Rapid functional genetics of the oligodendrocyte lineage using pluripotent stem cells

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Oligodendrocyte dysfunction underlies many neurological disorders, but rapid assessment of mutation-specific effects in these cells has been impractical. To enable functional genetics in oligodendrocytes, here we report a highly efficient method for generating oligodendrocytes and their progenitors from mouse embryonic and induced pluripotent stem cells, independent of mouse strain or mutational status. We demonstrate that this approach, when combined with genome engineering, provides a powerful platform for the expeditious study of genotype-phenotype relationships in oligodendrocytes.

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Oligodendrocytes generate myelin, a multilaminar structure that allows salutary propagation of action potentials in the central nervous system (CNS). The functional importance of oligodendrocytes is underscored by numerous neurological diseases characterized by myelin loss or dysfunction, including multiple sclerosis (MS), neuromyelitis optica, leukodystrophies (e.g., Pelizaeus–Merzbacher disease), and mental health disorders such as schizophrenia. A myriad of genes, regulatory elements, and single-nucleotide polymorphisms have been associated with oligodendrocyte dysfunction, but reverse genetics has not kept pace due to the cost and time of generating mutant mice and the lack of technology required to genetically manipulate primary oligodendrocytes.

To facilitate more rapid studies into the molecular mechanisms that underlie oligodendrocyte function and myelin disease, we report a new method for generating pure and highly scalable populations of myelogenic oligodendrocytes and their progenitor cells (oligodendrocyte progenitor cells (OPCs)) from pluripotent cell sources including mouse embryonic stem cells (mESCs) and induced pluripotent stem cells (iPSCs). Our previous method of generating OPCs from pluripotent mouse epiblast stem cells relied on a starting cell type that is challenging to grow, less accessible, and difficult to genetically manipulate. In contrast, our new method is highly efficient and universally reproducible across pluripotent stem cell lines from any wild-type or mutant genetic background, as well as from lines purposefully edited with CRISPR-Cas9 nuclease or other methods of genome engineering. Given the widespread accessibility of mESCs and iPSCs, this new protocol can be combined with existing methodologies such as CRISPR-Cas9 technology to enable in vitro molecular and cellular phenotyping of OPCs and oligodendrocytes with defined genotypes in <3 weeks.

**Results**

**Generation of myelogenic OPCs from patterned mESCs.** We initially selected four previously isolated germline-competent male mESC lines derived from independent mouse strains 129P2/Ola, C57BL/6, PO, and CBA/Ca. Prior to beginning the current studies, ES cell cultures were karyotyped (see Methods) and confirmed to express canonical markers of pluripotency, Oct4 and Nanog (Supplementary Figs. 1a, b and 2a). All four mESC lines were then differentiated to OPCs and oligodendrocytes using stage-specific small molecules and growth factors that mimic signaling events known to specify oligodendrocyte fate during development (see Fig. 1a for overview and Methods for the detailed protocol).

