Metformin Restores CNS Remyelination Capacity by Rejuvenating Aged Stem Cells

Highlights
- Aged OPCs fail to respond to differentiation signals
- Aged OPCs acquire many of the hallmarks of cell aging
- Fasting and the fasting mimetic metformin rejuvenate poor remyelination in aged rodents
- Metformin reverses age-related changes, making OPCs respond to differentiation factors

In Brief
Neumann et al. demonstrate that aging renders adult oligodendrocyte progenitor cells unresponsive to pro-differentiation factors, which can be reversed both in vitro and in vivo by calorie restriction (CR) and the CR mimetic metformin.
Metformin Restores CNS Remyelination Capacity by Rejuvenating Aged Stem Cells

Björn Neumann,1 Roey Baror,1 Chao Zhao,1 Michael Segel,1 Sabine Dietmann,1 Khalil S. Rawji,1 Sarah Foerster,1 Crystal R. McClain,1 Kevin Chalut,1,4 Peter van Wijngaarden,1,2,3,* and Robin J.M. Franklin1,5,*

1Wellcome – MRC Cambridge Stem Cell Institute, Jeffrey Cheah Biomedical Centre, Cambridge Biomedical Campus, University of Cambridge, Cambridge, CB2 0AW, UK
2Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, Melbourne, Australia
3Ophthalmology, Department of Surgery, University of Melbourne, Melbourne, Australia
4Department of Physics, University of Cambridge, Cambridge CB3 0HE, UK
5Lead Contact
*Correspondence: peterv@unimelb.edu.au (P.v.W.), rj1000@cam.ac.uk (R.J.M.F.)
https://doi.org/10.1016/j.stem.2019.08.015

SUMMARY

The age-related failure to produce oligodendrocytes from oligodendrocyte progenitor cells (OPCs) is associated with irreversible neurodegeneration in multiple sclerosis (MS). Consequently, regenerative approaches have significant potential for treating chronic demyelinating diseases. Here, we show that the differentiation potential of adult rodent OPCs decreases with age. Aged OPCs become unresponsive to pro-differentiation signals, suggesting intrinsic constraints on therapeutic approaches aimed at enhancing OPC differentiation. This decline in functional capacity is associated with hallmarks of cellular aging, including decreased metabolic function and increased DNA damage. Fasting or treatment with metformin can reverse these changes and restore the regenerative capacity of aged OPCs, improving remyelination in aged animals following focal demyelination. Aged OPCs treated with metformin regain responsiveness to pro-differentiation signals, suggesting synergistic effects of rejuvenation and pro-differentiation therapies. These findings provide insight into aging-associated remyelination failure and suggest therapeutic interventions for reversing such declines in chronic disease.

INTRODUCTION

The ability to regenerate oligodendrocytes, the myelin-forming cells of the CNS, contrasts with the poor capacity to regenerate neurons in most brain regions (Franklin and Ffrench-Constant, 2017). Generation of oligodendrocytes from oligodendrocyte progenitor cells (OPCs) occurs throughout life and contributes to myelin turnover (Young et al., 2013; Hill et al., 2018; Hughes et al., 2018; Tripathi et al., 2017) and adaptive myelination (Gibson et al., 2014; Mitew et al., 2018; Hughes et al., 2018) as well as to the regenerative process of remyelination that follows demyelination (Zawadzka et al., 2010). As with most regenerative processes, the efficiency of remyelination declines progressively with aging to the extent that it becomes so slow that it eventually fails (Oh et al., 2014; Sim et al., 2002). This has important implications for chronic demyelinating diseases such as multiple sclerosis (MS) that can extend over several decades. Delayed remyelination renders demyelinated axons susceptible to irreversible degeneration, a phenomenon that underpins the progressive neurological decline associated with the later stages of MS (Franklin et al., 2012).

The slowing of remyelination with aging is characterized by impaired recruitment of OPCs into the lesion area as well as their delayed differentiation into oligodendrocytes (Sim et al., 2002). Chronically demyelinated MS lesions either lack OPCs or, more commonly, contain OPCs that have failed to differentiate (Boyd et al., 2013; Chang et al., 2002; Kuhlmann et al., 2008). Increasing the number of OPCs in white matter lesions of aged animals does not improve remyelination (Woodruff et al., 2004), indicating that differentiation of OPCs into oligodendrocytes is the bottleneck for remyelination. The mechanisms that regulate OPC differentiation are dysregulated in the aging brain (Shen et al., 2008), in part because of age-related changes in the cells and molecules in the environment in which remyelination occurs (Cantuti-Castelvetri et al., 2018; Hinks and Franklin, 2000; Natraj et al., 2015). These changes can be overcome, in principle, by providing a more youthful systemic environment that is permissive for regeneration (Ruckh et al., 2012). Thus, remyelination can potentially be enhanced by pro-differentiation factors lacking in the aged brain. However, it remains unclear whether OPCs undergo intrinsic changes with aging that affect their responsiveness to differentiation signals.

RESULTS

Aged OPCs Differentiate Slowly and Do Not React to Pro-differentiation Compounds

We first asked whether age-related changes in OPCs contribute to the differentiation delay observed in aged animals during remyelination (Sim et al., 2002). Studies of OPC aging have been hampered by the technical challenges of culturing OPCs isolated from the aged adult rodent CNS. Thus, we first optimized existing protocols to establish cultures of adult OPCs from young adult (2–3 months) and aged (20–24 months) rats (Figure S1;
Figure 1. OPCs Lose Their Inherent Capacity for Differentiation and Their Responsiveness to Differentiation Factors with Aging

(A) Representative images of young adult (2–3 months old) and aged OPCs (20–24 months old) differentiated in the absence of growth factor or in the presence of T3. Increasing maturity was visualized using O4 (early), CNPase (intermediate), and MBP (mature), immunocytochemical markers of the oligodendrocyte (OL) lineage. Scale bars, 50 μm.

(B and C) Quantification of cells over time in culture: CNPase+/Olig2+ cells (B) and MBP+/Olig2+ cells (C). Statistical significance was determined using two-way ANOVA repeated measurements followed by Dunnett’s post test to compare each group against “aged T3.” All data are presented as mean ± SD (n = 3 biological repeats).

(D) Schematic of the experimental design.

(E) Representative images of the differentiation assay performed with young and aged OPCs. Newly formed oligodendrocytes were identified as MBP+/Olig2+ cells. Scale bars, 50 μm.

(legend continued on next page)
We used magnetic activated cell sorting (MACS) for A2B5 to isolate cells from young and aged adult brains (Figure S1A). The positively selected cells showed minor contamination for CD11b+ microglia (<0.8%) or MOG+ oligodendrocytes (<2%) with no significant difference between preparations from young and aged animals (Figures S1B–S1D). Using immunohistochemistry, we found that A2B5+ cells of both age groups co-expressed PDGFRα, NG2, Sox10, and Olig2, confirming their identity as OPCs (Figures S1E–S1I and S1N–S1R). In contrast, A2B5+ cells from young or aged adults never expressed mature lineage markers such as CNPase and MBP (Figures S1J and S1K), the microglia marker Cd11b (Figure S1L), or the astrocyte marker GFAP (Figure S1M), indicating that cultures were free of mature oligodendrocytes, microglia, and astrocytes. This enabled us to compare the differentiation efficiency of OPCs isolated from the young adult and aged CNS.

We next tested the relative differentiation ability of OPCs from young adults (hereafter referred to as young OPCs) and OPCs from old adults (aged OPCs) when grown in differentiation medium from which the growth factors that maintain proliferation were removed. Although 60% of young OPCs differentiated into mature oligodendrocytes (CNPase+ and MBP+), fewer than 20% of aged OPCs acquired these markers within the same period, revealing a slower inherent capacity for differentiation (Figures 1A–1C). We next assessed how adult OPCs responded to thyroid hormone (T3), a well-established promoter of OPC differentiation (Gao et al., 1998). Although T3 accelerated the differentiation of young OPCs, there was no significant effect on the differentiation of aged OPCs (Figures 1B and 1C). Similar results were obtained with other factors known to have pro-differentiation effects on OPCs derived from newborn animals or on pluripotent stem cells, such as 9-cis-retinoic acid (Huang et al., 2011), miconazole (Najm et al., 2015), and benzatropine (Deshmukh et al., 2013), all of which enhanced differentiation in young adult (Figures 1E and 1F) but not aged OPCs (Figures 1E, 1G, and S4).

Cultures of aged OPCs were generally of lower cell density, suggesting decreased survival. Because the density might influence the differentiation rate through secretion of paracrine factors, we asked whether increasing the cellular density might rescue the differentiation ability of aged OPCs or reduce the differentiation rate of young OPCs. Seeding half of the normal number of young OPCs or twice the number of aged OPCs did not result in a significant change in differentiation rates (Figures S2A–S2E), indicating that the differences in cellular density between young and aged cultures do not account for the reduced differentiation of aged OPCs.

Because some aged OPCs differentiated into MBP+ oligodendrocytes, we asked whether the non-differentiating cells were unable to differentiate or were differentiating at a slower rate. To test this, we cultured aged OPCs for 4 weeks in differentiation medium and found a significant increase in differentiation (Figures S2D and S2E), indicating that aged OPCs do not lose their general ability for differentiation but undergo intrinsic changes that significantly slow their differentiation program. These intrinsic changes also cause aging OPCs to become less responsive to factors that induce differentiation, which likely contributes to the failure of oligodendrocyte lineage differentiation, characteristic of many non-remyelinating chronic MS lesions (Kuhlmann et al., 2008).

Aged OPCs Exhibit Classical Hallmarks of Aging

Next we characterized molecular alterations responsible for the aged OPC phenotype using RNA sequencing (RNA-seq) to compare the transcriptomes of OPCs isolated from young and aged rats. Approximately 20% of all genes were differentially expressed with aging (1.5-fold change in expression, adjusted p value [p.adj] < 0.05). Among the genes more highly expressed in young adult OPCs were those that are characteristic of adult OPCs in 21-day-old mice (Marques et al., 2016; Figure 2A) and their self-renewal, including Pdgfra, Ascl1, and Ptprz1 (Emery, 2010; Figure 2B; Table S1). In contrast, aged OPCs expressed higher levels of the early differentiation markers Cnp1, Sirt2, and Enpp6 (Figure 2B). Because we did not find a higher proportion of MOG+ cells or those expressing more mature lineage markers, such as CNPase, in our aged OPC preparations compared with young OPCs (Figures S1J and S1K), we ruled out the possibility that these changes in the transcriptome were caused by contamination with oligodendrocytes. Thus, we concluded that aged OPCs lose their characteristic stem cell signature (Figures 2A and 2B). To identify the cellular processes that might contribute to the aged OPC state, we used Ingenuity pathway analysis on genes preferentially expressed in aged OPCs. We found enrichment of terms that are closely linked to organismal and stem cell aging, such as mitochondrial dysfunction, unfolded protein response (UPR), autophagy, inflammation, and nuclear factor κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) signaling (Figure 2C). Consistent with the predictions made on the basis of the RNA-seq data, we found increased mTOR activity in freshly isolated aged OPCs by detection of the phosphorylated forms of the downstream target p70S6-kinase (Figure 2D). mTOR activity is a crucial regulator of adult stem cell quiescence, activation, and differentiation (Mihaylova et al., 2014; Rodgers et al., 2014) and is linked to cellular aging (Laplante and Sabatini, 2012). Aging is associated with increased and dysregulated mTOR activity, which contributes to DNA damage and cellular senescence (Castillo et al., 2009; Chen et al., 2009; Yilmaz et al., 2006). We therefore predicted that both DNA damage and markers of senescence would increase with adult OPC aging. Consistent with this prediction, single-cell comet assays revealed that aged OPCs had significantly more DNA damage than young OPCs (Figures 2E and 2F). Using our RNA-seq data, we also found that aged OPCs expressed several genes associated with cellular senescence at significantly higher levels than young OPCs (Figure 2G; Tacutu et al., 2018). We found that aged OPCs had 8-fold higher mRNA levels of the senescence marker Cdkn2a (Figure 2H). Last, aged OPCs had lower levels of ATP.
and reduced cellular respiration (Figures 2I and 2J), likely reflecting a combination of mitochondrial dysfunction and reduced mitochondrial content. Thus, aged OPCs, like other adult stem cells, acquire a variety of hallmarks of aging that likely contribute to loss of their regenerative potential.

