Loss of Tuberous Sclerosis Complex1 in Adult Oligodendrocyte Progenitor Cells Enhances Axon Remyelination and Increases Myelin Thickness after a Focal Demyelination

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Although the mammalian target of rapamycin (mTOR) is an essential regulator of developmental oligodendrocyte differentiation and myelination, oligodendrocyte-specific deletion of tuberous sclerosis complex (TSC), a major upstream inhibitor of mTOR, surprisingly also leads to hypomyelination during CNS development. However, the function of TSC has not been studied in the context of remyelination. Here, we used the inducible Cre-lox system to study the function of TSC in the remyelination of a focal, lysolecithin-demyelinated lesion in adult male mice. Using two different mouse models in which *Tsc1* is deleted by Cre expression in oligodendrocyte progenitor cells (OPCs) or in premyelinating oligodendrocytes, we reveal that deletion of *Tsc1* affects oligodendroglia differently depending on the stage of the oligodendrocyte lineage. *Tsc1* deletion from NG2⁺ OPCs accelerated remyelination. Conversely, *Tsc1* deletion from proteolipid protein (PLP)-positive oligodendrocytes slowed remyelination. Contrary to developmental myelination, there were no changes in OPC or oligodendrocyte numbers in either model. Our findings reveal a complex role for TSC in oligodendrocytes during remyelination in which the timing of *Tsc1* deletion is a critical determinant of its effect on remyelination. Moreover, our findings suggest that TSC has different functions in developmental myelination and remyelination.

Key words: adult OPCs; mTOR; NG2; remyelination; TSC

Significance Statement

Myelin loss in demyelinating disorders such as multiple sclerosis results in disability due to loss of axon conductance and axon damage. Encouragingly, the nervous system is capable of spontaneous remyelination, but this regenerative process often fails. Many chronically demyelinated lesions have oligodendrocyte progenitor cells (OPCs) within their borders. It is thus of great interest to elucidate mechanisms by which we might enhance endogenous remyelination. Here, we provide evidence that deletion of *Tsc1* from OPCs, but not differentiating oligodendrocytes, is beneficial to remyelination. This finding contrasts with the loss of oligodendroglia and hypomyelination seen with *Tsc1* or *Tsc2* deletion in the oligodendrocyte lineage during CNS development and points to important differences in the regulation of developmental myelination and remyelination.

Introduction

Within the CNS, oligodendrocytes are tasked with the myelination of axons to facilitate rapid signal conduction and provide

trophic and metabolic support (Griffiths et al., 1998; Lappe-Siefke et al., 2003). Loss of myelin and oligodendrocytes in the adult CNS is a hallmark of demyelinating diseases such as multiple sclerosis (MS). Spontaneous endogenous remyelination occurs initially in some MS lesions, but often fails after repeated

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demyelinating attacks (Patrikios et al., 2006). Moreover, the myelin formed during remyelination is characteristically thinner than that formed during development and the remyelination capacity of the CNS diminishes with age (Shields et al., 1999; Goldschmidt et al., 2009). Therefore, much research has focused on elucidating the mechanisms underlying oligodendroglia maturation and myelination in both the developmental and the remyelination context to develop therapies to better promote remyelination (Keough and Yong, 2013).

The mammalian target of rapamycin (mTOR), and particularly mTOR complex 1 (mTORC1), has emerged as a key modulator of developmental oligodendrocyte maturation and myelination. Disrupting mTORC1 signaling perinatally in mice through the deletion of mTOR or the mTORC1 adaptor protein raptor results in delayed oligodendrocyte differentiation and hypomyelination (Bercury et al., 2014; Lebrun-Julien et al., 2014; Wahl et al., 2014; Zou et al., 2014). A similar phenotype was observed after deleting the small GTPase Rheb, a direct upstream activator of mTORC1 (Zou et al., 2014). Conversely, enhancing mTOR signaling during development is sufficient to increase myelin thickness: either deletion of the upstream inhibitor phosphatase and tensin homolog (PTEN) or constitutive activation of AKT results in increased mTOR signaling and hypermyelination (Flores et al., 2008; Harrington et al., 2010). Moreover, the hypermyelination phenotype due to constitutive AKT activation is mTOR dependent because it can be inhibited by rapamycin, a pharmacological inhibitor of mTOR (Narayanan et al., 2009).

The tuberous sclerosis complex (TSC), consisting of hamartin (TSC1) and tuberin (TSC2), acts upstream of Rheb to inhibit mTORC1 signaling (van Slegtenhorst et al., 1998; Inoki et al., 2003; Zhang et al., 2003). Given the evidence that mTOR signaling is a positive regulator of developmental myelination, inactivation of TSC might be expected to cause hypermyelination similar to the phenotype resulting from PTEN deletion or expression of constitutively activated AKT. It is thus surprising that several studies recently reported that deletion of *Tsc1* or *Tsc2* in oligodendrocyte progenitor cells (OPCs) during development in mouse models results in hypomyelination (Lebrun-Julien et al., 2014; Carson et al., 2015; Jiang et al., 2016). Disruption to the oligodendrocyte lineage was not explicitly examined when *Tsc1* was deleted using CNP-Cre, but mRNA expression of oligodendroglia transcription factors Olig2 and Sox10 were reduced, suggesting fewer cells in the lineage (Lebrun-Julien et al., 2014). Jiang et al. (2016) deleted *Tsc1* using Olig1-Cre and showed increased oligodendrocyte apoptosis attributed to elevated endoplasmic reticulum (ER) stress. In contrast, Carson et al. (2015) demonstrated a fate shift from the oligodendroglial to the astrocyte lineage after deleting *Tsc2* using an Olig2-Cre.

The phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway has not been studied extensively in remyelination. Systemic administration of the pharmacological mTOR inhibitor rapamycin inhibited remyelination in mice fed a cuprizone diet (Sachs et al., 2014). Surprisingly, deletion of PTEN from the oligodendroglial lineage during development had no effect on remyelination of a focal demyelinated lesion in the adult spinal cord, although the brains and spinal cords of these mice were already hypermyelinated due to developmental effects (Harrington et al., 2010).

The goal of the current study was to determine how deletion of *Tsc1* from the oligodendrocyte lineage affects remyelination of a focal demyelinating insult in the adult spinal cord. To date, TSC deletion in oligodendrocyte precursors before developmental myelination has resulted in hypomyelination, the opposite of what one might expect from hyperactivating mTORC1 signaling.

Here, we demonstrate that deletion of *Tsc1* from NG2⁺ adult OPCs augments endogenous remyelination, resulting in more rapid remyelination, which is consistent with the classical concept of the role of TSC1 inhibition of mTORC1. Furthermore, we show that this beneficial effect is stage specific such that deletion of *Tsc1* in differentiating proteolipid protein-positive (PLP⁺) oligodendrocytes fails to improve remyelination.

