Perineurial Glial Plasticity and the Role of TGF- β in the Development of the Blood–Nerve Barrier

Angela D. Morris,¹ Gwendolyn M. Lewis,² and Sarah Kucenas^{1,2}

¹Neuroscience Graduate Program and ²Department of Biology, University of Virginia, Charlottesville, Virginia 22904

Precisely orchestrated interactions between spinal motor axons and their ensheathing glia are vital for forming and maintaining functional spinal motor nerves. Following perturbations to peripheral myelinating glial cells, centrally derived oligodendrocyte progenitor cells (OPCs) ectopically exit the spinal cord and myelinate peripheral nerves in myelin with CNS characteristics. However, whether remaining peripheral ensheathing glia, such as perineurial glia, properly encase the motor nerve despite this change in glial cell and myelin composition, remains unknown. Using zebrafish mutants in which OPCs migrate out of the spinal cord and myelinate peripheral motor axons, we assayed perineurial glial development, maturation, and response to injury. Surprisingly, in the presence of OPCs, perineurial glia exited the CNS normally. However, aspects of their development, response to injury, and function were altered compared with wildtype larvae. In an effort to better understand the plasticity of perineurial glia in response to myelin perturbations, we identified transforming growth factor- β 1 as a partial mediator of perineurial glial development. Together, these results demonstrate the incredible plasticity of perineurial glia in the presence of myelin perturbations.

Key words: development; myelinating glia; perineurium; regeneration; zebrafish

Significance Statement

Peripheral neuropathies can result from damage or dysregulation of the insulating myelin sheath surrounding spinal motor axons, causing pain, inefficient nerve conduction, and the ectopic migration of oligodendrocyte progenitor cells (OPCs), the resident myelinating glial cell of the CNS, into the periphery. How perineurial glia, the ensheathing cells that form the protective blood–nerve barrier, are impacted by this myelin composition change is unknown. Here, we report that certain aspects of perineurial glial development and injury responses are mostly unaffected in the presence of ectopic OPCs. However, perineurial glial function is disrupted along nerves containing centrally derived myelin, demonstrating that, although perineurial glial cells display plasticity despite myelin perturbations, the blood–nerve barrier is compromised in the presence of ectopic OPCs.

Introduction

Formation of an efficient and functional PNS requires precise and coordinated interactions between several distinct cell populations (Gamble and Breathnach, 1965; Shanthaveerappa and Bourne, 1966; Bourne, 1968; Akert et al., 1976; Appel et al., 1995; Corfas et al., 2004; Kucenas et al., 2008b). Spinal motor nerves, the conduits by which information is conveyed from the CNS to

peripheral targets such as muscles, glands and organs, are composed of motor axons and their associated ensheathing glia (Appel et al., 1995; Corfas et al., 2004; Kucenas et al., 2008b). Given the complexity required to form and maintain a functional motor nerve, it is not surprising that aberrations may arise, resulting in disease states, such as peripheral neuropathies, including Charcot-Marie-Tooth disease and congenital peripheral amyelinopathy. Some forms of neuropathies attack the myelin sheath (Hughes, 2002). However, how and if remaining ensheathing glial cell populations, such as perineurial glia, compensate and maintain a functional nerve despite myelin perturbations, is not well understood.

During development, CNS-derived motor exit point (MEP) glia and neural crest-derived Schwann cells myelinate spinal motor axons (Jessen and Mirsky, 2005; Emery, 2010; Smith et al., 2014). CNS-derived perineurial glia exit the spinal cord and bundle several myelinated axons into a nerve fascicle, eventually differentiating into the mature perineurium, a component of the blood–nerve barrier (BNB) (Bourne, 1968; Kristensson and

Received Sept. 13, 2016; revised March 29, 2017; accepted March 31, 2017.

Author contributions: A.D.M., G.M.L., and S.K. designed research; A.D.M. and G.M.L. performed research; A.D.M., G.M.L., and S.K. analyzed data; A.D.M., G.M.L., and S.K. wrote the paper.

This work was supported by National Institutes of Health Grant NS072212 to S.K. and Grant NS051140 to G.M.L., March of Dimes 5-FY11-90 to S.K., and the Hartwell Foundation to S.K. We thank members of the S.K. laboratory for valuable discussions; Lori Tocke for zebrafish care; and Dr. Alev Erisir and Erisir laboratory members, Bonnie Sheppard, Dr. Stacey Criswell, and Dr. Yalin Wang (University of Virginia Advanced Microscopy Facility) for the transmission electron microscopy experiments.

The authors declare no competing financial interests.

Correspondence should be addressed to Dr. Sarah Kucenas, University of Virginia, Physical and Life Sciences Building, Room C312, PO Box 400328, Charlottesville, VA 22904-4328. E-mail: sk4ub@virginia.edu.

DOI:10.1523/JNEUROSCI.2875-16.2017

Copyright © 2017 the authors 0270-6474/17/374790-18\$15.00/0

Olsson, 1971; Akert et al., 1976; Rowitch, 2004; Jessen and Mirsky, 2005; Kucenas et al., 2008b; Clark et al., 2014; Smith et al., 2014). In both fish and mouse, perineurial glia are required for motor axon pathfinding from the spinal cord, restricting ectopic migration of motor neuron cell bodies into the periphery and are necessary for Schwann cell development and peripheral myelination (Kucenas et al., 2008b; Binari et al., 2013; Clark et al., 2014). Additionally, we recently discovered that perineurial glia are essential mediators of nerve regeneration by phagocytizing debris and forming glial bridges across injury sites (Lewis and Kucenas, 2014). Although we have begun to reveal the importance of perineurial glia in development, we still lack an understanding of how they respond in disease states, such as peripheral myelinopathies.

Following peripheral myelin perturbations in zebrafish, mice, and humans, oligodendrocytes are found ectopically along spinal motor root axons (Weinberg et al., 1975; Kucenas et al., 2009; Couplier et al., 2010; Lewis and Kucenas, 2014; Smith et al., 2014). However, nothing is known about how perineurial glia are affected by this change in glial composition. Here, we investigate whether perineurial glial development, maturation, function, and response to injury are altered in the presence of ectopically located oligodendrocyte progenitor cells (OPCs) and central myelin. By characterizing perineurial glial phenotypes in a zebrafish mutant with peripheral OPCs (*erbb3b*^{st48}) (Smith et al., 2014), we show that aspects of perineurial glial development and injury response behaviors are comparable with wildtype controls and that TGF- β 1 plays a role in perineurium maturation. Our results demonstrate the adaptability of perineurial glial cells despite the presence of ectopic OPCs. However, the functionality of the perineurium fails, further supporting the importance of Schwann cell–perineurial glial interactions during spinal motor nerve development and maintenance (Parmantier et al., 1999; Sharghi-Namini et al., 2006).

Materials and Methods

Fish husbandry. All animal studies were approved by the University of Virginia Institutional Animal Care and Use Committee. Zebrafish strains used in this study included *Tg(nkx2.2a:megfp)*^{vu17} (RRID:ZFIN_ZDB-ALT-080321-1) (Kirby et al., 2006; Kucenas et al., 2008b), *Tg(sox10(7.2):mrfp)*^{vu234} (RRID:ZFIN_ZDB-ALT-080321-3) (Kucenas et al., 2008b), *Tg(olig2:dsred2)*^{vu19} (RRID:ZFIN_ZDB-ALT-080321-2) (Kucenas et al., 2008b), *Tg(NBT:DsRed)* (RRID:ZFIN_ZDB-ALT-081027-2) (Peri and Nüsslein-Volhard, 2008), and *erbb3b*^{st48} (RRID:ZFIN_ZDB-ALT-050512-6) (Lyons et al., 2005). Table 1 describes the expression and abbreviations of all lines used in this study. Embryos were produced by pairwise matings, raised at 28.5°C in egg water, staged according to hours or days post fertilization (hpf and dpf, respectively), and embryos of either sex were used for all experiments (Kimmel et al., 1995). Embryos used for immunohistochemistry and microscopy were treated with 0.003% phenylthiourea in egg water to inhibit pigmentation.

Genotyping. Genomic DNA samples were scored for the *erbb3b*^{st48} mutation by digesting individual PCR products, amplified using primers 5'-GTGCTCTGTCAGAAATGGTAAA-3' and 5'-AGAAACCCCTGACTGGACCA-3', with the *Hpy8I* (*Mja*IV) restriction enzyme (Thermo Fisher Scientific) (Lyons et al., 2005).

In vivo imaging. At 24 hpf, all embryos used for live imaging were transferred to egg water containing phenylthiourea to inhibit pigment formation. At specified stages, embryos and larvae were anesthetized using 3-aminobenzoic acid ester (Tricaine), immersed in 0.8% low-melting point agarose, and mounted on their sides in glass-bottom, 35 mm Petri dishes (Electron Microscopy Sciences). Images were captured using either a 40 $\times$ (numerical aperture 1.2) or a 63 $\times$ (numerical aperture 1.2) water-immersion objective mounted on a motorized AxioObserver Z1 microscope (Carl Zeiss) equipped with a WaveFX-X1 spinning

Table 1. Descriptions and abbreviations of transgenic lines used in this study^a

Transgene name	Abbreviation	Description of expression
<i>Tg(nkx2.2a:megfp)</i> ^{vu1}	<i>nkx2.2a:megfp</i>	Membrane EGFP in <i>nkx2.2a</i> ⁺ cells (perineurial glia, floorplate, OPCs)
<i>Tg(sox10(7.2):mrfp)</i> ^{vu234}	<i>sox10:mrfp</i>	Membrane RFP in <i>sox10</i> ⁺ cells (Schwann cells, MEP glia, OPCs)
<i>Tg(olig2:dsred2)</i> ^{vu19}	<i>olig2:dsred</i>	Cytosolic DsRed in <i>olig2</i> ⁺ cells (motor neurons, axons, OPCs)
<i>Tg(NBT:DsRed)</i>	<i>nbt:dsred</i>	Cytosolic DsRed in <i>nbt</i> ⁺ cells (neurons, axons)

^aAll lines were stable, germline transgenics. Only cell types pertinent to this study are listed for each transgene.

disc confocal system (Quorum Technologies). For time-lapse imaging, z stacks were collected at specified time points and 3D datasets were compiled using MPEG-4 video compression at 10 frames per second and exported to QuickTime (Apple) to create movies. Image adjustments were limited to contrast enhancement and level settings using MetaMorph software (RRID:SCR_002368), Adobe Photoshop (RRID:SCR_014199), and ImageJ (RRID:SCR_003070).

Immunohistochemistry: whole mount. Larvae were fixed in AB Fix (4% PFA, 0.1% Triton X-100, 1 $\times$ PBS) for either 3 h at 23°C or overnight at 4°C, followed by a 5 min wash with PBSTx (1% Triton X-100, 1 $\times$ PBS), a 5 min wash with DWTx (1% Triton X-100 in distilled water), a 5 min wash with acetone at 23°C, a 10 min wash with acetone at –20°C, and three 5 min washes with PBSTx. Larvae were preblocked in 5% goat serum/PBSTx for at least 1 h and incubated in primary antibody for 1 h at 23°C and overnight at 4°C. The primary antibodies used in this study include the following: mouse anti-acetylated tubulin (1:5000, Sigma catalog #T7451, RRID:AB_609894), a rabbit antibody to Sox10 (1:5000, Thermo Fisher Scientific catalog #Kucenaslab_001, RRID:AB_2637056) (Binari et al., 2013), a mouse antibody to zonula occludens-1 (ZO-1; 1:200, Invitrogen catalog #33–9100, RRID:AB_2533147), and a rabbit antibody to anti-phospho-Smad3 (1:175, Abcam catalog #ab52903, RRID:AB_882596) (Casari et al., 2014). Larvae were washed extensively with 1 $\times$ PBSTx and stored in 50% glycerol-PBS at 4°C until imaging. Larvae were mounted on their sides in 0.8% low-melting point agarose on glass-bottom, 35 mm Petri dishes and imaged using the confocal microscope described above. Image adjustments were limited to contrast enhancement and level settings using MetaMorph software, Adobe Photoshop, and ImageJ.

