Fast generation of forebrain oligodendrocyte spheroids from human embryonic stem cells by transcription factors

Highlights
Develop a new protocol to generate forebrain OLs and OL-spheroids from hNPCs rapidly
Successful maturation and myelination of OLs in forebrain OL-spheroids within 60 days
OL-spheroids transplantation produces mature and functional OLs efficiently
Fast generation of forebrain oligodendrocyte spheroids from human embryonic stem cells by transcription factors

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SUMMARY
Oligodendrocyte spheroids (OL-spheroids) containing oligodendrocytes and neurons provide an accessible system to dissect demyelinating diseases and test therapeutic treatment. However, generation of human OL-spheroids is still technically challenging and time-consuming until now. Here, we presented evidence that overexpression of SOX10 and OLIG2 (SO) in human embryonic stem cells (hESCs)-derived ventral forebrain neural progenitors is sufficient to produce forebrain pre-oligodendrocytes (pre-OLs) and mature oligodendrocytes (OLs) within 20–40 days. More importantly, optimizing this procedure by overexpression of SO in ventral forebrain spheroids, we successfully generated OL-spheroids with pre-OLs, mature OLs, and neurons 40 days after OL-induction. We further demonstrated oligodendrocyte-neuron interactions and obvious axon myelination in OL-spheroids. Finally, over 30% cells developed into mature oligodendrocytes with forebrain identity and myelinate axons in mouse brain 3 months after transplantation. This study provides a strategy to generate forebrain OL-spheroids rapidly and efficiently which would facilitate development of new therapeutics for demyelinating disorders.

INTRODUCTION
Oligodendrocytes ensheath axons in the CNS, allowing rapid saltatory conduction and providing metabolic support to neurons. Dysfunction of OLs cause demyelination diseases such as multiple sclerosis, leukodystrophy, and periventricular leukomalacia in children which have no efficient and long-term treatment until now. Human pluripotent stem cells (hPSCs) have the capacity to differentiate into all neural lineages including neurons, astrocytes, and oligodendrocyte precursor cells (OPCs) and OLs (Kang et al., 2007), thus represent a promising source to model demyelinating diseases, drug screening, and OPCs-based cell therapy.

Interactions between OLs and neighboring neurons play key roles for axon myelination, impulse propagation, and trophic factors secretion during brain development (Fünschilling et al., 2012; Nave, 2010). Thus, generation of 3D OL-spheroids in vitro to recapitulate cell-cell interactions, microenvironments would provide new opportunities to identify pathological mechanisms underlying demyelinating diseases such as multiple sclerosis and vanishing white matter disease (van der Knaap et al., 2006; Wolswijk, 2000). Recently, researchers developed a novel protocol to produce human OL-spheroids with neurons, astrocytes, and OL-lineage cells which showed similar developmental stages transition to primary human OLs by transcription profiles (Marton et al., 2019). Other group generated hPSCs-derived “oligocortical spheroids” with MBP+ mature OLs which show initial myelin wrapping of axons. They also displayed that these oligocortical spheroids recapitulate human myelin disease phenotype (Madhavan et al., 2018). In these data, OL-spheroids showed complex cytoarchitectures and direct interactions between OLs and neuron which is required for myelination. However, it takes over 100–160 days to generate OL-spheroids with these protocols which are time-consuming and costly.

During neural development, OLs arise from diverse populations and developmental waves of oligodendrocyte precursor cells (OPCs) which derived from ventral or dorsal forebrain and spinal cord neural...
progenitors (NPs) in CNS (Cai et al., 2005; Fogarty et al., 2005; Kessaris et al., 2006; Vallstedt et al., 2005). Postnatal OPCs in spinal cord give rise to OLs with a higher myelin sheet length than OLs from the cortex on artificial fibers in vitro (Bechler et al., 2015) suggesting functional heterogeneities of OLs from different regions of CNS. Although there have been developed numerous methods to generate OLs from hPSCs, such as applied key insights from developmental biology studies (Goldman and Kuypers, 2015; Hu et al., 2009) or directly convert neural progenitor cells into OLs using transcription factors (Ehrlich et al., 2017; Garcia-Leon et al., 2018; Pawlowski et al., 2017), OLs generated by these protocols are mostly derived from spinal cord neural progenitors as a small morphogen RA which promote posterior fate determining was applied (Tao and Zhang, 2016).

Here, we develop a new protocol to differentiate forebrain OPCs with 2D and 3D culture system. By over-expression of transcription factor SOX10 and OLIG2 (SO) in hESCs-derived ventral forebrain neural progenitor cells (VF-NPCs), we generated O4+ OPCs in 2D culture and 3D OL-spheroids within 20–40 days. These OPCs developed into forebrain myelinating OLs after another 20 days maturation. Importantly, more than 30% neural progenitors become MBP+ OLs 3 months after OL-spheroids transplantation in vivo.

Application of the transcription factors overexpression platform and 3D OL-spheroids enabled us to develop rapid and efficient protocols for mature human oligodendrocytes.