Materials and Methods

Experimental animals. All mouse protocols were conducted in accordance with Rutgers University Institutional Animal Care and Use Committee. Mice homozygous for the floxed-*Tsc1* allele (The Jackson Laboratory 005680, RRID:IMSR_JAX:005680) were bred with either NG2-Cre^{ERT2} mice (The Jackson Laboratory 008538, RRID:IMSR_JAX:008538) to generate NG2-Cre; *Tsc1*^{fl/fl} mice, henceforth referred to as NG2-*Tsc1* conditional knock-out (cKO) mice or with PLP-Cre^{ERT2} mice (The Jackson Laboratory 005975, RRID:IMSR_JAX:005975) to generate PLP-Cre; *Tsc1*^{fl/fl}, henceforth referred to as PLP-*Tsc1*cKO mice. The resulting mice were on a mixed C57BL/6J, BALB/cJ, 129/SvJ background. The lines also carried a dual-reporter transgene (B6.129(Cg)-Gt(ROSA)26Sortm4^{(ACTB-tdTomato, EGFP)^{Luo}/J}, Jackson Laboratories, RRID:IMSR_JAX:007676) that allowed for assessment of recombination efficiency and specificity as all cells express tomato red (mTmG) in the absence of active Cre-recombinase and green fluorescent protein (GFP) when Cre-recombinase is active. Only males were used in the remyelination experiments to reduce background recombination in females due to endogenous estrogen.

For remyelination studies, tamoxifen was injected intraperitoneally (75 mg/kg) to induce recombination in adult (8–12 weeks old) male mice. Tamoxifen was dissolved in a 9:1 ratio of sesame oil:100% ethanol. NG2-*Tsc1*cKO mice were injected with tamoxifen for 5 consecutive days, followed by 4 d to clear the tamoxifen from the system before lysocleithin injections into the dorsal white matter (see below). Controls were either of the same genotype and injected with vehicle or were Cre negative (WT; *Tsc1*^{fl/fl}, dual-reporter mice) and injected with tamoxifen. Because these controls were not phenotypically distinct from each other, they were combined into a single control group in the subsequent analyses. PLP-*Tsc1*cKO or control Cre-negative mice were injected with tamoxifen for 6 consecutive days beginning 8 d after the lysocleithin injection. All controls were Cre-negative tamoxifen-injected animals to control for any effects of tamoxifen on remyelination.

To validate loss of TSC1 in the NG2-*Tsc1*cKO line, we injected tamoxifen (75 mg/kg) for 5 consecutive days into Cre-negative or Cre-positive mice at 4 weeks of age ($n = 3$ per genotype). Animals were taken for tissue harvesting 18 d after the last tamoxifen injection to mimic the timing of the analyses in the lesioned animals. NG2⁺ cells were isolated from brains and spinal cords using NG2 microbeads (Miltenyi Biotec 130-097-170) and MS columns (Miltenyi Biotec 130-042-201) and used for protein isolation. To validate TSC1 deletion in the PLP-*Tsc1*cKO line, Cre-positive or Cre-negative mice at PND 8 ($n = 3$ per genotype) were injected with tamoxifen (60 mg/kg) for 4 consecutive days. Animals were taken for tissue harvesting 5 d after the last tamoxifen injection to test for loss of TSC1 to resemble the time frame for the paradigm used for the lesion analyses in the PLP-*Tsc1*cKO line, in which animals were killed 8 d after the final tamoxifen injection. The O4⁺ oligodendrocytes were isolated from spinal cords using O4 microbeads (Miltenyi Biotec 130-094-543) and MS columns (Miltenyi Biotec 130-042-201) and used for protein isolation. Total protein was isolated from either the NG2⁺ or O4⁺ cells from these two deletion paradigms using RIPA lysis buffer (Thermo Scientific 89900) containing protease and phosphatase inhibitors and separated by SDS-PAGE as described previously (Tyler et al., 2009). TSC1 antibody (Cell Signaling Technology 6935, RRID:AB_10860420) was diluted 1:1000 and incubated overnight at 4°C.

Lysocleithin-induced demyelination. Lysocleithin (Sigma-Aldrich L4129) was injected into the dorsal column of the thoracic spinal cord of adult male mice 8–13 weeks of age. A dorsal laminectomy was performed at T10 of the spinal cord and a hole was made in the dura using a 26 gauge syringe needle. A 5 μ l Neurosyringe (Hamilton 65460–02) with a custom 33 gauge

needle (Hamilton 7803-05) was lowered into the dorsal white matter until the needle bevel was entirely within the cord. Then, 1 μ l of 1% lysolecithin dissolved in a 0.9% NaCl solution was injected at a rate of 0.25–0.3 μ l/min. The needle was left in place for 2 min after the injection to prevent liquid reflux. The injection site was marked with charcoal for identification.

Tissue preparation and immunostaining. NG2-*Tsc1*CKO animals and corresponding controls were taken for analysis at 7 d postlesion (dpl), when lesion size peaks; 14 dpl, a time point that corresponds to early remyelination; and 21 dpl, when more robust remyelination is present (Jeffery and Blakemore, 1995; Luo et al., 2014). Animals within the PLP-*Tsc1*CKO group and their corresponding controls were taken for analysis at 21 dpl. A total of three to six animals per genotype from the corresponding time point were used for all analyses described.

Mice were intracardially perfused with 10 ml of an ice-cold PBS solution containing phosphatase inhibitors and heparin followed by 40 ml of ice-cold 3% paraformaldehyde (PFA) at a rate of 2 ml/min. Spinal cord tissue 2 mm from either side of the injection site was dissected and drop-fixed in 3% PFA overnight. The tissue was then dehydrated with a 30% sucrose solution and subsequently frozen in OCT. Next, 12 μ m spinal cord cross-sections were taken throughout the lesion as defined by DAPI hypercellularity.