Sectioned tissue. For immunohistochemistry on sections, adults >3 months old were fixed in AB Fix as described above for 3 d at 23°C and 4°C overnight. The anterior and posterior ends were removed, such that only an ~0.5 cm region of the trunk remained. Trunk portions were embedded in 1.5% agar/30% sucrose and frozen in 2-methylbutane chilled by immersion in liquid nitrogen. Coronal sections (50 μ m for adults) were collected on microscope slides using a cryostat microtome. Sections were hydrated in 1 $\times$ PBS for 60 min at 23°C and preblocked in 2% goat serum/BSA/1 $\times$ PBS for 30 min. Sections were incubated in primary antibody overnight at 4°C. Sections were washed extensively with 1 $\times$ PBS, incubated for 3 h at 23°C with AlexaFluor-647 goat anti-mouse (1:600, Invitrogen), and washed with 1 $\times$ PBS for 30 min. Sections were mounted in Vectashield (Vector Laboratories) and imaged using the confocal microscope described above. Image adjustments were limited to contrast enhancement and level settings using MetaMorph software, Adobe Photoshop, and ImageJ. Fluorescence intensity measurements were collected using ImageJ. Six circular regions of interest (ROIs) were selected within *nkx2.2a*⁺/ZO-1⁺ regions of the perineurium and the area, integrated density and mean gray value were measured for each ROI. One background measurement was selected per image in an area that contained no fluorescence. The mean gray value was calculated for background ROIs and used to calculate the corrected total fluorescence for each individual ROI using the following equation: integrated density – (ROI area $\times$ mean fluorescence of background images). The corrected total fluorescence for each ROI was used to determine statistical significance between wildtype and *erbb3b*^{st48} labeling using GraphPad Prism software (RRID:SCR_002798).

Transmission electron microscopy. Adult wildtype and *erbb3b*^{-/-} zebrafish (~8 months old) were killed with Tricaine, fin tissue was collected for genotyping, and the trunk region was dissected and placed in fixative: 2% glutaraldehyde/4% PFA in 0.1 M sodium cacodylate. Adult zebrafish trunk samples were prepared for electron microscopy using microwave fixation (Panasonic model NNSD967S) and were kept on ice at 15°C to prevent sample heating. Samples were then kept in fixative for at least 2 h at 23°C. Samples were stored in 2.5% glutaraldehyde in 0.1 M sodium cacodylate at 4°C until genotyping results were available. Upon confirmation of genotype, trunk samples were mounted in 1.75% glutaraldehyde in gelatin/albumin (gelatin from porcine skin, Sigma Type A G1890; BSA Sigma A3912) embedding medium, and 200 μ m transverse sections were cut using a VT1000S vibrating blade microtome (Leica). The sections were then washed in 0.1 M sodium cacodylate, 2 times at 3 min per wash. The sections were removed from the gelatin/albumin embedding medium and transferred to a glass scintillation vial for secondary fixation; 1% osmium (OsO₄) in 0.1 M sodium cacodylate was added to the sections and incubated at ~23°C for ~1 h. The 1% OsO₄ in 0.1 M sodium cacodylate was extracted, and the samples were rinsed 3 times, 3 min per wash, in 0.1 M sodium cacodylate. The samples were dehydrated as follows: 50% ethanol for 3 min, 4% uranyl acetate in 70% ethanol for at least 1 h, 70% ethanol for 1 min, 90% ethanol for 5 min, 100% ethanol for 5 min, 2 times. Samples were rinsed in acetone 3 times, 2 min per rinse, and stored in a 1:1 EPON/acetone mixture at ~23°C overnight. The 1:1 EPON/acetone mixture was replaced with full EPON, and samples were incubated at ~23°C overnight. Samples used for coronal cross sections were embedded in full EPON, and the EPON was polymerized in a 60°C oven overnight. Ultrathin sections (70 nm) were obtained on a UCT ultramicrotome (Leica), transferred to copper grids, and counterstained with uranyl acetate. Coronal cross sections were visualized using a Zeiss Sigma HD variable pressure scanning electron microscope with a STEM detector. Images were taken at 25,000 $\times$ magnification for Figure 2F, G (top), 15,000 $\times$ magnification for Figure 7H, and 5000 $\times$ magnification for Figure 4C. For transverse sections, sections were flat embedded in between two ACLAR embedding sheets using full EPON and put in a 60°C oven overnight. Samples were excised from the ACLAR embedding sheets and reembedded in BEEM embedding capsules. The embedding capsules were placed in a 60°C oven overnight to polymerize the EPON. Following polymerization, the samples were removed from the capsule and were trimmed for ultrathin sectioning. Ultrathin transverse sections (70 nm) were obtained on an Ultracut UCT Ultramicrotome (Leica), transferred to copper grids, and visualized using a 1010 transmission electron microscope (JEOL). Images for Figures 2G (bottom), 4D, and 4E were taken at 1200 $\times$ magnification with a 16 megapixel CCD camera (Scientific Instruments and Applications).

Nerve transection. Nerve transections were performed using a Micro-Point Laser (Andor Technology) as previously published (Rosenberg et al., 2012; Banerjee et al., 2013; Binari et al., 2013; Lewis and Kucenas, 2013, 2014) attached to a spinning disk confocal system (Quorum Technologies) consisting of a nitrogen-pumped dye laser (wavelength 435 nm) controlled by MetaMorph version 7.7. Ablation laser settings ranged from power 15–20 depending on the age of the larvae and nerve position. One or two motor nerves per larva in hemisegments 10–16 were transected in all experiments. To transect nerves, a thin elliptical ROI was drawn digitally in MetaMorph over the image of the nerve, and the nerve was laser pulsed precisely within the ROI until the nerve appeared transected, whereby fluorescence did not refill the ROI in >10 s. Successful transections with this protocol were independently confirmed by identifying the presence of axonal degeneration in transgenic lines that label motor axons (data not shown). *In vivo* imaging of transected nerves was performed as described above.

Perineurium barrier integrity assay. Adult *sox10:mrfp* and *sox10:mrfp; erbb3b*^{-/-} zebrafish, ~8 months old, were anesthetized using Tricaine. Dextran conjugated to AlexaFluor-647 (10,000 MW, anionic, fixable; Invitrogen catalog #D22914) was diluted to a 2% working stock concentration, loaded into a micropipette glass capillary (World Precision Instruments TW100F-4), and injected into the muscle of the trunk, with at least 3 muscle injection sites per fish. Following injection of the fluores-

cent dye, the zebrafish were allowed to recover. At 1.5 h post injection (hpi), the zebrafish were killed using Tricaine, the anterior and posterior ends were removed, and the dissected trunk region was placed in 4% PFA for 3 d at 23°C and at 4°C overnight. Trunk portions were embedded in 1.5% agar/30% sucrose and frozen in 2-methylbutane chilled by immersion in liquid nitrogen. Coronal sections (50 μ m) were collected on microscope slides using a cryostat microtome. Sections were mounted in Vectashield (Vector Laboratories) and imaged using the confocal microscope described above. Image adjustments were limited to contrast enhancement and level settings using MetaMorph software, Adobe Photoshop, and ImageJ.

Drug treatments. Drug treatments were performed in 24-well plates with up to 12 larvae per well in 1.5 ml 25 μ M SB431542 in 0.5% DMSO in egg water, or in 1.5 ml 0.5% DMSO in egg water in dark conditions. Larvae were treated beginning at 6 dpf and placed in an incubator at 28.5°C for ~24 h. Fresh SB431542 or DMSO solutions were replenished at 7 dpf, larvae were placed back in the 28.5°C incubator in dark conditions for 24 h and then fixed in AB Fix (4% PFA, 0.1% Triton-X, 1 $\times$ PBS) at 8 dpf for 3 h at 23°C or overnight at 4°C. To validate SB431542 was working, one-cell stage embryos were treated with either 100 or 200 μ M SB431542 in 0.5% DMSO in egg water or 0.5% DMSO in egg water until ~24 hpf. Embryos were assayed for gross developmental changes as previously described (Sun et al., 2006).

Data quantification and statistical analysis. All graphically presented data represent the mean of the analyzed data. Statistical analyses and graphing were performed with GraphPad Prism software. The level of significance was determined by using an unpaired *t* test or a χ^2 test using a confidence interval of 95%.

Results

erbb3b^{-/-} spinal motor nerves as a model to study perineurial glial plasticity in response to myelin perturbations

The five main cellular components that comprise vertebrate spinal motor nerves (Fig. 1A) are motor axons, peripheral myelinating glial cells, such as MEP glia and Schwann cells, endoneurial components, perineurial glia, and epineurial cells (Fig. 1B) (Thomas, 1963; Gamble and Eames, 1964; Gamble and Breathnach, 1965; Gamble, 1966; Du Plessis et al., 1996; Jessen and Mirsky, 1999; Maurel and Salzer, 2000; Smith et al., 2014). In both mouse and zebrafish, perineurial glia originate from precursors in the p3 domain (floor plate) of the spinal cord and exit into the periphery via MEP transition zones (TZs) (Kucenas et al., 2008b; Clark et al., 2014). In zebrafish, perineurial glial migration is relatively stereotypic, with perineurial cells beginning to exit from the spinal cord at ~48 hpf, proliferating, and then extending and retracting along the motor nerve until full ensheathment of the motor nerve to the neuromuscular junction is achieved, beginning at ~4 dpf (Fig. 1C–E) (Kucenas et al., 2008b). We can visualize these migratory and extension behaviors using *in vivo*, time-lapse imaging of *nkx2.2a:megef;sox10:mrfp* embryos, where regulatory sequences of *nkx2.2a* and *sox10* drive expression of membrane-tethered EGFP and RFP in perineurial glia and peripheral myelinating glia, respectively (data not shown).

After ensheathment, perineurial glia ultimately differentiate into the mature perineurium, a component of the BNB, providing structural and permeability support to the nerve (Akert et al., 1976). Tight junctions have previously been used to reliably and reproducibly detect the differentiated perineurium (Kristensson and Olsson, 1971; Akert et al., 1976; Kucenas et al., 2008b; Binari et al., 2013; Clark et al., 2014; Lewis and Kucenas, 2014), and ZO-1 has been used as a marker for detecting these structures (Pummi et al., 2004; Kucenas et al., 2008b; Binari et al., 2013; Clark et al., 2014; Lewis and Kucenas, 2014). In zebrafish, ZO-1 labeling can be visualized at the MEP at 6 dpf; and as development

Figure 1. Perineurial glial development in zebrafish. **A**, Diagram of a zebrafish embryo identifying the CNS, which consists of the brain and spinal cord (red), and the peripheral spinal motor nerves (green). **B**, Diagram representing a cross sectional view of an adult peripheral motor nerve. Axons are wrapped by peripheral myelinating glial cells (myelin sheath, red) and are surrounded by the endoneurium (purple). Several myelinated axons are bundled by the perineurium (green) to form a fascicle. The epineurium (yellow) then encases several fascicles to form a nerve. **C–E**, All images are lateral views of the spinal cord, with dorsal to the top and anterior to the left. In wildtype *nkx2.2a:megfp;sox10:mrpf* zebrafish larvae, *nkx2.2a*⁺ perineurial glia (arrowheads) exit the CNS by ~52 hpf (**C**), proliferate (**D**), and extend (**E**) until full ensheathment of the motor nerve is achieved. Scale bar, 25 μ m.

proceeds, this labeling gradually extends distally along the nerve as perineurial glial cells continue to mature and become a component of the protective BNB (Kucenas et al., 2008b; Binari et al., 2013; Lewis and Kucenas, 2014).

