INVITED REVIEW

Dissecting the multifactorial nature of demyelinating disease

Karolina Kucharova, William B. Stallcup*
Sanford Burnham Prebys Medical Discovery Institute, Cancer Center; Tumor Microenvironment and Cancer Immunology Program, La Jolla, CA, USA

Funding: *This study was supported by National Institutes of Health R01 CA095287 (WBS) and Sanford Burnham Prebys Medical Discovery Institute Lab Funding Initiative (WBS).*

Abstract

Chondroitin sulfate proteoglycan-4 (CSPG4) is a surface component of two key cell types (oligodendrocyte progenitor cells (OPCs) and myeloid cells) present in lysolecithin-induced lesions in mouse spinal cord. Two types of CSPG4 manipulations have been used to study the roles of these cells in myelin damage and repair: (1) OPC and myeloid-specific ablation of CSPG4, and (2) transplantation of enhanced green fluorescent protein (EGFP)-labeled progenitors to distinguish between bone marrow-derived macrophages and resident microglia. Ablation of CSPG4 in OPCs does not affect myelin damage, but decreases myelin repair, due to reduced proliferation of CSPG4-null OPCs that diminishes generation of mature oligodendrocytes for remyelination. Ablation of CSPG4 in myeloid cells greatly decreases recruitment of macrophages to spinal cord lesions, resulting in smaller initial lesions, but also in significantly diminished myelin repair. In the absence of macrophage recruitment, OPC proliferation is greatly impaired, again leading to decreased generation of myelinating oligodendrocytes. Macrophages may promote OPC proliferation *via* phagocytosis of myelin debris and/or secretion of factors that stimulate OPC mitosis. Microglia are not able to substitute for macrophages in promoting OPC proliferation. An additional feature of lesions in myeloid-specific CSPG4 null mice is the persistence of poorly-differentiated platelet-derived growth factor receptor α (PDGFRα) $^{+}$ macrophages that may prolong damage.

Key Words: *myelin damage; myelin repair; chondroitin sulfate proteoglycan 4; oligodendrocyte progenitors; macrophages; microglia; Cre-Lox technology; bone marrow transplantation*

***Correspondence to:**
William B. Stallcup, Ph.D., stallcup@sbpdiscovery.org.

orcid:
0000-0001-7339-5742 (William B. Stallcup)

doi: *10.4103/1673-5374.230281*

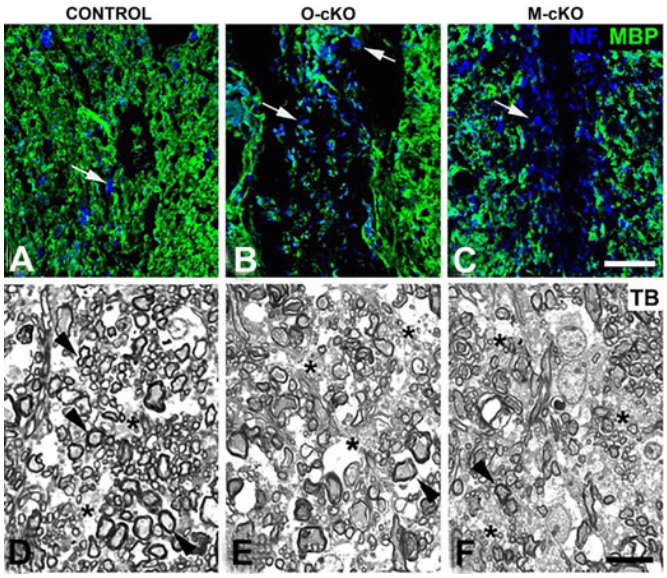
Accepted: *2018-01-25*

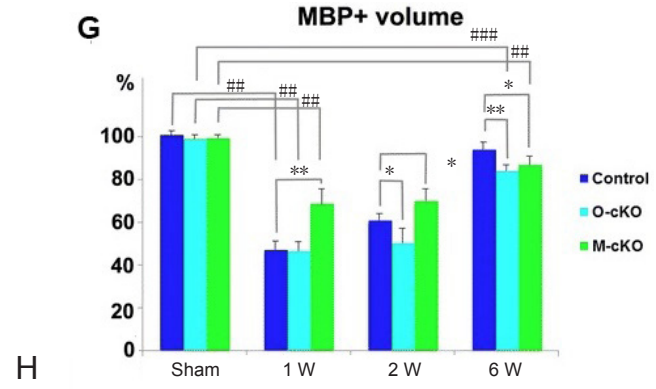
Chondroitin Sulfate Proteoglycan-4 (Cspg4) Proteoglycan as a Tool for Probing Myelin Damage and Repair