First, naive mESCs were transitioned to primed epiblast-like cells by culturing in suspension as spheres in the presence of a small-molecule inhibitor of the Janus kinase (JAK)/signal transducers and activators of transcription pathway. Cells were then specified to the neuroectodermal lineage using small molecules and recombinant proteins to inhibit both Activin/Nodal and bone morphogenetic protein signaling pathways. By day 5 of differentiation, cells robustly downregulated the pluripotency marker Oct4 and upregulated the early neuroectodermal lineage marker Pax6. Subsequent treatment with retinoic acid and sonic hedgehog (SHH) stimulated downregulation of Pax6 and emergence of Olig2 expression, a domain marker of the ventral developing neural tube from which OPCs are first specified in vivo. To facilitate outgrowth, expansion, and maturation of OPCs, day 9 ventralized spheres were seeded on polyornithine and laminin-coated culture plates (termed passage 0). Neuronal axons rapidly extended from the attached spheres followed by migratory and proliferative early OPCs expressing Olig2 and Sox10 (Fig. 1d, e, g and Supplementary Fig. 2c). Passageing of these cultures in the presence of fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and SHH enabled selective enrichment and continued maturation of OPCs co-expressing Olig2, Sox10, and Nkx2.2. At passage 1, 49 ± 4.32% of cells co-expressed all three of these OPC-defining transcription factors and adopted a bipolar morphology typical of bona fide OPCs (Fig. 1d–f, g and Supplementary Fig. 2d) and nearly all cells expressed at least one of the three markers (Fig. 1d). GFAP+ or βIII-Tubulin+ cells, demarcating astrocytes and neurons, respectively, accounted for <2% of the total number of cells at passage 1 (GFAP+, 0.74 ± 0.30%; βIII-Tubulin+, 0.54 ± 0.38%; Supplementary Fig. 2e, f), suggesting that nearly all cells were early-stage OPCs. Within two additional passages, cultures matured to a near-homogeneous population in which 81 ± 2.92% of cells co-expressed Nkx2.2, Sox10, and Olig2 and 93 ± 0.72% of cells expressed two of the three defining OPC markers across all four independent mESC lines without sorting or selection (Fig. 1d). These mESC-derived OPCs could be expanded in an additional 5–7 passages enabling rapid generation of billions of pure OPCs.

To confirm that mESC-derived OPCs could differentiate into terminally mature oligodendrocytes, the cells were treated with thyroid hormone (T3), a known stimulator of oligodendrocyte differentiation, in the absence of PDGF and FGF (Fig. 1a) and 17. Within 24–48 h, OPCs exhibited a multi-processed branched morphology and expressed the early oligodendrocyte cell surface antigen O4 (Fig. 1b and Supplementary Fig. 2g). By 72 h, cells took on a highly ramified morphology characteristic of mature oligodendrocytes (Fig. 1i) and expressed mature myelin proteins myelin basic protein (MBP) and proteolipid protein 1 (PLP1) (Fig. 1i and Supplementary Fig. 2g). To assess the capacity of mESC-derived OPCs to associate with and wrap axons (in vitro “myelination”), cells were co-cultured with dissociated dorsal root ganglion (DRG) sensory neurons for 10 days. OPCs gave rise to MBP+ oligodendrocytes with segmented tracts of MBP in close apposition with neurofilament-positive (NF+) DRG axons, indicative of axonal ensheathment and myelinating capability (Fig. 1j). Additionally, enhanced green fluorescent protein (eGFP)-labeled mESC-derived OPCs were injected into the developing brains of 2-day-old athymic nude mice. At 6 weeks post injection, cells exhibited eGFP+ segmented tracts co-localized to NF+ axons (Supplementary Fig. 3a, b). Taken together, these findings show that mESC-derived OPCs give rise to myelogenic oligodendrocytes in vitro and in vivo.

**Cellular profiling of mutant alleles.** The ability to reproducibly generate OPCs and oligodendrocytes from any mouse wild-type or mutant genetic background allows for the interrogation of cellular and molecular defects underlying spontaneous or engineered mutations within those cells. To validate the power of this approach, we generated iPSC lines from shiverer mice, which harbor a large homozygous deletion in the MBP gene that results in CNS hypomyelination. Additionally, we used CRISPR-Cas9 in wild-type mESCs (strain C57BL/6) to target myelin regulatory factor (MYRF), a transcription factor required for oligodendrocyte differentiation, to generate MYRF knockout (KO) mESCs (Fig. 2b). We then differentiated shiverer and MYRF KO pluripotent stem cell lines to OPCs and observed no difference in differentiation efficiencies compared to the wild-type mESC and iPSC control lines (Fig. 2c, d). To interrogate more mature cellular phenotypes, wild-type, shiverer, and MYRF KO OPCs were induced to differentiate into oligodendrocytes either in isolation or in co-culture with DRG neurons. Predictably, wild-type mESC-derived and iPSC-derived
OPCs differentiated over 72 h through an O4+ immature oligodendrocyte state (Fig. 2e) into mature PLP1+/MBP+ oligodendrocytes (Fig. 2f), which ensheathed DRG axons (Fig. 2g), indicative of myelination competency. In contrast, shiverer and MYRF KO cells exhibited stage-specific in vitro cellular phenotypes consistent with their previously defined in vivo pathology20–22. Shiverer OPCs readily gave rise to morphologically mature PLP1+ oligodendrocytes that failed to express MBP and thus had limited capacity to ensheathe DRG axons (Fig. 2f, g). MYRF KO OPCs arrested at the immature O4+ stage with no cell expressing mature oligodendrocyte markers or ensheathing DRG axons (Fig. 2e–g). Collectively, these data validate that our method can easily be used to rapidly phenotype oligodendrocyte cellular dysfunction across various genetic backgrounds.

