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In vivo knockdown of ErbB3 in mice inhibits Schwann cell precursor migration

Tomohiro Torii [a,1], Yuki Miyamoto [a,1], Shuji Takada [b,d], Hideki Tsumura [c], Miyuki Arai [c], Kazuaki Nakamura [a], Katsuya Ohbuchi [e], Masahiro Yamamoto [e,f], Akito Tanoue [a], Junji Yamauchi [a,d,*]

[a] Department of Pharmacology, National Research Institute for Child Health and Development, Setagaya, Tokyo 157-8535, Japan
[b] Department of Systems BioMedicine, National Research Institute for Child Health and Development, Setagaya, Tokyo 157-8535, Japan
[c] Laboratory Animal Facility, National Research Institute for Child Health and Development, Setagaya, Tokyo 157-8535, Japan
[d] Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo, Tokyo 113-8510, Japan
[e] Tsumura Research Laboratories, Tsumura & Co., Inashiki, Ibaraki 200-1192, Japan
[f] School of Medicine, Keio University, Shinjuku, Tokyo 160-8583, Japan

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ABSTRACT

The myelin sheath insulates neuronal axons and markedly increases the nerve conduction velocity. In the peripheral nervous system (PNS), Schwann cell precursors migrate along embryonic neuronal axons to their final destinations, where they eventually wrap around individual axons to form the myelin sheath after birth. ErbB2 and ErbB3 tyrosine kinase receptors form a heterodimer and are extensively expressed in Schwann lineage cells. ErbB2/3 is thought to be one of the primary regulators controlling the entire Schwann cell development. ErbB3 is the *bona fide* Schwann cell receptor for the neuronal ligand neuregulin-1. Although ErbB2/3 is well known to regulate both Schwann cell precursor migration and myelination by Schwann cells in fishes, it still remains unclear whether in mammals, ErbB2/3 actually regulates Schwann cell precursor migration. Here, we show that knockdown of ErbB3 using a Schwann cell-specific promoter in mice causes delayed migration of Schwann cell precursors. In contrast, littermate control mice display normal migration. Similar results are seen in an *in vitro* migration assay using reaggregated Schwann cell precursors. Also, ErbB3 knockdown in mice reduces myelin thickness in sciatic nerves, consistent with the established role of ErbB3 in myelination. Thus, ErbB3 plays a key role in migration, as well as in myelination, in mouse Schwann lineage cells, presenting a genetically conservative role of ErbB3 in Schwann cell precursor migration.

1. Introduction

In the embryonic stages of peripheral nervous system (PNS) development, Schwann cell precursors migrate along neuronal axons to their final destinations. After birth, Schwann cells wrap around individual axons, forming myelin sheaths. The myelin sheath is a morphologically differentiated Schwann cell plasma membrane that insulates axons and markedly increases the nerve conduction velocity. It also plays a role in protecting axons from various external factors such as physical stresses [1,2].

Many growth factors and adhesion molecules, which are derived from peripheral neurons, bind to cognate receptors that are expressed in Schwann cells, and thereby regulate certain processes involved in the myelination of axons [3–6]. Among these ligand–receptor interactions, the relationship between axonal neuregulin-1 (NRG1) type III variant and Schwann cell ErbB2/3 heterodimeric receptor is thought to be one of the primary units controlling Schwann cell development. In the PNS, NRG1 is specifically expressed in neurons and ErbB2/3 is specifically expressed in Schwann cells. NRG1 binds to ErbB3, whose structural change stimulates ErbB2 tyrosine kinase, to activate an array of intracellular signaling cascades. ErbB3 has a very low tyrosine kinase activity and ErbB2 has little ligand-binding activity [3,4]. It is well established that in zebrafish, Schwann cell ErbB2/3 receptor is critical not only for achieving myelination of posterior lateral line axons but also for regulating Schwann cell precursor migration [5]. In a series of mouse genetic studies, the ErbB2/3 receptor has also been

* Corresponding author at: Department of Pharmacology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan. Fax: +81 3 5494 7057.
E-mail address: yamauchi-j@ncchd.go.jp (J. Yamauchi).
[1] These authors equally contributed to this work.

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shown to play an essential role in PNS myelination [2–4]; however, the important question of whether the mammalian ortholog is actually involved in the migration of Schwann cell precursors still remains to be answered.