Mounted cryosections were rinsed in TBS, permeabilized in 1% Triton X-100 and 2% normal serum for 30 min, and then blocked in a solution containing 10% BSA and 10% normal serum for 1 h. Normal serum was from goat, rat, or donkey, depending on the species in which the secondary antibody was raised. Primary antibodies were incubated at 4°C overnight. Sections were washed in TBS with 0.05% Triton X-100 and incubated in secondary antibodies for 3 h. Both primary and secondary antibodies were diluted in a TBS solution containing 2% BSA, 2% serum, and 0.2% Triton X-100. For CC1 and MBP staining, antigen retrieval was performed before permeabilization; sections were placed in a 10 mM solution of sodium citrate and microwaved 3×40 s at medium power with 25 min in between microwaving sessions. For MBP staining, sections were first treated with 50 mM ammonium chloride to reduce autofluorescence and then delipidated with 100% ethanol for 10 min. The M.O.M. kit (Vector Laboratories BMK-2202, RRID:AB_2336833) was used for CC1 staining. After permeabilization, slides were first incubated in M.O.M. mouse Ig blocking reagent for 1 h followed by a 30 min incubation with CC1 antibody in M.O.M. diluent and then standard blocking solution and other primary antibodies. The following primary antibodies were used: CC1 (1:100, Millipore OP80, RRID:AB_2057371), Cleaved Caspase 3 (1:500, Cell Signaling Technology 9661, RRID:AB_2341188), GFP (1:500, Aves Laboratories GFP-1020, RRID:AB_10000240), Iba1 (1:750, Wako Technology 019-19741, RRID:AB_839504), MBP (1:1000, Covance SMI-99, RRID:AB_2314772), Olig2 (1:750, a gift from Dr. Charles Stiles, Harvard University, RRID:AB_2336878), PDGFR β (1:200, Abcam ab32570, RRID:AB_777165; 1:200 ab91066, RRID:AB_10563302), phospho-S6 (1:750, Cell Signaling Technology 4858, RRID:AB_916156), Sox10 (1:25, R&D Systems AF2864, AB_442208) and TSC1 (1:500, Bio-Rad AHP1001, RRID:AB_609656). Secondary antibodies were as follows: GaM 647 (1:750, Invitrogen A21235, RRID:AB_2535804), GaR AMCA (1:200, Jackson Laboratories, 111-155-144, RRID:AB_2337994), GaCk 488 (1:1000, Invitrogen A11039, RRID:AB_2534096), DaM 647 (1:750, Invitrogen A31571, RRID:AB_162542), DaGt AMCA (1:200, Jackson Laboratories 705-155-147, RRID:AB_2340409), DaCk 488 (1:700, Jackson Laboratories 703-545-155, RRID:AB_2340375), RtaM IgG2 β 647 (1:350, Abcam ab172327, RRID:AB_2650513).

For myelin oligodendrocyte glycoprotein (MOG) immunohistochemistry, mounted cryosections were rinsed, delipidated with 100% ethanol for 10 min, and then incubated in 3% H₂O₂ to eliminate endogenous peroxidase activity. Sections were permeabilized in 0.3% Triton X-100 and then blocked against nonspecific binding as described above. Sections were incubated with MOG primary antibody (1:1000, Abcam ab32760, RRID:AB_2145529) overnight followed by incubation in a biotinylated GaRb secondary antibody (1:500, Vector Laboratories BA-1000, RRID:AB_2313606) for 2 h, streptavidin (1 μ g/ml, Pierce 21126) for an additional 2 h. Positive signal was detected using the NovaRed substrate kit (Vector Laboratories SK-4800, RRID:AB_2336845). Sec-

tions were dehydrated and coverslipped with Cytoseal (Thermo Scientific 8312-4).

Toluidine blue and electron microscopy (EM). Animals were intracardially perfused with 40 ml of cold PBS followed by 40 ml of modified Karnovsky's fixative (2% paraformaldehyde/2.5% glutaraldehyde) at a rate of 2 ml/min. The spinal cord was removed and postfixed in the same fixative. The spinal cord was bisected through the middle of the lesion, embedded in 5% agarose in 0.1 M phosphate buffer, and sliced on a vibratome into 70- to 200- μ m-thick sections. Sections with clear lesions in the dorsal columns were postfixed in 1% osmium and 1.5% potassium ferrocyanide followed by 1% osmium tetroxide, dehydrated in graded acetone with 2% uranyl acetate, and resin embedded in Embed 812 (Electron Microscopy Sciences 14120) using a PelcoBiowave Pro tissue processor (Ted Pella). One-micrometer-thick sections were cut from a trapezoid positioned over the dorsal columns and mounted onto glass slides where they were then stained with a mixture of 1% toluidine blue, 1% methylene blue, and 1% sodium borate in water for $\sim$ 1 min. Ultrathin sections (65 nm) were cut at the same location within the lesion and mounted on Formvar-coated slot grids (EMS). Blocks were sectioned deeper into the lesion and another set of thick and ultrathin sections were collected for each sample. Ultrathin sections were viewed at 80 kV on a Tecnai G2 transmission electron microscope (FEI) and imaged with a side-mount AMT XR80S-B camera.

Experimental design and statistical analysis. Mouse strains, experimental and control groups, and sex are detailed above in the sections on experimental animals and tissue preparation. Initial lesion size was determined by Luxol fast blue staining of sections 80 μ m apart throughout the lesion. The area of demyelination in a given segment of the lesion was measured within each section and the volume estimated using the cylindrical rule (volume = area of selected section $\times$ distance between sections). Whole lesion volume was obtained by summing individual volumes. All immunofluorescence and MOG immunohistochemistry analyses were performed within the lesion borders, as defined by DAPI hypercellularity, on a minimum of 3 distinct sections, a minimum of 84 μ m apart with $n = 3$ animals per genotype. For MBP and MOG remyelination analyses, the area of the lesion that was immunopositive was measured in ImageJ and expressed as a percentage of the total lesion area as defined by DAPI hypercellularity. For MBP demyelination analysis at 7 dpl, the MBP-negative area was measured in ImageJ from a cross-section at lesion peak. For immunohistochemistry analysis, the experimental and control groups were compared with a Student's *t* test except for instances when multiple time points were being assessed, in which case an ANOVA was used to assess statistical significance.

For toluidine blue analysis, lesion borders were defined by the presence of darkly staining immune bodies and lack of organized axonal arrangement due to increased interstitial space. A minimum of two independent sections were analyzed for each animal. Three blinded, independent investigators ranked all cross-sections in a given set compared with one another. Rankings were based on the extent and density of remyelinated axons throughout the lesion, with a rank of 1 assigned to the cross-section with the least remyelination. To test for differences between remyelination rankings of the three raters, we used Friedman's χ^2 test, a nonparametric analog of the randomized block ANOVA, and tested for block effects in which each rater was treated as a block (Daniel, 1990). The Friedman test was implemented using SAS proc survey freq following the approach of Ipe (1987). To test for the association between the extent of remyelination and group, a binary variable was created from the remyelination ranks using the mid-rank as the cutoff between high and low ranks. We then modeled group membership using a log-binomial regression model with the binary version of the ranks as the predictor variable, and controlling for multiple rankers using the generalized estimating approach (Zeger and Liang, 1986). We assumed an independent working correlation and the sandwich estimator was used to estimate SEs. Exponentiating coefficients of this model produced the relative rates of membership in the control group compared with the cKO group for specimens that were ranked highly.

Areas for EM analysis were selected from toluidine blue images from animals that ranked along the median of their respective groups. Selected areas were in similar places within the lesion in all animals: well within

Figure 1. *Tsc1* deletion from NG2⁺ cells increases mTORC1 signaling, but does not affect oligodendrocyte differentiation. **A**, Timeline of tamoxifen administration, lysolecithin injection, and sample collection. **B**, Expression of GFP reporter in NG2-*Tsc1*cKO lesion at 14 dpl. Scale bar, 100 μ m. **C**, Colabeling of Sox10, CC1, and GFP in 14 dpl lesion of NG2-*Tsc1*cKO animal. Scale bar, 10 μ m. **D**, Representative images from control and NG2-*Tsc1*cKO animals of pS6RP expression in Sox10⁺ cells at 14 dpl. Scale bar, 100 μ m. **E**, Representative Western blot of TSC1 from control and NG2-*Tsc1*cKO animals. **F**, Colabeling of Sox10 and CC1 at 14 dpl in the lesions of control and NG2-*Tsc1*cKO animals. Scale bar, 100 μ m. **G**, **H**, Quantification of number of Sox10⁺ cells/mm² (**G**) and percentage of Sox10⁺ cells that were also CC1⁺ (**H**) within the lesion at 7 and 14 dpl. For 7 dpl, $n = 5$ control, $n = 3$ NG2-*Tsc1*cKO; for 14 dpl, $n = 4$ /group. All values are expressed as $\pm$ SEM.

the lesion borders but not in the necrotic lesion center. For g-ratio analysis, a minimum of 100 axons from two different areas of the lesion cross-section were analyzed from three animals per group. The resulting trend lines were then compared by linear regression analysis.