**Molecular profiling of mutant alleles.** To establish a temporal scorecard of gene expression from wild-type cells \( n = 4 \) independent mESC lines, we performed time of maximum (TOM) analysis of RNA-sequencing (RNA-seq) data to identify genes with stage-specific expression over 72 h of differentiation from OPCs to oligodendrocytes (Fig. 3a and see Supplementary Data 1 for full gene lists). These TOM gene sets showed strong overlap with previous RNA-seq data generated from in vivo isolated cells, suggesting that they define physiologically relevant cellular transitions (Supplementary Fig. 4)23. We used stage-specific TOM genes to define molecular correlates of cellular dysfunction observed in shiverer and MYRF KO cells. RNA-seq profiling of shiverer OPCs after 72 h of differentiation showed down-regulation of OPC-signature genes and up-regulation of oligodendrocyte-signature genes (Fig. 3b–d), with a few exceptions—notably, full-length expression of MBP was not observed, confirming that the shiverer mutation precludes proper MBP transcription. In addition, key myelination genes such as MAG and MOG were downregulated in shiverer oligodendrocytes relative to the differentiated control lines, consistent with the late-stage myelination defect identified in our cellular assays (Fig. 3d). RNA-seq profiling of 72 h differentiated MYRF KO OPCs showed clear down-regulation of the OPC-signature genes and a complete failure to upregulate mature oligodendrocyte-signature genes (Fig. 3b, c–e), with notable absence of MBP, MAG, MOG, PLP1, and, as expected, MYRF expression (Fig. 3e). This global molecular phenotype indicates that MYRF KO OPCs arrest at an early stage of differentiation that is consistent with their failure to progress beyond the immature O4+ oligodendrocyte stage in our cellular assays. Taken together, these results define OPC-specific and oligodendrocyte-specific expression signatures and underscore their utility in in vitro stage-specific molecular profiling of mutant alleles.

**Discussion**

In summary, we provide a transformative platform for rapidly generating highly scalable populations of OPCs and

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**Fig. 1** Reproducible generation of OPCs and oligodendrocytes from mESCs. **a** Graphical overview of the differentiation time course for generating OPCs and stage-specific oligodendrocytes from mESCs. **b** Quantification of immunocytochemistry for stage-specific markers demarcating the transition from pluripotency (Oct4) to neuroectoderm (Pax6) to ventral neural tube (Olig2) over 9 days. \( n = 4 \) independent biological replicates (mESC lines) with \( >25 \) colonies scored per cell line. Data are represented as means ± SEM. **c** Representative immunofluorescent images of starting mESCs (Oct4 and Nanog) and day 5 neuroectoderm (Pax6 and Sox1). Scale bar, 50 µm. **d** Quantification of OPC-defining transcription factors Olig2, Nkx2.2, and Sox10 at passages 1 and 3 of the differentiation protocol. \( n = 4 \) independent mESC lines; \( >114,500 \) cells scored per cell line. Data are represented as means ± SEM. **e** Representative images of starting mESCs (Oct4 and Nanog) and day 10 immunostaining of the immature oligodendrocyte marker O4, after treatment with T3. Scale bar, 50 µm. **f** Representative images of the CBA/Ca mESC line, and are representative of results obtained with C57BL/6, PO, and 129P2/Ola lines, which are shown separately in Supplementary Fig. 2.
oligodendrocytes. Until now, isolation of genotype-specific oligodendrocytes has been confined to pre-existing mouse strains. In contrast, our 3-week differentiation protocol harnesses the amenability of pluripotent stem cells to genetic modification and can be combined with existing technologies such as CRISPR-Cas9 to gain novel insight into developmental biology underlying normal and disease states in the oligodendrocyte lineage. Our method is also amenable to leverage the power of newer functional genetic tools, such as the dTag protein degrader system\textsuperscript{24}, to provide previously unprecedented temporal and selective control of any protein during oligodendrocyte development. As novel factors (genes, genetic variants, microRNAs, long noncoding RNAs, and epigenetic signatures) that regulate glial biology and disease continue to be identified, our method can be utilized for first tier proof-of-principle studies in lieu of more expensive and time-consuming approaches (e.g., generation and maintenance of mutant mouse colonies). Moreover, the ability to generate large populations of pure OPCs from normal and disease states provides the scalability necessary for biochemical, epigenetic, and proteomic analyses, and for large-scale genetic and/or chemical screens.