In this study, we have produced transgenic mice transcribing ErbB3 short-hairpin RNA (shRNA)-inserted artificial microRNA (miRNA) [7,8] under the control of a Schwann cell-specific myelin protein zero (MPZ, also called P0) promoter and found that knockdown of ErbB3 *in vivo* results in delayed migration of Schwann cell precursors. Also, shErbB3 transgenic mice exhibit reduced myelin thickness, as seen in studies using knockout mice. Therefore, signaling through the *erbb3* gene product is required for Schwann cell precursor migration, indicating that the role of the ErbB3 receptor in fish Schwann cell precursor migration is conserved in mammals.

2. Materials and methods

2.1. Antibodies

The following antibodies were purchased: polyclonal anti-ErbB2 and anti-ErbB3, and monoclonal anti-Ki67 antigen from Cell Signaling Technology (Danvers, MA, USA); polyclonal anti-Sox10 (Schwann cell lineage cell's marker) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal anti-p75 neurotrophin receptor (Schwann cell marker) from Promega (Madison, WI, USA); monoclonal anti-myelin basic protein (MBP) from Covance (Princeton, NJ, USA); monoclonal anti-actin from MBL (Nagoya, Japan); and peroxidase-conjugated secondary antibodies from Nacalai Tesque (Kyoto, Japan) or Wako (Osaka, Japan). Fluorescence-labeled secondary antibodies were obtained from Nacalai Tesque or Life Technologies (Carlsbad, CA, USA).

2.2. Cultures and migration assays of Schwann cell precursors

Primary Schwann cell precursors were prepared from dorsal root ganglia (DRGs) of male or female C57BL/6J mice on embryonic day 12.5 and cultured at 37 °C as previously described [9,11]. For migration assay, cell reaggregates were allowed to migrate in Sato medium containing 10 ng/ml NRG1 (R&D Systems, Minneapolis, MN, USA) on type I collagen-coated dishes or wells for 6 h [12]. The maximum distance from the center of the reaggregate was considered the migrating distance. To confirm cell viability under these experimental conditions, cells were stained with 0.4% trypan blue. Stained cells routinely accounted for less than 5% of all cells.

2.3. Schwann cell-neuronal cultures

Dissociated explants were established from male or female mice on embryonic day 12.5 [9,10]. In brief, DRGs were collected and dissociated using 0.25% trypsin and trituration. The cells were dispersed and plated onto collagen-coated coverslips (3×10^5 cells/22 mm-coverslip). The dissociated explants were maintained in MEM containing 10% FBS and 100 ng/ml NGF. Axonal processes and endogenous Schwann cells were allowed to grow and establish themselves for 5 days. Myelination was induced using 50 μg/ml ascorbic acid. The culture medium was changed every 2 or 3 days and cultures were maintained for an additional 2 weeks.

2.4. Generation of Schwann cell-specific shErbB3 transgenic mice

The mouse MPZ promoter (GenBank Acc. No. M62857) was amplified using C57BL/6J mouse genomic DNAs. Mouse ErbB3 shRNAs, designed using an RNAi Design program (http://rnaidesigner.lifetechnologies.com/rnaiexpress/), were inserted into the BLOCK-iT PolII miR RNAi expression vector (Cat. No. K4936-00; Life Technologies), followed by amplification with the 704-2010 bases. The ErbB3 nucleotide target sequences used were 5′-TACCC ATGACCACCTCACACT-3′ (ErbB3's 164-184 bases) and 5′-ATATCT GGCAGTCTTCTGGTC-3′ (ErbB3's 593-613 bases). The nucleotides encoding the promoter, shRNA-inserted artificial miRNA, and polyA signal units were successively inserted into the pCMV5 backbone as the subcloning vector. A DNA fragment (~2.7-kb) containing all nucleotide units was digested from the vector backbone with *Eco*RI and *Pst*I, purified, and injected into fertilized C57BL/6J oocytes [9,10]. Transgenic founder mice and established transgenic mice were identified by tail DNA's genomic PCRs with specific primers (5′-ATGGTGAGCAAGGGCGAGGAGCTG-3′ and 5′-CTTGTAC AGCTCGTCCATGCCGAGAGTGATC-3′ for the artificial miRNA sequence; 5′-GCTAACTGAAACACGGAAGGAGACAATACCGGAAG-3′ and 5′-CAGCTGCGCAGATCCATCAGAGATTTTGAGAC-3′ for the polyA sequence; and 5′-GTTGAAAATTGTGGATACTTTGACAC-3′ and 5′-TGAAGGGACATCTAACTACAATCAA-3′ for the *arf1* gene as the internal control), as well as by Southern blotting with *Hin*dIII-digested tail DNA hybridized to a radioisotope-labeled genomic probe for the artificial miRNA. The transgenic allele resulted in a hybridized band of ~1.4 kb. The scanned bands were densitometrically analyzed to identify their semi-quantification using an UN-SCAN-IT gel software (Silk Scientific, Orem, UT, USA). PCR was performed in 30 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 55–65 °C (depending on each primer's Tm value) for 1 min, and extension at 72 °C for 1 min. Transgenic founders were mated to wild type C57BL/6J mice and the transgene was stably maintained for at least 4 generations. The transgenic mice were fertile under standard breeding conditions. Male mice were used for experiments if it was possible to distinguish their sex.