Alternate-Day Fasting Enhances Remyelination in Aged Rats through Functional Rejuvenation of OPCs

On the basis of these findings, we hypothesized that reversal of these age-related changes in OPCs might be necessary to reinstate their differentiation potential. We therefore explored strategies known to alter the effects of aging as a potential strategy to improve OPC function and remyelination in aged animals. Dietary restriction is the most effective intervention known to alter the organismal aging process (Fontana and Partridge, 2015) and it does so in part by enhancing the function of aged adult stem cells (Cerletti et al., 2012; Mihaylova et al., 2018).

To test the effect of dietary restriction on remyelination, we subjected 12-month-old rats, an age when remyelination rate is substantially slower than in young adult rats (Sim et al., 2002; Shields et al., 1999), to alternate-day fasting (ADF) for 6 months (Figure 3A). We confirmed that OPCs from 12-month-old rats, like those from 24-month-old rats, were impaired in their differentiation and unable to respond to differentiation signals (Figure S3). At the end of the 6-month fasting period, we induced demyelination in cerebellar white matter by focal injection of ethidium bromide, a well-established in vivo model for studying remyelination (Woodruff and Franklin, 1999). We assessed the degree of...
A

EB CCP lesion:

MoTu WeTh Fr Sa Su

Fix at 7, 21, 50dpi

12 mo. old

18 mo. old

ADF

CON

Access to food

B

CON

ADF

C

CON

ADF

D

Remyelination ranking

***

0

5

10

15

CON

ADF

E

Remyelinated axons [%]

***

0

20

40

60

80

100

CON

ADF

F

CON-7dpi

ADF-7dpi

Nix2.2 KI67 Nuclei

Nix2.2 KI67 Nuclei

G

p=0.059

0

20

40

60

80

100

Nix2.2 cells/mm²

CON ADF

H

p=0.5185

0

5

10

15

Nix2.2 KI67/mm²

Aged ADF

I

CON-50dpi

ADF-50dpi

CC1 Olig2

CC1 Olig2

J

CON ADF

K

0

20

40

60

80

100

CC1/Olig2 [%]

7dpi 21dpi 50dpi

L

MBP Olig2

MBP Olig2

M

MBP+Olig2 [%]

CON ADF

(legend on next page)
remyelination 50 days post lesion (dpl) induction in semi-thin resin sections stained with toluidine blue, in which remyelinated axons are easily identifiable by their thin myelin sheaths (Blake-more, 1974; Figure 3B), which was confirmed by examination of electron micrographs (Figure 3C). Although remyelination in ad libitum-fed animals was restricted to the border of the lesion, animals undergoing fasting consistently exhibited nearly complete remyelination (Figures 3B and 3C). The clear difference in the extent of remyelination between the two groups was reflected by a blind ranking analysis of semi-thin sections of lesions as well as quantification of the percentage of remyelinated axons within the lesions (Figures 3B, 3D, and 3E). We also compared g ratios of control and treatment groups but found no differences, suggesting that the effect of ADF is on the extent rather than the quality of remyelination (Figure S5A). To further explore fasting-enhanced remyelination in aged animals, we characterized the recruitment, proliferation, and differentiation of OPCs during remyelination. At 7 dpl, there was no difference in the density of OPCs within lesions of animals subjected to fasting or ad libitum feeding (Figures 3F–3H). However, the density of mature oligodendrocytes (Olig2+/CC1+) was 2-fold greater in the lesions of fasting animals compared with those of controls at both 21 and 50 dpl (Figures 3I and 3J). The proportion of Olig2+ cells expressing CC1 was significantly higher in lesions of fasting rats at both time points relative to controls, suggesting enhanced OPC differentiation (Figure 3K).

The positive effects of fasting regimens on adult stem cells have been attributed to alterations within the stem cells and to changes in the stem cell niche or in the systemic environment (Cerletti et al., 2012; Igarashi and Guarente, 2016; Lee et al., 2000; Mihaylova et al., 2018; Yilmaz et al., 2012). We therefore reasoned that the observed effect of fasting on remyelination is likely the consequence of changes in a variety of cell types (such as astrocytes, inflammatory cells, and vascular cells as well as OPCs) associated with the lesion, caused by changes in the systemic environment. To determine whether the enhanced differentiation capacity of aged OPCs in fasting animals was at least in part due to intrinsic alterations within the OPCs, we isolated OPCs from unlesioned ADF and control aged animals. We found that OPCs derived from fasting animals differentiated into MBP+ oligodendrocytes more rapidly than those from age-matched control rats (Figures 3L and 3M) and with efficiency comparable with OPCs from young rats (Figure 1). Moreover, OPCs derived from fasting animals expressed higher levels of OPC self-renewal genes (Figure S4A), lower levels of differentiation genes (Figure S4A), and lower levels of Cdkn2a (Figure S4B) exhibited less DNA damage (Figures S4C and S4D); showed reduced levels of phosphorylated p70S6K (Figure S4E); and had higher ATP levels (Figure S4F). Thus, we concluded that ADF restored remyelination in part through the functional rejuvenation of aged OPCs. To address whether effects on other cell types also contributed to enhanced remyelination in ADF animals, we assessed the density of GFAP+ astrocytes and Iba1+ innate immune cells within the lesion during remyelination. In neither case was there a significant change between samples from fasting and control animals (Figures S5B–S5E). However, we found that removal of myelin debris was improved in animals undergoing fasting (Figures S5F–S5H), suggest functional changes in phagocytic cells within the lesion and a potential mechanism contributing to the improved remyelination outcome in ADF animals (Ruckh et al., 2012).

The Fasting Mimetic Metformin Restores the Differentiation Capacity of Aged OPCs

Because dietary restriction protocols are difficult to translate into a clinical context, we asked whether the small-molecule fasting mimetic metformin might replicate the effects of fasting. The fasting-mimicking effects of metformin, a drug widely prescribed for type 2 diabetes, are mediated via modulation of the AMPK pathway, a central nutrient signaling pathway. We found that metformin-treated cells exhibited increased expression of Pdgfra and Ascl1 (Figures S4G and S4H), expressed significantly less Cdkn2a (Figure S4I), and had less DNA damage, as indicated by comet assays (Figure S4J), suggesting that metformin...
is sufficient to ameliorate some of the hallmarks of aging and phenocopy at least some of the effects of ADF (Figures S4A–S4F).

We then asked whether exposure to metformin would also be sufficient to restore the potential of aged OPCs to differentiate and restore the responsiveness of aged OPCs to differentiation factors. Treatment with metformin prior to onset of differentiation increased the number of differentiated cells and enhanced the responsiveness of aged OPCs to pro-differentiation factors (Figures 4A and 4B), indicating functional rejuvenation of aged OPCs, but had no effect on differentiation of young OPCs (Figure S6). The mechanisms by which metformin rejuvenates aging OPCs are likely to be multiple but, at least in part, appear to be through the AMPK pathway because metformin increases the levels of phosphorylated AMPK (Figures 4C and 4D), whereas inhibition of AMPK signaling by dorsomorphin, a small-molecule inhibitor of the AMPK pathway, abrogated the effect of metformin on AMPK phosphorylation and aged OPC differentiation (Figures 4C, 4E, and 4F). Because dorsomorphin is also known to affect other kinases, we additionally used a genetic approach to specifically disrupt signaling through AMPK. Using the CRISPR/Cas9 system, we found that aged OPCs transfected with guide RNAs targeting Prkaa2 (AMPK) in aged OPCs using CRISPR/Cas9. We then asked whether exposure to metformin would also be sufficient to restore the potential of aged OPCs to differentiate and restore the responsiveness of aged OPCs to differentiation factors. Treatment with metformin prior to onset of differentiation increased the number of differentiated cells and enhanced the responsiveness of aged OPCs to pro-differentiation factors (Figures 4A and 4B), indicating functional rejuvenation of aged OPCs, but had no effect on differentiation of young OPCs (Figure S6). The mechanisms by which metformin rejuvenates aging OPCs are likely to be multiple but, at least in part, appear to be through the AMPK pathway because metformin increases the levels of phosphorylated AMPK (Figures 4C and 4D), whereas inhibition of AMPK signaling by dorsomorphin, a small-molecule inhibitor of the AMPK pathway, abrogated the effect of metformin on AMPK phosphorylation and aged OPC differentiation (Figures 4C, 4E, and 4F). Because dorsomorphin is also known to affect other kinases, we additionally used a genetic approach to specifically disrupt signaling through AMPK. Using the CRISPR/Cas9 system, we found that aged OPCs transfected with guide RNAs targeting Prkaa2 (AMPK) in aged OPCs using CRISPR/Cas9.

**Metformin Enhances Remyelination in Aged Rats**

Lesions in MS occur in an unpredictable manner and are not synchronized. This also implies that the regenerative process will not be synchronized within an individual. Therefore, any treatment targeting OPCs must not interfere with any specific stage of the remyelination process. We therefore asked whether the timing of metformin exposure during OPC differentiation might influence its effect. We found that exposure to metformin throughout the entire culture period produced the same effect as pre-incubation of aged OPCs with the drug before onset of differentiation by removal of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Figures 6A–6C). Finally, we tested whether metformin treatment could mimic ADF in vivo. Aged rats fed ad libitum received metformin in their drinking water for 3 months prior to induction of a focal demyelinating injury and during the time after lesion induction (Figure 6D). Metformin-treated animals exhibited remyelination that was comparable in extent with animals undergoing ADF and significantly higher than that in control animals, as judged by the ranking analysis and percentage of remyelinated axons (Figures 6E–6H), although there was no difference in g ratios of remyelinated axons between the groups (Figure S4).

**DISCUSSION**

MS is a chronic degenerative disease that typically becomes progressive. Epidemiological data indicate that age more than the duration of the disease influences when MS becomes progressive (Confavreux and Vukusic, 2006). The progressive phase is characterized by accumulative neurodegeneration. This is most likely the result of an unmet need for repair and a concomitant increase in the susceptibility of neurons and their axons for degeneration, which are both processes closely tied to aging. Thus, understanding how aging affects remyelination and how
Figure 5. Mitochondrial ATP Production Is Required for OPC Differentiation

(A) OPCs and differentiating pre-oligodendrocytes (POLs) were isolated from young rats (2–3 months) by MACS using A2B5 (OPCs) and O4 (pre-OL) antibodies.

(B) Representative graph depicting the fold change of the bOCR of OPCs and POLs under basal conditions and sequential treatment with oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone and antimycin A.

(C) Quantification of the bOCR normalized to POL (n = 2 biological replicates for each group, two-tailed t test).

(D) Quantification of normalized ATP measurements from freshly isolated OPCs and POLs. (n = 2 biological repeats for each group, circles depict technical repeats, two-tailed t test).

(E) Representative images of differentiation cultures from young OPCs after treatment with DMSO or increasing concentrations of rotenone (a mitochondrial complex I blocker). Newly formed oligodendrocytes were identified as MBP+/Olig2+ cells. Scale bar, 50 μm.

(F) Quantification of the differentiation assay (all data are presented as mean ± SD; statistical significance was determined using one-way ANOVA with Dunnett’s post test for each treatment group against DMSO; n = 3 biological replicates).

(G) Schematic illustration of the experiments using metformin and dorsomorphin.

(H) Relative change in the bOCR, measured after treatment of aged OPCs with metformin and/or dorsomorphin for 5 days in vitro. Dots represent technical replicates (n = 3 biological replicates for each age group, two-tailed t test).

(I) Intracellular ATP content of aged OPCs, normalized to cell numbers in the respective control group, treated with metformin alone or metformin and dorsomorphin (n = 3 biological repeats for each age group, two-tailed t test). Error bars represent SDs. *p < 0.05; **p < 0.01; ***p < 0.001; ns, p > 0.05.
these effects may be reversed is critical to devising regenerative interventions with which to combat progressive MS.