**Fig. 2** Cellular profiling of spontaneous and purposely generated mutant oligodendrocyte alleles. a Diagram indicating that shiverer mice harbor a ~20-kilobase (kb) homozygous deletion encompassing exons 2–7 of the MBP gene. A Sanger sequencing trace shows the breakpoint of the shiverer deletion. b Diagram indicating the location of the two gRNAs designed to target MYRF. A Sanger sequencing trace shows the location of the homozygous deletion of exon 1. c Quantification of transcription factors Olig2, Nkx2.2, and Sox10 at passage 3 of the differentiation protocol. $n = 3$ shiverer cell lines; $n = 3$ replicate wells per cell line; >179,500 cells scored per well. Data are represented as means ± SEM. d Fluorescent images of WT iPSC, shiverer, and MYRF KO OPCs expressing canonical OPC markers Olig2, Nkx2.2, and Sox10. Scale bar, 50 µm. e Cell surface immunostaining of the immature oligodendrocyte marker O4, after treatment with T3, of WT iPSC, shiverer, and MYRF KO OPCs. Scale bar, 50 µm. f Representative images of differentiated OPCs immunostained for mature oligodendrocyte markers MBP and PLP1, 72 h post treatment with T3 of WT iPSC, shiverer, and MYRF KO OPCs. Scale bar, 50 µm. g Representative images of OPC/DRG co-cultures stained for MBP and neurofilament (NF) at day 10 from WT iPSC, shiverer, and MYRF KO OPCs stained for PLP1 or MBP after being co-cultured for 10 days with NF+ embryonic rat DRGs. Scale bar, 50 µm.
**Fig. 3** Molecular profiling of spontaneous and purposely generated mutant oligodendrocyte alleles. **a** Row normalized heatmap with genes (rows) sorted by time of maximum (TOM). The four independent WT mESC lines for each time point are shown compared to gene expression of shiverer and MYRF KO OPCs 72 h post T3 induction. **b** Boxplot of OPC-signature genes prior to T3 induction and after differentiation with T3 for 24, 48, and 72 h. **c** Boxplot of oligodendrocyte-signature genes prior to T3 induction and after differentiation with T3 for 24, 48, and 72 h. **d** Row normalized heatmap of the top 30 dysregulated oligodendrocyte-signature genes for shiverer 72 h post T3 induction. Gene expression for the four WT mESC strains for each time point are shown compared to gene expression of shiverer OPCs 72 h post T3 induction. Note the lack of MBP expression (red star). **e** Row normalized heatmap of the top 30 dysregulated oligodendrocyte-signature genes for MYRF KO OPCs at 72 h post T3 induction. Gene expression for the four WT mESC strains for each time point are shown compared to gene expression of MYRF KO OPCs 72 h post T3 induction. Note the lack of MYRF expression (red star).
**Methods**

**Animal welfare.** All animal experiments were performed in accordance with procedures approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