2.5. Immunoblotting

Cells were lysed in lysis buffer A (50 mM HEPES-NaOH, pH 7.5, 20 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EDTA, 1 mM Na_3VO_4, and 10 mM NaF) containing biochemical detergents (0.5% NP-40, 1% CHAPS, and 0.3% SDS). Unless otherwise indicated, all lysis steps were performed at 4 °C [9,10]. The proteins in the cell supernatants were denatured, subjected to SDS–PAGE, and blotted to a polyvinylidene difluoride membrane using the TransBlot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The membranes were blocked with a Blocking One reagent (Nacalai Tesque), and immunoblotted using a primary antibody followed by a peroxidase-conjugated secondary antibody. The bound antibodies were detected using the chemiluminescence method (Nacalai Tesque or Wako) and scanned bands were analyzed densitometrically.

2.6. Immunofluorescence

Cells on dishes or wells were fixed in PBS containing 4% paraformaldehyde and permeabilized with PBS containing 0.1% Tween-20. Permeabilized cells were blocked with Blocking One, and incubated first with primary antibodies and then with fluorescence-labeled secondary antibodies. They were mounted for microscopic observation using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). The fluorescent images were captured using a DMI4000 or DMI4000B microscope system (Leica, Wetzlar, Germany) and analyzed using AF6000 software (Leica).

2.7. Immunohistochemistry

Tissues were perfused first with PBS and then with PBS containing 4% paraformaldehyde. They were postfixed with 4%

paraformaldehyde, replaced with 20% sucrose, and embedded in Tissue-Tek reagent (Sakura Finetechnical, Tokyo, Japan). Microtome sections were blocked with Blocking One, and incubated first with primary antibodies and then with fluorescence-labeled secondary antibodies. The glass coverslips were mounted with Vectashield. The fluorescent images were captured using a DM2500 microscope system (Leica) and analyzed using LAS software (Leica) or captured using a BX51 microscope system (Olympus, Tokyo, Japan) and analyzed using DP2-BSW software (Olympus).

2.8. Electron microscopy

Tissues were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1% cacodylate buffer, contrasted with 2% osmium tetroxide, dehydrated with an ethanol gradient, and treated with propylene oxide. Finally, samples were infiltrated and embedded in pure epoxy. Ultrathin sections were stained with uranyl acetate and lead staining solution. Images were taken with a JEM-1200EX electron microscope system (JEOL, Tokyo, Japan). The *g*-ratio is the value of axon diameter to outer myelinated axon diameter.

2.9. Ethics statement

Genetically modified/unmodified mice were produced and maintained in accordance with a protocol approved by the Japanese National Research Institute for Child Health and Development Animal Care Committee.

2.10. Statistical analysis

Values shown represent the mean ± SD from separate experiments. One-way analysis of variance (ANOVA) was performed followed by a Fisher's protected least significant difference (PLSD) test as a post hoc comparison. A p value less than 0.01 was considered significant.