Studies of OPC differentiation pathways have identified a number of candidate therapeutic agents to enhance remyelination (Deshmukh et al., 2013; Hubler et al., 2018; Mei et al., 2014; Najm et al., 2015), but none have been tested in aged cells or aged animals. This is important because aging is associated with remyelination failure, and age-related changes in stem cell...
function may render cells less responsive to candidate therapies. Indeed, our findings reveal that OPCs do indeed lose the capacity to respond to endogenous differentiation cues and candidate therapeutic agents with increasing age, with significant implications for therapies that aim to enhance remyelination.

Difficulties encountered in isolating and culturing OPCs from aged animals have previously limited our understanding of the influence of aging on these cells. Here we report methods for successful isolation and culture of OPCs from aged rats. Transcriptional analyses and in vitro assays revealed that aged OPCs exhibit many hallmarks of stem cell aging, including DNA damage and reduced mitochondrial function, which are known to impede cellular function (Oh et al., 2014). Importantly, using fasting or metformin to ameliorate hallmarks of aging, we show that the age-dependent loss of OPC potential is reversible, which is a pre-requisite for pharmacological interventions targeting endogenous OPCs to stimulate remyelination. Because exposure of metformin to purified cultures of aged OPCs led to functional rejuvenation, it is plausible that metformin could also have exerted a direct effect on OPCs in vivo. We show a direct effect of metformin on OPCs in culture and present evidence that this effect is AMPK-dependent and associated with an increase in mitochondrial function (Figure 4), which is required for differentiation (Figure 5). However, this is unlikely to be the only mechanism orchestrating this complex process because metformin has multiple functions, such as stimulating DNA repair pathways and activating autophagy. Even enhancement of a single cellular processes alone can lead to amelioration of several hallmarks of aging. For example, overexpression of Atg7, a key component of the autophagy pathway, improves proteostasis, as expected, but also improves mitochondrial function and reduces the levels of senescence markers (García-Prat et al., 2016). Similarly, the reduction of reactive oxygen species (ROS) in aged muscle stem cells by supplementation of an antioxidant has the same effects on these hallmarks of aging (Ho et al., 2017). The question that follows is whether restoring the function of aged OPCs alone could be sufficient to enhance remyelination in an aged animal.

Experiments in other stem cell systems indicate that rejuvenation of aged adult stem cells by inhibition of p38-MAPK signaling and exposure to a softer culture surface can be sufficient to restore regenerative ability in an aged animal (Cosgrove et al., 2014). Thus, it is possible that rejuvenation of aged OPCs by metformin is sufficient to promote remyelination in aged animals following demyelinating injuries. In addition, treatments such as metformin and fasting have wide-ranging systemic effects that together may be permissive for remyelination. For example, metformin is known to reduce inflammasome signaling, and reductions in this signaling have been reported to enhance remyelination in middle-aged mice (Cantuti-Castelvetri et al., 2018). Further, fasting has been shown to enhance muscle stem cell function and muscle regeneration relative to ad libitum feeding, even in young animals (Cerletti et al., 2012). It follows that the enhanced remyelination observed with metformin treatment is likely a composite of direct effects on OPCs together with effects on other cell types. Our in vitro work indicates that metformin acts synergistically with drugs identified previously to promote differentiation of OPCs. Importantly, when used in the absence of metformin treatment, these candidate remyelination drugs failed to mediate differentiation of aged OPCs. This points to the need for combination therapies to enhance remyelination in vivo because remyelination failure typically occurs in the context of aging.

Overcoming the effects of aging on OPCs is important in generating a permissive environment for remyelination. Interventions such as dietary restriction or drugs that mimic its effects will likely alter the function of OPCs and other cell types that contribute to remyelination. In addition, fasting and calorie restriction mimetics have been shown to suppress autoimmunity-mediated demyelination (Choi et al., 2016; Cignarella et al., 2018; Piccio et al., 2008) and may thus have dual benefits for the treatment of chronic demyelinating neurodegenerative diseases.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animal husbandry
  - Isolation of adult oligodendrocyte progenitor cells
  - Culture of adult oligodendrocyte progenitor cells
- **METHOD DETAILS**
  - Induction of white matter lesions and assessment of remyelination
  - Isolation of adult oligodendrocyte progenitor cells
  - Culture of adult oligodendrocyte progenitor cells
  - Immunofluorescence for tissue sections
  - Immunofluorescence for cells
  - Comet assay
  - RNA sequencing and downstream analysis
  - RNA isolation and qRT-PCR
  - ATP measurements
  - Oxygen Consumption rate measurements
  - Western Blot
  - Fluorescence Activated Cell Sorting
  - Oil-Red-O staining
  - CRISPR/Cas9 mediated knockdown of Prkaa2 (AMPK)
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Statistical analysis
- **DATA AND CODE AVAILABILITY**

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at [https://doi.org/10.1016/j.stem.2019.08.015](https://doi.org/10.1016/j.stem.2019.08.015).

ACKNOWLEDGMENTS

This work was supported by funding from the UK Multiple Sclerosis Society, MedImmune, The Adelson Medical Research Foundation, and a core support grant from the Wellcome Trust and MRC to the Wellcome Trust-Medical Research Council Cambridge Stem Cell Institute. P.v.W. was supported by the National Health and Medical Research Council of Australia (ECF #628928). We thank Dr. Daniel Morrison for assistance with electron microscopy, the NIHR Cambridge BRC Cell Phenotyping Hub for all flow cytometry work, and the staff at the Cambridge Advanced Imaging Centre.
AUTHOR CONTRIBUTIONS

Conceptualization, B.N., P.v.W., and R.J.M.F.; Methodology, B.N., R.B., and C.M.; Investigation, B.N., R.B., C.Z., M.S., K.S.R., and S.F.; Formal Analysis, B.N., R.B., C.Z., S.D., K.S.R., P.v.W., and R.J.M.F.; Writing, B.N., K.C., P.v.W., and R.J.M.F.; Funding Acquisition, R.J.M.F.; Supervision, P.v.W. and R.J.M.F.

DECLARATION OF INTERESTS

The authors declare that there are patent applications pending related to this work.

Received: January 28, 2019
Revised: June 27, 2019
Accepted: August 23, 2019
Published: October 3, 2019

REFERENCES

Blakemore, W.F. (1974). Pattern of remyelination in the CNS. Nature 249, 577–578.

Boyd, A., Zhang, H., and Williams, A. (2013). Insufficient OPC migration into demyelinated lesions is a cause of poor remyelination in MS and mouse models. Acta Neuropathol. 125, 841–859.

Cantuti-Castelveci, L., Fitzner, D., Bosch-Queralt, M., Well, M.T., Su, M., Sen, P., Ruhwedel, T., Mitkofski, M., Trendelenburg, G., Lutjohann, D., et al. (2018). Defective cholesterol clearance limits remyelination in the aged central nervous system. Science 359, 684–688.

Castillo, R.M., Squirrel, C.H., Chodosh, L.A., Williams, B.O., and Gutkind, J.S. (2009). mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. Cell Stem Cell 5, 279–289.

Cerletti, M., Jiang, Y.C., Finley, L.W.S., Haisig, M.C., and Wagers, A.J. (2012). Short-term calorie restriction enhances skeletal muscle stem cell function. Cell Stem Cell 10, 515–519.

Chang, A., Tourtellotte, W.W., Rudick, R., and Trapp, B.D. (2002). Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. Chang, A., Tourtellotte, W.W., Rudick, R., and Trapp, B.D. (2002). Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. Brain 129, 249–261.

Choi, I.Y., Piccio, L., Childress, P., Bollman, B., Ghosh, A., Brandhorst, S., Llewellyn, M.E., Delp, S.L., and Blau, H.M. (2014). Rejuvenation of the muscle stem cell population restores strength to injured aged muscles. Nat. Med. 20, 2232–2323.e6.

Collins, A.R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. Mol. Biotechnol. 26, 249–261.

Confavreux, C., and Vukusic, S. (2006). Age at disability milestones in multiple sclerosis. Brain 129, 595–605.

Cosgrove, B.D., Gilbert, P.M., Porpiglia, E., Mourkioti, F., Lee, S.P., Corbel, S.Y., Llewellyn, M.E., Delp, S.L., and Blau, H.M. (2014). Rejuvenation of the muscle stem cell population restores strength to injured aged muscles. Nat. Med. 20, 255–264.

Deshmukh, V.A., Tardif, V., Lyssiotis, C.A., Green, C.C., Kerman, B., Kim, H.J., Padmanabhan, K., Swoboda, J.O., Ahmad, I., Kondo, T., et al. (2013). A regenerative approach to the treatment of multiple sclerosis. Nature 502, 327–332.

Dugas, J.C., and Emery, B. (2013). Purification and culture of oligodendrocyte lineage cells. Cold Spring Harb. Protoc. 2013, 810–814.

Emery, B. (2010). Regulation of oligodendrocyte differentiation and myelination. Science 330, 779–782.

Fontana, L., and Partridge, L. (2015). Promoting health and longevity through diet: from model organisms to humans. Cell 161, 106–118, 257–258.

Franklin, R.J.M., and French-Constant, C. (2017). Regenerating CNS myelin - from mechanisms to experimental medicines. Nat. Rev. Neurosci. 18, 753–769.

Franklin, R.J.M., french-Constant, C., Edgar, J.M., and Smith, K.J. (2012). Neuroprotection and repair in multiple sclerosis. Nat. Rev. Neurol. 8, 624–634.

Gao, F.B., Apperly, J., and Raff, M. (1998). Cell-intrinsic timers and thyroid hormone regulate the probability of cell-cycle withdrawal and differentiation of oligodendrocyte precursor cells. Dev. Biol. 197, 54–66.

Garcia-Prat, L., Martinez-Vicente, M., Perdiguero, E., Oró, L., Rodriguez-Ureña, J., Rebollo, E., Ruiz-Bonilla, V., Gutierrez, S., Ballestar, E., Serrano, A.L., et al. (2016). Autophagy maintains stemness by preventing senescence. Nature 529, 37–42.

Gibson, E.M., Purger, D., Mount, C.W., Goldstein, A.K., Lin, G.L., Wood, L.S., Inema, I., Miller, S.E., Bieri, G., Zuchero, J.B., et al. (2014). Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. Science 344, 1252–304.

Hill, R.A., Li, A.M., and Grutzendorf, J. (2018). Lifelong cortical myelin plasticity and age-related degeneration in the live mammalian brain. Nat. Neurosci. 21, 683–695.

Hinks, G.L., and Franklin, R.J.M. (2000). Delayed changes in growth factor gene expression during slow remyelination in the CNS of aged rats. Mol. Cell. Neurosci. 16, 542–556.

Ho, T.T., Warr, M.R., Adelman, E.R., Lansinger, O.M., Flach, J., Verovskaya, E.V., Figueroa, M.E., and Passegué, E. (2017). Autophagy maintains the metabolism and function of young and old stem cells. Nature 543, 205–210.

Huang, J.K., Jarjour, A.A., Nait Oumesmar, B., Kerninon, C., Williams, A., Krezel, W., Kagechika, H., Bauer, J., Zhao, C., Baron-Van Evercooren, A., et al. (2011). Retinoid X receptor gamma signaling accelerates CNS remyelination. Nat. Neurosci. 14, 45–53.

Hubler, Z., Allimuthu, D., Bederman, I., Eilt, M.S., Madhavan, M., Allan, K.C., Shick, H.E., Garrison, E., T Karl, M., Factor, D.C., et al. (2018). Accumulation of 8,9-unsaturated sterols drives oligodendrocyte formation and remyelination. Nature 560, 372–376.

Hughes, E.G., Orthmann-Murphy, J.L., Langseth, A.J., and Bergles, D.E. (2018). Myelin remodeling through experience-dependent oligodendrogenesis in the adult somatosensory cortex. Nat. Neurosci. 21, 696–706.

Igarashi, M., and Guarente, L. (2016). mTORC1 and SIRT1 cooperate to foster expansion of gut adult stem cells during calorie restriction. Cell 166, 436–450.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Kuhlmann, T., Miron, V., Cui, Q., Wegner, C., Antel, J., and Brück, W. (2008). Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. Brain 131, 1749–1758.

Laplante, M., and Sabatini, D.M. (2012). mTOR signaling in growth control and disease. Cell 149, 274–293.

Lee, J., Duan, W., Long, J.M., Ingram, D.K., and Mattson, M.P. (2000). Dietary restriction increases the number of newly generated neural cells, and induces BNDF expression, in the dentate gyrus of rats. J. Mol. Neurosci. 15, 99–108.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.