Previously, we demonstrated that perturbing Schwann cell development prevents (Kucenas et al., 2009) or delays (Kucenas et al., 2008b; Lewis and Kucenas, 2014) perineurial glial migration from the spinal cord. Specifically, in *mont blanc* (*mob^{m610} -/-*) (Barrallo-Gimeno et al., 2004) and *mother superior* (*mos^{m188} -/-*) double mutant embryos, which harbor mutations in the transcription factors *tfap2a* and *foxd3*, respectively, there is a failure of neural crest induction; therefore, Schwann cells are absent along motor nerves (Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006; Arduini et al., 2009; Kucenas et al., 2009; Wang et al., 2011). In *colorless* (*cls^{tw11} -/-*) embryos and larvae, which harbor a mutation in the transcription factor *sox10* (Dutton et al., 2001), neural crest-derived Schwann cells migrate to nascent spinal motor nerves but die soon after associating with motor axons as *Sox10* is required for Schwann cell survival and differentiation (Kuhlbrodt et al., 1998; Britsch et al., 2001; Paratore et al., 2001; Kucenas et al., 2008b; Lewis and Kucenas, 2014). Time-lapse imaging of both mutants before 3 dpf revealed that perineurial glia failed to exit the CNS (Kucenas et al., 2009). However, imaging *cls*^{-/-} larvae at later time points in development revealed that perineurial glial migration was only delayed, not completely absent, along *cls*^{-/-} motor nerves (Lewis and Kucenas, 2014). Furthermore, in both *mob*^{-/-}; *mos*^{-/-} and *cls*^{-/-} larvae, the lack of all peripheral myelinating glia leads to the ectopic migration of OPCs into the periphery (Kucenas et al., 2008b, 2009). However, neither mutant is homozygous viable. Intriguingly, ectopically located oligodendrocytes and centrally derived myelin have previously been reported in the periphery of

zebrafish, mice, and humans (Weinberg et al., 1975; Kucenas et al., 2009; Couplier et al., 2010; Lewis and Kucenas, 2014; Smith et al., 2014). However, the impact that this abnormality has on remaining peripheral ensheathing glia, specifically perineurial glia, has not been investigated. To address this, we turned to another peripheral myelin mutant, *erbb3b*^{-/-} (Lyons et al., 2005), which has ectopic OPCs, centrally derived myelin in the periphery (Smith et al., 2014), is homozygous viable, and has normal swimming, feeding, and mating behaviors (Honjo et al., 2011).

During development, *erbb3*, a receptor tyrosine kinase expressed by Schwann cells and their precursors, forms a heterodimer complex with *erbb2* (Garratt et al., 2000a, b; Lyons et al., 2005; Brinkmann et al., 2008; Birchmeier, 2009; Newbern and Birchmeier, 2010; Perlin et al., 2011). This *erbb2*-*erbb3* complex then binds to the axonally expressed growth factor Neu-regulin I type III (Nrg1), which promotes Schwann cell survival, proliferation, and differentiation into myelinating glial cells (Lyons et al., 2005; Birchmeier, 2009; Newbern and Birchmeier, 2010; Perlin et al., 2011; Raphael et al., 2011; Pereira et al., 2012). In *erbb3b*^{-/-} embryos and larvae, Schwann cell migration, proliferation,

differentiation, and myelination along the mechanosensory posterior lateral line (PLLn) are disrupted (Riethmacher et al., 1997; Lyons et al., 2005; Torii et al., 2014) and OPCs ectopically exit the CNS and associate with and myelinate peripheral spinal motor nerve axons (Smith et al., 2014). Similarly, the loss of ErbB receptors in mice results in Schwann cell defects and hypomyelination of peripheral axons (Riethmacher et al., 1997; Brinkmann et al., 2008).

To validate that spinal motor nerves completely lack peripheral myelinating glial cells in *erbb3b*^{-/-} larvae, we performed *in vivo* imaging on *nkx2.2a:megfp;sox10:mrpf;erbb3b*^{-/-} and wildtype embryos, where *sox10* regulatory sequences drive expression of membrane-tethered RFP in CNS and PNS myelinating glia. In wildtype larvae imaged at 55 hpf, we observed RFP⁺ Schwann cells and MEP glia along wildtype spinal motor nerves (Fig. 2A). In contrast, at 53 hpf in *erbb3b*^{-/-} larvae, we observed spinal motor nerves that completely lacked RFP⁺ peripheral glia (Fig. 2B) (Smith et al., 2014). To confirm the presence of ectopically located OPCs along mutant nerves, we performed *in vivo*, time-lapse imaging of *nkx2.2a:megfp;olig2:dsred;erbb3b*^{-/-} larvae and observed *nkx2.2a*⁺/*olig2*⁺ cells exit the CNS at MEP TZs beginning at ~59.5 hpf (Fig. 2C). This combination of transgenes allowed us to confidently call these cells OPCs because previous studies demonstrate that OPCs express both *nkx2.2a* and *olig2*, and cells with this expression pattern are only ever observed within the CNS in wildtype larvae (Kucenas et al., 2008a). Once in the periphery, these ectopic OPCs migrated along motor axons until they ultimately associated with single axons and initiated ensheathment, as evidenced by the creation of thin, *nkx2.2a*⁺ rectangular tubes (data not shown), a phenotype consistent with glial ensheathment/myelination (Kucenas et al., 2009; Takada et al., 2010; Czopka et al., 2013; Snaidero et al., 2014; Nawaz et al., 2015).

Figure 2. OPCs ectopically exit the spinal cord in *erb3b*^{−/−} larvae. **A–D**, Lateral views of the spinal cord with dorsal to the top and anterior to the left. **A**, At 55 hpf in *nkx2.2a:megfp*; *sox10:mrfp* larvae, *sox10*⁺ peripheral glial cells (arrowheads) associate with spinal motor axons in the periphery. **B**, In contrast, *sox10*⁺ glial cells fail to associate with spinal motor axons in *nkx2.2a:megfp*; *sox10:mrfp*; *erb3b*^{−/−} larvae at 53 hpf. **C**, Frames captured from a 17 h time-lapse movie of a *nkx2.2a:megfp*; *olig2:dsred*; *erb3b*^{−/−} larva beginning at 57 hpf. Because the *olig2:dsred* transgene labels both motor axons and OPCs, an arrowhead is used to identify the OPC. An *nkx2.2a*⁺; *olig2*⁺ OPC (arrowhead) in the ventral spinal cord migrates toward the MEP TZ and ectopically exits the CNS. Bottom left corner, Numbers indicate the time that has elapsed from the first frame of the figure. **D**, Lateral view of 2 dpf *nkx2.2a*; *olig2:dsred* wildtype and (Figure legend continues.)

To further validate the reduction in peripheral myelinating glial cells in *erbb3b*^{-/-} larvae, we quantified the number of Sox10⁺ glial cells along motor axons by performing immunohistochemistry with a Sox10 antibody to label glia, including ectopically located OPCs, MEP glia, and Schwann cells. Using *nkx2.2a:megfp;olig2:dsred;erbb3b*^{-/-} or *nkx2.2a:megfp;erbb3b*^{-/-} larvae colabeled with an acetylated tubulin antibody, we were able to visualize spinal motor axons and associated Sox10⁺ glia (Fig. 2D). We then selected developmental stages that are important for perineurial glial development, including 2 dpf, which corresponds to perineurial glia exit from the CNS, 3 dpf, which is when perineurial glia are extending along motor axons, and 6–8 dpf, which is when perineurial glia begin to express ZO-1, and quantified the number of Sox10⁺ glia along individual spinal motor nerves (Kucenas et al., 2008b; Binari et al., 2013; Lewis and Kucenas, 2014). At all ages assayed, we observed a significant reduction in the number of Sox10⁺ cells associated with *erbb3b*^{-/-} motor nerves (2 dpf, average 1.04, *n* = 121; 3 dpf, average 1.31, *n* = 110; 6 dpf, average 1.59, *n* = 110; and 8 dpf, average 1.35, *n* = 110) compared with wildtype controls at similar developmental stages (2 dpf average 4.66, *n* = 176; 3 dpf average 4.84, *n* = 176; 6 dpf average 5.35, *n* = 110; 8 dpf average 5.50, *n* = 110) (Fig. 2E). Previously, we demonstrated that Sox10⁺ cells along *erbb3b*^{-/-} spinal motor nerves are *plp1a*⁺ at 4 dpf and express MBP at 8 dpf (Smith et al., 2014). To further validate that ectopic oligodendrocytes are present along *erbb3b*^{-/-} peripheral motor nerves, we examined spinal motor nerve ultrastructure in adult zebrafish using transmission electron microscopy. We observed myelinated spinal motor axons in both 8-month-old wildtype and *erbb3b*^{-/-} zebrafish (Fig. 2F, G, top). In a coronal cross section view of a wildtype zebrafish motor nerve, a distinct basal lamina was present around the myelin sheath, which is a defining feature of a Schwann cell (Fig. 2F). However, there was no distinct basal lamina surrounding the myelin sheath in *erbb3b*^{-/-} nerves (Fig. 2G). Furthermore, we observed Schwann cell processes associating with one axon in an adult wildtype zebrafish (Fig. 2F). However, in *erbb3b*^{-/-} motor nerves, we observed two morphologies of myelinating glial cells: single glial cell bodies were either associated with a single axon, reminiscent of a Schwann cell but lacking an organized basal lamina (Fig. 2G, top, bottom right), or multipolar glial cell bodies with multiple processes were found associated with several axons, suggestive of an oligodendrocyte cell body (Fig. 2G, bottom left). Therefore, the transmission electron microscopy data demonstrate ultrastructural characteristics that confirm myelinating glial cell changes along *erbb3b*^{-/-} motor nerves. Together, the alteration of peripheral myelinating glia and the ectopic presence of OPCs in the mutant larvae confirm that *erbb3b*^{-/-} spinal motor nerves are an ideal model for allowing us to assay the

plasticity and resilience of perineurial glia in an altered developmental environment.

Perineurial glia exit the spinal cord in the presence of ectopic oligodendrocytes

Using *erbb3b*^{-/-} motor nerves as a model for investigating perineurial glial plasticity, we sought to determine whether perineurial glial migration out of the spinal cord was affected in these mutants by conducting *in vivo*, time-lapse imaging experiments in *nkx2.2a:megfp;sox10:mrfp* wildtype and *erbb3b*^{-/-} larvae. Perineurial glia begin exiting the spinal cord at ~48 hpf (Kucenas et al., 2008b). However, because zebrafish development proceeds in an anterior to posterior fashion, perineurial glia may not exit along some nerves until after 48 hpf. Based on our imaging location, between 52 hpf and 55 hpf in wildtype larvae, we observed *nkx2.2a*⁺ perineurial glia migrate from the spinal cord and, once in the periphery, begin proliferating ~9.5 h later (data not shown). Similarly, *nkx2.2a*⁺ perineurial glial migration and proliferation occurred in *erbb3b*^{-/-} larvae in a manner that was indistinguishable from wildtype larvae (data not shown). Intriguingly, *nkx2.2a*⁺ perineurial glia proliferation occurred in *erbb3b*^{-/-} larvae in both the presence (Fig. 3A) and absence of ectopic OPCs (Fig. 3B).

Because perineurial glia migration from the CNS in *erbb3b*^{-/-} mutant larvae was indistinguishable from wildtype controls, we next investigated whether they had stereotypic extension down developing spinal motor nerves. Using *nkx2.2a:megfp;nbt:dsred* transgenic lines to label perineurial glia and motor axons (Fig. 4A), respectively, we imaged wildtype and *erbb3b*^{-/-} larvae and assayed perineurial extension by measuring the length of GFP expression from the MEP TZ to the horizontal myoseptum, a partition of connective tissue located between the dorsal and ventral body wall muscles. Although not statistically significant, we observed a consistent reduction in the extension length of perineurial glia in *erbb3b*^{-/-} larvae at all developmental stages evaluated (data not shown), demonstrating that perineurial glia are capable of migrating and extending along motor nerves in the absence of all peripheral myelinating glia and in the presence of ectopic OPCs.

