A Dual Role of \textit{erbB2} in Myelination and in Expansion of the Schwann Cell Precursor Pool

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Abstract. Neuregulin-1 provides an important axonally derived signal for the survival and growth of developing Schwann cells, which is transmitted by the ErbB2/ErbB3 receptor tyrosine kinases. Null mutations of the neuregulin-1, erbB2, or erbB3 mouse genes cause severe deficits in early Schwann cell development. Here, we employ Cre-loxP technology to introduce erbB2 mutations late in Schwann cell development, using a Krox20-cre allele. Cre-mediated erbB2 ablation occurs perinatally in peripheral nerves, but already at E11 within spinal roots. The mutant mice exhibit a widespread peripheral neuropathy characterized by abnormally thin myelin sheaths, containing fewer myelin wraps. In addition, in spinal roots the Schwann cell precursor pool is not correctly established. Thus, the Neuregulin signaling system functions during multiple stages of Schwann cell development and is essential for correct myelination. The thickness of the myelin sheath is determined by the axon diameter, and we suggest that trophic signals provided by the nerve determine the number of times a Schwann cell wraps an axon.

Key words: cre-loxP • neuregulin • myelin • glia • neuropathy

Introduction

Neural crest cells constitute a migratory and pluripotent cell population that emerges from the dorsal neural tube. Upon reaching their target sites, neural crest cells differentiate into various cell types, including Schwann cells, which is a population of glial cells that ensheathes axons of sensory and motoneurons (Le Douarin, 1982). Schwann cell precursors migrate and proliferate along preexisting axon tracts during development, and progress through a series of defined stages that are characterized by the expression of specific genes encoding proteins such as \textit{P0}, S100, SCIP, or Krox20 (Mirsy and Jessen, 1996; Zorick and Lemke, 1996). Proliferation, survival, and differentiation of Schwann cell precursors depend critically on signals that are provided by the associated axons. During the first week of postnatal life, the majority of Schwann cells destined to myelinate can already be distinguished morphologically in mice. They establish a 1:1 relationship with the accompanying axon and cease to proliferate, but remain capable of entering the cell cycle, a property important in adult regeneration processes (Zorick and Lemke, 1996). The myelination program in Schwann cells is characterized by the expression of specific transcription factor genes like SCIP (also known as \textit{Oc}-6 or \textit{Tst}-1) and Krox20 (also known as \textit{Egr}-2; Chavrier et al., 1988; He et al., 1989; Monuki et al., 1989; Wilkinson et al., 1989; Suzuki et al., 1990). Genetic analysis in mice shows that SCIP determines the correct onset of myelination, whereas Krox20 is required for proper ensheathment of the axon and for the expression of genes encoding myelin proteins (Topilko et al., 1994; Weinstein et al., 1995; Bermingham et al., 1996; Jae gle et al., 1996; Zorick et al., 1999). Myelin proteins are major constituents of the myelin sheath, and the molecular composition of the sheath as well as its appropriate thickness are essential for the normal functioning of a nerve fiber. Mutations in humans and rodents can cause neuropathies accompanied by myelin defects, and are typically associated with a reduced thickness of myelin and a disturbed transmission of the action potential along the affected nerves (Nave, 1994; Martini and Schachner, 1997; Scherer, 1997; Suter, 1997).