Marques, S., Zeisel, A., Codeluppi, S., van Bruggen, D., Mendoza Falcão, A., Xiao, L., Li, H., Häring, M., Hochgerber, H., Romanov, R.A., et al. (2016). Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. Science 352, 1326–1329.

Mei, F., Fancy, S.P.J., Shen, Y.A., Niu, J., Zhao, C., Presley, B., Miao, E., Lee, S., Mayoral, S.R., Redmond, S.A., et al. (2014). Micropillar arrays as a high-throughput screening platform for therapeutics in multiple sclerosis. Nat. Med. 20, 954–960.

Mihaylova, M.M., Sabatini, D.M., and Yilmaz, 0.H. (2014). Dietary and metabolic control of stem cell function in physiology and cancer. Cell Stem Cell 14, 292–305.

Mihaylova, M.M., Cheng, C.W., Cao, A.Q., Tripathi, S., Mana, M.D., Bauer-Rowe, K.E., Abu-Ramaleh, M., Clavain, L., Erdemir, A., Lewis, C.A., et al. (2018). Mtor signaling in growth control and disease. Cell 174, 274–293.

Lee, J., Duan, W., Long, J.M., Ingram, D.K., and Mattson, M.P. (2000). Dietary restriction increases the number of newly generated neural cells, and induces BNDF expression, in the dentate gyrus of rats. J. Mol. Neurosci. 15, 99–108.
(2018). Fasting activates fatty acid oxidation to enhance intestinal stem cell function during homeostasis and aging. Cell Stem Cell 22, 769–778.e4.

Mitew, S., Gobius, I., Fenlon, L.R., McDougall, S.J., Hawkes, D., Xing, Y.L., Buquila, H., Gundlach, A.L., Richards, L.J., Kilpatrick, T.J., et al. (2018). Pharmacogenetic stimulation of neuronal activity increases myelination in an axon-specific manner. Nat. Commun. 9, 306.

Najm, F.J., Madhavan, M., Zaremba, A., Shick, E., Karl, R.T., Factor, D.C., Miller, T.E., Nevin, Z.S., Kantor, C., Sargent, A., et al. (2015). Drug-based modulation of endogenous stem cells promotes functional remyelination in vivo. Nature 522, 216–220.

Oh, J., Lee, Y.D., and Wagers, A.J. (2014). Stem cell aging: mechanisms, regulators and therapeutic opportunities. Nat. Med. 20, 870–880.

Olive, P.L., and Banáth, J.P. (2006). The comet assay: a method to measure DNA damage in individual cells. Nat. Protoc. 1, 23–29.

Piccio, L., Stark, J.L., and Cross, A.H. (2008). Chronic calorie restriction attenuates experimental autoimmune encephalomyelitis. J. Leukoc. Biol. 84, 940–948.

Rodgers, J.T., King, K.Y., Brett, J.O., Cromie, M.J., Charville, G.W., Maguire, K.K., Brunson, C., Mastey, N., Liu, L., Tsai, C.R., et al. (2014). mTORC1 controls the adaptive transition of quiescent stem cells from G0 to G(Alert). Nature 510, 393–396.

Ruckh, J.M., Zhao, J.W., Shadrach, J.L., van Wijngaarden, P., Rao, T.N., Wagers, A.J., and Franklin, R.J.M. (2012). Rejuvenation of regeneration in the aging central nervous system. Cell Stem Cell 10, 96–103.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

Shen, S., Sandoval, J., Swiss, V.A., Li, J., Dupree, J., Franklin, R.J.M., and Casaccia-Bonnefill, P. (2008). Age-dependent epigenetic control of differentiation inhibitors is critical for remyelination efficiency. Nat. Neurosci. 11, 1024–1034.

Shields, S.A., Gilson, J.M., Blakemore, W.F., and Franklin, R.J.M. (1999). Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. Glia 28, 77–83.

Sim, F.J., Zhao, C., Penderis, J., and Franklin, R.J.M. (2002). The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. J. Neurosci. 22, 2451–2459.

Tacutu, R., Thornton, D., Johnson, E., Budovsky, A., Barardo, D., Craig, T., Diana, E., Lehmann, G., Toren, D., Wang, J., et al. (2018). Human Ageing Genomic Resources: new and updated databases. Nucleic Acids Res. 46 (D1), D1083–D1090.

Tripathi, R.B., Jackiewicz, M., McKenzie, I.A., Kougioumtzidou, E., Grist, M., and Richardson, W.D. (2017). Remarkable Stability of Myelinating Oligodendrocytes in Mice. Cell Rep. 21, 316–323.

Woodruff, R.H., and Franklin, R.J.M. (1999). Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotoxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: a comparative study. Glia 25, 216–229.

Woodruff, R.H., Fruttiger, M., Richardson, W.D., and Franklin, R.J.M. (2004). Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination. Mol. Cell. Neurosci. 25, 252–262.

Yilmaz, Ö.H., Valdez, R., Theisen, B.K., Guo, W., Ferguson, D.O., Wu, H., and Morrison, S.J. (2006). Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 441, 475–482.

Yilmaz, Ö.H., Katajisto, P., Lamming, D.W., Gültekin, Y., Bauer-Rowe, K.E., Sengupta, S., Birsoy, K., Dursun, A., Yilmaz, V.O., Selig, M., et al. (2012). mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. Nature 486, 490–495.

Young, K.M., Psachoulia, K., Tripathi, R.B., Dunn, S.J., Cossell, L., Attwell, D., Tohyama, K., and Richardson, W.D. (2013). Oligodendrocyte dynamics in the healthy adult CNS: evidence for myelin remodeling. Neuron 77, 873–885.

Zawadzka, M., Rivers, L.E., Fancy, S.P.J., Zhao, C., Tripathi, R., Damen, F., Young, K., Goncharevich, A., Pohl, H., Rizzi, M., et al. (2010). CNS-resident glial progenitor/stem cells produce Schwann cells as well as oligodendrocytes during repair of CNS demyelination. Cell Stem Cell 6, 578–590.
### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Olig2          | Millipore | Cat# AB15328; RRID:AB_2299035 |
| Anti-Sox-10 (N-20)  | Santa Cruz Biotechnologies | Cat# sc-17342; RRID:AB_2195374 |
| Anti-CD140a (Pdgfra) | BD Biosciences | Cat# 558774; RRID:AB_397117 |
| Anti-NG2 Chondroitin Sulfate Proteoglycan | Millipore | Cat# AB5320; RRID:AB_11213678 |
| Anti-A2B5, clone A2B5-105 | Millipore | Cat# MAB312; RRID:AB_94709 |
| Anti-Ki 67          | Abcam  | Cat# ab16667; RRID:AB_302459 |
| Anti-APC (Ab-7) Mouse mAb (CC-1) | Millipore | Cat# OP80; RRID:AB_2057371 |
| Anti-Oligodendrocyte Marker O4 | R&D Systems | Cat# MAB1326; RRID:AB_357617 |
| Anti-CNPase [11-5B] | Abcam  | Cat# ab6319; RRID:AB_2082593 |
| Anti-MBP (aa82-87)  | Bio-rad | MCA409S; RRID:AB_325004 |
| Anti-Nkx2.2         | DSHB   | Cat# 74.5A5; RRID:AB_531794 |
| Anti-Vimentin       | Bio-rad | Cat# MCA275R; RRID:AB_321302 |
| Anti-GFAP           | Abcam  | Cat# ab53554; RRID:AB_880202 |
| Anti-A2B5-PE        | Milteny Biotec | Cat# 130-098-038; RRID:AB_2660799 |
| Anti-Mouse IgM-PE   | Milteny Biotec | Cat# 130-099-127; RRID:AB_2661766 |
| Anti-MOG Biotinylated | R&D Systems | Cat# BAF2439; RRID:AB_2145536 |
| Mouse anti-rat Cd11b | Novus Bioscience | Cat# NB100-2143; RRID:AB_10001618 |
| Anti-rat CD11b/c PerCP/Cy5.5 | Biolegend | Cat# 201819; RRID:AB_2565948 |
| PerCP/Cy5.5 Mouse IgG2a, kappa Isotype Ctrl | Biolegend | Cat# 400257; RRID:AB_10695169 |
| SAv-Brilliant Violet 421 | Biolegend | Cat# 405226 |
| Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-21202; RRID:AB_141607 |
| Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-21203; RRID:AB_2535789 |
| Goat anti-Mouse IgM Heavy Chain Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-21042; RRID:AB_2535711 |
| Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 | Thermo Fisher Scientific | A-31571; RRID:AB_162542 |
| Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-21206; RRID:AB_2535792 |
| Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-21207; RRID:AB_141637 |
| Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 | Thermo Fisher Scientific | Cat# A-21447; RRID:AB_2535864 |
| Donkey anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-21208; RRID:AB_2535794 |
| Donkey anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-21209; RRID:AB_2535795 |

(Continued on next page)
| REAGENT or RESOURCE                      | SOURCE                     | IDENTIFIER          |
|-----------------------------------------|----------------------------|---------------------|
| Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific  | Cat# A-11055; RRID:AB_2534102 |
| Goat anti-Mouse IgG2b Cross-Adsorbed Secondary Antibody, Alexa Fluor 555         | Thermo Fisher Scientific   | Cat# A-21147; RRID:AB_2535783 |
| Anti-AMPK alpha 1 + AMPK alpha 2        | Abcam                      | Cat# ab80039; RRID:AB_1603618 |
| Anti-Phospho-AMPKα (Thr172) (40H9)     | Cell Signaling Technology  | Cat# 2535           |
| Anti-p70 S6 Kinase                     | Cell Signaling Technology  | Cat# 9202; RRID:AB_331676 |
| Anti-Phospho-p70 S6 Kinase (Thr389)     | Cell Signaling Technology  | Cat# 9205; RRID:AB_330944 |
| Anti-S6 Ribosomal Protein (S235/236)    | Cell Signaling Technology  | Cat# 2317; RRID:AB_2238583 |
| Anti-Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) | Cell Signaling Technology  | Cat# 4858; RRID:AB_916156 |
| Anti-beta-Actin Peroxidase conjugated   | Sigma-Aldrich              | Cat# A3854; RRID:AB_262011 |
| Donkey Anti-Mouse IgG Antibody,IRDye®680LT | LI-COR Biosciences        | Cat# 926-68022; RRID:AB_10715072 |
| Donkey Anti-Rabbit IgG Antibody,IRDye®680LT | LI-COR Biosciences        | Cat# 926-68032; RRID:AB_10706161 |
| Donkey Anti-Mouse IgG, IRDye 800CW      | LI-COR Biosciences         | Cat# 926-32212; RRID:AB_621847 |

### Chemicals, Peptides, and Recombinant Proteins

| Description                                                                 | Source                  | Cat# or Information |
|-----------------------------------------------------------------------------|-------------------------|---------------------|
| Recombinant Human FGF-basic                                                 | Peprotech               | 100-18B             |
| Recombinant Human PDGF-AA                                                   | Peprotech               | 100-13A             |
| Insulin, human recombinant, zinc solution                                   | Thermo Fisher Scientific | 12585014            |
| Sodium pyruvate                                                            | Thermo Fisher Scientific | 11360070            |
| apo-Transferin human                                                        | Sigma                   | Cat# T1147          |
| Putrescine                                                                  | Sigma                   | p5780               |
| Sodium Selenite                                                            | Sigma                   | S-5261              |
| Progesterone                                                                | Sigma                   | P8783               |
| Bovine Serum Albumin                                                        | Sigma                   | A4919               |
| T3                                                                          | Sigma                   | T6397               |
| Dorsomorphin                                                                | LC Laboratories         | D-3197              |
| Metformin                                                                   | Sigma                   | PHR1084-500MG       |
| Benztropine mesylate                                                        | Sigma                   | SML0847             |
| 9-cis-retinoic acid                                                         | Sigma                   | R4643               |
| Miconazole                                                                  | Sigma                   | M3512               |
| Isolation medium / (alternatively Hibernate A w/o Mg and Ca)                | This study / Brainbits  | Table S3 / HACAMG500 |

#### Critical Commercial Assays

| Description                                                                 | Source                  | Cat# or Information |
|-----------------------------------------------------------------------------|-------------------------|---------------------|
| ATPlite luminescence assay system                                           | Perkin Elmer            | 6016943             |
| Seahorse FluxPak XFp                                                        | Agilent                 | 103022-100          |
| Seahorse FluxPak XFe96                                                       | Agilent                 | 102601-100          |

#### Deposited Data

| Description                                                                 | Source                  | Information        |
|-----------------------------------------------------------------------------|-------------------------|--------------------|
| RNaseq data of young and aged OPCs                                           | This study              | GEO: GSE134765     |
**LEAD CONTACT AND MATERIALS AVAILABILITY**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Robin JM Franklin (rjf1000@cam.ac.uk). This study did not generate new unique reagents.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal husbandry
All animal procedures were performed in compliance with United Kingdom Home Office regulations. The animals were housed under standard laboratory conditions on a 12 h light/dark cycle with constant access to food and water. All animals were housed in pairs or groups of up to 4 animals.