Results

Deletion of *Tsc1* from adult OPCs increases mTORC1 signaling but does not affect oligodendrocyte differentiation

Demyelination in the dorsal white matter of the murine spinal cord was induced by a single injection of lysolecithin. This method was chosen to study remyelination for several reasons: (1) because it does not elicit a systemic immune response, so the primary repair response of oligodendrocyte progenitors can be more easily examined; (2) the processes of demyelination and

remyelination can be readily separated from one another; and (3) the kinetics of repair are well defined (Jeffery and Blakemore, 1995; Luo et al., 2014). Previous studies have shown that the lysolecithin-induced lesions are spontaneously remyelinated by resident NG2⁺ OPCs that migrate into the lesion, proliferate, and differentiate into myelin-producing oligodendrocytes (Zawadzka et al., 2010). To study the function of TSC in oligodendrocyte progenitors during remyelination, *Tsc1* was deleted from OPCs in adult mice before demyelination through a tamoxifen-inducible Cre recombinase expressed in NG2⁺ cells (NG2-*Tsc1*cKO). The paradigm for tamoxifen injections, induction of the lysolecithin lesion, and tissue harvest is described in the Materials and Methods and shown in Figure 1A.

We first confirmed successful recombination and functional disruption of the TSC1–TSC2 complex in the NG2-*Tsc1*cKO animals through GFP reporter expression, TSC1 protein expression, and the expression of phospho-S6 ribosomal protein (pS6RP), a downstream target of mTORC1/p70S6 kinase normally inhibited by TSC activity. GFP expression was observed throughout the lesion at 14 dpl in NG2-*Tsc1*cKO animals, colabeling $86.1 \pm 5.3\%$ of Sox10⁺ oligodendroglia and 86.5% of CC1⁺ mature oligodendrocytes (Fig. 1*B,C*). Moreover, the percentage of oligodendroglia expressing TSC1 in NG2-*Tsc1*cKO animals was $42.3 \pm 10.6\%$ of controls. The percentage of Sox10⁺ cells that expressed TSC1 was $30.3 \pm 7.6\%$ in NG2-*Tsc1*cKO animals, down from $71.5 \pm 3.0\%$ in control animals. TSC1 knockdown was confirmed by Western blot analysis; protein extracted from NG2⁺ cells of the brain and spinal cords of unlesioned adult mice that underwent a comparable tamoxifen injection paradigm revealed that TSC1 protein expression was markedly reduced in NG2-*Tsc1*cKO animals compared with controls (Fig. 1*E*). Furthermore, Sox10⁺ oligodendroglia in NG2-*Tsc1*cKO animals had increased staining for pS6RP, indicating increased mTORC1 activity (Fig. 1*D*). Together, these data suggest that Sox10⁺ cells were successfully recombined, with GFP reporter expression, loss of TSC1 and consequent disinhibition of mTORC1 signaling.

To determine whether the loss of *Tsc1* in the adult OPCs in the NG2-*Tsc1*cKO animals altered OPC differentiation, we analyzed CC1 expression, a marker of mature oligodendrocytes (Fig. 1*F*). The total number of oligodendrocyte lineage cells, as assessed by expression of the transcription factor Sox10, was not significantly different between control and NG2-*Tsc1*cKO animals at 7 and 14 dpl (Fig. 1*G*). Because *Tsc1* deletion was previously found to increase cell death in the oligodendrocyte lineage during development (Jiang et al., 2016), we assessed oligodendroglia expression of the apoptosis marker cleaved-caspase-3 at 14 dpl. Consistent with our findings on oligodendrocyte lineage cell number, we found no difference in the number of Sox10⁺ cells that expressed cleaved-caspase-3 with loss of *Tsc1* (control: 19.04 ± 3.74 cells/mm², NG2-*Tsc1*cKO: 15.88 ± 3.08 cells/mm²).

The percentage of Sox10⁺ oligodendroglia that were CC1⁺ mature oligodendrocytes also did not vary between genotypes; ~20% of oligodendroglia at 7 dpl and ~55% at 14 dpl were CC1⁺ in both the control and NG2-*Tsc1*cKO animals (Fig. 1*H*). Therefore, there was no indication of altered oligodendrocyte differentiation with loss of *Tsc1* in OPCs.

Tsc1 deletion in NG2⁺ OPCs and pericytes has no effect on initial lesion size or monocyte activation

NG2 is well known to be expressed in the adult OPCs that repair demyelinated lesions, but NG2 has also been described in pericytes and in some activated monocytes after injury (Kucharova et al., 2011). To determine whether the NG2-Cre induced recombination in these cell types in the lesions, we analyzed expression of the GFP reporter with Iba1, a marker of activated microglia and macrophages, and PDGFRβ to identify pericytes. We found no colocalization of GFP with Iba1 (Fig. 2*A*), consistent with the finding that NG2 is expressed in activated monocytes and not in quiescent microglia in the unperturbed CNS. Because tamoxifen was administered before the lysolecithin lesion in our paradigm, none of the quiescent monocytes would be expected to have an active NG2 promoter and thus would escape recombination of the floxed *Tsc1* allele. However, $54.6 \pm 4.37\%$ of the PDGFRβ⁺ pericytes colocalized with GFP indicative of active Cre and recombination within these cells (Fig. 2*B*). Although Cre is active in pericytes within this model, the expression of TSC1 within peri-

Figure 2. Initial lesion size and microglial/macrophage response are unchanged with *Tsc1* deletion from NG2⁺ cells. **A**, Representative image of Iba1 staining and GFP reporter in the lesion of an NG2-*Tsc1*cKO animal. Arrows indicate GFP⁺ cells, all of which are negative for Iba1. Scale bar, 50 μm. **B**, Left two panels depict colocalization of pericyte marker PDGFRβ with GFP reporter in NG2-*Tsc1*cKO lesion. Solid arrowheads identify colocalization; hollow arrowheads identify PDGFRβ⁺/GFP[−] cells. Rightmost panel shows low expression of TSC1 in PDGFRβ-positive pericytes in control animals. Scale bars, 50 μm. **C**, Representative images from control and NG2-*Tsc1*cKO animals of Luxol fast blue staining of the dorsal column at 7 dpl. Dotted line denotes borders of dorsal white matter. Scale bar, 100 μm. **D**, Immunostaining for Iba1 at 7 dpl in control and NG2-*Tsc1*cKO animals. Scale bar, 100 μm.

cytes in control sections was found to be very low (Fig. 2*B*). Moreover, upon examination, the number of CD3⁺ T cells and Iba1⁺ monocytes in unlesioned white matter did not differ between control and NG2-*Tsc1*cKO animals (data not shown). Given these data, it is not expected that deletion of TSC1 from pericytes would have a significant effect on the demyelination or remyelination of the lysolecithin lesion.