Early Schwann cell precursors rely on axonal signals for maintenance, proliferation, and differentiation. One neuronal signal that controls survival and proliferation is provided by Neuregulin-1, also named GGF, for glial growth factor (Raff et al., 1978; Lemke and Brockes, 1984; Good-earl et al., 1993; Marchionni et al., 1993). A number of names used for this factor are NDF (Neu differentiation factor), Heregulin, or ARIA (acetylcholine receptor inducing activity; for reviews see: Iroy and Yarden, 1997;
Burden and Y arden, 1997). Neuregulin-1 is produced by sensory and motoneurons as a transmembrane molecule inserted into axonal membranes (H o et al., 1995; B erming-
ham-M cd onogh et al., 1997; Y ang et al., 1998). This axon-
derived signal is recognized by Schwann cells via a recep-
tor tyrosine kinase composed of a heterodimer of ErbB2 and ErbB3, which signals through tyrosine phosphoryla-
tion (L evi et al., 1995; M orrissy et al., 1995; G rinski et al.
, 1996; S yrid et al., 1996; V artanian et al., 1997). This con-
scept is supported by genetic studies in the mouse. M ice with mutations in neuregulin-1, erbB2, or erbB3 all show severe reductions in the numbers of early Schwann cell precursors; at later developmental stages, erbB3 and erbB2 mutants lack Schwann cells (M eyer and Birchmeier,
1995; E rickson et al., 1997; M eyer et al., 1997; R ieth-
macher et al., 1997; B ritsh et al., 1998; W oldeyesus et al.,
1999; M orris et al., 1999).

The time period during which Schwann cell precursors critically depend on Neuregulin-1 for proliferation and survival ends with the transition from an early precursor to a more mature, differentiating Schwann cell (D ong et al., 1995; G rinski et al., 1996; S yrid et al., 1996; M ur-
phy et al., 1996). After this transition, the differentiating Schwann cells generate survival factors in an autocrine loop, and become independent of Neuregulin-1, although they are still able to respond to the factor (R osenbaum et al., 1997; C heng et al., 1998; M eyer et al., 1999; S yrid et al., 1999). Interestingly, neuregulin-1 continues to be ex-
pressed in sensory and motoneurons even in adulthood, and both differentiating and mature Schwann cells continue to express the Neuregulin receptor genes erbB2 and erbB3, albeit erbB2 is expressed at reduced levels (C hen et al., 1994; C orfas et al., 1995; G rinski et al., 1996). We investigate here the functions of the Neuregulin signaling system in myelinating Schwann cells by the use of a Cre-recombinase-induced erbB2 mutation. We observe severe defects in myelination, which results in the forma-
tion of abnormally thin myelin sheaths. This correlates with ataxia, tremor and wasting of the animals. Moreover, a postnatal loss of motor axons occurs. Thus, the Neuregulin recep-
tor tyrosine kinase signaling sequence together with an additional EcoRV site were inserted 5’ to exon 9 (see Fig. 1 A). The erbB2loxPneoloxP targeting vector was electroporated into E14.1 embryonic stem (ES) cells; homologous recombination events were observed (see Fig. 1 A). Two colonies that contained the erbB2loxP allele derived from independent parental erbB2loxPneoloxP clones were used for a generation of mice that carry this allele as described (R iethmacher et al., 1997). Homozygous erbB2loxP animals appeared normal and were fertile. To establish the erbB2 strain, erbB2loxP homozygotes were crossed with deleter mice (Schwenk et al., 1995). Cre-mediated deletion of the floxed exons p-n removes 362 nucleotides of coding sequence and, thus, introduces a frameshift mutation. The predicted protein encoded by the erbB2 allele contains the first 386 amino acids of ErbB2 followed by 51 amino acids from an altered reading frame. Mice heterozygous for the erbB2loxP allele were bred with erbB2+/− mice; all embryos with the genotype erbB2+/− identified at E11.5 were dead as judged by the absence of heartbeat and signs of resorption. Histological analysis of E10.5 erbB2+/− embryos showed the previously described pheno-
types, i.e., lack of trabeculation and abnormal cranial sensory ganglia. All analyses of conditional erbB2 animals were carried out on the mixed C57B/L6/129 background. A control for Krox20-cre/+; erbB2loxP/+ animals, littermates with a genotype Krox20-cre+; erbB2loxP+/− were rou-
tinely used. Littermates with genotypes Krox20-cre−/+; erbB2loxP+/−, or Krox20-cre−/+; erbB2loxPloxP were analyzed by light microscopy and thin myelin was not apparent.