Although the perineurial extension length was only slightly reduced in *erbb3b*^{-/-} larvae compared with wildtype controls, we observed distinct morphological differences in perineurial glia in *erbb3b*^{-/-} larvae at 8 dpf. We categorized these morphological observations into the following categories: (1) *nkx2.2a*⁺ perineurial glia with ensheathment comparable with wildtype, (2) patchy or discontinuous populations of perineurial glia, (3) perineurial glia that were thin and/or tubular, and (4) perineurial cells that were located only at the MEP TZ (Fig. 4A). In 8 dpf wildtype larvae, we observed the percentage of motor nerves with the following perineurial glial morphologies: 42% were fully ensheathed (26 of 61 nerves), 8% had nerves with patchy/discontinuous streams of cells (5 of 61 nerves), 25% had perineurial cells that were thin/tubular (15 of 61 nerves), and 25% had perineurial glia located only at the MEP TZ (15 of 61 nerves) (Fig. 4A,B). In contrast, the morphology of perineurial glia in 8 dpf *erbb3b*^{-/-} larvae was as follows: 20% with full ensheathment (12 of 60 nerves), 10% patchy/discontinuous (6 of 60 nerves), 47% with thin/tubular morphology (28 of 60 nerves), and 23% located only at the MEP TZ (14 of 60 nerves) (Fig. 4A,B). Thus, in 8 dpf wildtype larvae, there was a greater percentage of motor nerves with full perineurial glial ensheathment whereas in 8 dpf *erbb3b*^{-/-} larvae, there was a greater percentage of motor nerves with thin/tubular perineurial glial morphology.

(Figure legend continued.) *erbb3b*^{-/-} larvae labeled with a Sox10 antibody (blue, arrowheads). **E**, Quantification of Sox10⁺ glial cells along peripheral motor axons in wildtype and *erbb3b*^{-/-} larvae at 2, 3, 6, and 8 dpf. Sox10⁺ glial cells were significantly reduced in *erbb3b*^{-/-} larvae at all time points evaluated (*p* < 0.0001 for all time points evaluated). Statistical significance was measured using an unpaired *t* test. Error bars are representative of SEM. **F**, Transmission electron micrograph of a coronal section identifying a peripheral myelinating glial cell that has myelinated an axon in an adult wildtype zebrafish motor nerve. Arrowheads indicate presence of a basal lamina. **G**, Transmission electron micrograph of coronal (top panels) and transverse (bottom panels) sections of adult *erbb3b*^{-/-} zebrafish spinal motor axons. Bottom left, Pseudocolor represents the multipolar processes of a cell, which may be an endoneurial cell or peripherally located oligodendrocyte. Scale bars: **A–D**, 25 μm; **F**, **G**, 1 μm. ****p* < 0.0005.

Figure 3. Perineurial glial proliferation is normal in *erbb3b*^{−/−} larvae. All images are lateral views of the spinal cord, with dorsal to the top and anterior to the left. **A**, Frames captured from a 16 h time-lapse movie of a 55 hpf *nkx2.2a:megfp;sox10:mrpf;erbb3b*^{−/−} larva reveals that perineurial glial proliferation (dots) occurs despite the presence of an ectopically located OPC (arrowhead). **B**, Frames captured from a 13 h time-lapse movie of a 59 hpf *nkx2.2a:megfp;sox10:mrpf;erbb3b*^{−/−} larva reveals that perineurial glial proliferation (dots) also occurs in the absence of ectopically located OPCs. Bottom left corner, Numbers indicate the time that has elapsed from the first frame of the figure. Scale bars, 25 μ m.

Next, we investigated the ultrastructure of the perineurium using transmission electron microscopy in adult wildtype and *erbb3b*^{−/−} zebrafish. In both wildtype and *erbb3b*^{−/−} adults, we observed myelinated axons bundled into nerve fascicles by the perineurium (Fig. 4C). However, in *erbb3b*^{−/−} zebrafish, the morphology of the perineurium varied. There were areas of the perineurium in which the ultrastructural morphology appeared comparable with wildtype, as we observed concentric and compacted perineurial layers in both wildtype (Fig. 4D) and mutants (Fig. 4E, left). However, there were other areas of the mutant perineurium that appeared very disorganized (Fig. 4E, right): the layers lacked compaction, were discontinuous, and had a wavy appearance, and we never observed these phenotypes in wildtype animals. Together, these results demonstrate that, although certain perineurial glial migration and proliferation behaviors are comparable with wildtype larvae, perineurial glial morphology is altered in mutants with ectopic OPCs along spinal motor nerves.

Ectopically located OPCs affect perineurial glial response to injury

Although perineurial glial migration from the CNS is indistinguishable from wildtype larvae in mutants with ectopic OPCs, we wanted to investigate whether they were capable of responding to injury despite this change in cellular and myelin composition along spinal motor nerves. Peripheral nerves have the remarkable capacity to regenerate following nerve transection, whereby the nerve is divided and a regenerative program is induced, triggering Wallerian degeneration (Waller, 1850), debris clearance and the regrowth of axons from the proximal stump back to peripheral targets (Arthur-Farraj et al., 2012; Rosenberg et al., 2014). Previously, we identified perineurial glia as an essen-

tial component of the injury response (Lewis and Kucenas, 2014). After motor nerve transection, perineurial glia closest to the injury area extend processes toward the injury site, phagocytize debris, and form the first tissue bridge across the injury gap (Lewis and Kucenas, 2014). Recently, a study demonstrated that axonal regrowth was significantly impaired in *erbb3b*^{−/−} larvae following injury (Rosenberg et al., 2014), and we confirmed this finding in our assay of motor nerve transection as well (data not shown). Because of the impairment of axonal regrowth in *erbb3b*^{−/−} larvae, coupled with the presence of perineurial glia in the periphery, we hypothesized that perineurial glia did not perform important regenerative roles, including extending toward the injury site, forming phagocytic vesicles, and bridging injury gaps, leading to the failure of axonal regeneration in these mutants.

To test this hypothesis, we investigated whether perineurial glia were capable of extending toward an injury site in the presence of peripheral OPCs by selecting wildtype and *erbb3b*^{−/−} motor nerves that lacked full ensheathment by *nkx2.2a*⁺ perineurial glia, and mutant axonal tracts that also contained ectopically located OPCs. Using the motor nerve transection assay we described previously (Lewis and Kucenas, 2013, 2014) in 4 dpf *nkx2.2a:megfp;olig2:dsred* larvae, we transected a region of unensheathed motor nerve on the caudal motor tract ~10 to 15 μ m from the closest perineurial glial cell. As we previously reported, we observed perineurial glia in wildtype larvae rapidly extend highly motile membrane processes toward the injury site (Fig. 5A). By 3 h post transection (hpt), these processes had extended beyond the initial injury site and stretched toward the distal stump (Fig. 5A) (Lewis and Kucenas, 2014). In *erbb3b*^{−/−} larvae, perineurial glia initially extended membrane processes toward the injury site. However, in contrast to wildtype larvae, these

Figure 4. Perineurial glial morphology is altered in *erb3b^{-/-}* zebrafish. **A**, Images are lateral views of the spinal cord with dorsal to the top and anterior to the left. Morphologically, *nkx2.2a⁺* perineurial glia (arrowheads) in 8 dpf *erb3b^{-/-}* were different from wildtype, and we classified them into the following categories: perineurial glia ensheathment comparable with wildtype, perineurial glia that appeared patchy or discontinuous, perineurial glia thin/tubular, and perineurial glia only at the MEP TZ. Motor axons (red) are labeled using the *nbt:dsred* transgene. **B**, Quantification of *nkx2.2a⁺* perineurial glial morphology along spinal motor nerves in 8 dpf wildtype and *erb3b^{-/-}* larvae. **C**, Transmission electron micrographs of coronal cross sections of 8-month-old wildtype zebrafish perineurium (top, arrowheads) and 8-month-old *erb3b^{-/-}* zebrafish perineurium (bottom, arrowheads). **D**, Transmission electron micrograph of a transverse section of an adult wildtype perineurium (arrowheads). **E**, Transmission electron micrograph of a transverse section of an adult *erb3b^{-/-}* perineurium demonstrates that there are areas of the perineurium (left, arrowheads) that appear comparable with wildtype in (**D**), and there are areas of the *erb3b^{-/-}* perineurium that appear disorganized (right, arrowheads). Scale bars: **A**, 25 μ m; **C–E**, 1 μ m.

processes eventually stalled, retracted, or became misdirected and failed to reach the injury even by 3 hpt (Fig. 5B). We quantified these data by plotting perineurial membrane extension toward the injury site as a percentage of the initial distance between the injury site and the closest perineurial glial membrane (i.e., distance traveled toward injury site/initial distance between perineurial glia and injury site), with a value of 1 representing perineurial processes reaching the injury site, a value of >1 representing perineurial processes with extension beyond the injury site, and values <1 representing perineurial processes that did not reach the injury site within the time investigated after injury. In *erbb3b*^{-/-} larvae, we observed that perineurial process extension toward injury sites was significantly reduced by 120 min post transection (mpt) compared with wildtype, demonstrating a failure of membrane extension across the injury site (Fig. 5C) (Lewis and Kucenas, 2014). Surprisingly, the initial response (15 mpt to 1 hpt) of perineurial glia to the injury in *erbb3b*^{-/-} larvae was indistinguishable from wildtype larvae, demonstrating that perineurial glia in mutant larvae are capable of initially responding to injury cues. From these data, we conclude that ectopically located OPCs impair the maintained extension of perineurial processes to an injury (Lewis and Kucenas, 2014).

Because perineurial glial process extension toward the injury site is impaired after transection of the caudal axonal tract containing ectopically located OPCs, we decided to injure the rostral axonal tract, which we never observe populated by ectopic OPCs, to investigate whether perineurial–perineurial interactions were perturbed in the absence of all myelinating glial cells. Specifically, we investigated whether perineurial glia were capable of phagocytizing debris and forming a glial bridge across injury sites along *erbb3b*^{-/-} nerves (Lewis and Kucenas, 2014). We selected motor nerves that were ensheathed by perineurial glia but lacked ectopically located OPCs, and transected the rostral motor nerve tract in 6 dpf *nkx2.2a:megef; olig2:dsred* wildtype and *erbb3b*^{-/-} larvae (Lewis and Kucenas, 2013, 2014). Following transection, we assayed the perineurial response and, similar to our previous findings, we observed wildtype perineurial glia located proximally and distally to the injury site respond to the injury by extending processes toward the lesion, form phagocytic vesicles and bridge the injury gap (Fig. 6A) in 90% of cases ($n = 10$) (Lewis and Kucenas, 2014). Intriguingly, the perineurial response in *erbb3b*^{-/-} larvae was similar (Fig. 6B). Perineurial glia extended processes toward the injury site from both the proximal and distal stumps, formed phagocytic vesicles, and bridged the injury gap

Figure 5. Perineurial glial recruitment to the injury site is impaired on axonal tracts with centrally derived OPCs. All images are lateral views of the spinal cord with dorsal to the top and anterior to the left. **A**, Frames captured from a 3 h time-lapse movie of a 4 dpf *nkx2.2a:megef; olig2:dsred* wildtype larva. After axotomy, perineurial glia (arrowheads) extended processes into the injury site following motor nerve transection. **B**, In contrast, in frames captured from a 3 h time-lapse movie of a 4 dpf *nkx2.2a:megef; olig2:dsred; erbb3b*^{-/-} larva, perineurial glia (arrowheads) initially extended robust processes toward the injury site following transection of an axonal tract containing ectopically located OPCs. However, the processes failed to reach the injury. Bottom right corner, Numbers indicate time elapsed (mpt) from the initial panel of the figure, which was immediately before axotomy. **C**, Quantification of the extension of perineurial processes toward transection sites in 4 dpf wildtype and *erbb3b*^{-/-} larvae shows perineurial glia extend processes significantly farther in wildtype by 120 mpt ($p = 0.0132$) and 180 mpt ($p = 0.0478$). Because the initial distance between the injury site and glial cells varied slightly with each trial, membrane extension was plotted as a percentage of the initial distance between the injury site and the closest perineurial glial membrane (distance traveled toward injury site/initial distance between perineurial glia and injury site), where values >1 represent processes that have extended beyond the initial injury site. Dotted ellipse indicates approximate ablated ROI. Error bars are representative of SEM. Scale bar, 25 μ m. * $p < 0.05$.

(Fig. 6B) in 75% of cases ($n = 4$, not significantly different from wildtype). Together, results from these injury studies demonstrate that the presence of ectopic OPCs and central myelin affect perineurial glial responses to injury, but that peripheral myelinating glia are not required for perineurial glia to clear debris and form a glial bridge.