Because tamoxifen was administered before the injection of lysolecithin and because pericytes can regulate the blood–brain barrier, we sought to determine whether there were differences in initial lesion size or in number of microglia/macrophages within the demyelinated lesions. To address initial lesion size, we calculated total lesion volume by Luxol fast blue staining at 7 dpl, when lesion size is maximal before the onset of remyelination. Although there was some variability in lesion volume across all animals, there was no significant difference in lesion volume between groups at 7 dpl (control: 0.86 ± 0.3 mm³; NG2-*Tsc1*cKO:

$0.60 \pm 0.23 \text{ mm}^3$; Fig. 2C). Moreover, similar results were obtained when MBP staining was quantified in cross-sections taken at lesion peak at 7 dpl; there was no significant difference between controls and NG2-*Tsc1* animals in the MBP⁺ area (control: $0.44 \pm 0.06 \text{ mm}^2$; NG2-*Tsc1* KO: $0.40 \pm 0.17 \text{ mm}^2$). We further analyzed Iba1 staining within the lesion to assess potential differences in microglial/macrophage infiltration. There were no consistent changes in Iba1⁺ cell number or intensity with genotype (Fig. 2D). These data support the conclusion that *Tsc1* deletion in NG2⁺ cells before the lesion induction had no impact on the initial lesion size or on microglia/monocyte infiltration into the lesion.

***Tsc1* deletion in adult OPCs accelerates myelin protein expression during remyelination**

To address whether deletion of *Tsc1* in adult OPCs affected remyelination, we first performed immunohistochemistry for MBP and MOG at 14 and 21 dpl and quantified the percentage of the lesion that expressed MBP or MOG (Fig. 3A). Analysis revealed a main effect of genotype for both MBP and MOG with greater myelin protein expression in NG2-*Tsc1* KO animals compared with controls across the two time points ($F_{(1,14)} = 15.79$, $p = 0.001$, ANOVA and $F_{(1,11)} = 7.02$, $p = 0.023$, ANOVA, respectively; Fig. 3B). Moreover, an increase in the percentage of the lesioned area expressing MBP was seen at 14 dpl in the NG2-*Tsc1* KO (post hoc comparisons using the Sidak test indicated significant differences, adjusted $p = 0.003$; Fig. 3B). Because nearly the entire lesioned area was MBP⁺ at 14 dpl in the NG2-*Tsc1* KO animals, the percentage of MBP⁺ area increased only marginally from a mean of $90.4 \pm 1.8\%$ at 14 dpl to $95.5 \pm 3.6\%$ at 21 dpl. Conversely, within the control lesions, MBP immunoreactivity increased from an average of $69.0 \pm 4.7\%$ of lesioned area at 14 dpl to $83.5 \pm 4.9\%$ at 21 dpl (Fig. 3B). Therefore, at 21 dpl, the difference in MBP⁺ area was no longer statistically different between the NG2-*Tsc1* KO animals and control animals because the lesions in both groups were nearly entirely MBP⁺. Because MOG is a late marker of mature oligodendrocytes and myelinogenesis, we expected MOG expression to occur in later stages of remyelination. Accordingly, the mean percentages of MOG⁺ area at both 14 dpl and 21 dpl were markedly lower than the percentages of MBP⁺ area in both groups. NG2-*Tsc1* KO animals had a trend toward a greater percentage of MOG⁺ lesioned area, although this was not significantly different at either time point. However, between 14 and 21 dpl, the mean percentage of lesion that was MOG⁺ increased significantly in NG2-*Tsc1* KO animals (post hoc comparisons using the Sidak test indicated significant differences, adjusted $p = 0.029$), but not in control animals, suggesting that MOG expression was increasing more rapidly in NG2-*Tsc1* KO animals (Fig. 3B). Therefore, the lesions in NG2-*Tsc1* KO animals expressed both MBP and MOG at a faster rate than the control lesions. Collectively, these data support increased remyelination in the NG2-*Tsc1* KO animals as early as 14 dpl.

Accelerated remyelination with *Tsc1* deletion from OPCs

To further assess remyelination, we analyzed toluidine blue-stained semithin cross-sections of the lesion at 21 dpl (Fig. 4A). Cross-sections from both control and NG2-*Tsc1* KO animals were ranked from least remyelinated (rank of 1) to most remyelinated. When comparing similar areas of the lesion, NG2-*Tsc1* KO animals consistently ranked higher than controls based on more remyelinated axons ($p = 0.012$, see Materials and Methods for description of statistical analysis; Fig. 4B). An animal that

ranked in the bottom half of the rankings was $3 \times$ more likely to be a control animal than an NG2-*Tsc1* KO animal. These data support increased remyelination with *Tsc1* deletion.

The prior analyses demonstrated an increase in remyelination in the NG2-*Tsc1* KO lesions. To determine whether the differences in early remyelination between control and NG2-*Tsc1* KO animals also altered myelin thickness, we examined remyelinated areas of the lesion using EM (Fig. 4C). Although the area of remyelination was larger in the NG2-*Tsc1* KO animals, there was no change in the percentage of myelinated axons within the remyelinated areas of control and NG2-*Tsc1* KO animals; $\sim 20\%$ of axons were unmyelinated in both groups (control: $22.64 \pm 7.81\%$, NG2-*Tsc1* KO: $18.00 \pm 4.41\%$). However, g-ratio analysis revealed that the myelinated axons in the NG2-*Tsc1* KO animals had significantly lower g-ratios and thus thicker myelin (Fig. 4D). The g-ratio for each axon was plotted as a function of its axon diameter; linear regression analysis revealed that the trend line for the NG2-*Tsc1* KO animals was significantly lower than that for the control animals, indicating thicker myelin across the range of axon diameters (y -intercepts: control = 0.73 ; NG2-*Tsc1* KO = 0.71 ; $F_{(1,3554)} = 137.75$, $p < 0.0001$, linear regression analysis). The slopes of these lines were not significantly different, but they trended toward significance ($F_{(1,3553)} = 3.57$, $p = 0.059$, linear regression analysis), suggesting that the effect of *Tsc1* deletion on myelin thickness was not the same across all axon diameters. Indeed, when g-ratios were averaged across small-diameter ($< 1.5 \mu\text{m}$) or large-diameter ($\geq 1.5 \mu\text{m}$) axons, there was a significant decrease in g-ratio in the NG2-*Tsc1* KO animals in the large-diameter axons (Holm-Sidak test, $p = 0.019$), but not in the small-diameter axons (Fig. 4E).