RESULTS

Generation and characterization of VF-NPCs from hESCs

To emulate a differentiation route analogous to how OPCs are generated in the brain, neural differentiation was induced by the dual SMAD-inhibitors from day 4–7 after suspension of hESCs colonies. Then, the SHH signaling pathway was activated by SHH agonist SAG (day 10–15) to obtain VF-NPCs (Figures 1A and 1B). Immunofluorescent staining was used to analyze the efficiency of ventral forebrain neural progenitor fate specification. About 90% of the cells expressed FOXG1, a forebrain-specific maker, and less than 5% of the cells were PAX6+ (a dorsal forebrain marker) suggesting potential ventral forebrain identity of these NPCs (Figures 1C, 1D, and 1G). In addition, these VF-NPCs highly expressed with neural progenitor marker SOX1 (92.81 ± 1.35%) and SOX2 (98.14 ± 0.50%) (Figures S1C – S1E). A previous paper suggested OPC progenitors from MGE is under control of transcriptional factors NKX2.1 which expressed specifically in ventral forebrain (Tekki-Kessaris et al., 2001). The immunostaining of NKX2.1 and the statistical data displayed that 74.14 ± 4.81% of these NPCs were immuno-positive for NKX2.1. Immunostaining pictures showed most of NKX2.1+ cells were double stained with FOXG1 confirmed fate of ventral forebrain identity (Figure 1D). We then investigated properties of these VF-NPCs by immunostaining of OLIG2, SOX10, and NKX2.2 (Figures 1E and 1F) which are considered oligodendrocyte lineage makers (Liu et al., 2007). Statistical data showed that 22.63 ± 3.40% are NKX2.2+, and 24.56 ± 2.00% are OLIG2+ of total cells (Figure 1H). However, we did not find SOX10 immunopositive cells which are important for the terminal specification of oligodendrocytes from VF-NPCs (Qi et al., 2001) (Figures 1F and 1H). Altogether, hESCs-derived NPCs in our study show identity of ventral forebrain, the main origin of OPCs in the brain, but only few expressed with OL lineage markers 15 days after neural differentiation in vitro.

Rapid generation of forebrain oligodendrocytes from hESCs-derived VF-NPCs by SOX10 and OLIG2 overexpression

As mentioned above, SOX10 and OLIG2 are important transcription factors for OL lineage specification during neural differentiation (Liu et al., 2007). Thus, we want to know if over expression of SOX10 and OLIG2 in VF-NPCs would promote forebrain OLs generation. We amplified and cloned the cDNA sequences of human SOX10 and OLIG2 into PL552 plasmid, respectively. In order to trace transgenes, expression of SOX10 and OLIG2 is reported by mCherry and EGFP, respectively. SOX10 and OLIG2 expression were validated in 293T first by plasmid transfection which showed successful expression of mCherry and EGFP (Figures S1A and S1B). To further express SOX10 and OLIG2 in human VF-NPCs, we use lentivirus as a vector which showed strong integration into both dividing and nondividing cells previously (Gill and Denham, 2020). The schematic diagram and the culture medium in each stage of this experiment were shown in Figure 2A. The forebrain VF-NPCs derived from hESCs were treated with LV-SOX10-mCherry and LV-OLIG2-EGFP (LV-SO) or lentivirus-EGFP (LV-EGFP) particles (Figures 2A–2C). Most of the VF-NPCs showed mCherry/EGFP expression 2–3 days after LV-SO or LV-EGFP treatment (Figures 2D and 2E). Statistical data showed that 78.66 ± 2.23% of these human VF-NPCs were mCherry positive and 66.87 ± 2.09% were EGFP positive in LV-SO group (Figure 2F). The EGFP positive cells to total were...
72.79 ± 3.44% in LV-EGFP group (Figure 2F). Intriguingly, EGFP⁺-mCherry⁺ double-positive cells to total were 52.87 ± 2.61% which is slightly lower than EGFP⁺ or mCherry⁺ single positive cells (Figure 2F).

We next counted SOX10 and OLIG2 expression in LV-SO group, respectively. Statistical data revealed that 80.26 ± 2.44% of total cells were SOX10 positive which is very similar with the ratio of mCherry⁺ in LV-SO group, suggesting very few endogenous SOX10 expression in 15 days VF-NPCs (Figure 2G). Indeed, we did not find any SOX10 immunopositive cells in LV-EGFP group at this stage. (Figures 2E and 2G) further confirm that endogenous VF-NPCs do not express SOX10 at this stage. On the other hand, the ratio of OLIG2 positive cells was significantly increased from 17.03 ± 1.26% to 78.32% ± 3.23% by LV-SO treatment (Figure 2G). These results revealed that LV-SO treatment greatly improved the expression of oligodendrocyte lineage transcription factors SOX10 and OLIG2 in human VF-NPCs and may facilitate forebrain OPCs generation.