**Materials and Methods**

**Generation of a Targeting Vector and erbB2loxP Strain of Mice**

The isolation of genomic erbB2 DNA derived from the 129 mouse strain has been described (B ritsch et al., 1998). Oligonucleotides encoding the loxP sequence together with an additional EcoRV site were inserted 5’ of exon p. A neomyein cassette flanked by loxP sites was inserted 3’ of exon n (see Fig. 1 A). The erbB2loxPneoloxP targeting vector was electroporated into E14.1 embryonic stem (ES) cells; homologous recombination events were enriched by selection with G418, and identified by Southern blot hy-
bridization using an external genomic probe located 5’ to exon r (data not shown). A control previously (T orres and K ühn, 1997), independent ES cell clones heterozygous for the erbB2loxPneoloxP allele (see Fig. 1 A) were electroporated with pICcre; colonies were screened by Southern blot hybridization using probe 1 (see Fig. 1 A). Two colonies that contained the erbB2loxP allele derived from independent parental erbB2loxPneoloxP clones were used for a generation of mice that carry this allele as described (R iethmacher et al., 1997). Homozygous erbB2loxP animals appeared normal and were fertile. To establish the erbB2 strain, erbB2loxP homozygotes were crossed with deleter mice (Schwenk et al., 1995). Cre-mediated deletion of the floxed exons p-n removes 362 nucleotides of cod-
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tinely used. Littermates with genotypes Krox20-cre−/+; erbB2loxP+/−, or Krox20-cre−/+; erbB2loxPloxP were analyzed by light microscopy and thin myelin was not apparent.

**Determination of Recombination Specificity and Efficiency**

Tissues from 6-wk-old mice double heterozygous for a reporter-lacZ allele and Krox20-cre were stained with 5-bromo-4-chloro-3-indolyl β-galacto-
topyranoside (X-gal) as described (A kagi et al., 1997). Blue staining in-
dicative of Cre-mediated recombination was observed in peripheral nerves, hair follicles, and cartilage in which Krox20 expression has been described (Levi et al., 1996). Bundles of fibers from sciatic nerves were teased, wholemount stained with 4’,6-diamidino-2-phenylindole (DAPI), and cleared in 80% glycerol in PBS. Alternatively, the nerve sheaths were disrupted to enhance fixation and allow access of X-gal to Schwann cells. The nerves were osmicated and embedded in resin (see also below), and 1-μm sections were counterstained with neutral red and eosin. For immu-
nohistochemical analysis, teased nerves from Krox20-cre−/+; reporter-lacZ animals were fixed for 20 min at 4°C in 4% paraformaldehyde (PFA), blocked with 10% goat serum in PBS containing 0.5% Triton X-100, and incubated with monoclonal anti-mycelin basic protein antibody (Boehr-
inger Mannheim) and rabbit polyclonal anti-β-galactosidase antibody (ICN Cappel). FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (Dianova) were used as secondary antibodies and nuclei were counterstained with DAPI. Genomic DNA of tissues from adult Krox20-cre−/+; erbB2loxPloxP animals was used as Southern blot hybridization; spinal roots were pooled from several animals to allow detection of erbB2 alleles.

**Histology, Electron Microscopy, and Axon/Schwann Cell Counts**

Nerves were isolated from animals perfused with 2.5% glutaraldehyde in phosphate buffer, postfixed, and contrasted with osmium tetroxide as de-
scribed (Topilko et al., 1994). For light microscopy, nerves were embed-
ed in Technovit 7100 resin (K ulzer); 1-μm sections were stained with toluidine blue. Preparation for electron microscopy was done as described (Topilko et al., 1994). Numbers of axons in the fifth lumbar segment (L5) ventral roots were determined from composite light micrographs. The axon numbers determined in individual mutant animals were 549, 679, 721, 739, 773, 778, 800, 855, 887, 1,014 and 1,06; in control animals, 956, 968, 974, 1,026, 1,092 and 1,103 axons were counted. Neuron numbers in the dorsal root ganglia were determined as described (R iethmacher et al., 1997). Numbers of Schwann cell nuclei associated with nerves were deter-
mined from 1-μm sections.