To determine whether the increase in myelin thickness represented accelerated remyelination or sustained hypermyelination, we next quantified EM images of the lesions in control and NG2-*Tsc1* KO animals at 49 dpl, a time point when remyelination should be complete. At this time, the g-ratios of NG2-*Tsc1* KO animals were no longer significantly smaller than controls (Fig. 4F), indicating that, by the culmination of remyelination, there were no differences in myelin thickness between groups.

Deletion of *Tsc1* from PLP⁺ oligodendrocytes has no effect on oligodendrocyte number or myelin protein expression in remyelinating lesions

Tsc1 deletion from NG2⁺ OPCs resulted in an increased rate of remyelination without changing the number of mature oligodendrocytes. In addition to the presence of NG2⁺ OPCs in the adult CNS, premyelinating oligodendrocytes are present in and around MS lesions, particularly in chronically demyelinated MS lesions. Therefore, we investigated whether *Tsc1* deletion later in the lineage, once the cells have differentiated into premyelinating oligodendrocytes, would have the same beneficial effect as *Tsc1* deletion from OPCs. To delete *Tsc1* from differentiating oligodendrocytes, we injected tamoxifen into mice that expressed both the floxed *Tsc1* allele and a tamoxifen-inducible Cre recombinase expressed from the PLP promoter (PLP-*Tsc1* KO). Within the adult spinal cord, PLP is expressed only in differentiating oligodendrocytes, not in OPCs (Guo et al., 2009). To induce recombination in the largest number of premyelinating oligodendrocytes, we injected tamoxifen from 8–13 dpl, during the peak of lesion repair associated with oligodendrocyte differentiation but before significant remyelination has occurred (Fig. 5A). To confirm *Tsc1* deletion, we assessed TSC1 protein expression in Sox10⁺ cells at 21 dpl (Fig. 5B). TSC1 expression in Sox10⁺

oligodendroglia in the PLP-*Tsc1* cKO animals was $38.9 \pm 10.8\%$ of control levels, confirming *Tsc1* knock-down (Fig. 5C). The percentage of Sox10⁺ cells that expressed TSC1 was $30.6 \pm 8.5\%$ in PLP-*Tsc1* cKO animals, similar to what was seen in the NG2-*Tsc1* cKO animals. This contrasted with the $78.8 \pm 2.8\%$ of Sox10⁺ cells that expressed TSC1 in control animals. TSC1 knock-down in animals expressing the PLP-Cre and the floxed-*Tsc1* allele was further determined through Western blot analysis. O4⁺ cells were harvested from the spinal cords of adolescent control and PLP-*Tsc1* cKO animals that had received tamoxifen during development. Within the resulting protein lysates, TSC1 was markedly decreased in the PLP-*Tsc1* cKO animals (Fig. 5D), demonstrating effective recombination and *Tsc1* deletion in PLP-Cre; *Tsc1*^{fl/fl} animals that received tamoxifen injections.

Because *Tsc1* deletion occurs once the cells have initiated differentiation in the PLP-*Tsc1* cKO model, we expected no change in the number of mature oligodendrocytes between groups. As anticipated, neither the total number of Olig2⁺ cells (~ 140 cells/mm²) nor the percentage of Olig2⁺ cells that was CC1⁺ ($\sim 70\%$) differed between control and PLP-*Tsc1* cKO animals (Fig. 5E,F).

We then assessed remyelination through myelin protein expression at 21 dpl (Fig. 5G). Both genotypes had significant remyelination, with MBP and MOG expression covering $>75\%$ of the lesion area. There was no significant difference in the percentage of lesioned area that was positive for either myelin protein (Fig. 5H).

Tsc1 deletion from differentiated oligodendrocytes reduces remyelination

To further investigate alterations in remyelination, we ranked toluidine blue-stained semithin cross-sections from each lesion (Fig. 6A). Surprisingly, we found that control animals ranked higher than PLP-*Tsc1* cKO animals in the extent of remyelination. An animal from the top half of ranks was more than twice as likely to be a control animal than a PLP-*Tsc1* cKO animal ($p = 0.039$, see Materials and Methods for details on statistical analysis; Fig. 6B). Therefore, *Tsc1* deletion from differentiated oligodendrocytes had a negative effect on remyelination.

The reduction in remyelination in the PLP-*Tsc1* cKO animals was confirmed by ultrastructural EM analysis (Fig. 6C). When examining areas of remyelination, there was no change in the number of myelinated axons between control and PLP-*Tsc1* cKO animals (control: $14.1 \pm 2.8\%$, PLP-*Tsc1* cKO: $21.4 \pm 1.0\%$). However, the g-ratios of the PLP-*Tsc1* cKO animals were significantly larger than in control animals, indicating that the myelin sheaths on the remyelinated axons were thinner. Linear regression analysis of g-ratios plotted in relation to the corresponding axon diameter revealed that, whereas the slopes of the two trend lines were nearly identical (control: 0.049 ± 0.0036 ; PLP-*Tsc1* cKO: 0.049 ± 0.0035), the y intercept of the PLP-*Tsc1* cKO line was significantly higher than the control line (control: 0.69 ± 0.005 ; PLP-*Tsc1* cKO: 0.73 ± 0.006 ; $F_{(1,1928)} = 106.69$, $p < 0.0001$, linear regression anal-

Figure 3. Increased myelin protein expression in NG2-*Tsc1* cKO animals during remyelination. **A**, Immunostaining for myelin proteins MBP and MOG in the lesion of control and NG2-*Tsc1* cKO animals. Representative images for MBP and MOG are from 14 and 21 dpl, respectively. Scale bars, 100 μ m. **B**, Analysis of the percentage of the lesioned area that was MBP⁺ or MOG⁺ at 14 and 21 dpl. Two-way ANOVA analyses revealed a significant main effect of genotype on MOG expression (* $p < 0.05$) and MBP expression (** $p < 0.01$) with a significant difference in MBP expression at 14 dpl between control and NG2-*Tsc1* cKO animals (** $p < 0.01$). $n = 3 - 5$ animals/group/time point. All values are expressed as $\pm$ SEM.

ysis). Therefore, myelin was consistently thinner in the PLP-*Tsc1* cKO animals compared with controls across the range of axon diameters. To determine whether this effect on myelin thickness persisted, we also performed g-ratio analysis on electron micrographs of the lesion at 49 dpl (Fig. 6D). At this time, no differences in myelin thickness were seen between control and PLP-*Tsc1* cKO animals, indicating that the hypomyelination observed at 21 dpl was a transient effect of *Tsc1* deletion from differentiated oligodendrocytes.