Perineurial glia have an increase in ZO-1 labeling along *erbb3b*^{-/-} spinal motor nerves

In addition to developmental and injury response roles, perineurial glia maintain and protect the nerve by maturing into a component of the BNB. A hallmark of perineurial differentiation is the

Figure 6. Perineurial glia form phagocytic vesicles and glial bridges in the absence of all myelinating glial cells. All images are lateral views of the spinal cord, with dorsal to the top and anterior to the left. **A**, Frames captured from 1 h of a 3 h time-lapse movie of a 6 dpf *nkx2.2a:megfp; olig2:dsred* wildtype larva demonstrate that perineurial glia form phagocytic vesicles and glial bridges that span the injury site following axotomy. **B**, Similarly, frames captured from a 3 h time-lapse movie in a 6 dpf *nkx2.2a:megfp; olig2:dsred; erb3b^{-/-}* larva show that perineurial glia form phagocytic vesicles and glial bridges that span the injury site in the absence of all myelinating glia. Bottom right corner, Numbers indicate time elapsed (mpt) from the initial panel of the figure, which was immediately before axotomy. Scale bars, 25 μ m.

formation of tight junctions between adjacent perineurial cells, which is required for the mature permeability function of this structure (Kristensson and Olsson, 1971; Pummi et al., 2004; Kucenas et al., 2008b; Clark et al., 2014). In zebrafish, ZO-1 labeling of tight junctions along spinal motor nerves is evident at 6 dpf beginning at the MEP TZ. As development proceeds, ZO-1 labeling is observed further distally along the nerve, and this is a sign of progressive perineurial differentiation (Kucenas et al., 2008b; Lewis and Kucenas, 2014). Intriguingly, a previous study demonstrates that oligodendrocytes are involved in supporting the integrity of the blood–brain barrier (BBB) by increasing the formation of tight junctions and expression of ZO-1 within BBB components (Seo et al., 2014). Because *erb3b^{-/-}* larvae have ectopically located OPCs along spinal motor nerves, we hypothesized that their presence in the periphery may impact perineurial glial development by altering ZO-1 expression. Using an antibody to ZO-1 in 6 and 8 dpf larvae, we labeled fixed tissue, imaged motor nerves in both *nkx2.2a:megfp* wildtype (Fig. 7A) and *nkx2.2a:megfp; erb3b^{-/-}* (Fig. 7B) larvae, and assayed the extension of perineurial tight junctions by measuring the length of ZO-1 labeling along spinal motor nerves, starting from the MEP TZ. From these studies, we observed no statistically significant difference in ZO-1 extension at 6 dpf (data not shown). However, by 8 dpf, we observed a significant increase in the extension of perineurial glial ZO-1 labeling along *erb3b^{-/-}* nerves with ectopic OPCs (average = 63.83 μ m, n = 61 nerves) compared with wildtype (average = 45.62 μ m, n = 61 nerves) and *erb3b^{-/-}* nerves without ectopic OPCs (average = 36.47, n = 5 nerves) (Fig. 7C).

To determine whether ZO-1 labeling was also enhanced in motor nerves of *erb3b^{-/-}* adults, we labeled coronal sections of *nkx2.2a:megfp; sox10:mrfp* *erb3b^{-/-}* and wildtype adults (>3 months old) with an antibody to ZO-1 (Fig. 7D,E). In these studies, we observed abnormally large deposits of ZO-1 immunoreactivity within *nkx2.2a⁺* perineurial layers around *erb3b^{-/-}* nerves (Fig. 7E) compared with wildtype controls, which appeared to

have more diffuse ZO-1 labeling in the perineurium (Fig. 7D). To quantify this observation, we measured the fluorescence intensity of ZO-1 labeling within six *nkx2.2a⁺/ZO-1⁺* regions of the perineurium in both wildtype and *erb3b^{-/-}* nerves and, after correcting for background fluorescence, analyzed the average corrected fluorescence intensity of each ROI in both groups (Fig. 7F). We observed a statistically significant increase in the average ROI corrected fluorescence intensity of ZO-1 labeling in the perineurium in adult *erb3b^{-/-}* nerves (average = 28,189 arbitrary units [A.U.], n = 54 ROIs) compared with wildtype controls (19,469 A.U., n = 30 ROIs) (Fig. 7F). Because we observed large deposits of ZO-1 labeling within the perineurium of *erb3b^{-/-}* adults, we next categorized the corrected fluorescence intensity for each ROI into one of the following: 0–25,000, 25,001–50,000, or 50,001–75,000 A.U. (Fig. 7G). In wildtype controls, we observed that the majority of ROIs were within the lowest fluorescence intensity category: 73% of ROIs fall within 0–25,000 A.U. (22 of 30 ROIs), 23% of ROIs (7 of 30 ROIs) were within 25,001–50,000 A.U., and 3% of ROIs (1 of 30 ROIs) were within the highest fluorescence intensity category of 50,001–75,000 A.U. In contrast, the *erb3b^{-/-}* ROIs had the following distribution: 52% (28 of 54 ROIs) were within 0–25,000 A.U., 41% (22 of 54 ROIs) were within 25,001–50,000 A.U., and 7% (4 of 54 ROIs) were within 50,001–75,000 A.U., demonstrating that there were patches of significantly higher tight junctional proteins. To confirm that the less diffuse and increased level of ZO-1 labeling we observed within the *erb3b^{-/-}* perineurium was due to an increase in tight junctions, we performed transmission electron microscopy to investigate the perineurium ultrastructure in 8-month-old wildtype and mutant zebrafish. We observed tight junctions in both wildtype and *erb3b^{-/-}* zebrafish (Fig. 7H). However, there were discrete areas of the perineurium that had clusters of more tight junctions present within the mutant perineurium (Fig. 7H, bottom).

Because of the altered distribution of tight junctions observed in *erb3b^{-/-}* adults, we investigated the barrier function of this

Figure 7. Perineurial glia have more tight junctional proteins in *erbB3b* mutant zebrafish. **A, B**, Lateral views of 8 dpf *nkx2.2a:megfp* wildtype and *erbB3b*^{-/-} larvae, with dorsal to the top and anterior to the left, labeled with an antibody to ZO-1 (blue). **A**, At 8 dpf, ZO-1 labeling (arrowhead) is primarily localized near the MEP TZ in wildtype larvae. **B**, In 8 dpf *erbB3b*^{-/-} larvae, ZO-1 labeling (arrowhead) extended further ventrally toward the horizontal myoseptum. **C**, Quantification of the extension length of ZO-1 labeling along peripheral motor nerves in 8 dpf *nkx2.2a:megfp* wildtype and *erbB3b*^{-/-} larvae. The ZO-1 labeling along *erbB3b*^{-/-} motor nerves that contain ectopically located OPCs extended significantly further toward the horizontal myoseptum compared with 8 dpf wildtype larvae ($p < 0.0001$) and 8 dpf *erbB3b*^{-/-} motor nerves that did not contain OPCs ($p = 0.0339$). Statistical significance was measured using an unpaired t test. **D, E**, Coronal cross sections of peripheral motor nerves in adult *nkx2.2a:megfp;sox10:mrpf* wildtype and *erbB3b*^{-/-} zebrafish labeled with an antibody to ZO-1 (blue). **D**, ZO-1 labeling (arrowhead) appeared more punctate within wildtype perineurium. **E**, In contrast, ZO-1 labeling (arrowhead) in *erbB3b*^{-/-} adult perineurium was more heavily concentrated in discrete regions. **F**, Quantification of the average fluorescence intensity, corrected for background, of ZO-1 labeling in A.U. within *nkx2.2a*^{+/+}/ZO-1⁺ ROIs in adult *nkx2.2a:megfp;sox10:mrpf* wildtype and *erbB3b*^{-/-} zebrafish. Six ROIs were selected per nerve (wildtype, $n = 5$ nerves; *erbB3b*^{-/-}, $n = 9$ nerves). There was a statistically significant increase in the average corrected fluorescence intensity of ZO-1 labeling within the *erbB3b*^{-/-} adult perineurium ROIs ($n = 54$ ROIs) compared with wildtype control ROIs ($n = 30$ ROIs) ($p = 0.0094$). **G**, Categorization of the corrected fluorescence intensity of the ROIs. Error bars in **C, F**, and **G** are representative of SEM. **H**, Transmission electron micrograph of a coronal cross section of an adult wildtype (top) and an adult *erbB3b*^{-/-} (bottom) perineurium identifying tight junctions (arrowheads). Scale bars: **A, B, D, E**, 25 μm ; **H**, 1 μm . * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; ns, not significant.

Figure 8. Perineurial barrier integrity is compromised in adult *erbb3b*^{-/-} zebrafish. All images are cross sections of 8-month-old *sox10:mrfp* wildtype and *erbb3b*^{-/-} zebrafish injected with a Dextran-647 dye (blue) into the muscle. **A**, In wildtype, the Dextran-647 dye (blue, arrowheads) remains localized to the perimeter of the motor nerve. **B**, In *erbb3b*^{-/-}, the Dextran-647 dye (blue, arrowheads) infiltrates into the motor nerve. Scale bar, 25 μ m.

structure by injecting a fluorescent dextran dye into the muscle of adult WT and *erbb3b* mutants. Fluorescent dextrans have previously been used in mammals to investigate the barrier function of the BBB and choroid plexus epithelial cells (Ek et al., 2003; Liddel et al., 2009; Saunders et al., 2015). Therefore, we injected a 10,000 MW Dextran-647 into the muscle of 8-month-old wildtype and *erbb3b*^{-/-} zebrafish and fixed harvested trunk tissue at 1.5 hpi. We imaged coronal cross sections and observed that the Dextran-647 remained localized to the nerve perimeter in wildtype adults, demonstrating that the perineurial barrier was functional (Fig. 8A). However, in *erbb3b*^{-/-} adults, we observed Dextran-647 infiltration into the nerve, demonstrating that the integrity of the perineurial barrier was compromised (Fig. 8B). Together, these results demonstrate that the increase in ZO-1 labeling observed along spinal motor nerves in 8 dpf *erbb3b*^{-/-} larvae is redistributed into clusters of tight junctions within the *erbb3b*^{-/-} adult perineurium; and although clusters of tight junctions are present, the barrier function of the *erbb3b*^{-/-} perineurium is compromised.

TGF- β 1 partially mediates the increase in ZO-1 labeling along *erbb3b*^{-/-} motor nerves

A previous study implicated OPCs in promoting the formation of tight junctions in the BBB via TGF- β 1, as depleting TGF- β 1 specifically in OPCs decreased ZO-1 labeling in this tissue (Seo et al., 2014). Upon ligand binding, the TGF- β type II receptor phosphorylates the Type I receptor (Kitisin et al., 2007). The activated Type I receptor then phosphorylates Smad effector proteins, initiating the modulation of gene expression (Sun et al., 2006; Kitisin et al., 2007). Because TGF- β 1 is important for promoting ZO-1 expression in the BBB, we hypothesized that TGF- β 1 signaling would be present along peripheral spinal motor nerves in zebrafish larvae.