**Determination of Apoptosis and Proliferation Rates in Nerves**

A animals between postnatal day 3.5 and 15 (P3.5 and P15, respectively) were injected once intraperitoneally with bromodeoxyuridine (BrDU; 100 μg/g body weight) and killed 80 min after injection. To increase numbers of BrDU-positive cells in nerves from 5-wk-old and adult animals, multiple BrDU injections (1 each day, over 4 d) were used. A animals were perfused

\[ \text{X-gal} \]
with 4% PFA in PBS, nerves were dissected, postfixed for 2–4 h, and embedded in OCT compound (Sakura). Frozen sections (6 μm) were processed for TUNEL staining using the ApopTag kit (Oncor); BrdU-positive nuclei were detected using a monoclonal anti-BrdU antibody (Sigma Chemical Co.). Sections were counterstained with DAPI (Sigma Chemical Co.) to determine overall numbers of Schwann cell nuclei.

**Protein Extraction and Western Blot Analysis**

Sciatic nerves were dissected, flash-frozen in liquid nitrogen, and stored at −70°C. Total protein lysates of nerves were prepared by sonication in Laemmli sample buffer (Laemmli, 1970). Samples were separated in 12.5% acrylamide gels under reducing conditions and transferred to nitrocellulose membranes. Western blotting was performed using rabbit polyclonal antibodies directed against Krox20, SCIP, or PMP22, or a mouse mAb directed against P-cad for detection, an ECL kit (Amersham) was used.

**Preparation of Digital Images**

Southern and Western blots, hybridized with 32P-labeled probes, or incubated with ECL reagents, respectively, were exposed to Kodak Bio-Max film and scanned into Adobe Photoshop 3.0. PhosphorImage scans of Southern blots were analyzed using the software package TINA 2.08e (Schwenk et al., 1995). The resulting combination was detected in the spinal cord.

**ErbB2 Is Required for Correct Myelination of Peripheral Nerves**

Conditional mutant animals with the genotype Krox20-cre+/--; erbB2flox/+/-- were viable, but displayed various behavioral abnormalities within the first weeks of postnatal life. Alterations included kinked or serpentine tails, gait abnormalities, difficulties in hindlimb movement, and wasting associated with weight loss and premature death (5 out of a group of 37 mutants died within 6 mo). Such behavioral abnormalities were observed in all conditional mutants, but were variable in the time of onset and severity. Control animals, for instance animals with the genotype Krox20-cre++; erbB2+/+/, did not display these behavioral phenotypes.

Examination of the sciatic nerves from Cre-induced mutants showed no gross changes in morphology or histology at P3.5 (Fig. 3 A, compare to B), indicating that the Schwann cell precursor pool in this nerve is established. However, sciatic nerves of mutants at P15 were translucent and thin. Histologically, a strikingly reduced thickness of myelin sheaths was apparent (Fig. 3, C and D). Reduced thickness of the myelin sheaths persisted, and was also observed at 6 or 14 mo (Fig. 3, E–J, Fig. 4, arrowheads, and data not shown). Myelin of normal thickness (Fig. 3 E, arrow) was found only in 1.7 ± 1.0% of all myelinated axons in the sciatic nerve. Thus, the Krox20-cre-induced erbB2 mutation reproducibly affected virtually all myelinating Schwann cells. Thin myelin was not observed in control mice with a genotype Krox20-cre+/--; erbB2+/+/--; or Krox20-cre+/--; erbB2flox/+/+; or Krox20+/+--; erbB2flox/+/+; or Krox20+/+--; erbB2+/+/. Electron micrographs were used to quantify myelin thickness (Fig. 4). In 6-mo-old animals, the thickness of myelin was reduced two- to threefold. This is reflected in G ratios (ratios of axon diameters to fiber diameters) of 0.83 ± 0.04 in mutant, and 0.68 ± 0.04 in control sciatic nerves (P < 10−6). Hypomyelination in sciatic, sural, saphenous nerves and nerves innervating lower leg muscles were observed in all mutant animals examined (28 animals). In the spinal roots, the phenotype was more dramatic (see below). The thin myelin was due to the presence of fewer wraps of myelin around the axon.