Discussion

Here, we show that disruption of TSC, an upstream inhibitor of mTORC1 signaling, has differential effects on remyelination depending on when in the oligodendrocyte lineage it is deleted. These findings are summarized in Figure 7. Deletion of *Tsc1* from NG2⁺ OPCs has no effect on OPC differentiation, but accelerates remyelination. NG2-*Tsc1* cKO animals have higher MBP expression early during remyelination, an increased rate of MOG accumulation, increased extent of remyelination throughout the lesion area, and smaller g-ratios for remyelinated axons compared with control animals. These data are indicative of an increase in both area of remyelination and thickness of myelin on remyelinated axons at 21 dpl. There were no changes in the initial lesion size or in the infiltration of monocytes/microglia into the lesions, supporting the conclusion that the increase in remyelination was due to loss of TSC1 in the oligodendrocytes. Because the increase in remyelination is seen without an increase in the number of mature oligodendrocytes, greater remyelination may be due to either an increase in the number of CC1⁺ mature oligodendrocytes that contribute to remyelinating denuded axons or, alternatively, to an increase in the amount of myelin produced by each myelinating oligodendrocyte. Prior evidence suggests that mTOR signaling plays a role in the amount of myelin produced per oligodendrocyte. Hyperactivating the mTOR pathway through the constitutive activation of AKT causes hypermyelination without increasing the number of mature

Figure 4. Remyelination is accelerated in NG2-*Tsc1*cKO animals. **A**, Low (top) and high (bottom) magnification images of toluidine blue-stained semithin sections of control and NG2-*Tsc1*cKO animals at 21 dpl. Low-magnification images capture the entire lesioned dorsal white matter; high-magnification images show remyelinating axons at the lesion border. Scale bars: top, 100 μm ; bottom, 20 μm . **B**, Rank analysis of toluidine blue images by three independent, blinded rankers with higher ranks indicating greater remyelination. Each symbol represents an independent section; two to three independent sections/animal were used in the ranking. Lines indicate median rank. $n = 6$ control animals, $n = 5$ NG2-*Tsc1* cKO animals. **C**, Representative EMs from lesioned spinal cord of control and NG2-*Tsc1*cKO animals at 21 dpl. Arrows and arrowheads denote axons of similar axon diameter representing axons of large ($\geq 1.5 \mu\text{m}$) and small ($< 1.5 \mu\text{m}$) diameter, respectively. Scale bar, 4 μm . **D**, Quantification of myelin thickness through g-ratio analysis at 21 dpl. The g-ratios of individual axons were plotted in relation to their diameter. Control animals are represented by the solid trend line; NG2-*Tsc1*cKO animals by the dashed trend line. **** $p < 0.0001$, linear regression analysis. **E**, G-ratios were compared within small-diameter ($< 1.5 \mu\text{m}$) and large-diameter ($\geq 1.5 \mu\text{m}$) axons. * $p < 0.05$, Holm–Sidak test. All values are expressed as $\pm$ SEM. **F**, G-ratios were quantified in control and NG2-*Tsc1*cKO animals at 49 dpl. Linear regression analysis revealed no significant differences between the two trend lines (control, solid trend line; NG2-*Tsc1*cKO, dashed trend line). At both time points, g-ratio measurements were performed on > 100 axons from two different regions of the remyelinated lesion in each animal. $n = 3/\text{group}$.

oligodendrocytes (Flores et al., 2008). Similarly, deletion of PTEN, an upstream inhibitor of mTOR signaling, results in no differences in remyelination despite a $> 50\%$ reduction in oligodendroglia number (Harrington et al., 2010). In both cases, the hypermyelination or normal remyelination, respectively, cannot be attributed solely to oligodendrocyte number and

must therefore result from increased myelin production by oligodendrocytes.

Given that *Tsc1* deletion from OPCs accelerates remyelination without affecting oligodendrocyte differentiation, we reasoned that *Tsc1* deletion from differentiating premyelinating oligodendrocytes would have a similar effect. Therefore, we were surprised

Figure 5. *Tsc1* deletion from differentiated oligodendrocytes has no effect on oligodendrocyte number or myelin protein expression. **A**, Timeline of lysolecithin injection, tamoxifen injections, and sample collection. **B**, Representative images from control and PLP-*Tsc1* cKO animals of TSC1 expression in Sox10⁺ cells. Solid arrowheads identify colocalization; hollow arrowheads identify Sox10⁺ cells that are negative for TSC1. Scale bar, 50 μ m. **C**, Quantification of the number of Sox10⁺ cells that have high TSC1 expression in PLP-*Tsc1* cKO animals compared with controls (** $p < 0.01$). $n = 4$ /group. **D**, Representative Western blots of TSC1 expression in O4⁺ cells isolated from control and PLP-*Tsc1* cKO animals. β -actin is used as a loading control. **E**, Colabeling of Olig2 and CC1 in lesions from control and PLP-*Tsc1* cKO animals. Scale bar, 50 μ m. **F**, Quantification of the total number of Olig2⁺ cells and CC1⁺/Olig2⁺ cells. $n = 4$ /group. **G**, Immunostaining for myelin proteins MBP (top) and MOG (bottom). Solid lines denote lesion boundaries; dotted lines denote boundaries of dorsal white matter. Scale bars, 100 μ m. **H**, Quantification of the percentage of lesioned area that was MBP⁺ or MOG⁺ at 21 dpl in control and PLP-*Tsc1* cKO animals. $n = 5$ control, 4 PLP-*Tsc1* cKO. NS, Not significant. All values are expressed as $\pm$ SEM.

Figure 6. Remyelination is slower in PLP-*Tsc1* cKO animals. **A**, Representative images of toluidine blue-stained semithin sections from 21 dpl control and PLP-*Tsc1* cKO animals at low (top) and high (bottom) magnification. Low-magnification images capture the entire lesioned dorsal white matter; high-magnification images show remyelinating axons at the lesion border. Scale bars: top, 100 μm ; bottom, 20 μm . **B**, Rank analysis of remyelination in toluidine blue images with the least remyelinated section receiving a rank of 1. Each symbol represents an individual section; two independent sections were analyzed per animal. Each color represents a different ranker. Lines indicate median rank. $n = 6$ control animals, $n = 4$ PLP-*Tsc1* cKO animals. **C, D**, Representative EMs from control and PLP-*Tsc1* cKO animals and g-ratio scatterplots at 21 dpl (**C**) and 49 dpl (**D**). Scale bar, 4 μm . Control animals are represented by the solid trend line; PLP-*Tsc1* cKO animals by the dashed trend line. Linear regression analysis revealed a significantly higher y-intercept for the PLP-*Tsc1* cKO trend line at 21 dpl, **** $p < 0.0001$. No differences were seen between control and PLP-*Tsc1* cKO animals in g-ratios across axon diameters at 49 dpl.