3. Results

The aim of this study was to determine whether the ErbB3 receptor regulates Schwann cell precursor migration in mammals. Therefore, we injected a linearized transgene containing the shRNA sequence for ligand-binding ErbB3 receptor under the control of a MPZ promoter into fertilized mouse eggs, according to the standard method of transgenic mouse production (Fig. 1A). The shErbB3 nucleotide sequence is inserted within an miRNA backbone and is transcribed as artificial miRNA in an MPZ promoter-dependent manner. This knockdown construct is often called shRNAmir [7,8]. MPZ promoter is active in Schwann cell lineage cells [13,14]. We succeeded in generating three founder mice, each harboring multiple transgenes, which were mated to wild type mice. In only one transgenic line, the transgene was stably maintained for several

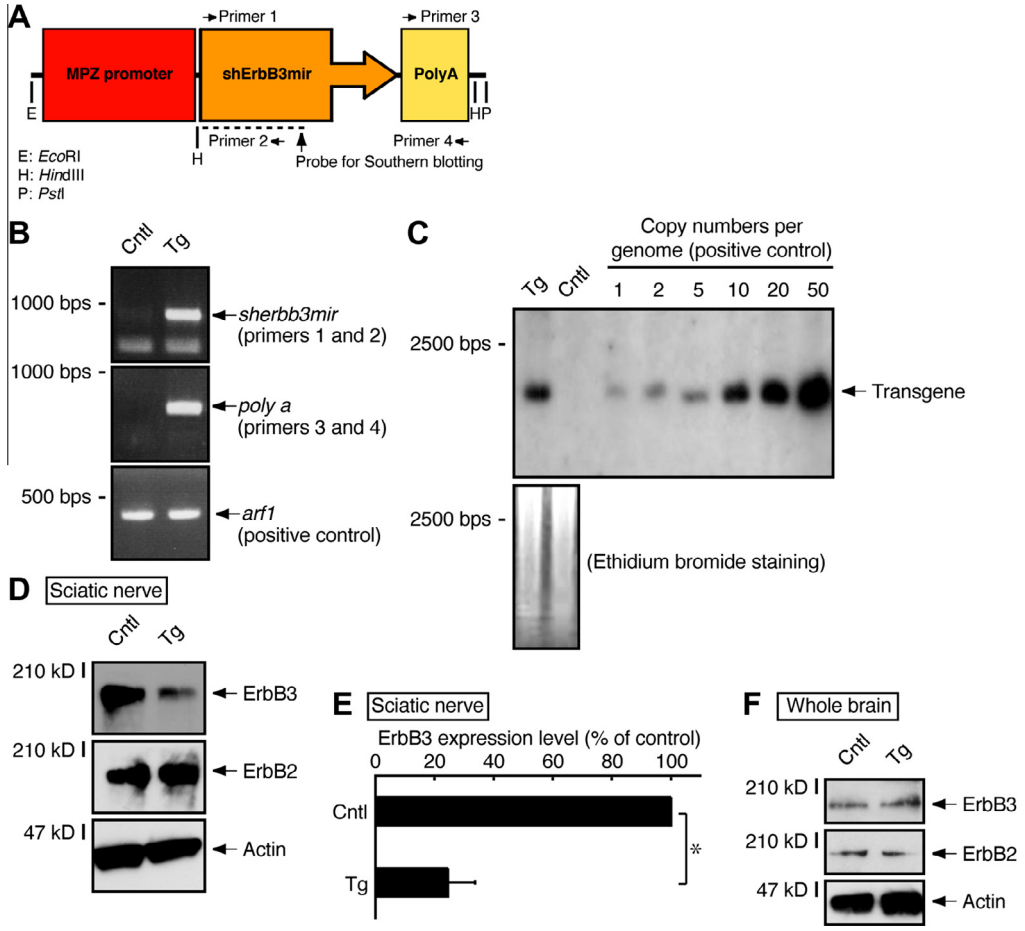

Fig. 1. Knockdown effect of ErbB3 in shErbB3 transgenic mice. (A) Schematic diagram of the shErbB3mir transgene. The positions of genomic PCR primers (arrows), Southern blotting probe (dashed lines), and restriction enzymes (single large characters) are shown. (B) Representative genomic PCR photographs for control mice (Cntl) and transgenic mice (Tg) are shown. The *arf1* gene is the positive genomic PCR control. (C) Genomic DNAs were digested with *Hin*dIII and Southern blotting was performed using radioisotope-labeled shErbB3mir fragments as the probe. The number of transgene copies (~10 copies) per genome was measured in comparison with that in positive control fragments. Ethidium bromide staining is also shown. (D and E) Immunoblottings for ErbB receptors and control actin were performed using sciatic nerve tissue lysates. ErbB3 bands were scanned and their relative intensities are statistically shown ($^*p < 0.01$; $n = 3$). (F) Immunoblots for ErbB receptors and actin using whole brain lysates are shown.

generations. The transgene was routinely identified using genomic PCR with tail DNAs (Fig. 1B). In Southern blotting, the number of transgenes was roughly 10 copies per genome (Fig. 1C). In the sciatic nerve lysates, expression levels of ErbB3, but not ErbB2, were approximately 70% lower in transgenic mice compared to their littermate controls (Fig. 1D and E). In contrast, no effect of ErbB3 knockdown was seen in the whole brain (Fig. 1F), indicating that the knockdown occurs in the peripheral nervous system only.