Then, we detected pre-oligodendrocytes (Pre-OLs) by O4 immunostaining 20 days after LV-SO treatment. The representative pictures showed obvious O4⁺ cells in LV-SO group, but none O4⁺ cells in the control group (Figure 2H). The statistical data showed that 19.24 ± 3.18% cells were O4⁺ OPCs (Figure 2J). Furthermore, FOXG1 and O4 double staining result showed that 81.58 ± 3.94% of the O4 immunopositively cells...
Figure 2. LV-SO infection and oligodendrocyte commitment in hESCs derived VF-NPCs
(A) Schematic and timeline of OPC differentiation protocol by 2D culture.
(B) Schematic of the lentiviral expression vector: LV-SOX10-mCherry and LV-OLIG2-EGFP (LV: lentivirus).
(C) Schematic of the control lentiviral expression vector: LV-EGFP.
(D) Immunofluorescence images of OLIG2, EGFP, mCherry and SOX10, EGFP, mCherry in VF-NPCs after 3 days of LV-SOX10-mCherry and LV-OLIG2-EGFP infection. Nuclei were counterstained with Hoechst (blue). Scale bar, 50μm.
(E) Immunofluorescence images of OLIG2, EGFP and SOX10, EGFP in VF-NPCs after 3 days of LV-EGFP induction. Nuclei were counterstained with Hoechst (blue). Scale bar, 50μm.
(F) Quantification of EGFP⁺, mCherry⁺, EGFP⁺/mCherry⁺ cells 3 days after LV-SOX10-mCherry and LV-OLIG2-EGFP induction, and quantification of EGFP⁺ cells after 3 days of control LV-EGFP infection. n = 10–12 randomly selected fields from three replicated independent experiments.
(G) Quantification of SOX10 and OLIG2 in LV-SO group and LV-EGFP group. n = 10–12 randomly selected fields from three replicated independent experiments.
(H) Representative immunofluorescence images of O4 in LV-SO group and control LV-EGFP group 20 days after lentivirus infection. Nuclei were stained with Hoechst (blue). Scale bar, 50μm.
(I) Immunostaining of pre-OL in LV-SO group 20 days after virus infection. Pre-OL marker O4 and forebrain maker FOXG1 were co-expressed. Scale bar: 50μm.
(J) Quantification of O4⁺ cells in LV-SO group and LV-EGFP group in H. n = 25–30 randomly selected fields in each group.
(K) Immunofluorescence images for O4 and mCherry in single LV-SOX10-mCherry infected cells, the above panel; Immunofluorescence images for O4 and EGFP in single LV-OLIG2-EGFP infected cells, the bottom panel. Scale bar: 50μm.
(L) Representative immunofluorescence images for MBP of OL in LV-SO group and control LV-EGFP group. Nuclei were stained with Hoechst (blue). Scale bar, 50μm.
(M) Immunofluorescent staining images for MBP (magenta) and the neuronal marker Tuj-1 (cyan). Scale bar, 50μm.
(N) Quantification of O4⁺/FOXG1⁺ cells out of O4⁺ cells in LV-SO group and LV-EGFP group. n = 10–15 randomly selected fields in LV-SO group.
(O) Quantification of the density of MBP⁺ cells in LV-SO group and LV-EGFP group in L. n = 10–12 randomly selected fields in each group.
(P) Quantification of MBP⁺ cells that interacting with Tuj-1⁺ neurons out of total MBP⁺ cells. (n = 3 replicated experiments). Data are presented as mean ± SEM. Unpaired two-tailed Student’s t test was used to assess differences between two groups, ****p < 0.0001 in G, J, O.

Next, we cultured these progenitor cells for another two weeks. Mature OLs which detected by MBP immunostaining only appeared in LV-SO group at this stage compared with LV-EGFP (Figure 2L). We quantified the density of MBP⁺ cells in each group. There were about 32.75 ± 6.15 MBP⁺ cells per mm² in LV-SO group compared with zero in the LV-EGFP group (Figure 2O). We next investigate interactions between OLs and nearby neurons by MBP⁺ and Tuj-1⁺ double immunofluorescent staining as some of the VF-NPCs become neurons in our differentiation system. The representative image showed obvious MBP immunopositively signals wrapping around Tuj-1⁺ axons (Figure 2M). Then, the proportion of MBP⁺ cells overlapped with more than 1μm Tuj1⁺ fibers was calculated (Marton et al., 2019). Statistical data showed that 6.15 ± 2.84% of total cells were NKX2.2 or OLIG2 immunopositively, respectively. We did not find any SOX10⁺ cells in these VF-NPC-spheroids (Figure S2H). These results were consistent with VF-NPCs in 2D culture system. After LV-SO induction, LV-OLIG2-EGFP and LV-SOX10-mCherry were co-labeled with FOXG1 indicating forebrain OPCs (Figures 21 and 2N). Interestingly, we found LV-SOX10 or LV-OLIG2 alone in VF-NPCs did not produce O4 positive OPCs, suggesting single SOX10 or OLIG2 may not be sufficient to differentiate VF-NPCs into OPCs (Figure 2K).