Extensive research has identified multiple factors that contribute to the failure of remyelination in multiple sclerosis (Franklin, 2002). This pathological complexity is due at least in part to the fact that several different cell types participate in disease progression. In addition to oligodendrocytes and neurons (axons), immune cells and other elements of the microenvironment also play important roles in myelin damage and repair. Defining in more depth the respective contributions of these various cell types to demyelination and remyelination will be required for a better understanding of multiple sclerosis that can lead to improved treatment of patients. Toward this end, we have identified chondroitin sulfate proteoglycan-4 (CSPG4, also known as the NG2 proteoglycan) as a surface component of three cell types present in lysolecithin-induced demyelinated lesions in mouse spinal cord. Oligodendrocyte progenitor cells (OPCs), myeloid cells (macrophages and microglia), and microvascular pericytes in demyelinated lesions all exhibit enhanced CSPG4 expression one week after lysolecithin microinjection (Kucharova et al., 2011). CSPG4 thus not only serves as a marker for recognizing these cells, but also provides a means of manipulating the cells for experimental purposes. We have utilized two types of manipulations to study the respective roles of CSPG4-expressing cell types in myelin damage and repair.

Cell type-specific CSPG4 ablation

We have used Cre-Lox technology to ablate CSPG4 specifically in two cell types, OPCs and myeloid cells. By crossing CSPG4 floxed mice (Chang et al., 2012) with *Olig2-Cre* mice (Schüller et al., 2008) and with *LysM-Cre* mice (Stockmann et al., 2008), we have created OPC-specific CSPG4 null mice (*CSPG4fl/fl*;*Olig2-Cre*) and myeloid-specific NG2 null mice (*CSPG4fl/fl*;*LysM-Cre*), respectively. These have allowed us to define the functional role of CSPG4 in the biology of OPCs and myeloid cells during lysolecithin-induced spinal cord demyelination and remyelination. In addition, these mouse models allow us to study the effects of reduced participation of these two cell types on demyelination and remyelination (Kucharova and Stallcup, 2015).





	Control	O-cKO	M-cKO
Sham-operated animals			
Number of Olig2$^+$ cells	115.27 ± 3.8	111.02 ± 5.7	117.63 ± 5.4
Number of Olig2$^+$ / PDGF-Rα^+ OPCs	14.55 ± 1.7	12.31 ± 2	14.46 ± 3.5
% of circulating cells with CD11b	16%	N.D.	18%
1 week after demyelination			
Number of Iba1$^+$ macrophages/microglia	117.19 ± 22.3	120.1 ± 28.1	39.64 ± 19.8**
Number of Olig2$^+$ / PDGF-Rα^+ OPCs	42.92 ± 1.5	32.4 ± 1.7***	23.02 ± 7.2 ***
Mitotic index of Olig2$^+$ / PDGF-Rα^+ OPCs(%)	22.91 ± 3.5	13.49 ± 1.5**	11.93 ± 2.1***
6 weeks after demyelination			
Number of APC$^+$ oligodendrocytes	62.14 ± 6.7	44.48 ± 8.6*	43.17 ± 4.1**