In addition, we observed occasionally large-caliber axons lacking myelin that were still surrounded by a Schwann cell basal lamina in the sciatic nerve (Fig. 4 C,
Such Schwann cells frequently ensheathed several axons (Fig. 3, G and I, arrows). These profiles were more frequent in nerves innervating muscle than in cutaneous sensory nerves. Extensive patches of amelinated axons were observed in some Cre-induced mutants (Fig. 3 I). The histology of nonmyelinating bundles appeared normal (Fig. 4, asterisks).

In early postnatal stages or in the embryo, a more severe phenotype was observed in dorsal and ventral spinal roots. These nerves display a severe lack of Schwann cells in all animals with Krox20-cre–induced erbB2 mutations at P3.5 and at P15 (10 examined; Table I, Fig. 5, and data not shown). In accordance, myelin was absent in ventral roots, whereas thin myelin sheaths were surrounding a small proportion of axons in dorsal roots. This coincides with the early onset of Krox20-cre expression in these cells, revealing the early requirement for ErbB2 in establishment of the Schwann cell precursor pool. As animals reached adulthood, Schwann cell numbers increased, but the myelin sheaths formed were thin (Fig. 5). Localized patches of amelinated axons persisted. Moreover, a single perineurium surrounds the entire spinal root in control animals, whereas perineurial sheaths compartmentalized the roots into six to seven branches in mutants (Fig. 5, arrowheads).
We determined the absolute numbers of Schwann cells in sciatic nerves and spinal roots. In the sciatic nerves of animals at all stages tested (P3.5, P15, and 6 mo), Schwann cell numbers were comparable in the control and Cre-induced mutant mice (Table I). In contrast, in dorsal and
ventral roots, the numbers of cells were severely reduced. At P3.5, a 10-fold reduction was observed in the ventral, and a 5-fold reduction in the dorsal roots of the mutant mice. This reduction was still apparent at P15, and again more pronounced in ventral than in dorsal roots (Table I). At 6 mo, numbers were similar in the dorsal and ventral roots of control and mutant mice. Thus, compensatory mechanisms exist that allow repopulation of spinal roots.
Schwann cell proliferation and apoptosis determine the absolute numbers of Schwann cells, and were analyzed using BrdU incorporation and TUNEL labeling, respectively. Compared with the controls, the numbers of BrdU-positive cells were increased in the sciatic nerves of mutants at P3.5 and 5 wk (Fig. 6 A). The numbers of apoptotic nuclei were marginally increased at P3.5 (Fig. 6 B). In adults (age 6 mo), proliferation as well as apoptosis frequencies were below detection limits. Increased cell proliferation was also observed in ventral roots of 5-wk-old animals.

To assess the effect of the conditional erbB2 mutation induced by Krox20-cre on Krox20 and SCIP protein levels, sciatic nerve extracts were analyzed by Western blotting (Fig. 6 C). The level of Krox20 protein was similar in the nerves of control and Krox20-cre-induced erbB2 mutants. The level of SCIP protein was slightly increased in mutant nerves at P15, but levels attained baseline in control and mutant nerves at later stages. Levels of myelin protein were reduced in conditional mutants, in accordance with the histology.

### Table I. Effect of Krox20-cre–induced Mutation of erbB2 on Schwann Cell Number

<table>
<thead>
<tr>
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<th>Sciatic</th>
<th>Ventral root</th>
<th>Dorsal root</th>
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<tr>
<td></td>
<td>Mutant</td>
<td>Control</td>
<td>Mutant</td>
</tr>
<tr>
<td>P3.5</td>
<td>564 ± 31</td>
<td>588 ± 27</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>P15</td>
<td>395 ± 12</td>
<td>409 ± 21</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>P 6 mo</td>
<td>209 ± 33</td>
<td>204 ± 20</td>
<td>40 ± 13</td>
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Schwann cell nuclei were counted from semi-thin sections of nerves at the indicated ages; values are displayed as numbers of nuclei per section. The number of nuclei within spinal roots at P3.5 was determined from sections of lumbar blocks (L4-L5 level); at later stages, L5 roots were examined. Data are shown as mean ± SD.
Neuropathology in Krox20-cre–induced erbB2 Mutants