Rapid generation of forebrain OL-spheroids from hESCs by SOX10 and OLIG2 overexpression

After we generated mature OLs by LV-SO overexpression within 60 days with 2D culture system successfully, we wondered if we could generate human OL-spheroids rapidly using the same strategy. To testify if LV-SO treatment promotes forebrain oligodendrocyte production in 3D spheroids, we modified 2D OLs differentiation protocol. Briefly, hESCs clones were dissociated and aggregated to generate embryonic bodies, then they were exposed to dual SMAD inhibitors SB, DMH1, and SHH pathway activator SAG to generate VF-NPC-spheroids. The VF-NPCs-spheroids were treated with LV-SO or LV-EGFP at day 13 and were kept in OPC induction medium for 10 days to induce human forebrain OL-spheroids. From day 23 to day 60, these OL-spheroids were kept with OL differentiation medium to promote oligodendrocyte differentiation and maturation (Figure 3A).
Figure 3. Characterization of forebrain human oligodendrocytes spheroids (OL-spheroids) derived from hESCs

(A) Schematic and timeline of OL-spheroids differentiation protocol.
(B–D) Immunostaining for O1, O4, and MBP in OL-spheroids at day 50–60. Scale bar, 20 μm.
(E and F) Immunostaining for MBP in day 60 and day 86 OL-spheroids. Scale bar, 100 μm.
(G) Immunofluorescence images for MBP (magenta) and the neuronal marker MAP-2 (cyan). Scale bar, 50 μm.
(H) MBP+ cell density of day 60 and day 86 OL-spheroids.

[Additional notes and descriptions as needed]
expressed in most cells of the peripheral areas of the VF-NPC-spheroids (Figure S2I). Quantification of singly and/or doubly infected cells showed that 11.10 ± 1.23% cells were infected by LV-OLIG2-EGFP, 13.70 ± 1.16% cells were infected by LV-SOX10-mCherry, and 6.32 ± 0.71% cells were infected by LV-OLIG2-EGFP and LV-SOX10-mCherry (Figure S2J). Besides, representative images also showed multiple SOX10+ and OLIG2+ cells in VF-NPCs-spheroids after LV-SO infection suggesting OLs production potentials (Figures S2K and S2L). To investigate OPC/OL generation, we stained O1/O4 for pre-OLs and MBP for mature OLs. Interestingly, we found obvious O1+, O4+, and MBP+ cells in the edge of LV-SO-treated VF-NPC-spheroids 60 days after differentiation in vitro (Figures S3B–S3D), suggesting successful generation of human oligodendrocyte spheroids (OL-spheroids) from hESCs. However, none of O1+, O4+, or MBP+ cells were observed in control group (LV-EGFP-treated VF-NPC-spheroids) at the same stage (Figures S3A–S3C). To determine the abundance of mature oligodendrocytes, we quantified the density of MBP+ cells with these day-60 OL-spheroids (Figure 3E). The statistical data showed 5.39 ± 0.66 MBP+ cells in each square millimeter (Figure 3H). We next questioned whether MBP+ cells will increase after longer culture time. Then, OL-spheroids were cultured for another 26 days, and MBP+ cells in OL-spheroids were stained and quantified. The result displayed that MBP+ cells per mm2 significantly increased to 25.00 ± 3.34 in day 86 OL-spheroids (Figures 3F and 3H), suggesting higher productivity of mature OL in OL-spheroids with longer cell culture. Next, we want to know if these 60-day OL-spheroids also have mature neurons, and if these MBP+ cells could myelinate neuron fibers. The immunostaining data showed a lot of MAP-2+ neurons interacting with MBP+ cells from 3 replicated experiments, 17 of them showing cellular interactions with MAP-2+ neuron fibers (73.19 ± 3.27%) in OL-spheroids (Figures 3G-g and 3I). To further explore if these MBP+ cells could form myelin sheath, we did electron microscopy with OL-spheroids. The representative images showed structurally well-organized myelin wrapping around axons in the edge areas suggesting axon myelination in OL-spheroids as early as day 60 after hESCs differentiation (Figure 3J). The immunofluorescent staining data showed many FOXG1 immunopositively cells in these OL-spheroids (Figure 3K). The quantified data showed 73.19 ± 3.27% of total cells were FOXG1 positive (Figure 3L). And FOXG1 and MBP double staining and quantified data showed 87.88 ± 5.43% of MBP+ cells co-labeled with FOXG1 (Figures 3M and 3N), supporting forebrain identity. Taking together, these results indicate that SOX10 and OLIG2 overexpression strongly promote forebrain OL-spheroids generation from hESCs within 60 days in vitro.