**Isolation of fibroblasts.** Mouse embryonic fibroblasts (MEFs) were isolated at embryonic day 13.5 (E13.5) from embryos generated through timed natural matings between *shiverer* homozygous mice (C3Fae-SWV-Mbp^−/−; Jackson Laboratory). E13.5 embryos were collected and digested for 15 min in 0.25% Trypsin-EDTA (Thermo Fisher) and either frozen or seeded at 3 × 10^5 cells/cm^2 wells pre-plated with an iMEF feeder layer and cultured in doxycycline-containing mESC medium, supplemented with 10^3 U/ml LIF 3 days prior to differentiation. On day 0 of the differentiation protocol, mESC or iPSC colonies were passaged free of the iMEF feeder layer by treatment with 1.5 mg/ml collagenase type IV (Thermo Fisher) followed by dissociation to single cells with 0.25% Trypsin-EDTA (Thermo Fisher). iMEF-free mESCs and iPSCs were seeded at 7.8 × 10^6 cells/cm^2 on low glialoma’s Transfection Kit or Lenti-X Packaging Single Shots (all from Clontech). Mammalian Transfection Kit or Lenti-X Packaging Single Shots (all from Clontech). On day 3, cells were dissociated with 0.25% Trypsin-EDTA and 5.6 × 10^6 mESCs were fed with mESC medium supplemented with 2 µg/ml puromycin (Thermo Fisher), and 2 mM glutamax and supplemented with 0.2 µM JAK inhibitor 1 (Calbiochem). On day 2, the medium was changed and cultures were fed with 50/50 (vol/vol) KSR medium and mESCs, supplemented with 100 ng/ml noggin (R&D Systems), 20 µg/ml SB431542 (Sigma), and 2 µM dorsomorphin (EMD). On days 8 and 9, the medium was changed and cultures were fed with 50/50 (vol/vol) KSR medium and mESCs, supplemented with 100 ng/ml noggin, 20 ng/ml SHH, 20 ng/ml FG2 and 20 ng/ml PDGF-AA (eGFp+ OOCs). Individual supernatants containing virus were harvested and filtered with a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Millipore) 24 and 48 h later.

**Generation of mIPSCs.** MEFs or TTFs were seeded at 1.3 × 10^5 cells/cm^2 on Nunclon-α plates, allowed to attach overnight, and infected with 50/50 (vol/vol) of pLVX-Tet-On-Puro Advanced and pHAGE2-TetOminCMV-STEMMCA-W-loop (kindly gifted from Gustavo Mostoslavsky) and pLV-Egfp plasmids according to the manufacturer’s protocol using the Lenti-X HT Packaging Mix and Lenti-Phos or Cal-Phos mammalian transfection kit (all from Clontech). The 293T cells (5.0 × 10^6 cells/cm^2) (Clontech) were cultured on rat tail collagen I-coated plasticware (BD Biosciences) and transfected 16 h later in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% FBS, 2 mM glutamax, 1× nonessential amino acids, and 1× 2-mercaptoethanol. iPSC generation) or neural medium supplemented with 20 ng/ml FG2 and 20 ng/ml PDGF-AA (for eGFp+ OOCs). Individual supernatants containing virus were harvested and filtered with a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Millipore) 24 and 48 h later.

