR green dye): forward (F), GCT TTG ATT CGA GAC AGC TTC TAC T; reverse (R), TGC TGG GTT GGT GGT GCT A. PMP22: F, GGG ATC CTG TTC CTG CAC AT; R, TGC CAG AGA TCA GTC GTG TGT. TaqMan probe: TCC ACC ATC GTC AGC CAA TGG CT. PO (detected by SYBR green dye): F, CCC TGG CCA TTG TGG TTT AC; R, CCA TTC ACT GGA CCA GAA GGA G. Periaxin (detected by SYBR green dye): F, AAG GAA TCT TTG TCC GCG AG; R, CTC AAA GAA CAC ACG GGC G. Data were collected during each PCR cycle and analyzed using the Sequence Detection software (v. 1.6.3, Applied Biosystems). Results were expressed as molar ratio of mRNA to 18S rRNA (detected with the Pre-Developed TaqMan Assay Reagent kit, Applied Biosystem). Statistical significance was determined using the two-tailed unpaired Student's t test.

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