To test if this protocol is applicable for other cell lines, we initiate OL-spheroid differentiation with another iPSC line from a healthy adult. Consistent with previous results, forebrain maker FOXG1 (87.66 ± 2.13%), NKX2.1 (80.91 ± 1.66%) were highly and dorsal forebrain marker PAX6 (1.09 ± 0.43%) was lowly expressed in iPSC-derived VF-NPCs, while oligodendrocyte lineage makers NKX2.2 (19.10 ± 2.96%), OLIG2 (21.67 ± 1.76%), and SOX10 (1.85 ± 0.22%) were relatively low (Figures S4A–S4H). After LV-SO induction, there are obvious O1+ and O4+ cells which appeared in iPSC-derived OL-spheroids (Figures S4I and S4J). Quantification of the intensity of MBP+ cells in the iPSC-derived OL-spheroids showed 11.93 ± 3.17 cells per mm2 (Figures S4K and S4L). In addition, O4+ or MBP+ cells showed obvious co-localization with FOXG1 (Figures S4M and S4N).

**OL-spheroids give rise to functional myelin following engraftment in brains of newborn mice**

To assess the functional maturation of OPCs from OL-spheroids, we transplanted 40-day OL-spheroids from control (LV-EGFP) and LV-SO-treated group into newborn SCID mouse. 12 weeks after transplantation, the animals were scarified, and the brain slices were immunostained by MBP and human-specific marker human Nuclei (hN). The representative pictures showed a great number of hN+ cells co-labeled with MBP in LV-SO treated (Figures 4B-b and Video S1) but very few in LV-EGFP-treated (Figure 4A) OL-spheroids 3 months after transplantation. Statistical data showed that 35.54 ± 6.14% transplanted human cells (hN+) differentiated into MBP+ OLs from LV-SO-treated OL-spheroids compared with 0.20 ± 0.03% from LV-EGFP group in vivo (Figure 4C). Of note, hN/FOXG1 double staining and the...
statistical data displayed that 77.49 ± 3.50% hN⁺ of the transplanted cells from human OL-spheroids co-stained with FOXG1 suggest forebrain identity (Figures 4D and 4E). To further investigate if these MBP⁺ cells are functional, we stained human cell marker Stem121 with MBP, and NF-H which is used to stain neuron fibers. The result showed prominent co-localization of human cells with MBP and NF-H suggesting potential myelination of axons by human OLs in mouse brain (Figure 4F). To confirm axon myelination of SO-induced OL in vivo, we did immunoelectron microscopy by which the transplanted OLs were stained and indicated by transgenes EGFP. The representative pictures in Figure 4G showed high density of EGFP immunoparticles located on the myelin sheaths wrapping axons, suggesting axon myelination in vivo.

**DISCUSSION**

Oligodendrocytes and their interactions with neuron are of critical importance for CNS function and play a key role in many neurological diseases. Protocols for efficient generation of human oligodendrocyte spheroid showing O4⁺, O1⁺, and MBP⁺ cells are extremely long after in vitro differentiation (Madhavan et al., 2018; Marton et al., 2019). In their protocols, organoid-containing NPCs were rapidly and efficiently derived from hPSC. However, NPCs were then hindered by the rate-limiting steps of oligodendrocyte specification and differentiation in 3D culture which cause OL-spheroids differentiation technically challenging and time-consuming, and limiting the application of these cells for research and regenerative medicine.

In this study, we presented in vitro evidence that overexpression of OLIG2 and SOX10 in hESCs-derived VF-NPC spheroid induced forebrain OL-spheroids within 40 days. There are 5.39 ± 0.66 MBP⁺ cells each...
square millimeter of the 60-day forebrain OL-spheroids which is the similar level with 110–160-day OL-spheroids (Marton et al., 2019). More importantly, our data show that the induced OPCs in the OL-spheroids differentiate into mature OLs and form myelination as early as 60 days after differentiation in vitro (Figure 3J) which is much earlier than other protocols (Kim et al., 2019). However, we did not examine functional subdomains of myelinated axons, such as the paranodal axo-glial junction (PNJ) and the node of ranvier which was shown in spinal cord myeloid recently (James et al., 2021). Of note, we found that most of the O1+ and O4+ cells mainly distribute in the edges of oligodendroglia spheroids which were also shown in other studies (James et al., 2021; Kim et al., 2019). We thought this probably related to the penetration and infection range of the virus where the edges of the spheroids are more accessible to infection. Actually, EGFP+ and/or mCherry+ double or single-stained cells (Figure S2I) mainly distributed in the edges of spheroids. Besides, better oxygen and nutrients in the peripheral of OL-spheroid than the core areas may also contribute to easier oligodendrocyte induction and maturation in the edge of the OL-spheroid.