**Production of lentivirus.** Lentivirus was generated for pLVX-Tet-On-Puro Advanced, pHAGE2-TetOminCMV-STEMMCA-W-loop (kindly gifted from Gustavo Mostoslavsky) and pLV-Egfp plasmids according to the manufacturer’s protocol using the Lenti-X HT Packaging Mix and Lenti-Phos or Cal-Phos mammalian transfection kit (all from Clontech). The 293T cells (5.0 × 10^6 cells/cm^2) (Clontech) were cultured on rat tail collagen I-coated plasticware (BD Biosciences) and transfected 16 h later in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% FBS, 2 mM glutamax, 1× nonessential amino acids, and 1× 2-mercaptoethanol. On day 1, the medium was changed and cultures were fed with KSR medium supplemented with 0.2 µM JAK inhibitor 1 (Calbiochem). On day 2, the medium was changed and cultures were fed with 50/50 (vol/vol) KSR medium and mESCs, supplemented with 100 ng/ml noggin (R&D Systems), 20 µg/ml SB431542 (Sigma), and 2 µM dorsomorphin (EMD). On days 8 and 9, the medium was changed and cultures were fed with 50/50 (vol/vol) KSR medium and mESCs, supplemented with 100 ng/ml noggin, 20 ng/ml SHH, 20 ng/ml FG2 and 20 ng/ml PDGF-AA (R&D Systems). At this point, the medium was changed and cultures were fed with 50/50 (vol/vol) KSR medium and mESCs, supplemented with 100 ng/ml noggin, 20 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA. On day 10, the medium was changed and cultures were fed with 200 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA. The medium was changed every other day with “day 10” medium. Cells were passaged at 80–90% confluence and seeded at 2.5 × 10^5 cells/cm^2. Cultures of mESCs-derived or iPSC-derived OPCs were cultured for one passage without FG2 in neural medium supplemented with 200 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA. To facilitate enrichment and maturation of the OPC population, mESCs-derived or iPSC-derived OPCs were cultured for one passage without FG2 in neural medium supplemented with 200 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA. To facilitate enrichment and maturation of the OPC population, mESCs-derived or iPSC-derived OPCs were cultured for one passage without FG2 in neural medium supplemented with 200 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA. To facilitate enrichment and maturation of the OPC population, mESCs-derived or iPSC-derived OPCs were cultured for one passage without FG2 in neural medium supplemented with 200 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA. To facilitate enrichment and maturation of the OPC population, mESCs-derived or iPSC-derived OPCs were cultured for one passage without FG2 in neural medium supplemented with 200 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA. To facilitate enrichment and maturation of the OPC population, mESCs-derived or iPSC-derived OPCs were cultured for one passage without FG2 in neural medium supplemented with 200 ng/ml SHH, 20 ng/ml FG2, and 20 ng/ml PDGF-AA.
OPC-DRG co-culture. All cells were cultured at 37 °C and 5% CO₂, unless otherwise noted. OPC-DRG co-cultures were prepared as described previously. Briefly, DRG neurons were isolated and dejunctioned from E15 Sprague–Dawley rats and seeded at 7 x 10⁵ cells per 18-mm collagen-coated cover slips. DRGs were cultured in medium supplemented with 100 ng/ml of nerve growth factor (Sero tec), 2 mM Uridine (Sigma), and 2 mM 5-fluoro-2-deoxyuridine (Sigma) for 3 weeks. DRGs were washed extensively with 1x PBS before plating 9-10 x 10⁶ OPCs per cover slip. Co-cultures were cultured in neural medium for 10 days before fixation.

Immunohistochemistry of OPC-DRG co-cultures. Cells were prepared for immunostaining by fixation in 100% ice-cold methanol for 20 min. Cultures were rinsed with 1x PBS and blocked for non-specific binding with filtered 5% normal donkey serum in 0.1% Triton X-100, 2% donkey serum, and 1x PBS and incubated with appropriate fluorescently labeled Alexa Fluor secondary antibody (Thermo Fisher; 1:500) for 1 h at room temperature. Primary PLP1 and MBF antibodies were diluted in 2% normal donkey serum in 0.1% saponin and incubated overnight at 4°C. Samples were rinsed with 1x PBS and incubated with appropriate fluorescently labeled Alexa Fluor secondary antibodies (Thermo Fisher; 1:500) for 1 h at room temperature. For nuclear staining, samples were incubated with 1 μg/ml DAPI (Sigma) for 5 min. Primary antibodies used were: MBP (Abcam, ab7349; 1:100), PLP1 (kindly gifted from Bruce Trapp; 1:100), and NF cocktail (Covance, SMI-311 and SMI-312; 1:100).