Figure 1 Myelin repair in NG2 null mice.
(A–F) Six weeks after lysolecithin microinjection into the dorsal spinal cord white matter of *CSPG4 fl/fl* (control), *CSPG4fl/fl*;*Olig2-Cre* (O-cKO), and *CSPG4fl/fl*;*LysM-Cre* (M-cKO) mice, animals were euthanized for determination of the extent of myelin repair. (A–C) Tissue sections were immunolabeled for myelin basic protein (MBP, green) and neurofilament protein (NF, blue), allowing visualization of myelinated axons and axons lacking association with myelin (arrows). (D–F) Toluidine blue-stained (TB) semi-thin sections enabled identification of well-myelinated axons (arrowheads) and unmyelinated axons (asterisks). Controls: A, D; O-cKO: B, E; M-cKO: C, F. Bars: 30 μm in C, 10 μm in F. (G) MBP-positive volumes are evaluated in control, O-cKO, and M-cKO mice at 1, 2, and 6 weeks after lysolecithin microinjection. One hundred percent myelination is defined by the level of MBP labeling in sham-operated control mice. Demyelination is reduced by myeloid-specific, but not by OPC-specific NG2 ablation. On the other hand, remyelination is retarded in both lines of NG2-null animals. Statistically significant differences are indicated by $*P < 0.05$ and $**P < 0.01$ when values were compared between controls and NG2 null mice at the same time point. $\#\#P < 0.01$ and $\#\#\#P < 0.001$ indicate statistically significant differences between mice of the same genotype at different time points. (H) Key parameters for Olig2$^+$PDGFRα$^+$ OPCs, APC$^+$ oligodendrocytes, and circulating CD11b$^+$ monocytes/macrophages were quantified in control, O-cKO, and M-cKO mice ($n = 4$ for each group) from 1–6 weeks after lysolecithin microinjection. Values represent as the mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (Reproduced with permission from Kucharova and Stallcup, 2015). PDGFRα:platelet-derived growth factor receptor α; OPCs: oligodendrocyte progenitor cells; Iba1: ionized calcium binding adapter molecule 1; W: week(s).

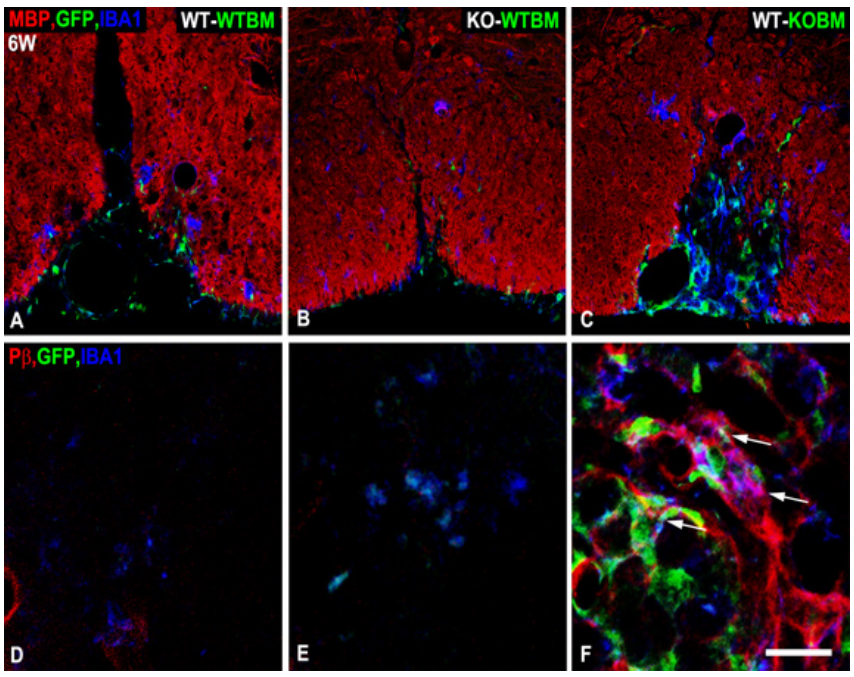


Figure 2 Persistence of atypical NG2 null macrophages in lesions in WT-KOBM mice.
Sections from spinal cord lesions at 6-weeks after lysolecithin injection were evaluated for myeloid cell abundance by use of the EGFP marker (green) and immunolabeling for Iba-1 (blue). (A–C) Immunolabeling for MBP (red) allows visualization of the extent of myelin repair. A: WT-WTBM; B: KO-WTBM; C: WT-KOBM. Iba-1 positive EGFP positive macrophages remain prominent in areas of the WT-KOBM spinal cord in which myelin repair is incomplete. (D–F) Immunolabeling for Iba-1 (blue) and PDGFRβ (red) in conjunction with the EGFP marker identifies atypical macrophages that express PDGFRβ. Many persistent macrophages in lesions in WT-KOBM mice co-express Iba-1, EGFP, and PDGFRβ (arrows). Scale bars: 100 μm in A–C, 25 μm in D–F. (Reproduced with permission from Kucharova K and Stallcup, 2017).
WT-KOBM: Transplant bone marrow from β-actin EGFP germline CSPG4 null donors into irradiated wild type recipients; EGFP: enhanced green fluorescent protein; Iba-1: ionized calcium binding adapter molecule 1; MBP: myelin basic protein; WT-WTBM: transplant bone marrow from wild type donors into irradiated wild type recipients; KO-WTBM: Transplant bone marrow from wild type donors into irradiated CSPG4 null recipients; PDGFRβ: platelet-derived growth factor receptor β; CSPG4: chondroitin sulfate proteoglycan-4.