For alternate day fasting (ADF) 12 months old female SD rats were restricted from food every other day. ADF animals had access to food on Tuesday, Thursday, Saturday and Sunday and all food was removed from their cages on Monday, Wednesday and Friday. The food was removed and returned in the mornings. The weight of each animal was weekly monitored. The fasting paradigm was interrupted for the first three days after surgery when all animals had free access to food.

For metformin treatment 15 months old female SD rats that were fed *ad libitum* received metformin (Glucophage) in their drinking water (300mg/kg bodyweight per day). Metformin treatment was interrupted for two days before and three days after surgery and then commenced to the end of the study (21 days after lesion induction). Fluid consumption was continuously monitored to adapt dosages. All animals were randomly allocated to experimental groups.

Isolation of adult oligodendrocyte progenitor cells
Adult male and female rats (2-24 months) were decapitated after lethal injection with phenobarbital. The brains were removed quickly and placed into ice-cold isolation medium (Table S3; alternatively Hibernate A Brainbits). The telencephalon and cerebellum were dissected in isolation medium, meninges, and the olfactory bulb were mechanically removed and the brain tissue was mechanically minced into 1mm³ pieces. The tissue pieces were spun down at 100 g for 1min at RT and the tissue was washed in HBSS- (no Mg²⁺ and Ca²⁺, GIBCO). Each half of the brain was mixed with 5ml of dissociation solution (34U/ml papain (Worthington), 20 μg/ml DNase Type IV (GIBCO) in isolation medium). The brain tissue was dissociated on a shaker (50rpm) for 40 min at 35 °C. The digestion was stopped by addition of ice cold HBSS-. The tissue was centrifuged (200 g, 3 min, RT), the supernatant completely aspirated and the tissue resuspended in isolation medium supplemented with 2% B27 and 2mM sodium-pyruvate (trituration solution). The tissue was allowed to sit in this solution for 5min. To obtain a single cell suspension the tissue suspension was triturated 10 times using first a 5ml serological pipette and subsequently three fire polished glass pipettes (opening diameter > 0.5mm). After each trituration step the tissue suspension was allowed to sediment (approximately 1-2 min) and the supernatant (approximately 2ml), containing the cells, was transferred into a fresh tube. After each round of trituration 2ml of fresh trituration solution were added. To remove accidentally transferred undigested tissue bits, the collected supernatant was filtered through 70 μm cell strainers into tubes that contained 90% isotonic Percoll (GE Healthcare, 17-0891-01, in 10xPBS pH7.2 (Lifetech). The final volume was topped up with phenol-red free DMEM/F12 with HEPES (GIBCO) and mixed to yield a homogeneous suspension with a final Percoll concentration of 22.5%. The single cell suspension was separated from remaining debris particles by gradient density centrifugation (800 g, 20min, RT, without break). The myelin debris and all layers without cells were discarded and the brain cell containing phase (last 2ml) and cell pellet were resuspended in HBSS+ and combined in a fresh 15ml tubes and centrifuged (300 g, 5min, RT). The cell pellet was resuspended in red blood cell lysis buffer (Sigma, R7757) and incubated for 1min at RT to remove red blood cells. 10ml of HBSS+ were added to this cell suspension and spun down (300 g, 5min, RT). The pelleted cells were resuspended in 0.5ml modified Milteny washing buffer (MWB, 2mM EDTA, 2mM Na-Pyruvate, 0.5% BSA in PBS, pH 7.3) supplemented with 10ng/ml human recombinant insulin (GIBCO). To this cell suspension 2.5μg mouse-anti-rat-A2B5-IgM antibody (Millipore, Table S4) were added for every 10 million cells. After 25 min incubation, gently shaking at 4 °C, 7ml of MWB were added. The solution was centrifuged (300 g, 5min, RT) and the pellet resuspended in 80 μL MWB supplemented with 20 μL rat-anti-mouse-IgM antibody (Milteny, 130-047-302) per 10 million cells. The cells were incubated for 15 min, slowly shaking at 4 °C. The secondary antibody was again washed out with 7ml MWB and the sample was centrifuged (300 g, 5min, RT). The cell pellet was resuspended in 0.5ml and MACS was performed according to the recommendations of the supplier. Briefly, a MS column (Milteny, 130-042-201) was inserted into MiniMACS Separator (Miltenyi; 130-042-102) and pre-wet with 0.5ml MWB. Resuspended cells were put onto one MS column. Subsequently the column was washed three times using 500 μL MWB for each wash. Finally, A2BS positive cells were flushed out the column with 1ml pre-warmed, CO₂ and O₂ pre-equilibrated OPC medium.

Culture of adult oligodendrocyte progenitor cells
Isolated OPCs were seeded onto 12mm glass coverslips in 24 well plates (VWR) or into 96 well-plates (InVitro-Sciences) coated with PDL (Sigma). After isolation, OPCs were left to recover in OPC medium (60μg/ml N-Acetyl cysteine (Sigma), 10μg/ml human recombinant insulin (GIBCO), 1mM sodium pyruvate (GIBCO), 50μg/ml apo-transferrin (Sigma), 16.1μg/ml putrescine (Sigma), 40ng/ml sodium selenite (Sigma), 60ng/ml progesterone (Sigma), 330μg/ml bovine serum albumin (Sigma)) supplemented with b-FGF and PDGF (30ng/ml each, Peprotech). OPCs were incubated at 37 °C, 5% CO₂ and 5% O₂. The medium was completely exchanged to OPC medium with 20ng/ml bFGF and PDGF after overnight culture to remove any dead cells. After 3d the cell culture medium was switched to promote further proliferation (OPC medium+20ng/ml bFGF and PDGF) or differentiation (OPCM + 40ng/ml T3). During differentiation or proliferation experiments 66% of the medium were replaced every 48h and growth factors or other small molecules were added fresh to the culture. The culture medium used was 500 μL for cultures in 24 well plate wells and 150 μL for cultures in 96 well plate wells. For differentiation assays the medium was in some instances supplemented with 40ng/ml thyroid-hormone (T₃, Sigma), 50nM 9-cis retinoic acid (9cRA, Sigma), 1μM miconazole (Sigma, M3512) or 1.5μM benzotropine.
(Sigma, SML0847). Otherwise used small molecules: 100µM metformin (Sigma), rotenone (Sigma, R8875), 1µM dorsomorphin (LC Laboratories, D-3197).

METHOD DETAILS

Induction of white matter lesions and assessment of remyelination

For studies involving demyelination, female Sprague Dawley rats (Harlan Laboratories) 18 months of age were used. The rats were anesthetized with buprenorphine (0.03mg/kg, s.c.) and 2.5% isoflurane. Demyelination was induced by stereotaxic injection of 4µl of 0.01% ethidium bromide (EB) into the caudal cerebellar peduncles (CCPs), as previously described (Woodruff and Franklin, 1999). EB was delivered at a rate of 1 µl/min. After EB delivery, the injection needle remained in position for additional 4 min.

To assess remyelination the rats were transcardially perfused with 4% glutaraldehyde and 0.4 mM CaCl2 in PBS. The cerebellum was cut in to transverse 1mm thick sections. The tissue was fixed in 2% osmium-tetroxide at 4°C overnight, dehydrated through a series of washes in ethanol and propylene-oxide and embedded in resin. From the resin blocks 1µm thick sections were cut and stained with 1% toluidine blue. To compare the extent of remyelination in different experimental groups we first used a blinded ranking analysis. Single blocks from each animal from which sections containing the largest area of lesion were identified and used for subsequent analysis. In resin sections, remyelinated axons can be readily distinguished from normally myelinated axons outside the lesion by the thinness of the myelin sheath. Within the lesion, remyelinated axons can be distinguished from demyelinated axons because the former have myelin sheaths recognizable as a dark staining rim around the axon. Sections from each animal was examined by an observer blind to the experimental group form which the animal came. The highest rank was given to the animal exhibiting the highest proportion of remyelinated axons. If it was not possible to differentiate two animals using this method then they were given the same rank. In this method, no attempt is made to assign a value to the proportion of remyelination, but simply to establish how a section from an individual animal ranks relative to others. Additional analysis was undertaken by counting the number of remyelinated and demyelinated axons within the lesion by electron microscopy, where remyelinated axons can be identified using the same morphological criteria used in semi-thin section light microscopy. For electron microscopy (EM), ultrathin sections of lesion sites were cut and transferred onto copper grids. The sections were stained with uranyl acetate and imaging was performed using a Hitachi-H600 Transmission Electron Microscope.

G ratio was measured with open source software Fiji (ImageJ, https://imagej.net/Fiji) on transverse electron micrographs at 4,000 – 6,500 magnification with internal calibration. The G ratio was calculated as the ratio of the diameter of axon over that of myelin on the same axons, which is inversely correlated to myelin thickness, that a maximum G ratio of 1 results from unmyelinated or demyelinated axons.

Isolation of adult oligodendrocyte progenitor cells

Adult male and female rats (2-24 months) were decapitated after lethal injection with phenobarbital. The brains were removed quickly and placed into ice-cold isolation medium (Table S3; alternatively Hibernate A Brainbits). The telencephalon and cerebellum were dissected in isolation medium; meninges, and the olfactory bulb were mechanically removed and the brain tissue was mechanically minced into 1mm3 pieces. The tissue pieces were spun down at 100 g for 1min at RT and the tissue was washed in HBSS- (no Mg2+ and Ca2+, Gibco). Each half of the brain was mixed with 5ml of dissociation solution (34U/ml papain (Worthington), 20 µg/ml DNase Type IV (Gibco) in isolation medium). The brain tissue was dissociated on a shaker (50rpm) for 40 min at 35°C. The digestion was stopped by addition of ice cold HBSS-. The tissue was centrifuged (200 g, 3 min, RT), the supernatant completely aspirated and the tissue resuspended in isolation medium supplemented with 2% B27 and 2mM pyruvate (trituration solution). The tissue was allowed to sit in this solution for 5min. To obtain a single cell suspension the tissue suspension was triturated 10 times using first a 5ml serological pipette and subsequently three fire polished glass pipettes (opening diameter > 0.5mm). After each trituration step the tissue suspension was allowed to sediment (approximately 1-2 min) and the supernatant (approximately 2ml), containing the cells, was transferred into a fresh tube. After each round of trituration 2ml of fresh trituration solution were added. To remove accidentally transferred undigested tissue bits, the collected supernatant was filtered through 70 µm cell strainers into tubes that contained 90% isotonic Percoll (GE Healthcare, 7-0891-01, in 10xPBS pH7.2 (Lifetech)). The final volume was topped up with phenol-red free DMEM/F12 with HEPES (Gibco) and mixed to yield a homogeneous suspension with a final Percoll concentration of 22.5%. The single cell suspension was separated from remaining debris particles by gradient density centrifugation (800 g, 20min, RT, without break). The myelin debris and all layers without cells were discarded and the brain cell containing phase (last 2ml) and cell pellet were resuspended in HBSS+ and combined in a fresh 15ml tubes and centrifuged (300 g, 5min, RT). The cell pellet was resuspended in red blood cell lysis buffer (Sigma, R7757) and incubated for 1min at RT to remove red blood cells. 10ml of HBSS+ were added to this cell suspension and spun down (300 g, 5min, RT). The cell pellets were resuspended in 0.5ml modified Milteny washing buffer (MWB, 2mM EDTA, 2mM Na-Pyruvate, 0.5% BSA in PBS, pH 7.3) supplemented with 10ng/ml human recombinant insulin (Gibco). To this cell suspension 2.5µg mouse-anti-rat-A2B5-IgM antibody (Millipore; Table S4) were added for every 10 million cells. After 25 min incubation, gently shaking at 4°C, 7ml of MWB were added. The solution was centrifuged (300 g, 5min, RT) and the pellet resuspended in 80 µL MWB supplemented with 20 µL rat-anti-mouse-IgM antibody (Milteny, 130-047-302) per 10 million cells. The cells were incubated for 15 min, slowly shaking at 4°C. The secondary antibody was again washed out with 7ml MWB and the sample was centrifuged (300 g, 5min, RT). The cell pellet was resuspended in 0.5ml and MACS was performed according to...
the recommendations of the supplier. Briefly, a MS column (Milteny, 130-042-201) were inserted into MiniMACS Separator (Miltenyi; 130-042-102) and pre-wet with 0.5ml MWB. Resuspended cells were put onto one MS column. Subsequently the column was washed three times using 500 µL MWB for each wash. Finally, A2BS positive cells were flushed out the column with 1ml pre-warmed, CO₂ and O₂ pre-equilibrated OPC medium.