to find that deletion of *Tsc1* from PLP⁺ oligodendrocytes had the opposite effect and slowed remyelination. Although there was no obvious change in myelin protein expression, both the remyelination rank and the thickness of the myelin on the remyelinated axons were less than controls at 21 dpl. It is unclear why deletion of *Tsc1* from different stages of the oligodendrocyte lineage has differential effects. One possible explanation lies in the modula-

tion of mTORC2 by TSC and differential function of mTORC1 and mTORC2 signaling in oligodendroglial maturation. Although TSC has classically been described as an upstream inhibitor of mTORC1, it is also an activator of mTORC2, albeit through unknown mechanisms (Huang et al., 2008). It is plausible that mTORC1 and mTORC2 play interconnected but distinct roles in the regulation of OPC differentiation and myelina-

tion. Recent studies have suggested that mTORC1 has a larger role during developmental myelination; inhibition of mTORC1 signaling through the deletion of *Rptor* results in reduced oligodendrocyte differentiation and myelination during development, similar to the phenotype seen with mTOR deletion (Bercury et al., 2014; Lebrun-Julien et al., 2014). The function of mTORC2 is less clear. Deletion of *Rictor* and consequent abrogation of mTORC2 signaling has no effect on developmental myelination, although there is an increase in mature oligodendrocytes (Bercury et al., 2014). This suggests the possibility that a greater number of oligodendrocytes are needed to produce normal levels of myelin when mTORC2 signaling is disrupted. Moreover, mTORC2 phosphorylates AKT at Ser473, a site targeted in the constitutively active AKT mutant that results in developmental hypermyelination (Flores et al., 2008). Together, these studies suggest that mTORC2 signaling may have a subtle but important role in the regulation of myelin production by oligodendrocytes.

It is plausible that *Tsc1* deletion in OPCs and the consequent increase in mTORC1 signaling may promote CC1⁺ oligodendrocyte ensheathment and myelination of axons. *In vitro* studies indicate that mTOR is important, not only in the differentiation of OPCs to oligodendrocytes, but also in the maturation of immature oligodendrocytes to mature oligodendrocytes that express myelin proteins (Tyler et al., 2009; Guardiola-Diaz et al., 2012). Therefore, even though *Tsc1* deletion from OPCs has no effect on the number of CC1⁺ oligodendrocytes, it might be important for the full maturation of these cells. Conversely, when *Tsc1* is deleted from PLP⁺ oligodendrocytes, the deletion may happen too late to influence the later stages of oligodendrocyte maturation, rendering the increase in mTORC1 signaling inconsequential. Concurrently, the acute reduction in mTORC2/Akt signaling at the onset of remyelination in our paradigm may result in reduced myelin production by these cells, accounting for the reduction in remyelination seen in PLP-*Tsc1* cKO animals. Although mTORC2 signaling would also be reduced in the NG2-*Tsc1* cKO animals, the increase in mTORC1 signaling may compensate by increasing the number of existing CC1⁺ oligodendrocytes that initiate remyelination of the denuded axons.

Our finding that OPC disruption of TSC, an upstream inhibitor of mTORC1, accelerates and enhances remyelination is in agreement with previous studies showing that systemic inhibition of mTOR signaling through rapamycin treatment inhibits remyelination in the cuprizone model (Sachs et al., 2014). Both of these studies implicate mTOR signaling in remyelination: in our current study, we show that deletion of an upstream inhibitor of mTORC1 accelerates remyelination; in the prior study, mTOR

Figure 7. Stage-dependent effects of *Tsc1* deletion on remyelination. Comparison of remyelination in control (middle row) animals and NG2-*Tsc1* cKO (top row) or PLP-*Tsc1* cKO (bottom row) animals reveal differential effects of *Tsc1* deletion in the two different paradigms. Before lysolecithin (left column), the NG2-*Tsc1* cKO receive tamoxifen, so NG2⁺ OPCs are recombined and express the Cre reporter GFP. At 21 dpl (right column), a time point associated with robust remyelination, the nonremyelinated lesioned area is smaller and the larger-diameter remyelinated axons within the lesion have thicker myelin in the NG2-*Tsc1* cKO animals compared with control. PLP-*Tsc1* cKO animals received tamoxifen 8–13 dpl; therefore, the remyelinating oligodendrocytes are now GFP⁺ recombined cells from which the *Tsc1* allele has been excised. At 21 dpl, the nonremyelinated area of the lesion is larger and the remyelinated axons have thinner myelin in the PLP-*Tsc1* cKO animals compared with controls. Green-colored cells represent cells that have undergone recombination and the GFP reporter is consequently expressed.

was inhibited and thus caused delayed remyelination. However, in disagreement with our findings, another study found that deletion of the upstream inhibitor PTEN does not affect remyelination (Harrington et al., 2010). It should be noted that PTEN was deleted during development in this latter study and resulted in hypermyelination throughout the CNS. It is unclear what effect developmental hypermyelination had on the ability of OPCs to respond to and repair a demyelinated lesion. Indeed, PTEN deletion markedly reduced adult OPC proliferation and thus the number of oligodendroglia during remyelination (Harrington et al., 2010). Therefore, although remyelination was not increased with PTEN deletion, repair mechanisms may have been hindered by altered environmental cues from the surrounding hypermyelinated white matter or by intrinsic changes within OPCs due to developmental PTEN deletion.

We have shown that deletion of *Tsc1* from adult OPCs is beneficial to remyelination. However, others have shown that deletion of *Tsc1* or *Tsc2* from OPCs during development is

detrimental, resulting in hypomyelination as a consequence of reduced oligodendroglial number (Carson et al., 2015; Jiang et al., 2016). The most likely explanation for why deletion of a *Tsc* gene from OPCs results in cell death or a lineage shift during development but not during remyelination is that adult OPCs are a distinctly different population from their developmental counterparts. A higher percentage of adult OPCs *in vitro* express O4, an antigen associated with late progenitors (Wolswijk and Noble, 1989). Adult OPCs are also more morphologically complex than developmental OPCs and differentiate more readily in culture (Wolswijk and Noble, 1989; Lin et al., 2009). Finally, microarray analysis has revealed that adult and developmental OPCs have distinct gene profiles, with adult OPCs expressing higher levels of myelin gene transcripts (Moyon et al., 2015). Together, these data suggest that adult OPCs are more mature than their developmental counterparts. Therefore, it is probable that *Tsc1* deletion in NG2⁺ adult OPCs before a demyelination insult results in recombination in cells that are at a later stage in the lineage than those targeted by the Olig1-Cre or Olig2-Cre during development. These more mature progenitors may be less likely to shift lineage identity and differentiate into astrocytes and may be less susceptible to ER stress. In any case, NG2⁺ adult OPCs are clearly a distinct population from the OPCs seen in development and may simply respond differently to disruption of the TSC1–TSC2 complex, a conclusion supported by our findings that *Tsc1* deletion from adult OPCs has no effect on cell death or oligodendroglia number and ultimately increases remyelination.

The findings presented here suggest a complex role for *Tsc1* in remyelination. This emphasizes the importance of carefully considered therapeutics to enhance remyelination in diseases such as MS because what is beneficial to oligodendroglial development at one stage may be detrimental in another stage. The mTOR signaling pathway consistently appears to be an attractive target to enhance remyelination. It was shown previously to be essential for proper developmental myelination and for remyelination during active cuprizone treatment (Bercury et al., 2014; Sachs et al., 2014; Wahl et al., 2014). Previous studies have shown that promoting PI3K/Akt/mTOR signaling enhances developmental myelination. Here, we have presented the first evidence that disruption of the mTORC1 inhibitor TSC accelerates remyelination.

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