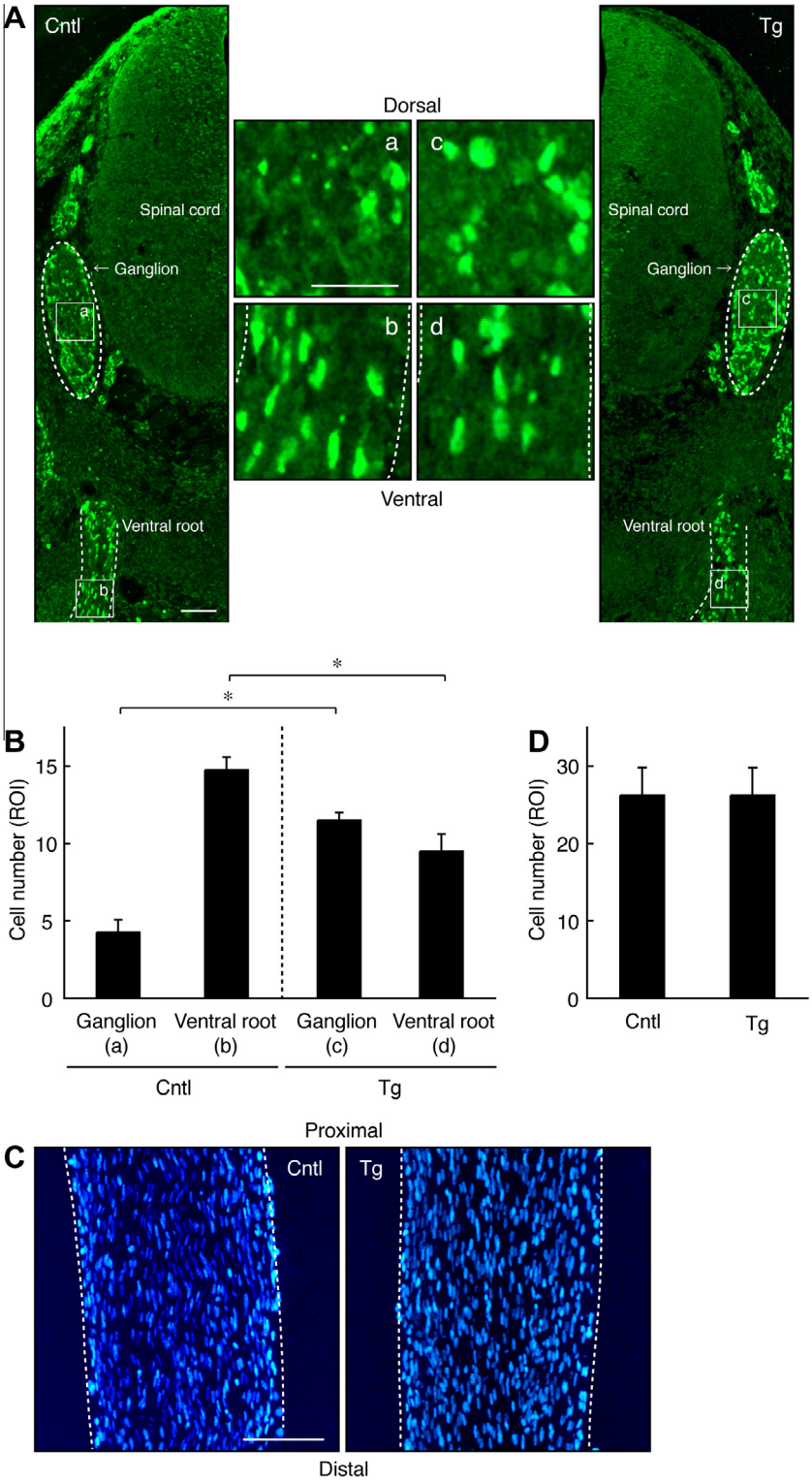

Fig. 2. ShErbB3 transgenic mice exhibit delayed migration from ganglia to ventral roots. (A) Dorsoventral sections of control mice (Cntl) and transgenic mice (Tg) at embryonic day 12.5 were stained with an anti-Sox10 antibody. Central panels a–d are enlarged images of the positions indicated by white squares a–d in the left and right panels. Peripheral nerve outlines are indicated by white dotted lines. (B and D) The number of Sox10- or DAPI-positive cells in each 200 μm-square field was counted and is shown in the graph ($^*p < 0.01$; $n = 4$). (C) Vertical sections of sciatic nerves at postnatal day 7 were stained with DAPI (blue). Scale bars indicate 100 μm.