Several recent studies have laid the foundation for the use of OL lineage conversion by transcriptional factors in regenerative therapies for neurological disorders (Yavarpour-Bali et al., 2020). Some papers have been shown that mouse or human fetal fibroblasts can be directly converted into 20%–30% O4+ OPCs by forced expression of Sox10, Olig2, and Zfp536 or Nkx6.2 after 15–20 days (Chanoumidou et al., 2021; Najm et al., 2013; Yang et al., 2013). Further studies showed that ectopic expression of SOX10, OLG2, and NNX6.2 was efficient to induce OLs from human PSCs-derived NPCs within few days in 2D culture (Ehrlich et al., 2017; Garcia-Leon et al., 2018; Pawlowski et al., 2017). These induced OL (iOL) not only ensheathed the neuronal processes of hPSC-induced neuron but also efficiently myelinated of MBP loss transgenic mouse and remyelinated the adult demyelinated spinal cord. In our study, we found transplanted cells from OL-spheroid survive and efficiently differentiated into mature OLs and myelinated neuron axons in the SCID mouse brain (Figure 4F). These data suggested potential functional roles of iOL in regenerative medicine.

Interestingly, our data showed single SOX10 is not sufficient to generate forebrain OLs which is inconsistent with previous studies (Garcia-Leon et al., 2018; Pawlowski et al., 2017). We hypothesize that these might be due to different initial hNPCs used for OPC lineage conversion. In their paper, they induced NPCs by dual SMAD inhibition combined with the caudalizing and ventralizing factors, RA and SHH agonist which resulted in 51.7% ± 4.6% OLIG2+ NPCs (Garcia-Leon et al., 2018). In our study, to mimic conditions present in ventral forebrain oligodendrocyte progenitor region, we combined dual SMAD inhibition with ventralizing factor, SHH agonist (SAG) which only produced 24.56 ± 2.00% and 12.62 ± 1.61% OLIG2+ NPCs in 2D culture and VF-NPC spheroid, respectively (Figures 1 and S2D–S2F). OLG2 which is necessary for OPCs generation, specification, and differentiation is believed to orchestrate OL differentiation by inducing the expression of NKX2.2 and later that of SOX10 (Sim et al., 2011). Loss-of-function experiments showed that oligodendrocytes are completely lost in the spinal cord in the Olig2 null mice, and induced expression of Olig2 can generate more oligodendrocytes and less neurons and astrocytes significantly (Du et al., 2006; Lopez Juarez et al., 2016; Lu et al., 2002). Thus, lower OLIG2 may impede OL specification at the initial OL induction stage. Besides, SOX10 alone robustly induced OPC (O4+) which failed to differentiate into myelin-expressing cells and died eventually suggesting importance of SOX10 during maturation of OPCs (Rodrigues et al., 2017). In our study, OL could be originating from SOX10 and OLIG2 double-positive cells. Thus, the efficiency of lentiviral-SO double-infected cells (mCherry+/GFP+) at the initial stage may affect the percentage of O4+ and MBP+ cells in 2D culture or OL-spheroid. Optimizing the lentivirus vectors such as using a polycistronic lentiviral expression vector may help to increase SO expression with limited virus in the future.

In summary, we demonstrated that overexpression of SOX10 and OLIG2 in hPSC-derived VF-NPCs allows a very efficient and rapid generation of hPSCs-derived forebrain OLs, especially OL-spheroids containing oligodendrocyte and neurons, which can myelinate axons in vivo. The interactions between oligodendrocyte and neurons and myelination of neuron fibers using iPSCs from patient to model demyelinating disease can be evaluated with this 3D OL-spheroids. These fast generated hPSC-derived OL-spheroids have the potential to model and understand mechanisms of underlying neurodegenerative disease, screen drugs for congenital or acquired demyelinating diseases in CNS, and even provide better cell source for stem cell-based therapy.
Limitations of the study
In this study, we generate forebrain OL-spheroid with pre-OLs and mature OLs within 60 days from hPSCs-derived VF-NPCs treated with SO; however, if any other important transcription factors can improve human OL-spheroids differentiation efficiency within short time need to be investigated in further. In addition, we only differentiate OL-spheroid using healthy hESCs or iPSCs in this study. To further apply this OL-spheroid differentiation strategy into disease modeling, drug screening, or stem cell therapy, we should test this method with more cell lines including iPSCs lines from patient with demyelination, or transplantation of these cells into animals with disease models in the future.

STAR METHODS
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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105172.

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AUTHOR CONTRIBUTIONS
M.X. and Y.C. conceived this study and designed experiments. L.M. and Y.M. performed cell culture, cell differentiation, immunoelectron microscopy, and immunofluorescence. L.M. performed cell counting and statistics. P.X. and Z.Y. performed plasmid construction. Z.W. preformed lentivirus packaging. X.J. and L.M. performed cell transplant. M.X., L.M., Y.M., Y.C., W.Z., and D.Z. contributed to the preparation of resources and approved the paper.