To investigate this hypothesis, we fixed *nkx2.2a:megef;erbb3b*^{-/-} and wildtype control larvae at 8 dpf and labeled with an antibody to phosphorylated Smad3 (pSmad3), a downstream effector of TGF- β 1 signaling (Kitisin et al., 2007). We observed specific pSmad3 labeling along 68.18% of wildtype spinal motor nerves ($n = 66$ nerves) (Fig. 9A,C) and 63.6% of *erbb3b*^{-/-} spinal motor nerves ($n = 66$ nerves) (Fig. 9B,C) at 8 dpf. Although the percentage of nerves that were pSmad3⁺ was not significantly different between mutant and wildtype larvae at this stage, we observed more robust pSmad3 labeling along *erbb3b*^{-/-} larval motor nerves compared with wildtype. Therefore, we categorized pSmad3 expression along motor nerves as strong, moderate, low, and none (Fig. 9A,B). In wildtype larvae, we observed 13.64% (9 of 66 nerves) with strong pSmad3 expression, 13.64% (9 of 66 nerves) with moderate pSmad3 expression, 40.91% (27 of 66 nerves) with low pSmad3 expression, and 31.82% (21 of 66 nerves) with no pSmad3 expression (Fig. 9C). Along *erbb3b*^{-/-} nerves, we observed 27.27% (18 of 66 nerves) with strong pSmad3 expression, 19.7% (13 of 66 nerves) with moderate pSmad3 expression, 16.67% (11 of 66 nerves) with low pSmad3 expression, and 36.36% (24 of 66 nerves) with no pSmad3 expression (Fig. 9C). Overall, for nerves that were positive for pSmad3 labeling, we observed a greater percentage of nerves with low expression in wildtype controls compared with a greater percentage of nerves with strong expression in *erbb3b*^{-/-} larvae (Fig. 9C). To more objectively quantify this observation, we measured the fluorescence intensity of pSmad3 labeling by outlining pSmad3⁺ regions along spinal motor nerves in 8 dpf wildtype (Fig. 9A, bottom panels) and *erbb3b*^{-/-} (Fig. 9B, bottom panels) larvae. After correcting for background fluorescence, we observed a statistically significant increase in the fluorescence intensity in *erbb3b*^{-/-} larvae (average = 66717 A.U., $n = 66$ nerves) compared with wildtype control (average = 35369 A.U., $n = 66$ nerves) (Fig. 9D). Additionally, to confirm that the ectopic OPCs were contributing to the increase in pSmad3 labeling we observed along *erbb3b*^{-/-} motor nerves, we compared pSmad3 labeling along wildtype and *erbb3b*^{-/-} nerves that were populated by myelinating glia. We observed a significantly larger proportion of nerves that were pSmad3⁺ in the mutants, with ~75% of *erbb3b*^{-/-} nerves ($n = 32$ nerves) containing ectopic OPCs that were pSmad3⁺ compared with wildtype in which only 25% of nerves ($n = 37$ nerves) were pSmad3⁺ (Fig. 9E). In the small number of *erbb3b*^{-/-} nerves that did not have ectopic oligodendrocytes, we observed that ~60% of nerves did not have pSmad3 labeling (data not shown). These results demonstrate that ectopic OPCs are contributing to the increase in pSmad3 labeling observed in *erbb3b*^{-/-} larvae.

Because nerves that were positive for pSmad3 expression in 8 dpf *erbb3b*^{-/-} larvae had stronger labeling (Fig. 9C,D), we hypothesized that using a selective inhibitor of the TGF- β 1 receptor (SB431542), previously used in *Xenopus* (Ho et al., 2006), zebrafish (Ho et al., 2006; Sun et al., 2006), and mouse (Seo et al., 2014), would inhibit TGF- β signaling and decrease the extent of ZO-1 labeling more significantly along mutant nerves compared with wildtype. Because TGF- β is important for early embryogenesis, we validated that SB431542 was effectively working by treating one-cell stage zebrafish embryos for ~24 h with 100 or 200 μ M SB431542 dissolved in 0.5% DMSO or 0.5% DMSO alone. In inhibitor-treated embryos, we observed concentration-dependent morphological changes in 24 hpf embryos that were consistent with previously reported findings (data not shown) (Sun et al., 2006).

Figure 9. Phosphorylated Smad3 levels are increased along *erb3b*^{-/-} larval spinal motor nerves. All images are lateral views of 8 dpf *nkx2.2a:megfp* wildtype and *erb3b*^{-/-} larvae, with dorsal to the top and anterior to the left, labeled with an antibody to pSmad3 (red) and tubulin (blue). **A, B**, At 8 dpf, pSmad3 labeling (red, arrowheads), along wildtype and *erb3b*^{-/-} spinal motor axons, can be classified into the following categories: Strong, Moderate, Low, and None. Bottom panels, The area used to quantify the average corrected fluorescence intensity of pSmad3 labeling is outlined. *Area used for calculating the background fluorescence. **C**, Quantification of pSmad3 labeling. **D**, Average fluorescence intensity of pSmad3 in A.U., corrected for background fluorescence, in 8 dpf wildtype and *erb3b*^{-/-} larvae. There is significantly greater pSmad3 fluorescence intensity along *erb3b*^{-/-} motor nerves compared with wildtype controls ($p = 0.0158$). Statistical significance was measured using an unpaired *t* test. Error bars are representative of SEM. **E**, Quantification of motor nerves with myelinating glia that are pSmad3⁺ versus pSmad3⁻. Scale bars, 25 μ m. * $p < 0.05$.

To validate that SB431542 was inhibiting TGF- β 1 at later stages, we treated 6 dpf wildtype zebrafish with 25 μ M SB431542 dissolved in 0.5% DMSO or with 0.5% DMSO alone for $\sim$ 48 h, fixed at 8 dpf, and performed immunohistochemistry with an antibody to pSmad3. In wildtype larvae exposed to 0.5% DMSO from 6 to 8 dpf, we observed pSmad3 labeling along $\sim$ 53.03% of motor nerves ($n = 66$ nerves) (Fig. 10A). However, in 8 dpf wildtype larvae treated with 25 μ M SB431542, only 30.91% of motor nerves ($n = 55$ nerves) had pSmad3 labeling (Fig. 10B), a significant decrease compared with the DMSO control. Next, we categorized pSmad3 labeling along spinal motor nerves into strong, moderate, low, and no expression for both the DMSO and SB431542 groups. In the DMSO group, $\sim$ 12.12% (8 of 66 nerves) had strong expression, 15.15% (10 of 66 nerves) had moderate expression, 25.76% (17 of 66 nerves) had low expression, and 46.97% (31 of 66 nerves) had no pSmad3 expression (Fig. 10C). In comparison, the SB431542 group had 1.82% (1 of 55 nerves) with strong expression, 5.45% (3 of 55 nerves) with moderate, 23.64% (13 of 55 nerves) with low expression, and 69.09% (38 of 55 nerves) with no expression (Fig. 10C).

After validating that SB431542 was functional in our hands, we proceeded to treat *nkx2.2a:megfp* wildtype and *erbb3b*^{-/-} larvae from 6 to 8 dpf with 0.5% DMSO or 25 μ M SB431542 dissolved in 0.5% DMSO (Fig. 10D–G). At 8 dpf, we investigated the morphology of the *nkx2.2a:megfp*⁺ perineurial glial cells and observed that there were significantly more perineurial glia that appeared patchy/discontinuous in *erbb3b*^{-/-} treated with 25 μ M SB431542 compared with *erbb3b*^{-/-} treated with 0.5% DMSO (data not shown). To investigate the effects that inhibiting TGF- β 1 had on ZO-1 labeling, we treated larvae from 6 to 8 dpf, fixed the larvae at 8 dpf, performed immunohistochemistry using an antibody to ZO-1 and imaged all groups (Fig. 10D–G). After imaging, we measured the extension length of ZO-1 labeling from the MEP TZ ventrally toward the horizontal myoseptum. In these studies, we observed a statistically significant reduction in the extension length of ZO-1 labeling in 8 dpf wildtype larvae treated with SB431542 (average 30.95, $n = 140$ nerves) compared with 8 dpf larvae treated with DMSO (average 40.26, $n = 102$ nerves) (Fig. 10H). Similarly, there was a statistically significant reduction in the extension length of ZO-1 labeling along spinal motor nerves in 8 dpf *erbb3b*^{-/-} larvae treated with SB431542 (average 24.72, $n = 108$ nerves) compared with DMSO-treated *erbb3b*^{-/-} (average 38.00, $n = 146$ nerves) (Fig. 10H). Intriguingly, the extension length of ZO-1 labeling in 8 dpf *erbb3b*^{-/-} treated with SB431542 (average 24.72, $n = 108$ nerves) was significantly decreased compared with 8 dpf wildtype treated with SB431542 (average 30.95, $n = 140$ nerves) (Fig. 10H). Together, these results demonstrate that pSmad3 expression is stronger along *erbb3b*^{-/-} motor nerves, and inhibiting TGF- β 1 signaling in these mutants has a statistically significant effect on perineurial glial maturation compared with TGF- β 1 inhibition in wildtype controls.

Discussion

Spinal motor nerves are important conduits for relaying information from the CNS to the PNS. Forming a functional motor nerve requires precise interactions between various cell populations, including glial cells. Previously, we have shown that Schwann cells require perineurial glia for migration, differentiation, and myelination (Kucenas et al., 2008b; Binari et al., 2013) and, reciprocally, perineurial glia require Schwann cells for proper development and response to injury (Parmantier et al., 1999; Sharghi-Namini et al., 2006; Kucenas et al., 2008b, 2009;

Lewis and Kucenas, 2014). These studies demonstrate that peripheral glial–glial interactions are essential during motor nerve assembly, but what happens in disease states in which myelin sheath perturbations occur? Are the remaining ensheathing glial cells, specifically perineurial glia, able to compensate for this aberration and maintain a functional nerve?

Here, we provide evidence that, despite the presence of centrally derived myelin along *erbb3b* mutant motor nerves, perineurial glia are capable of exiting the CNS, extending along peripheral motor nerves during development and expressing ZO-1, a marker of tight junctions and perineurial glial maturation. Our ultrastructural data corroborate our *in vivo* imaging and immunohistochemistry findings (i.e., the perineurium is present in *erbb3b*^{-/-} adults). However, in some areas, perineurial morphology is comparable with wildtype, whereas other areas are disorganized or patchy (Fig. 4E, right) and we observed large pockets of high levels of tight junctions (Fig. 7H, bottom). Additionally, the perineurium barrier function is compromised in *erbb3b*^{-/-} zebrafish as we observed a fluorescent dye infiltrate within the motor nerve (Fig. 8B), and we hypothesize that the leaky barrier may be due to the observed morphological alterations described above. Future studies further characterizing the development of the perineurial barrier will help provide a better understanding about mechanisms underlying how it is formed and ultimately functions. However, despite the complete absence of peripheral myelinating glial cells, perineurial glia are remarkably plastic in response to injury by forming phagocytic vesicles and glial bridges spanning the injury site. Although the presence of OPCs from the spinal cord does not have detrimental effects on certain perineurial glial developmental and injury response behaviors, the barrier integrity is ultimately impaired. Therefore, normal peripheral glial–glial interactions are important for development and maintenance of spinal motor nerves (Parmantier et al., 1999; Sharghi-Namini et al., 2006).

Is *erbb3b* an important mechanism for mediating perineurial glial development and maturation?

The molecular mechanisms involved in perineurial glial development and maturation are poorly understood. Desert hedgehog (Dhh), derived from Schwann cells, is important for perineurium maturation as *Dhh*^{-/-} mice have a patchy, disorganized perineurium with abnormal tight junction formation (Parmantier et al., 1999). However, it is unknown when, and if, hedgehog signaling is present along the peripheral nerves in *erbb3b*^{-/-} mutants because neural crest-derived Schwann cells fail to associate with motor nerves (Fig. 2B) (Smith et al., 2014).