To investigate whether ErbB3 regulates Schwann cell precursor migration in mouse embryos, we produced a tissue slice that was cut along a dorsoventral axis. A dorsoventral root from a ganglion is one of the major migration roots in Schwann cell precursors [1,2]. At embryonic day 12.5, Schwann cell lineage cell's marker Sox10-positive cells in ErbB3 shRNA transgenic mice exhibited delayed migration along a route from the ganglion to the ventral root (Fig. 2A). The number of Sox10-positive cells in ErbB3 shRNA transgenic mouse ganglia was greater than that in the controls (panel c compared to panel a in Fig. 2A; 3rd lane compared to 1st lane in Fig. 2B). In contrast, the opposite phenomenon was observed in the ventral roots (panel d compared to panel b in Fig. 2A; 4th lane compared to 2nd lane in Fig. 2B). After birth, the number of DAPI-positive cells in the sciatic nerves was comparable in transgenic mice and the controls (Fig. 2C and D). Both Schwann cells and neuronal axons are contained in the sciatic nerves where DAPI-positive nucleus are mainly present in Schwann cells. Thus, ErbB3 is required for Schwann cell precursor migration *in vivo*; however, it is unlikely that ErbB3 knockdown affects cell proliferation in transgenic mice (Fig. S1). Next, we isolated Schwann cell precursors from mouse embryos and asked whether Erb3 is involved in their migration *in vitro*. Schwann cell precursors were reaggregated and allowed to migrate out onto dishes. The reaggregates from ErbB3 shRNA transgenic mice exhibited decreased migration, compared to the controls (Fig. 3A and B), consistent with our finding that ErbB3 is required for Schwann cell precursor migration *in vivo*.

To confirm that ErbB3 contributes to Schwann cell myelination in this transgenic mouse, we performed ultrastructural analysis of sciatic nerves. Myelination in the PNS begins within couples of days after birth and remains active until around 2 months of age [1,2]. Electron microscopic analysis illustrated that sciatic nerves from 3.5-day-old shErbB3 transgenic mice exhibited decreased myelin thickness compared to those from the controls (Fig. 4A). The decreased myelin thickness in transgenic mice is clearly evident from quantification of the *g*-ratio, that is the numerical ratio between the axon diameter and the outer myelinated fiber diameter (Fig. 4B). Thinner myelin layers yield larger *g*-ratio values.