Bone marrow transplantation

We have used bone marrow transplantation to discriminate between the actions of macrophages and microglia in myelin damage and repair. By transplanting bone marrow (BM) from *β-actin EGFP* germline CSPG4 null (KO) donors into irradiated wild type (WT) recipients, we created chimeric WT-KOBM mice that lack CSPG4 in EGFP-labeled bone marrow-derived macrophages, but retain CSPG4 in host cells, including microglia and OPCs. Conversely, by transplanting bone marrow from *β-actin EGFP* wild type donors into irradiated germline CSPG4 null recipients, we created chimeric KO-WTBM mice that lack CSPG4 in OPCs and microglia, but retain CSPG4 in EGFP-labeled macrophages. WT-WTBM chimeric mice provide controls for lysolecithin-based experiments with WT-KOBM and KO-WTBM mice in which various aspects of spinal cord demyelination and repair are quantified (Kucharova and Stallcup, 2017).

Importance of CSPG4 in OPC Function

Because OPCs produce the mature oligodendrocytes needed for remyelination of demyelinated axons (Gensert and Goldman, 1997), we would expect deficits in OPC generation to have a negative impact on remyelination. Our finding that ablation of CSPG4 diminishes OPC proliferation (Kucharova and Stallcup, 2010; Kucharova et al., 2011) thus suggests that CSPG4 loss should also impair remyelination. Using the lysolecithin model, negative effects on OPC proliferation (40% decrease) and abundance (25% decrease) at 1-week post-injury, with resulting deficits in mature oligodendrocyte number (30% decrease) at 6-weeks post-injury, are clearly observed in spinal cord lesions in *CSPG4fl/fl*;*Olig2-Cre* mice. These data are based on quantification of OPC and oligodendrocyte numbers (**Figure 1H**).

It is important to note in **Figure 1H** that numbers of OPCs and oligodendrocytes do not differ between sham-operated control, OPC-specific, and myeloid-specific CSPG4 null mice. Similarly, levels of myelination are the same in the three lines of mice at the starting point (**Figure 1G**, Sham). Differences seen in the three mouse lines therefore occur in response to the demyelinating event. Figure 1G shows that although the initial extent of demyelination at 1-week is not affected by OPC loss of CSPG4, CSPG4-dependent OPC deficits cause myelin repair in *CSPG4fl/fl*;*Olig2-Cre* mice to lag behind that seen in controls at 2-weeks. Even at 6-weeks post-injury, when repair is virtually complete in control mice, myelin volume is not fully restored in *CSPG4fl/fl*;*Olig2-Cre* mice. This is illustrated more dramatically at 6-weeks post-injury in **Figure 1A, D** and **B, E** by the deficit in well-myelinated axons in *CSPG4fl/fl*;*Olig2-Cre* mice, compared to controls. Significantly, the overall number of axons, regardless of size, is also reduced by 40% in lesions in *CSPG4fl/fl;Olig2-Cre* mice, reflecting the importance of myelination for neuronal cell survival (Kucharova and Stallcup, 2015).

The effects of CSPG4 loss on OPCs are also seen in the bone marrow transplantation studies. In the lysolecithin model, the absence of CSPG4 from OPCs in KO-WTBM chimeric mice results in a 30% decrease in OPC number at one-week post-injury. The resulting deficit in generation of mature oligodendrocytes translates into a significant decrease in the extent of myelin repair observed at 6-weeks after lysolecithin microinjection (Kucharova and Stallcup, 2017).