**Culture of adult oligodendrocyte progenitor cells**

Isolated OPCs were seeded onto 12mm glass coverslips in 24 well plates (VWR) or into 96 well-plates (InVitro-Sciences) coated with PDL (Sigma). After isolation, OPCs were left to recover in OPC medium (60µg/ml N-Acetyl cysteine (Sigma), 10µg/ml human recombinant insulin (GIBCO), 1mM sodium pyruvate (GIBCO), 50µg/ml apo-transferrin (Sigma), 16.1µg/ml putrescine (Sigma), 40ng/ml sodium selenite (Sigma), 60ng/ml progesterone (Sigma), 330µg/ml bovine serum albumin (Sigma)) supplemented with b-FGF and PDGF (30ng/ml each, Peprotech). OPCs were incubated at 37°C, 5% CO₂ and 5% O₂. The medium was completely exchanged to OPC medium with 20ng/ml bFGF and PDGF after overnight culture to remove any dead cells. After 3d the cell culture medium was switched to promote further proliferation (OPC medium+20ng/ml bFGF and PDGF) or differentiation (OPCM + 40ng/ml T3). During differentiation or proliferation experiments 66% of the medium were replaced every 48h and growth factors or other small molecules were added fresh to the culture. The culture medium used was 500 µL for cultures in 24 well plate wells and 150 µL for cultures in 96 well plate wells. For differentiation assays the medium was in some instances supplemented with 40ng/ml thyroid-hormone (T3, Sigma), 50nM 9-cis retinoic acid (9cRA, Sigma), 1µM miconazole (Sigma, M3512) or 1.5µM benzotropine (Sigma, SML0847). Otherwise used small molecules: 100µM metformin (Sigma, PHR1084-500MG), rotenone (Sigma, R8875), 1µM dorsomorphin (LC Laboratories, D-3197).

**Immunofluorescence for tissue sections**

Rats received a lethal dose of pentobarbital and were transcardially perfused with 4% paraformaldehyde (PFA) in PBS. The brains were removed and post-fixed for 2h at RT with 4% PFA. After a rinse in PBS the tissue was incubated in 20% sucrose solution (in PBS) overnight. The tissue was then imbedded in OCT- medium (TissueTek) and stored at −80°C. 12 µm sections were obtained using a cryostat. Tissue sections were air-dried and stored at −80°C. Cryostat cut sections were dried for 45 min at RT. For antigen-retieval the slides were submerged in preheated citrate buffer pH 6.0 (Sigma) in a water bath at 95°C for 15 min. The slides were washed three times with PBS (5min, RT) and blocked in 0.3% PBST with 5%NDS for 1h at RT. Primary antibodies (Table S4) were diluted in 0.1% PBST with 5%NDS and incubated overnight at 4°C. The slides were washed 3 times for 10min with PBS. Next, secondary antibodies in blocking solution were applied at a concentration of 1:500 for 2h at RT. Slides were washed 3 times with PBS for 10 min each, whereby the first wash contained Hoechst 33342 nuclear stain (2 µg/ml). The slides were mounted with coverslips using FluoSave (CalBiochem). Image acquisition was performed using a Leica-SP5 microscope (Leica) and LAS software (Leica) or a Zeiss Observer A1 inverted microscope (Zeiss) and Zeiss Axiovision software. Further image processing and analysis was performed using the ImageJ software package (Schindelin et al., 2012).

**Immunofluorescence for cells**

Cultured cells were rinsed with PBS before fixation with 4% PFA (10 min, RT). Subsequently, the cells were washed three times with PBS (5 min, RT, shaking). If permeabilisation was required, the cells were incubated with PBST (0.1% Triton X-100 in PBS) for 20 min at RT. The samples were then blocked in PBS supplemented with 10% normal donkey serum (NDS). Primary antibodies (Table S4) were diluted in PBS with 5% NDS and incubated overnight at 4°C in a humidified chamber. Excess antibodies were washed off with three washes in PBS (10 min, RT, shaking). The primary antibodies were then labeled with secondary antibodies (Table S4) diluted in PBS with 5% normal donkey serum. Again, excess antibody was washed off with three washes PBS (10 min, RT, shaking). If visualization of nuclei was required the first wash contained 2 µg/ml Hoechst 33342 (Sigma). If coverslips were used, they were mounted onto Polysine glass slides (VWR) in a drop of Fluosave (Calbiochem). Image acquisition was performed using a Leica-SP5 microscope (Leica) and LAS software (Leica) or a Zeiss Observer A1 inverted microscope (Zeiss) and Zeiss Axiovision software. Further image processing and analysis was performed using the ImageJ software package (Schindelin et al., 2012).

**Comet assay**

For comet assays, a single cell gel electrophoresis based assay for detecting DNA damage, approximately 5000 OPCs were resuspended in 100µl PBS and mixed with 300 µl 1% low melting point agarose (37°C). Alternatively, when OPCs were cultured prior to the assay, the cells were detached using TrypLE 1x Select (GIBCO) for 8 min at 37°C. The comet assay was then performed as described in Olive and Baneth (2006). Briefly, OPCs were centrifuged at 300 g for 5 min. at room temperature and the cell pellet was resuspended with 100µl PBS and then mixed with 300µl molten low-melting point agarose pre-incubated at 37°C. The cell-agarose suspension was then applied gently onto polylysine slides that were pre-treated with 1% agarose and allowed to solidify at 4°C. The slides were submersed in alkaline cell lysis buffer (0.3M NaOH, 100mM EDTA, 0.1% (w/v) N-Lauroylsarcosine (Sigma, 61745), 1.2M NaCl in ddH2O) for 16 h at 4°C in the dark. The samples were then electrophoresed in alkaline electrophoresis buffer (0.03M NaOH, 2mM EDTA, pH > 12.3, pre-chilled at 4°C) for 25 min at RT with 1V/cm, whereby cm represents the distance between the electrodes. Finally, electrophoresed and propidium iodide stained DNA was visualized using a Zeiss Axiovision Fluorescence microscope (Carl Zeiss), and 50-100 nuclei per animal were visually scored according to published protocols (Collins, 2004). Statistical significance was
determined comparing respective damage categories between experimental groups by a two-tailed unpaired t test. A significant result was assumed for \( p < 0.05 \).

RNA sequencing and downstream analysis

RNA was isolated from freshly purified young adult (2-3 months) and aged (20-24 months) OPCs using QIAGEN RNAeasy Micro kit (QIAGEN) and RNA was stored at \(-80 \, ^\circ \text{C}\). RNA quality was assessed by Qubit measurement and posterior RNA nanochip/picochip Bioanalyzer. Ribosomal RNA was depleted with rat-specific oligos (InDA-C technology). Sequencing libraries were prepared using 10-100ng total RNA and the Nugen Ovation RNA-Seq Systems 1–16 for Model Organisms Kit (0349-32). Sequencing was performed on the Illumina HiSeq4000 in a pair-end 150 base pair format. Adaptor sequences were removed and reads were quality-trimmed using TrimGalore. Trimmed reads were aligned to the rat reference genome (RGSC6.0/rn6) by using TopHat2 (Kim et al., 2013) (http://ccb.jhu.edu/software/tophat, version: 2.0.13) guided by Ensembl gene models. Raw counts per gene regions were obtained by featureCounts. Replicates were evaluated, counts were normalized and differential expression of transcripts was evaluated by the R Bioconductor DESeq2 package (Love et al., 2014). Expression levels were further normalized by transcript length (per kB). Transcript annotations were based on Ensembl (Release 82). GO term analysis was performed using the goseq package. For ingenuity pathway analysis we used differentially expressed genes with an adjusted p value cutoff (\( p_{\text{adj}} < 0.05 \)).

RNA isolation and qRT-PCR

RNA was isolated from freshly purified OPCs or from cultured OPCs according to the Directzol RNA MicroPrep Kit (Zymo Research; R2061). All RNA samples were stored at \(-80 \, ^\circ \text{C}\) prior to further processing. cDNA was generated using the QuantiTect Reverse Transcription Kit’s according to the instructions of the manufacturer (QIAGEN; 205310). For RT-qPCR, primers (see Table S5) were used at a concentration of 400\( \mu \text{M}\). The efficiency of each primer was greater than \(-95\%\) as determined for each primer pair by serial dilutions of OPC cDNA. cDNA, primers, and the Syber Green Master Mix (QIAGEN; 204141) were mixed as instructed by the manufacturer, and RT-qPCR and melting curve analysis were performed on Life Technologies’ QuantaStudio 6 Flex Real-Time PCR System. Fold changes in gene expression were calculated using the delta delta Ct method in Microsoft Excel. Statistical significance was determined using two-tailed unpaired t tests assuming equal variances.

ATP measurements

For the comparison of freshly isolated cell populations (young, aged and ADF aged) cells were spun down (300 g, 5min). The supernatant was removed and the cell pellets were frozen down at \(-80 \, ^\circ \text{C}\) and stored until analysis. The relative ATP content was measured using the ATPlite luminescence Assay System (Perkin Elmer). Cell pellets were resuspended in 100\( \mu \text{l}\) OPC medium, alternatively freshly cultured cells in 100\( \mu \text{l}\) OPC medium were used. To this suspension 100\( \mu \text{l}\) of lysis buffer were added and the samples shaken at 600rpm for 5min on a horizontal shaker. 50\( \mu \text{l}\) of substrate solution were added and the samples were shaken at 600rpm in a horizontal shaker for another 5min. The luminescence signal was recorded using an Infinite pro 200 Tecan plate reader (Tecan) and the Magellan software. The luminescence was measured for 1s intervals. The counts were normalized to cell numbers.

Oxygen Consumption rate measurements

OPCs or pre-oligodendrococytes were seeded onto PDL coated Seahorse cell culture plates (Agilent). The oxygen consumption rate was recorded using the manufacturers standard protocol for mitochondrial stress tests (Agilent). For the comparison of young and aged cells or OPCs and POLs, the cells were cultured overnight prior to the assay. For the comparison of aged cells treated with metformin and dorsomorphin the cells were cultured for 5d in the presence of the drugs. The cells were cultured 1h before the assay in modified assay medium (1.5mM sodium pyruvate, 2mM L-Glutamine, 2mM Glucose, 1% SATO in XF base medium, pH 7.4). The OCR measurements were carried out using a Seahorse XFP or a Seahorse XF96 analyzer. The basal OCR was calculated as the difference between the average of the measurements taken under untreated conditions and the average of the measurements taken after the injection of rotenone and antimycin A. All OCR values were then normalized to the cell number. The final OCR values were normalized to one treatment group and the results are presented as the relative basal oxygen consumption rate between the groups. The concentrations of the small molecules in the assay were: oligomycin (1\( \mu \text{M}\), FCCP (0.5\( \mu \text{M}\)) rotenone (0.5\( \mu \text{M}\)) and antimycin A (0.5\( \mu \text{M}\)).