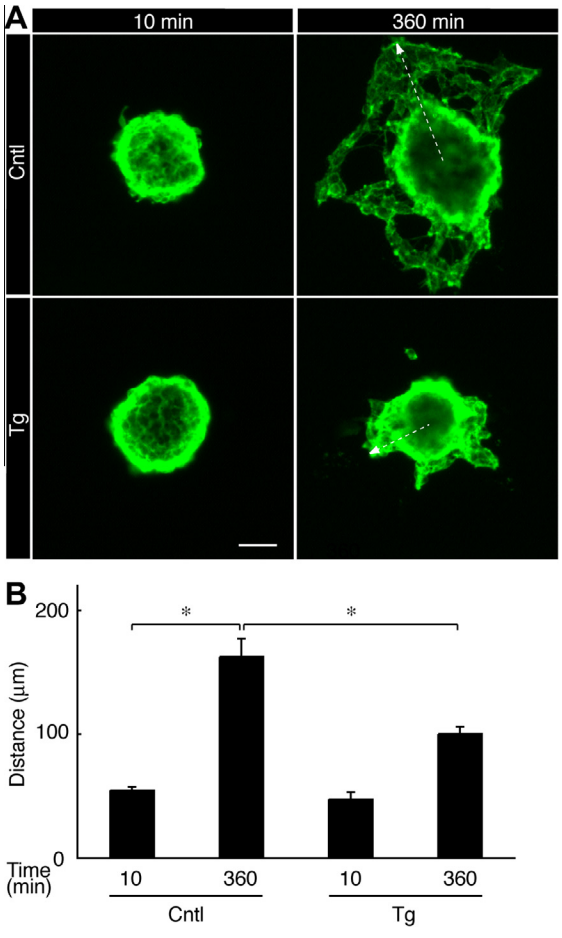

Fig. 3. Knockdown of ErbB3 inhibits migration from Schwann cell precursor reaggregates. (A and B) Schwann cell precursors from control mice (Cntl) and transgenic mice (Tg) were reaggregated and allowed to migrate from reaggregates for 6 h. Cells were stained with an anti-p75 neurotrophin receptor antibody (green). The distance from the center of the reaggregate was considered the migrating distance and was measured. The representative migrating direction and distance are shown in a white dotted arrow. The distance is shown in the graph (*$p < 0.01$; $n = 3$). Scale bar indicates 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

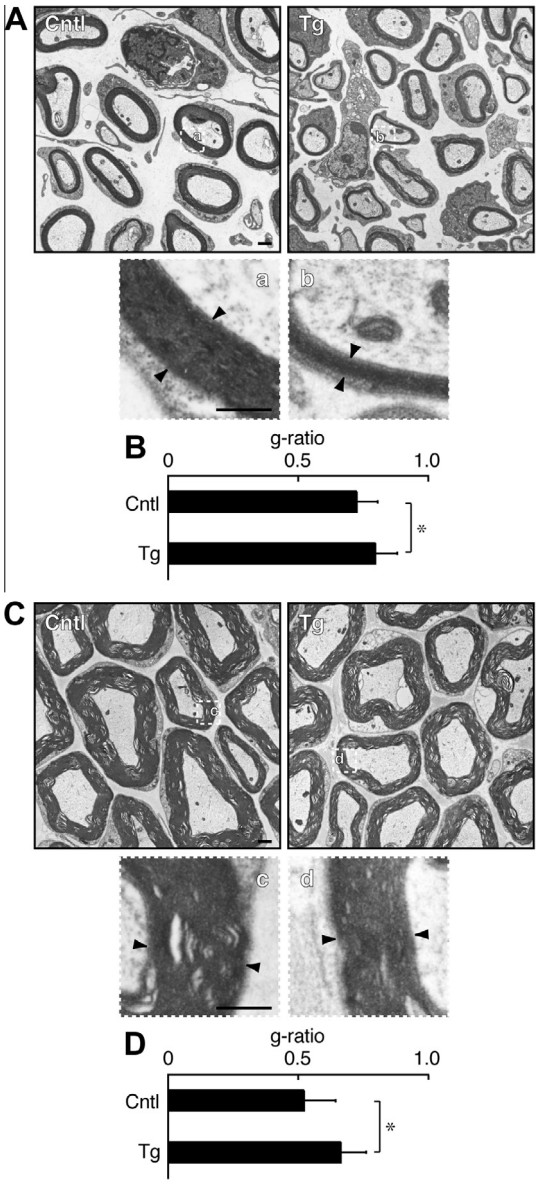

Fig. 4. ShErbB3 transgenic mice exhibit delayed myelination. Electron micrographs of sciatic nerve cross sections in control mice (Cntl) and transgenic mice (Tg) at postnatal day 3.5 (A) and 56 (C) are shown. (A and C) Lower panels a–d are enlarged images of the positions indicated by white dotted squares a–d in the upper panels. Distance between arrowheads indicates representative myelin sheath thickness. Scale bars indicate 1 μm. (B and D) The ratios of axon diameters to outer myelinated axon diameters are shown (*$p < 0.01$; n = more than 50).

Similar results were observed in 56-day-old transgenic mice (Fig. 4C and D). We finally established Schwann cell-neuronal cultures from transgenic mice and the controls to confirm that ErbB3 knockdown reduces myelination *in vitro*. After around 2-week-culture following addition of ascorbic acid, we stained myelin segments with an antibody against MBP, which is the myelin marker protein. Knockdown of ErbB3 inhibited myelination by more than 80% (Fig. S2), consistent with our finding that ErbB3 is required for Schwann cell myelination *in vivo*.