In this study, we used a zebrafish line with a mutation in the *erbb3b* receptor tyrosine kinase as a model for perturbing peripheral myelinating glial cells (Lyons et al., 2005), leading to the ectopic migration of OPCs (Smith et al., 2014), to investigate whether perineurial glia are affected by the presence of centrally derived myelin. However, it is unknown whether perineurial glial cells express *erbb3b*, and if so, whether it is the perturbation in *erbb3b*, or the presence of ectopic OPCs and lack of peripheral myelinating glia, that is responsible for our results. To investigate this, we used a zebrafish line with a mutation in *neuregulin 1 type III* (*nrg*^{z26-/-}) (Perlin et al., 2011), an axonally expressed growth factor that binds to the *erbb2-erbb3* heterodimer receptor complex on peripheral myelinating glial cells to initiate the peripheral myelination signaling cascade (Chen et al., 2006; Brinkmann et al., 2008). In *nrg*^{-/-} larvae, peripheral myelinating glia fail to associate with spinal motor nerves and OPCs ectopically exit the CNS (data not shown), but *erbb3b* is intact. Intriguingly, we ob-

Figure 10. Inhibiting TGF- β 1 decreases ZO-1 labeling along spinal motor nerves. All images are lateral views of 8 dpf *nkx2.2a:megfp* wildtype and *erbb3b*^{-/-} larvae, with dorsal to the top and anterior to the left. **A**, pSmad3 labeling (arrowheads) is evident along motor nerves in 8 dpf *nkx2.2a:megfp* wildtype larvae treated with 0.5% DMSO from 6 to 8 dpf. **B**, pSmad3 labeling (arrowheads) was reduced along motor nerves treated with 25 μ M SB431542 from 6 to 8 dpf. **C**, Quantification of pSmad3 labeling in 8 dpf *nkx2.2a:megfp* wildtype larvae treated with 0.5% DMSO or 25 μ M SB431542 between 6 and 8 dpf. **D**, **E**, At 8 dpf, *nkx2.2a:megfp* wildtype (**D**) and *erbb3b*^{-/-} (**E**) larvae exposed to 0.5% DMSO from 6 to 8 dpf have ZO-1 labeling (arrowhead) from the MEP TZ, and it extends ventrally toward the horizontal myoseptum. **F**, **G**, When treated with 25 μ M SB431542, ZO-1 labeling (arrowhead) is reduced in 8 dpf *nkx2.2a:megfp* wildtype (**F**) and *erbb3b*^{-/-} (**G**) larvae. **H**, Quantification of the extension length of ZO-1 labeling from **D**–**G**. There is a statistically significant reduction in the extension length of ZO-1 labeling along spinal motor nerves in 8 dpf wildtype larvae treated with 25 μ M SB431542 compared with wildtype larvae treated with 0.5% DMSO ($p = 0.0001$). Similarly, 8 dpf *erbb3b*^{-/-} larvae treated with 25 μ M SB431542 had a significant reduction in ZO-1 labeling compared with *erbb3b*^{-/-} larvae treated with 0.5% DMSO ($p < 0.0001$). Intriguingly, there is also a statistically significant reduction in the extension length of ZO-1 labeling along spinal motor nerves in 8 dpf *erbb3b*^{-/-} larvae treated with 25 μ M SB431542 compared with wildtype larvae treated with 25 μ M SB431542 ($p = 0.0144$). Statistical significance was measured using an unpaired t test. Error bars are representative of SEM. Scale bars, 25 μ m. * $p < 0.05$; *** $p < 0.0005$; ns, not significant.

served perineurial glial migration, proliferation, and extension behaviors in *nrg*^{-/-} larvae (data not shown) that are similar to those we observed in *erbb3b*^{-/-} larvae. Although future studies are needed to definitively rule out whether perineurial glia express *erbb3b*, the results we observed in *nrg*^{-/-} larvae are consistent with the hypothesis that loss of *erbb3b* does not directly affect perineurial glia and, instead, centrally derived myelinating glial cells are what influence perineurial glial developmental and regenerative behaviors in *erbb3b*^{-/-} larvae.

Do ectopically located OPCs impair perineurial glial injury responses?

Peripheral nerves demonstrate remarkable regenerative capabilities, a phenomenon that the CNS lacks (Faroni et al., 2015). Following PNS axonal transection, Schwann cells transdifferentiate to help with regenerative processes and, as we have recently shown, perineurial glia are essential components also involved in injury responses (Lewis and Kucenas, 2013, 2014). In this study, we demonstrate that transecting a nerve containing ectopically located OPCs impairs perineurial membrane extension toward an injury site. However, perineurial–perineurial injury response behaviors, such as forming phagocytic vesicles and a glial bridge spanning the transection site, are not impaired on axonal tracts that do not contain ectopically located OPCs. This raises the intriguing possibility that OPCs have remarkable control over regeneration and that it is not necessarily the permissive environment of the PNS, but rather the permissive qualities of the peripheral ensheathing glial cells themselves, which are responsible for the remarkable repair that typically occurs in the PNS. Because it has previously been reported that oligodendrocytes may impede CNS regeneration by expressing axonal growth inhibitors (Caroni and Schwab, 1988; Fawcett, 2006; Busch and Silver, 2007; Faroni et al., 2015), the lack of peripheral myelinating glial cells, combined with the presence of OPCs along spinal motor nerves, may be a combination that impairs certain perineurial glial regenerative behaviors in *erbb3b*^{-/-} larvae that are required for PNS repair. Future studies investigating whether OPCs located along *erbb3b*^{-/-} nerves secrete or express signaling cues that negatively affect perineurial cells are exciting next steps for understanding the regenerative potential of both the CNS and PNS.

Although mechanisms involved in perineurial glial regeneration behaviors are largely unknown, one potential mechanism that may be involved is TGF- β 1. A previous study demonstrated that applying a neutralizing antibody to TGF- β 1 via perineurial administration after axonal transection improved sciatic nerve regeneration (Davison et al., 1999). This report, in combination with our observation of phosphorylated Smad3, a downstream signaling transducer of TGF- β 1, along spinal motor nerves, leads us to hypothesize that inhibiting TGF- β 1 may enhance perineurial glial injury responses. Future studies aimed at investigating the role of TGF- β 1 as well as identifying additional signaling cues that mediate the various perineurial injury responses may reveal novel therapeutic targets for treating peripheral nerve injuries and promoting regeneration.

Do ectopic OPCs increase zonula occludens-1 labeling within the BNB?

Above we show that perineurial glia have an increase in the extension of ZO-1 labeling along *erbb3b*^{-/-} motor nerves compared with wildtype controls, leading us to hypothesize that the presence of OPCs along spinal motor nerves may be influencing this increase by helping to promote the integrity of the BNB. A

recent study demonstrated that OPCs help support and promote the integrity of the BBB, as evidenced by an increase in ZO-1 labeling and the formation of tight junctions by endothelial cells, both *in vitro* and *in vivo* (Seo et al., 2014). OPCs secrete TGF- β 1 in the CNS, activating the MEK/ERK signaling cascade, eventually leading to tight junction protein expression by endothelial cells of the BBB (Seo et al., 2014). We investigated the involvement of TGF- β 1 on ZO-1 expression in the BNB by inhibiting TGF- β 1 signaling in both wildtype and mutant larvae. We observed a significant decrease in the extension of ZO-1 labeling in *erbb3b*^{-/-} larvae compared with wildtype. This result demonstrates that OPCs are partially responsible for the increase in extension of ZO-1 labeling observed in *erbb3b*^{-/-}. Additionally, we observed a decrease in the extent of ZO-1 labeling along wildtype motor nerves treated with the TGF- β 1 inhibitor as well, although the reduction was not as great as in *erbb3b*^{-/-} larvae. This suggests that TGF- β 1 is involved in wildtype perineurial glial development as well. Schwann cells are known to express TGF- β 1 during development (Scherer et al., 1993; Feng and Ko, 2008) and, because of this, may be a likely source of this ligand for perineurial glia.

Together, our work reinforces the importance of glial–glial interactions along spinal motor nerves during development and in response to injury. Intriguingly, our data reveal that perineurial glia can adapt to the presence of ectopically located, centrally derived OPCs for certain developmental behaviors and injury responses, demonstrating their incredible plasticity in response to myelinopathies and axonal transections. Furthermore, we identified that TGF- β 1 has an influence on ZO-1 expression, a marker of perineurial differentiation (Kristensson and Olsson, 1971; Puumi et al., 2004). Although perineurial glial cells demonstrate remarkable plasticity to myelin perturbations, their barrier function is compromised, resulting in a nerve that is left unprotected and vulnerable. Therefore, our data further support the importance of Schwann cell–perineurial glial interactions in forming and maintaining functional peripheral nerves (Parman-tier et al., 1999; Sharghi-Namini et al., 2006).

References

- Akert K, Sandri C, Weibel ER, Peper K, Moor H (1976) The fine structure of the perineurial endothelium. *Cell Tissue Res* 165:281–295. [Medline](#)
- Appel B, Korzh V, Glasgow E, Thor S, Edlund T, Dawid IB, Eisen JS (1995) Motoneuron fate specification revealed by patterned LIM homeobox gene expression in embryonic zebrafish. *Development* 121:4117–4125. [Medline](#)
- Arduini BL, Bosse KM, Henion PD (2009) Genetic ablation of neural crest cell diversification. *Development* 136:1987–1994. [CrossRef Medline](#)
- Arthur-Farraj PJ, Latouche M, Wilton DK, Quintes S, Chabrol E, Banerjee A, Woodhoo A, Jenkins B, Rahman M, Turmaine M, Wicher GK, Mitter R, Greensmith L, Behrens A, Raivich G, Mirsky R, Jessen KR (2012) c-Jun reprograms Schwann cells of injured nerves to generate a repair cell essential for regeneration. *Neuron* 75:633–647. [CrossRef Medline](#)
- Banerjee S, Isaacman-Beck J, Schneider VA, Granato M (2013) A novel role for Lh3 dependent ECM modifications during neural crest cell migration in zebrafish. *PLoS One* 8:e54609. [CrossRef Medline](#)
- Barrallo-Gimeno A, Holzschuh J, Driever W, Knapik EW (2004) Neural crest survival and differentiation in zebrafish depends on mont blanc/ tfap2a gene function. *Development* 131:1463–1477. [CrossRef Medline](#)
- Binari LA, Lewis GM, Kucenas S (2013) Perineurial glia require Notch signaling during motor nerve development but not regeneration. *J Neurosci* 33:4241–4252. [CrossRef Medline](#)
- Birchmeier C (2009) ErbB receptors and the development of the nervous system. *Exp Cell Res* 315:611–618. [CrossRef Medline](#)
- Bourne GH (1968) The structure and function of nervous tissue, Vol 5. San Diego: Academic.
- Brinkmann BG, Agarwal A, Sereda MW, Garratt AN, Müller T, Wende H, Stassart RM, Nawaz S, Humml C, Velanac V, Radyushkin K, Goebbels S,