Western Blot

Cells were lysed in IP lysis buffer (Thermo Scientific) supplemented with 1% Halt protease inhibitor (Thermo Scientific, 87786) for 10 min on ice. The lysates were spun down for 10min at 4 \( {\text{C}} \) and 10, 000 g in a table top centrifuge. The supernatant was stored at \(-80 \, ^\circ \text{C}\). Protein quantification was carried out using Pierce BCA protein assay kit (Thermo Scientific) measured with a NanoDrop2000. Equal amounts of protein (15-20\( \mu \text{g}\)) were loaded mixed with 4X Bolt\( ^{\text{TM}} \) LDS Sample Buffer (Thermo Fisher; B0007) and 10X Bolt reduction agent and boiled to 70 °C for 10min. Protein was run on Bolt 4%–12% Bis-Tris Plus Gels (Thermo Fisher; NW04120BOX) in Bolt MOPS SDS running buffer (Thermo Scientific, B0001) for 32min at 200V. Protein was transferred for 60min at 20V to a nitrocellulose membrane (Immobilon FL 0.45µm pore size, Millipore) membrane using the Mini Blot module (Thermo Scientific, B1000) and Bolt transfer buffer with 10% methanol and 1% Bolt antioxidant (Thermo Scientific, BT005) according to
the manufacturer’s instructions. Membranes were blocked in 50% Odyssey blocking buffer TBS (Li-Cor, 927-50100) in TBS. All primary antibodies were used in a dilution of 1:1000 in 0.1% TBS-Tween with 50% Odyssey blocking buffer (TBS) in TBS. The membranes were incubated shaking in antibody solution overnight at 4°C. The membranes were washed twice in 0.1% TBS-Tween. Secondary antibodies were added in a concentration of 1:10000 in 50% Odyssey blocking buffer in 0.1% TBS-Tween. Secondary antibodies were incubated at room temperature for 1h in the dark. The membranes were washed three times in 0.1% TBS-Tween. Fluorescent antibody signal was detected using the Odyssey (Li-Cor) and Image Studio v4.0 software. For luminescent signal detection the membranes were incubated with ECL solution (Amersham ECL Western Blot analysis system, GE Healthcare) and the signal was detected as described for fluorescent signals. For PGC1a detection we used a secondary antibody anti-mouse-IgG coupled to HRP (CST) and the

**Fluorescence Activated Cell Sorting**

Freshly isolated OPCs were fixed in ice-cold 4% PFA for 10min and washed in FACS buffer (0.5% BSA in PBS). The cells were stained with primary antibodies (Anti-A2B5-PE, anti-MOG-biotin and anti-rat-Cd11b-PerCP-Cy5.5 and appropriate isotype controls; see Table S4) for 30min at 4°C. With exception of A2B5 staining, which was carried out for 10min at 4°C. Cells were washed with FACS buffer and stained with secondary antibody (Streptavidin-BV421) for 15min at 4°C in the dark. Cells were washed and resuspended in FACS buffer. Cells were analyzed using an Attune-NXT (Thermo Scientific) equipped with 405, 488 and 561 lasers. For compensation, beads (OneComp) were used for single stains for each fluorophore. The compensation matrix was automatically calculated and applied by the Attune software. Gates for the quantification of A2B5, Cd11b and MOG positive cells were set according to appropriate FMOs. A minimum of 30,000 cell singlets were recorded and used for quantification with FlowJo software (v10).

**Oil-Red-O staining**

12µm cryostat cut sections were dried for 45 min at RT. Tissue sections were dehydrated in 100% 1,2 propanediol (Sigma) for 2 times 5 min. The slides were stained at 60C in prewarmed 0.5% Oil Red O solution (ORO, Sigma). Then, the samples were placed into 85% 1,2 propanediol (v/v in distilled water) for 8 min to differentiate the staining. The slides were then rinsed three times with distilled water and mounted with jelly mounting media. Image acquisition was performed with a Nikon microscope. Digital images were converted into 8-bit greyscale images. The pixel values were inverted so that a more intense staining corresponded to higher pixel values. The staining was quantified using ImageJ software, measuring the mean gray value for the lesion area.

**CRISPR/Cas9 mediated knockdown of Prkaa2 (AMPK)**

Using Phusion polymerase (Thermo Fisher; F530S) a T7 promoter Cas9 PCR product was amplified from the AAV-CMV-Cas9 plasmid (Addgene plasmid # 106431, Juan Ipiszua Belmonte’s laboratory). The product was purified and used as a template to generate capped mRNA using the HiScribe mRNA kit with tailing (NEB, E2060S). All guide RNAs were designed using CHOPCHOP (https://chopchop.cbu.uib.no). DNA Templates containing a T7 promoter site for the generation of non-targeting and prkaa2 gRNAs were assembled by oligo annealing to a gRNA scaffold sequence using Klenow fragment (NEB, M0210S). gRNAs were then produced by in vitro transcription using T7 polymerase (NEB, M0251S). gRNAs were purified using RNA extraction columns (Zymo, R2060). Capped Cas9 mRNA and gRNAs were transfected into aged OPCs (seeded at 30,000 cells per 96 well to achieve approximately 80%–90% confluency) using Lipofectamine LTX (Thermo Scientific, 15338100, 0.4µl LTX, 0.1µl Plus reagent, 100ng Cas9 mRNA, 50ng gRNAs per 96 well in Opti-MEM, final volume 10µl). The medium was completely replaced after 16h.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis**

All statistical analysis was performed in GraphPad Prism (GraphPad Software, Inc.) or R. Prior to the analysis of parametric data we performed Shapiro-Wilk tests to ensure normality of the data. For data derived from the quantification of immunohistochemical staining, comparisons between two groups were performed with an unpaired t test assuming two-tailed distribution and equal variances. In cases where more than two groups were compared to each other, a one-way analysis of variance (ANOVA) was performed assuming equal variances, followed by an appropriate post-test to compare individual groups. For ranking analysis of remyelination, the non-parametric Mann-Whitney test was used to determine whether two groups differed in their extent of remyelination. A comparison of the extent of remyelination between three groups was performed using a Kruskal-Wallis test followed by Dunn’s post-test to compare individual groups. For qRT PCR data two groups were compared to each other using unpaired two-tailed t test. For data derived from comet assays damage categories would be tested between treatment groups using unpaired two-tailed t tests. For all statistical tests, differences were considered significant at p < 0.05.

**DATA AND CODE AVAILABILITY**

The RNA sequencing data of young and aged oligodendrocyte progenitor cells (OPCs) generated during this study are available at GEO: GSE134765.
Supplemental Information

Metformin Restores CNS Remyelination Capacity
by Rejuvenating Aged Stem Cells

Björn Neumann, Roey Baror, Chao Zhao, Michael Segel, Sabine Dietmann, Khalil S. Rawji, Sarah Foerster, Crystal R. McClain, Kevin Chalut, Peter van Wijngaarden, and Robin J.M. Franklin
A

Mechanical trituration and enzymatic digestion

Filtration and density gradient centrifugation

MACS (A2B5+ selection)

B

Young

Aged

C

Young

Aged

D

% of cells

E

Nuclei

A2B5

Fdgfr2

Merge

Sox10

Olig2

Nuclei

Cnpase

Olig2

MBP

Olig2

Nuclei

GFAP

Nuclei

A2B5

NG2

Pdgfr2

Merge

A2B5-PE

A2B5-PE

A2B5+MOG- 
A2B5+MOG+ 
MOG+A2B5- 
Cd11b+

0
2
4
6
8
10
50
60
70
80
90
100

Young

Aged

12h

2h

7d

12h

12h

2h

7d

12h
Figure S.1. Related to Figure 1. Isolation and culture of adult OPCs from aged rats. (A), Schematic illustration of the adult OPC isolation workflow. (B-C), Representative density plots of FACS data from freshly isolated cells stained for A2B5 (OPC marker) and Cd11b (microglia marker) (B), and A2B5 and MOG (oligodendrocyte marker) (C). (D), Quantification of FACS data presented in B and C (n=2 biological repeats). (E-H), A2B5+ sorted cells are positive for OPC markers A2B5 and Pdgfra. (I), More than 90% of A2B5 cells are positive for Sox10 and Olig2. (J-K), A2B5 sorted cells do not express more mature lineage markers such as CNPase or MBP. (L-M), The A2B5+ population does not contain significant contamination from other glia cells such as Cd11b+ microglia (L), or GFAP+ astrocytes (M). (N), In the presence of PDGF-AA and b-FGF, A2B5+ isolated cells form colonies of mostly bipolar cells that express the OPC markers A2B5, NG2 and Pdgfra. (O-R), Higher magnification of A2B5, NG2, Pdgfra cells. Images in E-Q are taken from cultures of aged OPCs (≥18 months). Scale bar = 50µm.
Aged 

**A**

Young (2-3mo) or Aged (12-13mo) OPCs → Normal density (1x) or Double density (2x) → 3d +PDGF/bFGF → 5d +T3

**B**

Aged 1x Aged 2x Young 1x Young 0.5x

**C**

MBP/Olig2 [%] 0 20 40 60 80

Aged 1x Aged 2x Young 1x Young 0.5x

**D**

Aged 5d Aged 28d

**E**

MBP/Olig2 [%] 0 10 20 30 40 50

Aged 5d Aged 28d

Young (2-3mo) or Aged (12-13mo) OPCs

Normal density (1x) or Double density (2x) → 3d +PDGF/bFGF → 5d +T3
Figure S.2. Related to Figure 1. Density changes do not alter the differentiation rate of OPCs.

(A) Schematic of the experimental design. Young (2-3 months old) or aged OPCs (18-20 months) were recovered in growth factor-supplemented (PDGF-AA and b-FGF) medium for 3d and were then subjected to pro-differentiation compounds for 5d. Young OPCs were seeded in the regular or half the density. Aged OPCs were seeded in the regular or double density. (B), Representative images of the differentiation assay. Newly formed oligodendrocytes were identified as Olig2⁺MBP⁺ cells. (C), Quantification of the differentiation assay. All data are presented as mean ± SD (n=3 biological replicates, One Way ANOVA with Dunnett’s multiple comparison test for each group against each other). (D) Aged OPCs (18-20 months) were cultured as described above. Differentiation was assessed 5d or 28d after removal of growth factors and addition of T3. Newly formed oligodendrocytes were identified as Olig2⁺MBP⁺ cells. (E) Quantification of the differentiation assay. All data are presented as mean ± SD (n=3 biological replicates, two-tailed t-test). All scale bars = 50µm. *P<0.05, **P<0.01, ***P<0.001.
A

Young (2-3mo) or Middle Aged (12-13mo) OPCs

3d +PDGF/bFGF

5d no GF

Differentiation factors

B

Middle aged no GF

Middle aged +T3

Middle aged +9-cis-RA

Middle aged +Benztropine

Middle aged +Miconazole

Young +T3

MBP Olig2

MBP Olig2

MBP Olig2

MBP Olig2

MBP Olig2

MBP Olig2

C

MBP+Olig2 [%]