To assess whether neuron numbers were altered in Krox20-cre–induced erbB2 mutant mice, we counted axons in the L5 ventral root, which contains mainly motor axons. The numbers of such axons were frequently reduced in mutant animals, amounting to 20% loss on average. From 11 mutant animals, and 6 control animals examined, the number of axons in the mutant was $808 \pm 152$, compared with $1,020 \pm 65$ in control nerves ($P < 0.002$). The numbers of neurons in L5 dorsal root ganglia were similar in the control and mutant animals (not shown).

Discussion

The role of Neuregulin-1 as a growth and survival factor during Schwann cell development is well established (Zorick and Lemke, 1996; Jessen and Mirsky, 1999). Neuregulin-1 provides an axonally derived signal important in establishing the Schwann cell precursor pool, which is received in precursor cells by the Neuregulin-1 receptors ErbB2 and ErbB3 (for review see Adlkofer and Lai, 2000). We show here that in addition to its function in the establishment of the precursor pool, the Neuregulin signaling system also plays a role in myelination: abnormally thin myelin sheaths are formed in mice with conditional mutations of erbB2 in myelinating Schwann cells. To introduce the conditional erbB2 mutation, we used a Krox20-cre allele, but it should be noted that thin myelin is also observed when the mutation is introduced by a P0-cre transgene (Garratt, A.N., unpublished observations). The conditional erbB2 mutations also result in an altered behavior of the affected animals, which display movement abnormalities and loss of motoneurons.

The Role of ErbB2 in Myelination

Mutations that cause an abnormal myelin thickness and neuropathy have been extensively characterized (for reviews see Nave, 1994; Martini and Schachner, 1997; Scherer, 1997; Suter, 1997; Warner et al., 1999). Peripheral hypo- or hypermyelination are observed in patients with Charcot Marie Tooth disease, and can be caused by mutations in genes encoding the myelin proteins PM P22 and P0, the gap junction protein connexin-32 or the transcription factor Krox20. Even incorrect PM P22 dosage can cause changes in myelination and neuropathy. Mice with targeted or spontaneous mutations in these genes display similar phenotypes. Thus, the thickness of the myelin sheath is strictly controlled, and molecular mechanisms that cause pathological changes are manifold. We show here that hypomyelination and peripheral neuropathy can arise by a novel molecular mechanism, the ablation of the ErbB2 receptor tyrosine kinase. Mutations in signaling molecules have not been observed in patients with hereditary neuropathy. However, afflicted individuals frequently do not carry mutations in known disease causing genes. ErbB receptor tyrosine kinases modulate the activity of various signaling pathways and are known to affect expression and activity of transcription factors (for reviews see Dougall et al., 1994; Schweitzer and Shilo, 1997; A Iroy and Y arden, 1997; Fromm and B urden, 1998; M oghal and Sternberg, 1999). We tested whether the conditional erbB2 mutation affects the levels of transcription factors known to regulate myelination. Schwann cells arrest wrapping of axons in the absence of Krox20 (Topilko et al., 1994; Zorick et al., 1999). In vitro, Neuregulin-1 does not affect Krox20 expression directly, but influences the transition from a precursor to a committed Schwann cell that expresses Krox20 (Murphy et al., 1996). In accordance,
levels of Krox20 protein are not altered in sciatic nerves of mice with Krox20-cre-induced loss of erbB2. It should be noted that the conditional erbB2 ablation occurs after the transition from a precursor to a committed Schwann cell.

In SCIP mutant mice, myelination is delayed. SCIP is expressed in precursors and in Schwann cells during the myelination process, but is downregulated when myelination is completed (Monuki et al., 1989; Jaegle et al., 1996; Zorick et al., 1999). Downregulation of SCIP is delayed in the sciatic nerves of conditional erbB2 mutant mice. This correlates with the prolonged growth phase of Schwann cells, and might not reflect a direct effect of ErbB2 on SCIP expression.