- Fischer TM, Franklin RJ, Lai C, Ehrenreich H, Birchmeier C, Schwab MH, Nave KA (2008) Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. *Neuron* 59:581–595. [CrossRef Medline](#)
- Britsch S, Goerich DE, Riethmacher D, Peirano RI, Rossner M, Nave KA, Birchmeier C, Wegner M (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev* 15:66–78. [CrossRef Medline](#)
- Busch SA, Silver J (2007) The role of extracellular matrix in CNS regeneration. *Curr Opin Neurobiol* 17:120–127. [CrossRef Medline](#)
- Caroni P, Schwab ME (1988) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron* 1:85–96. [CrossRef Medline](#)
- Casari A, Schiavone M, Facchinello N, Vettori A, Meyer D, Tiso N, Moro E, Argenton F (2014) Developmental biology. *Dev Biol* 396:81–93. [CrossRef Medline](#)
- Chen S, Velardez MO, Warot X, Yu ZX, Miller SJ, Cros D, Corfas G (2006) Neuregulin 1-erbB signaling is necessary for normal myelination and sensory function. *J Neurosci* 26:3079–3086. [CrossRef Medline](#)
- Clark JK, O'Keefe A, Mastracci TL, Sussel L, Matisse MP, Kucenas S (2014) Mammalian Nkx2.2⁺ perineurial glia are essential for motor nerve development. *Dev Dyn* 243:1116–1129. [CrossRef Medline](#)
- Corfas G, Velardez MO, Ko CP, Ratner N, Peles E (2004) Mechanisms and roles of axon-Schwann cell interactions. *J Neurosci* 24:9250–9260. [CrossRef Medline](#)
- Coulpier F, Decker L, Funalot B, Vallat JM, Garcia-Bragado F, Charnay P, Topilko P (2010) CNS/PNS boundary transgression by central glia in the absence of Schwann cells or Krox20/Egr2 function. *J Neurosci* 30:5958–5967. [CrossRef Medline](#)
- Czopka T, French-Constant C, Lyons DA (2013) Individual oligodendrocytes have only a few hours in which to generate new myelin sheaths in vivo. *Dev Cell* 25:599–609. [CrossRef Medline](#)
- Davison SP, McCaffrey TV, Porter MN, Manders E (1999) Improved nerve regeneration with neutralization of transforming growth factor-beta1. *Laryngoscope* 109:631–635. [CrossRef Medline](#)
- Du Plessis DG, Mouton YM, Muller CJ, Geiger DH (1996) An ultrastructural study of the development of the chicken perineurial sheath. *J Anat* 189:631–641. [Medline](#)
- Dutton KA, Pauliny A, Lopes SS, Elworthy S, Carney TJ, Rauch J, Geisler R, Haffter P, Kelsh RN (2001) Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* 128:4113–4125. [Medline](#)
- Ek CJ, Habgood MD, Dziegielewska KM, Saunders NR (2003) Structural characteristics and barrier properties of the choroid plexuses in developing brain of the opossum (*Monodelphis domestica*). *J Comp Neurol* 460:451–464. [CrossRef Medline](#)
- Emery B (2010) Regulation of oligodendrocyte differentiation and myelination. *Science* 330:779–782. [CrossRef Medline](#)
- Faroni A, Mobasser SA, Kingham PJ, Reid AJ (2015) Peripheral nerve regeneration: experimental strategies and future perspectives. *Adv Drug Deliv Rev* 82:160–167. [CrossRef Medline](#)
- Fawcett JW (2006) Overcoming inhibition in the damaged spinal cord. *J Neurotrauma* 23:371–383. [CrossRef Medline](#)
- Feng Z, Ko CP (2008) Schwann cells promote synaptogenesis at the neuromuscular junction via transforming growth factor-1. *J Neurosci* 28:9599–9609. [CrossRef Medline](#)
- Gamble HJ (1966) Further electron microscope studies of human foetal peripheral nerves. *J Anat* 100:487–502. [Medline](#)
- Gamble HJ, Breathnach AS (1965) An electron-microscope study of human foetal peripheral nerves. *J Anat* 99:573–584. [Medline](#)
- Gamble HJ, Eames RA (1964) An electron microscope study of the connective tissues of human peripheral nerve. *J Anat* 98:655–663. [Medline](#)
- Garratt AN, Britsch S, Birchmeier C (2000a) Neuregulin, a factor with many functions in the life of a Schwann cell. *Bioessays* 22:987–996. [CrossRef Medline](#)
- Garratt AN, Voiculescu O, Topilko P, Charnay P, Birchmeier C (2000b) A dual role of erbB2 in myelination and in expansion of the Schwann cell precursor pool. *J Cell Biol* 148:1035–1046. [CrossRef Medline](#)
- Ho DM, Chan J, Bayliss P, Whitman M (2006) Inhibitor-resistant type I receptors reveal specific requirements for TGF- β signaling in vivo. *Dev Biol* 295:730–742. [CrossRef Medline](#)
- Honjo Y, Payne L, Eisen JS (2011) Somatosensory mechanisms in zebrafish lacking dorsal root ganglia. *J Anat* 218:271–276. [CrossRef Medline](#)
- Hughes RA (2002) Peripheral neuropathy. *Br Med J* 324:466–469. [CrossRef](#)
- Jessen KR, Mirsky R (1999) Schwann cells and their precursors emerge as major regulators of nerve development. *Trends Neurosci* 22:402–410. [CrossRef Medline](#)
- Jessen KR, Mirsky R (2005) The origin and development of glial cells in peripheral nerves. *Nat Rev Neurosci* 6:671–682. [CrossRef Medline](#)
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* 203:253–310. [CrossRef Medline](#)
- Kirby BB, Takada N, Latimer AJ, Shin J, Carney TJ, Kelsh RN, Appel B (2006) In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. *Nat Neurosci* 9:1506–1511. [CrossRef Medline](#)
- Kitisin K, Saha T, Blake T, Golestaneh N, Deng M, Kim C, Tang Y, Shetty K, Mishra B, Mishra L (2007) Tgf-Beta signaling in development. *Sci STKE* 2007:cm1. [CrossRef Medline](#)
- Kristensson K, Olsson Y (1971) The perineurium as a diffusion barrier to protein tracers. *Acta Neuropathol* 17:127–138. [CrossRef Medline](#)
- Kucenas S, Snell H, Appel B (2008a) nkx2.2a promotes specification and differentiation of a myelinating subset of oligodendrocyte lineage cells in zebrafish. *Neuron Glia Biol* 4:71–81. [CrossRef Medline](#)
- Kucenas S, Takada N, Park HC, Woodruff E, Broadie K, Appel B (2008b) CNS-derived glia ensheath peripheral nerves and mediate motor root development. *Nat Neurosci* 11:143–151. [CrossRef Medline](#)
- Kucenas S, Wang WD, Knapik EW, Appel B (2009) A selective glial barrier at motor axon exit points prevents oligodendrocyte migration from the spinal cord. *J Neurosci* 29:15187–15194. [CrossRef Medline](#)
- Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M (1998) Sox10, a novel transcriptional modulator in glial cells. *J Neurosci* 18:237–250. [Medline](#)
- Lewis GM, Kucenas S (2013) Motor nerve transection and time-lapse imaging of glial cell behaviors in live zebrafish. *J Vis Exp* 20:76. [CrossRef Medline](#)
- Lewis GM, Kucenas S (2014) Perineurial glia are essential for motor axon regrowth following nerve injury. *J Neurosci* 34:12762–12777. [CrossRef Medline](#)
- Liddelow SA, Dziegielewska KM, Ek CJ, Johansson PA, Potter AM, Saunders NR (2009) Cellular transfer of macromolecules across the developing choroid plexus of *Monodelphis domestica*. *Eur J Neurosci* 29:253–266. [CrossRef Medline](#)
- Lyons DA, Pogoda HM, Voas MG, Woods IG, Diamond B, Nix R, Arana N, Jacobs J, Talbot WS (2005) erbb3 and erbb2 are essential for Schwann cell migration and myelination in zebrafish. *Curr Biol* 15:513–524. [CrossRef Medline](#)
- Maurel P, Salzer JL (2000) Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI 3-kinase activity. *J Neurosci* 20:4635–4645. [Medline](#)
- Montero-Balaguer A, Lang MR, Sachdev SW, Knappmeyer C, Stewart RA, De La Guardia A, Hatzopoulos AK, Knapik EW (2006) The mother superimutation ablates foxd3 activity in neural crest progenitor cells and depletes neural crest derivatives in zebrafish. *Dev Dyn* 235:3199–3212. [CrossRef Medline](#)
- Nawaz S, Sánchez P, Schmitt S, Snaidero N, Mitkovski M, Velte C, Brückner BR, Alexopoulos I, Czopka T, Jung SY, Rhee JS, Janshoff A, Witke W, Schaap IA, Lyons DA, Simons M (2015) Actin filament turnover drives leading edge growth during myelin sheath formation in the central nervous system. *Dev Cell* 34:139–151. [CrossRef Medline](#)
- Newbern J, Birchmeier C (2010) Nrg1/ErbB signaling networks in Schwann cell development and myelination. *Semin Cell Dev Biol* 21:922–928. [CrossRef Medline](#)
- Paratore C, Goerich DE, Suter U, Wegner M, Sommer L (2001) Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development* 128:3949–3961. [Medline](#)
- Parmentier E, Lynn B, Lawson D, Turmaine M, Namini SS, Chakrabarti L, McMahon AP, Jessen KR, Mirsky R (1999) Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. *Neuron* 23:713–724. [CrossRef Medline](#)
- Pereira JA, Lebrun-Julien F, Suter U (2012) Molecular mechanisms regulat-

- ing myelination in the peripheral nervous system. *Trends Neurosci* 35: 123–134. [CrossRef Medline](#)
- Peri F, Nüsslein-Volhard C (2008) Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. *Cell* 133:916–927. [CrossRef Medline](#)
- Perlin JR, Lush ME, Stephens WZ, Piotrowski T, Talbot WS (2011) Neuronal Neuregulin 1 type III directs Schwann cell migration. *Development* 138:4639–4648. [CrossRef Medline](#)
- Pummi KP, Heape AM, Grénman RA, Peltonen JT, Peltonen SA (2004) Tight junction proteins ZO-1, occludin, and claudins in developing and adult human perineurium. *J Histochem Cytochem* 52:1037–1046. [CrossRef Medline](#)
- Raphael AR, Lyons DA, Talbot WS (2011) ErbB signaling has a role in radial sorting independent of Schwann cell number. *Glia* 59:1047–1055. [CrossRef Medline](#)
- Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* 389:725–730. [CrossRef Medline](#)
- Rosenberg AF, Wolman MA, Franzini-Armstrong C, Granato M (2012) In vivo nerve-macrophage interactions following peripheral nerve injury. *J Neurosci* 32:3898–3909. [CrossRef Medline](#)
- Rosenberg AF, Isaacman-Beck J, Franzini-Armstrong C, Granato M (2014) Schwann cells and deleted in colorectal carcinoma direct regenerating motor axons towards their original path. *J Neurosci* 34:14668–14681. [CrossRef Medline](#)
- Rowitch DH (2004) Glial specification in the vertebrate neural tube. *Nat Rev Neurosci* 5:409–419. [CrossRef Medline](#)
- Saunders NR, Dziegielewska KM, Møllgård K, Habgood MD (2015) Markers for blood–brain barrier integrity: how appropriate is Evans blue in the twenty-first century and what are the alternatives? *Front Neurosci* 9:13. [CrossRef Medline](#)
- Scherer SS, Kamholz J, Jakowlew SB (1993) Axons modulate the expression of transforming growth factor-betas in Schwann cells. *Glia* 8:265–276. [CrossRef Medline](#)
- Seo JH, Maki T, Maeda M, Miyamoto N, Liang AC, Hayakawa K, Pham LD, Suwa F, Taguchi A, Matsuyama T, Ihara M, Kim KW, Lo EH, Arai K (2014) Oligodendrocyte precursor cells support blood–brain barrier integrity via TGF- β signaling. *PLoS One* 9:e103174. [CrossRef Medline](#)
- Shanthaveerappa TR, Bourne GH (1966) Perineurial epithelium: a new concept of its role in the integrity of the peripheral nervous system. *Science* 154:1464–1467. [CrossRef Medline](#)
- Sharghi-Namini S, Turmaine M, Meier C, Sahni V, Umehara F, Jessen KR, Mirsky R (2006) The structural and functional integrity of peripheral nerves depends on the glial-derived signal desert hedgehog. *J Neurosci* 26:6364–6376. [CrossRef Medline](#)
- Smith CJ, Morris AD, Welsh TG, Kucenas S (2014) Contact-mediated inhibition between oligodendrocyte progenitor cells and motor exit point glia establishes the spinal cord transition zone. *PLoS Biol* 12:e1001961. [CrossRef Medline](#)
- Snaidero N, Möbius W, Czopka T, Hekking LH, Mathisen C, Verkleij D, Goebbels S, Edgar J, Merkler D, Lyons DA, Nave KA, Simons M (2014) Myelin membrane wrapping of CNS axons by PI(3,4,5)P3-dependent polarized growth at the inner tongue. *Cell* 156:277–290. [CrossRef Medline](#)
- Sun Z, Jin P, Tian T, Gu Y, Chen YG, Meng A (2006) Activation and roles of ALK4/ALK7-mediated maternal TGF β signals in zebrafish embryo. *Biochem Biophys Res Commun* 345:694–703. [CrossRef Medline](#)
- Takada N, Kucenas S, Appel B (2010) Sox10 is necessary for oligodendrocyte survival following axon wrapping. *Glia* 58:996–1006. [CrossRef Medline](#)
- Thomas PK (1963) The connective tissue of peripheral nerve: an electron microscope study. *J Anat* 97:35–44. [Medline](#)
- Torii T, Miyamoto Y, Takada S, Tsumura H, Arai M, Nakamura K, Ohbuchi K, Yamamoto M, Tanoue A, Yamauchi J (2014) In vivo knockdown of ErbB3 in mice inhibits Schwann cell precursor migration. *Biochem Biophys Res Commun* 452:782–788. [CrossRef Medline](#)
- Waller A (1850) Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibres. *Philos Trans R Soc Lond B Biol Sci* 140:423–429.
- Wang WD, Melville DB, Montero-Balaguer M, Hatzopoulos AK, Knapik EW (2011) Tfap2a and Foxd3 regulate early steps in the development of the neural crest progenitor population. *Dev Biol* 360:173–185. [CrossRef Medline](#)
- Weinberg HJ, Spencer PS, Raine CS (1975) Aberrant PNS development in dystrophic mice. *Brain Res* 88:532–537. [CrossRef Medline](#)