DECLARATION OF INTERESTS
All authors declare no competing interests.
INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Hoechst             | Sigma  | Cat# 33324; RRID:AB_2869394 |
| Rabbit anti-PAX6    | Biolegend | Cat#901301; RRID:AB_2565003 |
| Mouse anti-NKX2.1   | Millipore | Cat# MAB5460; RRID:AB_571072 |
| Rabbit anti-FOXG1   | Abcam  | Cat# ab18259; RRID:AB_732415 |
| Rabbit anti-Olig2   | Millipore | Cat# AB9610; RRID:AB_570666 |
| Rabbit anti-Sox10   | Abcam  | Cat# ab155279; RRID:AB_2650603 |
| Chicken anti-EGFP   | Abcam  | Cat# ab13970; RRID:AB_300798 |
| Rat anti-mCherry    | Invitrogen | Cat# M11217; RRID:AB_2536611 |
| Mouse anti-O1       | Millipore | Cat#1451014; RRID:AB_2574269 |
| Mouse anti-O4       | R&D    | Cat# MAB1326; RRID:AB_357617 |
| Rat anti-MBP        | Abcam  | Cat# ab7349; RRID:AB_305869 |
| Mouse anti-Tuj-1    | Sigma  | Cat# T8660; RRID:AB_477590 |
| Mouse anti-MAP-2    | Sigma  | Cat# M1406; RRID:AB_477171 |
| Chicken anti-NF-H   | Millipore | Cat# AB5539; RRID:AB_1121611 |
| Mouse anti-human Nuclei | Millipore | Cat# MAB1281; RRID:AB_94090 |
| Mouse anti-Stem121  | TAKARA | Cat# Y40410; RRID:AB_2801314 |
| Alexa Fluor 488 donkey anti-rabbit | Invitrogen | Cat#A21206; RRID:AB_2535792 |
| Cy™3 AffiniPure donkey anti-mouse | Jackson | Cat#715-165-151; RRID:AB_2315777 |
| Alexa Fluor® 405 Donkey anti-mouse | Abcam | Cat#ab175658; RRID:AB_2687445 |
| Alexa Fluor® 647 AffiniPure Donkey Anti-Rat | Jackson | Cat#712-605-153; RRID:AB_2340694 |
| Alexa Fluor 647 donkey anti-rabbit | Invitrogen | Cat#A31573; RRID:AB_2340694 |
| Alexa Fluor 488 goat anti chicken | Thermo Fisher | Cat#A32931; RRID:AB_2762843 |
| Cy™3 AffiniPure donkey anti-rat | Jackson | Cat#712-165-153; RRID:AB_2340667 |
| Alexa Fluor 647 donkey anti Mouse | Abcam | Cat#Ab150111; RRID:AB_2890625 |
| Alexa Fluor 405 donkey anti rabbit | Thermo Fisher | Cat#A48258; RRID:AB_2890547 |

| Bacterial and virus strains | This paper | N/A |

| Chemicals, peptides, and recombinant proteins |        |            |
|-----------------------------------------------|--------|------------|
| bFGF                                         | R&D    | Cat#233-FB-01M |
| SB431542                                      | Stemgent | Cat#04-0010-10 |
| DMH1                                         | Tocris | Cat#4126/10 |
| B27 without vitamin A                          | Gibco  | Cat#12587001 |
| Phosphate buffered saline (PBS)               | Sigma-Aldrich | Cat# D8537 |
| Nonsynthetic amino acids (NEAA)                | Gibco  | Cat#11140050 |
| Glutamax                                      | Gibco  | Cat# 350050038 |
| Penicillin/streptomycin solution (P/S)         | Gibco  | Cat#10378016 |
| DMEM/F12                                      | Gibco  | Cat# 21331020 |
| Matrigel                                      | Coming | Cat#354277 |
| TrypLE express                                | Gibco  | Cat# 12604013 |

(Continued on next page)
## Resource Availability

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Man Xiong (man_xiong@fudan.edu.cn).

### Reagent or Resource Source Identifier

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| Dimethylsulfoxide (DMSO) | Sigma-Aldrich | Cat# D2650 |
| Triton X-100 | AppliChem | Cat# A4975,0100 |
| SAG | Millipore | Cat#S66660-5MG |
| L-Ascorbic acid (AA) | Sigma-Aldrich | Cat#A4544-25G |
| cAMP | Sigma-Aldrich | Cat#A9501-1G |
| insulin-like growth factor 1 (IGF1) | Peprotech | Cat#100-11(500ug) |
| Trace Elements B |Coming |Cat#25-022-C1 |
| 3,3',5-Triiodo-L-thyronine (T3) | Sigma-Aldrich | Cat#T2877 |
| Recombinant Human NT-3 (NT-3) | Peprotech | Cat#450-03 |
| biotin | Sigma-Aldrich | Cat#B4501 |
| CaCl2 | Sigma-Aldrich | Cat#C1016 |
| 2*HEPES | Sigma-Aldrich | Cat#H3375 |
| PFA | Sigma-Aldrich | Cat#P6148 |
| Recombinant Human PDGF-AA | Peprotech | Cat#100-13A |

### Critical commercial assays

- One-step RT-qPCR kits: TAKARA Cat# RR064A
- Plasmid Midi Preparation Kit: AXYGEN Cat#AP-MD-P-10AxyPrep