The number of myelin wraps a Schwann cell forms is determined by the diameter of the accompanying axon. Thus, axons not only provide signals that control growth or differentiation, but also instructive signals that determine the size of the membrane surface of Schwann cells and, thus, the number of myelin wraps. In general, the number of myelin wraps might be controlled by trophic signals provided by the axon. Neuregulin provides such a signal, as wrap numbers are reduced in conditional erbB2 mutants. Large diameter axons, because of the enlarged surface area, can present greater amounts of trophic factors and might, therefore, instruct the Schwann cell to form more wraps. Other trophic signals, in addition to the one provided by the Neuregulin signaling system, might be operative, and the sum of these signals would determine myelin thickness and the number of wraps. It is interesting to note that the Drosophila gene DPTEN that encodes a protein and PIP3 lipid phosphatase controls both cell and body size (Goberdhan et al., 1999).

**Neuregulin Signaling System in Myelinating Schwann Cells**

The balance of cell death and growth in peripheral nerves during the early postnatal phase reflects an adjustment of the numbers of Schwann cell precursors (Grinspan et al., 1996; Syroid et al., 1996; Zorick et al., 1999). The correct 1:1 relationship between axons and myelinating Schwann cells is thus attained, supernumerary cells are removed and areas deficient in Schwann cells are replenished. In early postnatal nerves, Schwann cell survival is regulated in part by access to axonally derived Neuregulin-1, and axotomy-induced death of pre- and perisynaptic Schwann cells can be rescued by exogenous Neuregulin-1 (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). In our conditional mutants, the importance of ErbB2/Neuregulin-1 signaling in Schwann cell survival and growth is particularly apparent in spinal roots. There, Krox20 is expressed earlier (~E11), and the Krox20-cre driven mutations are induced earlier than in other peripheral nerves (Topilko et al., 1994; Voiculescu et al., 2000). In the spinal roots, Schwann cells are almost completely absent late in development and into the perinatal period. However, this deficiency is compensated at later stages.

The apoptotic response of Schwann cells to denervation is pronounced in early postnatal life but declines with increasing age (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). In culture, Schwann cells from the postnatal nerve survive without Neuregulin-1, but secrete survival-promoting growth factors like PDGF, neurotrophin-3, and insulin-like growth factor in an autocrine manner (Porter et al., 1987; Mier et al., 1999; Syroid et al., 1999). An autocrine expression of particular isoforms of neuregulin-1 also has been reported (Osenbaum et al., 1997; Carroll et al., 1997). In adult mice with Krox20-cre-induced erbB2 mutations, mature myelinating Schwann cells survive, and we do not detect apoptotic nuclei in the nerves. Thus, neither paracrine nor autocrine
activation of ErbB2 is essential for survival of mature myelinated Schwann cells.

Regeneration of Schwann Cells

The ability of Schwann cells to regenerate is enormous. In experimentally induced allergic neuritis, myelinating Schwann cells are destroyed, but can be effectively regenerated (Saida et al., 1980). This regenerative capacity is particularly evident in mice that express diphertheria toxin A-chain in myelinating Schwann cells. In such animals, myelinating Schwann cells die and are continuously regenerated through proliferation of the nonmyelinating compartment (Messing et al., 1992). In conditional erbB2 mutant mice, we can still detect single Schwann cells that wrap several large-caliber axons in the adult. Such lesions are not repaired.

We observe a pronounced reduction of Schwann cell numbers in spinal roots of mice with Krox20-cre-induced erbB2 mutations. This severe lack of Schwann cells is compensated during postnatal life, although local patches devoid of Schwann cells remain unrepaired. Interestingly, Krox20 is not expressed in satellite cells of dorsal root ganglia (Murphy et al., 1996); nevertheless, proliferation is increased in dorsal root ganglia of conditional erbB2 mutants (Garratt, A.N., unpublished observations). Thus, satellite cells might replenish Schwann cells in the mutants, particularly in the projections of dorsal root ganglia neurons (dorsal roots) where regeneration is faster than in ventral roots.