Importance of CSPG4 in Myeloid Cell Function

Previous findings from spinal cord demyelination experiments using germline CSPG4 null mice have suggested that loss of CSPG4 greatly reduces the number of myeloid cells associated with demyelinated lesions (Kucharova et al., 2011). This result is confirmed by our subsequent examination of lysolecithin-induced spinal cord lesions in *CSPG4fl/fl*;*LysM-Cre* mice. Although numbers of circulating CD11b-positive monocytes/macrophages are similar in sham-operated control and *CSPG4fl/fl*;*LysM-Cre* mice, lesions in these mice at 1-week post-injury contain 70% fewer myeloid cells than lesions in control mice (**Figure 1H**). This deficit in myeloid cell recruitment to lesions is associated with changes in both the initial extent of myelin damage and the subsequent extent of myelin repair. At one-week after lysolecithin microinjection, lesions in *CSPG4fl/fl*;*LysM-Cre* mice are only about half the size of lesions in control mice (**Figure 1G**), likely due to the involvement of myeloid cells in causing myelin damage. In spite of this reduced extent of myelin damage, remyelination of axons in CSPG4fl/fl;LysM-Cre mice lags behind remyelination in control mice and is still incomplete at 2 and 6-weeks post-injury. Compared to controls (**Figure 1A, D**), numbers of well-myelinated axons in *CSPG4fl/fl;LysM-Cre* mice (**Figure 1C, F**) are reduced to a similar extent to that seen in *CSPG4fl/fl;Olig2-Cre* mice (**Figure 1B, E**). These results indicate an important role for myeloid cells in myelin repair, in addition to their contribution to myelin damage. One intriguing function of myeloid cells appears to be their ability to support expansion of the OPC population, as reflected by a 50% decrease in OPC proliferation and a 40% reduction in OPC abundance seen in lesions in *CSPG4fl/fl*;*LysM-Cre* mice at 1-week post-injury

(**Figure 1H**). These decreases are even greater than the OPC deficits observed in *CSPG4fl/fl;Olig2-Cre* mice, indicating that loss of myeloid cells due to myeloid-specific ablation of NG2 is more deleterious to expansion of the OPC pool than loss of CSPG4 by the OPCs themselves. Myeloid cells may promote OPC proliferation by at least two mechanisms: (1) phagocytosis of myelin debris that is inhibitory to OPC proliferation and/or (2) production of cytokines/growth factors that stimulate OPC mitosis.

We have noted two drawbacks of the Cre/Lox approach for evaluating CSPG4 function in myeloid cells. First, LysM-Cre mediated ablation of CSPG4 does not allow discrimination between the effects of ablation on macrophages and microglia, since CSPG4 is ablated in both populations. Second, because CSPG4 expression is quite transient in myeloid cells, it has been difficult to determine the efficiency of LysM-Cre mediated CSPG4 ablation in these cells. The bone marrow transplantation approach offers improvement in both of these areas. Because CSPG4 is ablated in the germline in these experiments, we know that CSPG4 is completely lost in cells derived from recipient mice in KO-WTBM chimeras and in cells derived from donor mice in WT-KOBM chimeras. In addition, ionized calcium binding adapter molecule 1 (Iba-1) positive bone marrow-derived macrophages are marked with the EGFP tracer, while Iba-1 positive resident microglia are not.

Examination of WT-KOBM mice at 1-week after lysolecithin microinjection reveals that the absence of CSPG4 in macrophages results in a 5-fold decrease in macrophage number in spinal cord lesions, confirming our results with *CSPG4fl/fl*;*LysM-Cre* mice. An additional finding is that the abundance of resident microglia is increased 6-fold in WT-KOBM lesions, possibly as compensation for decreased macrophage recruitment. Double labeling for Iba-1 and myelin basic protein in WT-WTBM mice demonstrates that macrophages and microglia are both active in phagocytosis of myelin debris, while evaluation of WT-KOBM and KO-WTBM chimeras, respectively, shows that CSPG4 ablation diminishes phagocytic activity in both macrophages and microglia. Interestingly, the increased numbers of microglia in lesions in WT-KOBM mice are not able to replace the effects of macrophages in promoting expansion of the OPC pool, as evidenced by the greatly reduced abundance of lesional OPCs in these chimeras. This may suggest that phagocytosis of myelin debris is less important for OPC expansion than other microenvironmental macrophage contributions that affect OPC proliferation and/or recruitment. As previously shown in **Figure 1A–C** for cell type-specific CSPG4 ablation, double immunolabeling for myelin basic protein and neurofilament protein at 6-weeks after lysolecithin injection identifies myelinated axons. In the case of WT-KOBM chimeras, myelin repair is only 35% complete, compared to 88% complete in control WT-WTBM mice, confirming the effect of macrophage loss on myelin repair. The persistent myelin damage seen in WT-KOBM mice in **Figure 2C** occurs in conjunction with elevated numbers of bone marrow-derived macrophages in the lesion. In contrast, macrophage numbers have returned to baseline levels in control WT-WTBM (**Figure 2A**) and KO-WTBM (**Figure 2B**) mice at this time point. Although somewhat low in number at 6-weeks compared to 1-week, these persistent macrophages in WT-KOBM mice are characterized by their expression of platelet-derived growth factor receptor β (PDGFRβ) (**Figure 2F**), a marker that is absent in lesions in WT-WTBM (**Figure 2D**) and KO-WTBM (**Figure 2E**) mice. Activation of PDGFRβ has been previously linked to maintenance of macrophages in a less differentiated state (Reiterer and Yen, 2007), possibly contributing to aberrant functions that distinguish these immature cells from mature macrophages.