NS

***

MBP Olig2

MBP Olig2

MBP Olig2

MBP Olig2

MBP Olig2

MBP Olig2

MA w/o GF +T3 +9cis +Mic +Benz YA+T3
Figure S.3. Related to Figure 1 and 3. Middle aged OPCs lose their inherent differentiation potential and responsiveness to pro-differentiation compounds. (A), Schematic of the experimental design. Young (2-3 months old) or middle aged OPCs (12-13 months old) were recovered in growth factor-supplemented (PDGF-AA and b-FGF) medium for 3d and were then subjected to pro-differentiation compounds for 5d. (B), Representative images of the differentiation assay. Newly formed oligodendrocytes were identified as Olig2\(^+\)MBP\(^+\) cells. Scale bar = 50\(\mu\)m. (C), Quantification of the differentiation assay. All data are presented as mean ± SD (n=3 biological replicates, One Way ANOVA with Dunnett’s multiple comparison test for each group against the middle aged group differentiating in the absence of growth factors “MA”). *P<0.05, **P<0.01, ***P<0.001.
Figure S.4. Related to Figure 3 and 4. ADF and treatment of aged OPCs with metformin in vitro reverses hallmarks of ageing. Animals were exposed to alternate day fasting or fed ad libitum as described in Figure 3. At 18 months of age OPCs were isolated from both experimental groups. (A), qRT-PCR for OPC genes and (B), Cdkn2a. All data are presented as mean ± SD (n=3 biological replicates, two-tailed t-test). (C), Representative images of comet assay data. (D), Quantification of comet assays. All data are presented as mean ± SD (n=3 biological replicates, two tailed t-test comparing each damage category between the two groups, i.e. Aged Low vs ADF Low). (E), Representative Western Blot data from acutely isolated OPCs from aged (18 months) and ADF animals (18 months). n=2 biological repeats for each experimental group. (F), Quantification of normalised intracellular ATP content (n=5 biological repeats for each experimental group). (G), OPCs were isolated from aged animals (≥18 months) and cultured in the presence of growth factors for 5 days. Some cells were treated with 100µM metformin with each medium change during the first 5 days (“metformin” days 2 and 4), whereas control cells (“Aged”) received medium alone. (H), qRT-PCR for OPC genes (Pdgfra, Ascl1 and Sox6) and (I), Cdkn2a. All data are presented as mean ± SD (n=3 biological replicates for each group, two-tailed t-test). (J), Quantification of comet assays. All data are presented as mean ± SD (n=3 biological replicates for each group, two-tailed t-test comparing each damage category between the two experimental groups, i.e Aged Low and Metformin low). *P < 0.05, **P<0.01, ***P<0.001, ns P>0.05
Figure S.5. Related to Figure 3. Additional analysis about remyelinating lesions. (A), G-ratio analysis of ADF, Control and Metformin remyelinated axons. (B), Representative images of lesions stained for the reactive astrocyte marker GFAP at 7,21 and 50dpl. (C), Quantification of the area within a lesion covered by GFAP. (D), Representative images of lesions stained for the macrophage and microglia marker lba1 at 7,21 and 50dpl. (E), Quantification of the area within a lesion covered by lba1. (F, H), Representative images of lesions stained Oil-Red-O to visualise myelin debris at 7dpl (F) and 21dpl (H). (G, I), Quantification of the myelin debris load in a lesion at 7dpl (G, n= 4 biological repeats, two-tailed t-test) and 21dpl (I, n=4 biological repeats, two-tailed t-test). All scale bars: 100µm. ns: P>0.05
Figure S.6. Related to Figure 4. Metformin does not accelerate the differentiation of young OPCs. (A) Young OPCs (2-3 months old) were recovered in growth factor-supplemented (PDGF-AA and b-FGF) medium for 3d and were then differentiated for 5d. Metformin treated cells were exposed to metformin throughout the whole culture period. Newly formed oligodendrocytes were identified as Olig2^+MBP^+ cells. (B) Quantification of the differentiation assay. All data are presented as mean ± SD (n=3 biological replicates, two-tailed t-test). All scale bars = 50µm. ns: P>0.05.
SI Table 1. Log2 transformed RNAseq expression data for characteristic OPC genes between young and aged OPCs. Related to Figure 2A.

SI Table 2. Ingenuity Pathway analysis results for genes significantly higher expressed in aged versus young OPCs. Related to Figure 2C.
**SI Table. 3. Formulation of isolation medium. Related to STAR Methods.** All chemicals were purchased from Sigma. pH was adjusted to 7.3 and the medium was filtered (0.22µm) and stored at 4ºC.

| Amino Acids                        | µM  | MW    | mg/5l |
|------------------------------------|-----|-------|-------|
| Glycine                            | 400 | 75.07 | 150.1 |
| L-Alanine                          | 22  | 89.09 | 9.8   |
| L-Arginine hydrochloride           | 483 | 174.2 | 420.7 |
| L-Asparagine-H2O                   | 5.5 | 150.13| 4.1286|
| L-Cysteine hydrochloride-H2O       | 7.7 | 313.2 | 12.06 |
| L-Histidine hydrochloride-H2O      | 200 | 209.6 | 209.6 |
| L-Isoleucine                       | 802 | 131.2 | 526.1 |
| L-Leucine                          | 802 | 131.2 | 526.1 |
| L-Lysine hydrochloride             | 798 | 146.2 | 583.3 |
| L-Methionine                       | 201 | 149.2 | 149.9 |
| L-Phenylalanine                    | 400 | 165.2 | 330.4 |
| L-Proline                          | 67  | 115.13| 38.569|
| L-Serine                           | 400 | 105   | 210   |
| L-Threonine                        | 798 | 119   | 474.8 |
| L-Tryptophan                       | 78  | 204.2 | 79.6  |
| L-Tyrosine disodium salt dihydrate | 398 | 181.2 | 360.6 |
| L-Valine                           | 803 | 117.2 | 470.6 |

| Vitamins                           |      |       |       |
|------------------------------------|------|-------|-------|
| Choline chloride                   | 28   | 139.62| 19.55 |
| D-Calcium pantothenate             | 8    | 238.27| 9.53  |
| Niacinamide                        | 30   | 122   | 18.3  |
| Pyridoxine hydrochloride           | 20   | 206   | 20.6  |
| Thiamine hydrochloride             | 10   | 337   | 16.9  |
| i-Inositol                         | 40   | 180.2 | 36    |

| Inorganic Salts                    |      |       |       |
|------------------------------------|------|-------|-------|
| Ferric Nitrate (Fe(NO3)3“9H2O)     | 0.25 | 404   | 0.5   |
| Potassium Chloride (KCl)           | 5360 | 74.55 | 1997.9|
| Sodium Bicarbonate (NaHCO3)        | 880  | 84    | 369.6 |
| Sodium Chloride (NaCl)             | 89000| 58    | 25810 |
| Sodium Phosphate dibasic (Na2HPO4) | anhydrous | 906 | 120 | 543.6 |
| Zinc sulfate (ZnSO4-7H2O)          | 0.67 | 287.56| 0.9633|

| Other Components                   |      |       |       |
|------------------------------------|------|-------|-------|
| D-Glucose (Dextrose)               | 25000| 180.2 | 22525 |
| Sodium Pyruvate                    | 227  | 110.04| 124.9 |
| MOPS                               | 10000| 269.3 | 13465 |
### SI Table 4. Antibodies used in the study. Related to STAR Methods

AF: Alexa Fluor, PE: Phycoerythrin, BV: Brilliant Violet, SA: Streptavidin

| Antibody       | Class | Host species | Dilution (in FACS) | Dilution (in vitro) | Dilution (in vivo) | Source       | Catalogue number |
|----------------|-------|--------------|--------------------|---------------------|--------------------|--------------|------------------|
| Anti-Olig2     | IgG   | Rabbit       | -                  | 1:1000              | 1:500              | Millipore    | AB9610           |
| Anti-Sox10     | IgG   | Goat         | -                  | 1:100               | 1:100              | Santa Cruz   | sc-17342         |
| Anti-Pdgfra    | IgG   | Rat          | -                  | 1:300               | -                  | BD           | 558774           |
| Anti-NG2       | IgG   | Rabbit       | -                  | 1:500               | -                  | Pharmingen   | MAB5320          |
| Anti-A2B5      | IgM   | Mouse        | -                  | 1:500               | -                  | Millipore    | MAB312           |
| Anti-Ki67      | IgG   | Rabbit       | -                  | -                   | 1:500              | Abcam        | Ab166667         |
| Anti-CC1 (APC) | IgG   | Mouse        | -                  | -                   | 1:300              | Calbiochem   | OP80             |
| Anti-O4        | IgM   | Mouse        | -                  | 1:1000              | -                  | R&D          | PZO              |
| Anti-CN Pase   | IgG   | Mouse        | -                  | 1:1000              | -                  | Abcam        | Ab6319           |
| Anti-MBP       | IgG   | Rat          | -                  | 1:500               | -                  | Serotec      | MCA4095          |
| Anti-Nkx2.2    | IgG   | Mouse        | -                  | -                   | 1:200              | DHSB         | 745A5            |
| Anti-Cd11b/c   | IgG   | Mouse        | -                  | 1:200               | -                  | Serotec      | MCA275R          |
| Anti-GFAP      | IgG   | Goat         | -                  | 1:2000              | -                  | Abcam        | Ab53554          |
| Anti-A2B5-PE   | IgM   | Mouse        | 1:10               | -                   | -                  | Milteny      | IS5-20C4         |
| Mouse IgM-PE   | IgM   | Mouse        | 1:10               | -                   | -                  | Milteny      | IS5-20C4         |
| Anti-MOG-Biotin| IgG   | Goat         | 1:40               | -                   | -                  | R&D          | BAF2439          |
| IgG-biotin Control | IgG  | Donkey      | 1:40               | -                   | -                  | Biolegend    | Ox-42            |
| Anti-Cd11b-PerCP-Cy5.5 Mouse | IgG | Mouse | 1:1000 | - | - | Biolegend | MOPC1-73 |
| SA-BV421       | -     | -            | 1:800              | -                   | -                  | Biolegend    | 405226           |
| Anti-Mouse AF-488 | IgG | Donkey      | -                  | 1:1000              | 1:500              | Invitrogen   | A21202           |
| Anti-Mouse AF-594 | IgG | Donkey      | -                  | 1:1000              | 1:500              | Invitrogen   | A21203           |
| Anti-Mouse AF-488 | IgM | Goat       | -                  | 1:1000              | 1:500              | Invitrogen   | A21042           |
| Antibodies for Western Blot | Class | Host species | Dilution (WB) | Source | Catalogue number |
|----------------------------|-------|--------------|---------------|--------|------------------|
| Anti-AMPK IgG              | Mouse | 1:1000       | Abcam         | Ab80039 |
| Anti-phospho-AMPK IgG      | Rabbit| 1:1000       | CST           | 2353   |
| Anti-p70S6K IgG            | Rabbit| 1:1000       | CST           | 9205   |
| Anti-phospho-p70S6K IgG    | Rabbit| 1:1000       | CST           | 9205   |
| Anti-S6 Ribosomal Proteins | Mouse | 1:1000       | CST           | 2317   |
| Anti-phospho-S6 IgG        | Rabbit| 1:1000       | CST           | 4858   |
| Anti-PGC1a IgG             | Mouse | 1:1000       | Calbiochem    | ST1202  |
| Anti-beta-actin-HRP IgG    | Mouse | 1:20000      | Sigma         | A3854  |
| IRDye680 LT anti-mouse IgG| Donkey| 1:10000      | Li-Cor        | 926_68022 |
| IRDye680 LT anti-rabbit IgG| Donkey| 1:10000      | Li-Cor        | 926_38073 |
| IRDye800CW anti-rabbit IgG| Donkey| 1:10000      | Li-Cor        | 926_32212 |
| IRDye800CW anti-mouse IgG | Donkey| 1:10000      | Li-Cor        | 926_32213 |
| Anti-Mouse-IgG-HRP         | Horse | 1:1000       | CST           | 7076   |
**SI Table 5. qRT primer sequences used in this study. Related to STAR methods.** All primers were purchased from Sigma except Cdkn2a, which was designed using Primer3.

| Gene  | Forward primer sequence (5’-3’)                      | Reverse primer sequence (5’-3’)                  | Source     |
|-------|------------------------------------------------------|--------------------------------------------------|------------|
| Pdgfra| GAGATTATGAATGTGCTGCC                                 | TTTCTCGTGAAACAGAAATGC                            | Sigma      |
| Ascl1 | AAACAAGGGAAGGAAAGGAAAG                             | CATTGAATCTAAGTCCTGGTG                            | Sigma      |
| Sox6  | TCCCAATTTCCTCCACATGAC                                | GTTATCACCTGGCTTTGAT                              | Sigma      |
| Enpp6 | AATTGTCTCTCTTTTGACC                                 | CTTTCTGGACATCAGATAGC                             | Sigma      |
| Cnp1  | TTTCAAGAAAGAGCTTCGAC                                | TAAGATCTCCTCACCACATC                             | Sigma      |
| Cdkn2a| TCGTGCGGTATTGTCCGTTAT                                | TAGTCTCGGGTGCCAGAAG                              | This study |
| Tbp   | CATCATGAGAATAAGAGAGCC                               | GGATTGTCTTCTCAGTTG                              | Sigma      |
| Prkaa2| ccaattatcgacaccggag                                 | acgtgctcagtcgacag                               | This study |