Transplantation of OPCs. OPCs were seeded at 2.9 x 10⁵ cells/cm² on Nuncolon-A wells coated with poly(γ-ornithine) followed by laminin, allowed to attach overnight, and infected with 100 (vol) of plv-eGFP lentivirus supplemented with protamine sulfate (8 μg/ml; Sigma). Cells were incubated in lentivirus for 4 h followed by the addition of new medium. After an additional 20 h of incubation, the medium was changed with neurobasal medium supplemented with 20 ng/ml PDGF and 20 ng/ml PDGF-AA. OPCs were passaged before transplantation. On postnatal day 2, N/L/J (Jackson Laboratory) mice were cryoanesthetized and injected with 5 x 10⁴ eGFP+ OPCs in 0.5 μl Hank’s balanced salt solution (Thermo Fisher) at a depth of 1.5 mm, 1 mm lateral of the sagittal suture and midway between bregma and lambda. On postnatal day 42 (6 weeks), transplanted mice were anesthetized with isoflurane to effect and transcardially perfused with 4% PFA (Electron Microscopy Sciences) followed by incubation in 4% PFA for 16 h at 4 °C. Tissue was then washed in 1x PBS followed by incubation in 30% sucrose (Sigma) at 4 °C. Brains were embedded in optimum cutting temperature formulation (Tissue-Tek), sectioned at 20 μm on a Leica CM 1900 cryostat, and mounted on SuperFrost Plus slides (Fisher Scientific). For fluorescent staining, slides were incubated in permeabilization buffer (0.25% Triton X-100, 2% donkey serum, and 1x PBS) and NF cocktail (Covance, SMI-311R, SMI-312; 1:500) and GFP (Thermo Fisher, A1122; 1:500) primary antibodies overnight. Slides were washed with 1x PBS and incubated with appropriately labeled Alexa Fluor secondary antibodies (Thermo Fisher; 1:500) for 2 h at room temperature in 0.1% Triton X-100, 2% normal donkey serum, and 1x PBS. Samples were rinsed with 1x PBS and incubated with 1 μg/ml DAPI (Sigma) for 5 min.

RNA-seq and analysis. Cells were lysed directly in 1 ml of TRIzol (Thermo Fisher) and stored at −80°C. Once all samples were collected, samples were thawed on ice and RNA was separated with chloroform using Phase Lock Gel tubes (5 μl). RNA was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. One microgram of each sample was then subject to ribosome depletion, fragmented, and library prepared using the Truseq Stranded Total RNA Kit with Ribo Zero Gold (Illumina). The resulting library was sequenced on an Illumina HiSeq 2500 in the University Sequencing Core, Cleveland, OH, USA. Total RNA-seq reads were aligned to a mouse reference genome (GRCm38; GSE7944) and read depth (<10x) was used to calculate FPKM. Data were filtered to remove low-quality, low-expression, and low-confidence sequences from the notochord.

Sequencing shiverer deletion. Genomic DNA was isolated from shiverer tail tips and Sanger sequenced with the following forward and reverse primers, CAGG GCTGGAGTCGAGATCAAGTCTGCACGTTGTTGTCTTACTCTAGTGA. Sequence transcript was compared to the mouse reference genome in order to identify the shiverer deletion sequence.

Data availability

The data that support the findings of this study are available from the corresponding author upon request. RNA-seq datasets were deposited to Gene Expression Omnibus database GSE9744.

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Author contributions
A.M.L. and P.J.T. conceived and designed the project, and wrote the manuscript with input from all authors. A.M.L. and H.E.O. designed the gRNAs and generated CRISPR edited lines. A.M.L. generated shiverer iPSC lines and performed iPSC and mESC in vitro differentiation experiments. A.M.L., M.M., and J.M.C. performed co-culture experiments. O.G.C. analyzed RNA-seq data. M.S.E., K.C.A., H.E.S., B.L.L.C., and M.M. performed in vivo transplants and analyses.

Additional information
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Competing interests: P.J.T. is on the scientific advisory board of Cell Line Genetics. The remaining authors declare no competing interests.

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