4. Discussion

Schwann cell precursors proliferate, migrate to their final destinations, and differentiate for myelination of the peripheral neuronal axons. In zebrafish, a transparent experimental model animal, genetic studies have illustrated that the ligand-binding ErbB3 receptor is required for Schwann cell precursor proliferation and migration along peripheral axons, as well as for myelination [5,15]. In mice, complete knockout of ErbB3 results in embryonic lethality with a complete loss of Schwann cell precursors; its effects are similar to those of various peripheral neuropathy symptoms involving Schwann cell precursor loss [16,17], indicating that ErbB3 is essential for cell growth and/or survival of the precursors. Although ErbB3 is known to play several roles in Schwann cell lineage cell development, it remains unclear whether ErbB3 is actually required for migration in mammals. Here, we show that ErbB3 knockdown does not lead to lethality; rather, it delays Schwann cell precursor migration. This finding is consistent with the results of our *in vitro* migration assay. Therefore, our results indicate that the role of ErbB3 in migration is conserved in mammals.

Schwann cell-specific ErbB3 shRNA transgenic mice are not lethal, probably since ErbB3 expression is not completely blocked in these transgenic mice. A similar phenotype of mildly reduced myelin thickness is also seen in the Schwann cell developmental stage following migration. On the other hand, like mice with complete ErbB3 knockout, mice with knockout of the cognate ligand, NRG1 type III, display embryonic lethality with a loss of Schwann cell precursors [18]. Since peripheral neurons express NRG1 type III, peripheral neuron-specific NRG1 type III shRNA transgenic mice may allow us to analyze the role of NRG1 type III, as the ErbB3 ligand, in Schwann cell precursor migration. Transgenic mice transcribing shRNA using a tissue-specific promoter are called shRNAmir transgenic mice [7,8]. Although tissue-specific RNA interference does not completely suppress expression of the target molecule, it is useful for analyzing the role of an intended gene *in vivo* [7,8]. In particular, it may be helpful to produce shRNAmir transgenic mice before evaluating the intended gene using conditional knockout mice, since the length of time required to produce transgenic mice is much shorter than that required to produce knockout mice.

In addition to neuregulin-1, growth factors such as neurotrophin-3 [2,6,19] and insulin-like growth factor 1 [2,6,20] are known to be provided by peripheral neurons. They bind to the respective cognate receptors on Schwann cells and promote Schwann cell precursor migration, although these roles have only been demonstrated in *in vitro* experimental systems. Transgenic mice transcribing shRNA using each peripheral neuron- or Schwann cell-specific promoter may be useful in determining whether the relationship between previously unknown growth factors and receptors actually contributes to migration *in vivo*.

It is clear that ErbB3 is required for Schwann cell precursor migration, but the downstream signal transducers are not thoroughly understood. A possible signal transducer seems likely to be non-receptor type protein tyrosine phosphatase 11 (PTPN11, also called Shp2). PTPN11 is a binding partner of the ErbB family, and PTPN11 mutant mice display not only reduced myelin thickness but also impaired Schwann cell precursor migration [21]. PTPN11 dephosphorylates the negative phosphorylated tyrosine residues of non-receptor type Src family tyrosine kinases including Fyn, resulting in the activation of Src family kinases *in vitro* and *in vivo* [22]. Fyn is an important regulator not only in the development of oligodendrocytes, myelin-forming glial cells in the central nervous system, but also in Schwann cell development [9,23]. It is conceivable that PTPN11 couples with ErbB2/3 to activate Fyn, leading to the promotion of Schwann cell precursor migration. In addition, it is noteworthy that, in PTPN11 mutant precursors, the activity of extracellular signal-regulated kinase (ERK), the major intracellular signal convergent molecule, is greatly inhibited [21]. ERK plays a key role in migration in many cell types by phosphorylating many actin and tubulin cytoskeletal proteins and their associated molecules [24]. Thus, ERK may act downstream of ErbB2/3 and PTPN11, contributing to Schwann cell precursor migration.

In this study, we show that in mice, the ligand-binding ErbB3 is required for Schwann cell precursor migration, and thus that ErbB3 plays a conservative role in PNS glial cell migration. Further studies will clarify the detailed molecular mechanisms by which ErbB2/3 regulates migration. It would be interesting to investigate whether the ErbB2/3 receptor and the underlying signaling pathway may be also involved in migration following injury, since cell migration is often observed following injury as well as during development. Such studies on the mechanisms controlling migration may help us to elucidate a paradigm for remyelination processes and nerve regeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.156.

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