Conclusions

Our use of both cell type-specific CSPG4 ablation and germline CSPG4 ablation coupled with bone marrow transplantation has allowed us to accomplish two objectives in the context of myelin damage and repair. First, we have been able to demonstrate important roles for CSPG4 in establishing populations of both OPCs and myeloid cells in demyelinated lesions in mouse spinal cord. Second, we have generated mouse models that allow us to assess the consequences of reduced OPC and myeloid cell participation in the processes of demyelination and remyelination.

In the case of OPCs, our findings demonstrate that reduced numbers of OPCs lead to diminished capability for myelin repair. This is hardly surprising, given the fact that newly-generated oligodendrocytes are required for axon remyelination. However, both of our CSPG4 ablation paradigms also support an important role for CSPG4 in expansion of the OPC pool, and thus for generating a population of oligodendrocytes that is sufficient for carrying out myelin repair. While our data firmly support diminished proliferation of OPCs as a likely reason for insufficient generation of oligodendrocytes in lesions in both OPC-specific and myeloid-specific CSPG4 null mice, it is also possible that impaired OPC differentiation as a result of CSPG4 ablation may contribute to the oligodendrocyte deficiency (Franklin, 2002). This possibility remains to be investigated.

In the case of myeloid cells, both experimental approaches support a key role for CSPG4 in establishing substantial myeloid populations in demyelinated lesions. We have also observed dramatic reductions in myeloid cell abundance in brain tumors in CSPG4 null mice (Yotsumoto et al., 2015; Cejudo-Martin et al., 2016), suggesting a general requirement for CSPG4 in recruitment or maintenance of myeloid cells at sites of inflammation. Reduced myeloid cell numbers result in a decrease in the initial extent of axon demyelination, due to reduced myeloid cell participation in myelin damage. More interesting, however, is the diminished myelin repair observed in myeloid cell deficient mice. This appears to stem, at least in part, from the ability of myeloid cells to stimulate expansion of the OPC population, *via* phagocytosis of inhibitory myelin debris and/or production of factors that promote OPC proliferation. An additional factor contributing to poor myelin repair may be the actions of atypical, incompletely-differentiated macrophages that persist in lesions in WT-KOBM mice. The identity and function of aberrant immune cells will be critical issues in improving therapy for chronic demyelinating pathologies.

Acknowledgments: *We thank Ms. Regina Kapono for her help with preparing this manuscript.*
Author contributions: *KK and WBS were responsible for planning and executing experimental protocols, analyzing data, and writing and editing the manuscript.*
Financial support: *This study was supported by National Institutes of Health R01 CA095287 (WBS) and Sanford Burnham Prebys Medical Discovery Institute Lab Funding Initiative (WBS).*
Conflicts of interest: *None declared.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer review reports:
Reviewer 1*: Maximilian Michael Saller, Ludwig-Maximilians-Universitat Munchen, Germany.*
Comments to authors*: The manuscript provides an interesting summary of the proteoglycan CSPG4 function in the turnover and repair of myelin after lysolecithin treatment in mice.*
Reviewer 2: *Qun Li, Johns Hopkins Medicine, USA.*
Comments to authors: *Development of oligodendrocyte progenitor cells (OPCs) contributes to remyelination in multiple sclerosis (MS) and other demyelination events. In this article, the authors apply type-specific NG2 ablation models and show that genetically deleting NG2 in OPCs and myeloid cells diminishes capability for OPC proliferation and myelin repair after lesion. In addition, the authors investigate the distinct functions of macrophages and microglia for demyelination and remyelination in subacute (1 week) and chronic (6 weeks) injuries using germline NG2 ablation coupled with bone marrow transplantation. Action of atypical and incompletely-differentiated macrophages for myelination inability is also reported. The mechanisms that NG2 in OPCs and myeloid cells contribute to myelin repair are relevantly discussed. The manuscript is well organized, and authors clearly elucidate their viewpoints.*

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