Neuropathology in Conditional erbB2 Mutant Mice

The conditional mutation of erbB2 in myelinating Schwann cells results in a moderate loss of motoneurons. Since erbB2 is not mutated in cells of the spinal cord, this is caused by indirect mechanisms. Axonal retractions were previously noted in mice with defective myelination (Giese et al., 1992; Adlkofer et al., 1995; Griffiths et al., 1998; Frei et al., 1999). In mice with Krox20-cre-induced mutations, motoneurons are lost, but sensory neuron numbers are not altered. We observe thin myelin in all those animals and in all nerves. A lack of myelin surrounding large-caliber axons is, however, mainly found in nerves innervating muscle, and is variable in extent in different animals, as is the extent of motoneuron loss. Thus, not thin myelin, but a local lack of myelin in parts of the nerve might cause this damage.

Multiple Functions of Neuregulin in the Schwann Cell Lineage

Neuregulin-1 and its receptors serve several functions in the Schwann cell lineage. The first stage in which this signaling system is required occurs early during development of the lineage. In neuregulin-1, erbB2, or erbB3 mutant mice, the population of early Schwann cell precursors is already diminished as they start to populate the spinal nerves (Meyer and Birchmeier, 1995; Erickson et al., 1997; Riethmacher et al., 1997; Britsch et al., 1998; Morris et al., 1999; Woldeyesus et al., 1999). Effective migration of the precursors along the axon might be responsible. It is interesting to note that neural crest cells that form sympathetic ganglia require neuregulin-1, erbB2, and erbB3 for migration (Britsch et al., 1998). However, increased apoptosis and/or a lack of proliferation contribute to the severe Schwann cell phenotype apparent at late stages in null mutants, since precursors that form do not expand. The severe reduction of Schwann cells in spinal roots of conditional erbB2 mutants appears to be caused by cell death, since Krox20 expression and, thus, the Krox20-cre-induced mutation occurs after precursors reach the roots.

Importantly, we demonstrate here that erbB2 is also required for correct myelination in Schwann cells. The thin myelin observed in conditional erbB2 mutant mice is stable, and not repaired. Similarly, local deficits in Schwann cell numbers in peripheral motor nerves are not repaired. Thus, this and other studies provide evidence for a role of erbB2 during repair of dysmyelinated lesions and regeneration of damaged Schwann cells (Carroll et al., 1997; Kwon et al., 1997; Li et al., 1997; Chen et al., 1998). Therefore, Neuregulin/ErbB signaling functions during the entire life span of a myelinating Schwann cell.

We want to thank Ralf Grossfeld and Dieto Riethmacher for help in the initial phase of this project, Gary Lewin for advice, Sven Buchert and Andrea Rehaus for expert technical assistance, as well as Klaus-Armin Nave, Rhonda Miraky and Kristjan Jessen for helpful discussions. We are also indebted to Sandra Gobels and Klaus-Armin Nave (ZMBH, Heidelberg, Germany) for providing us with a P<sub>P</sub>-cre transgenic strain. The following provided antibodies: Greg Lemke (anti-SCIP; Salk Institute, San Diego, CA), Pascale Gilardi (anti-Krox20; Ecole Normale Superieure, Paris, France), Ueli Suter (anti-PM22; Swiss Federal Institute of Technology, Zurich, Switzerland), Juan A. Archelegos (anti-Po; Graz University Clinic, Graz, Austria), and Michael Wegner (anti-SCIP antibodies and also COS cell transfecteds of Krox20 and SCIP expression plasmids; ZMNH, Hamburg, Germany). We also thank Volker Brinkmann for preliminary analysis of mutant nerves by electron microscopy, and Walter Birchmeier for critical reading of the manuscript.

This work was funded by a grant from the European Commission T.M.R. Programme (to A.N. Garratt), and by The Volkswagen Stiftung (to C. Birchmeier).

Submitted: 29 November 1999
Revised: 25 January 2000
Acepted: 26 January 2000

References