### Experimental Models: Cell lines

- H9: Zhang et al., 2001 N/A
- iPSC: This paper N/A
- MEF: This paper N/A
- HEK293 cells: ATCC Cat# CRL-3216

### Experimental models: Organisms/strains

- Mouse:SCID: Bred in house N/A

### Oligonucleotides

- SOX10-V1-F: 5'-ATGGCGGAGGACGAGCATCTATC-3' This paper N/A
- SOX10-V1-R: 5'-CTTATGGCCGGACAGTGTG-3' This paper N/A
- OLIG2-V1-F: 5'-AGACTCTCTCAACTCGGCT-3' This paper N/A
- OLIG2-V1-R: 5'-ATCAGGTCCGGAGAGAACA-3' This paper N/A

### Recombinant DNA

- plasmid FUGW: Lois et al., 2002 Addgene Plasmid #14883
- psPAX2: Didier Trono Addgene Plasmid #12260
- VSV.G: Reya et al., 2003 Addgene Plasmid #14888

### Software and algorithms

- ImageJ: Schneider et al., 2012 https://imagej.nih.gov/ij/
- Prism (Version 9.0.1): GraphPad N/A

### Other

- 6-well cell culture plates: Thermo Fisher Cat#140675
- Corning CellBIND Surface 100 mm Culture Dish: Corning Cat#3296
Materials availability
All plasmids and cell lines generated in this study are available upon request.

Data and code availability
This study did not generate original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. All data produced in this study are included in the published article and its supplementary information, or are available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Mice
The animal experiment was approved by Research Ethics Board of Children’s Hospital of Fudan University (Agreement number: 2021-197). Server combined immune-deficiency (SCID) mice were housed under standard conditions of 12-h light/12-h dark cycles with ad libitum access to dry food and water cycle. Neonatal (P0–P3) SCID mice were randomized divided into two groups: SCID mice transplanted with oligodendrocyte spheroids in LV-EGFP group (LV-EGFP, n = 9 in total, 4 female and 5 male mice), and SCID mice transplanted with oligodendrocyte spheroids in LV-SO group (LV-SO, n = 10 in total, 5 female and 5 male mice). Oligodendrocyte spheroids were digested two days before transplantation, then were transplanted into the corpus callosum and cerebral cortex in one injection in each hemisphere (Coordinates: AP 1mm, ML 1mm, VRT 1mm). 12 weeks after transplantation, transplanted mice were anesthetized with Chloral hydrate, then perfusion fixed with cold normal saline (NS) followed by 4% paraformaldehyde. Brains were extracted after 2 hours, then dehydrate in 20% sucrose solution overnight and followed by 30% sucrose solution in PBS.

Cells
hESCs maintenance and passage
All the studies were approved by Fudan University. Human ESCs (H9, passages 25–50, WiCell Agreement No. 16-W0060). H9 colonies were maintained on a layer of mitotically inactivated mouse embryonic fibroblasts in human ESC medium consisting of DMEM/F12 (Gibco), 20% knockout serum replacement (Invitrogen), 1% nonessential amino acids (NEAA, Gibco), 1% GlutaMAX (Gibco), 100 μM β-mercaptoethanol (Sigma-Aldrich), 1% antibiotic/antimycotic (Gibco), freshly supplemented with 5 ng/mL b-FGF (R&D) (Reya et al., 2003). H9 Colonies were passaged using dispase when they became 80% confluent. Regular tests for mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza) were negative.

METHOD DETAILS

Neural differentiation
When the H9 colonies were became 80% confluent, chemically digest them with dispase, and suspended with human ESC medium in T-25 flask (Corning) to forming embryoid bodies. Four days after, change medium with neural induction medium (NIM) containing of Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Gibco), N2 supplement, nonessential amino acids (Life technologies) in the presence of 2 μM SB431542 (Stemgent), 2 μM DMH1 (Torcris) for 3 days. On day 7, embryoid bodies was allowed to attach in 6-well plates (thermo fisher) in NIM supplemented with 5% fetal bovine serum (Gibco) for 15 hours. Then change medium with NIM supplemented with 2 μM SB431542 (Stemgent), 2 μM DMH1 (Torcris) and 1 μM SAG (Millipore) for 5–6 days.

Oligodendrocyte differentiation
The attached neural progenitors were collected and cultured in suspension from day 15 to day 20 which the culture medium was changed every other day with OIM which consisted of DMEM-F12 with 1:200 N2 supplement, 1:100 B27 supplement lacking vitamin A, 1 μM SAG (Millipore), 10 ng/mL hPDGF-AA (Peprotech), 10 ng/mL NT3 (Peprotech), 10 ng/mL IGF-I (Peprotech), 200 μM AA (Sigma), 1:1,000 Trace Elements B (Corning), 10 ng/mL T3 (Sigma). Then, the neural spheres were cultured with DM medium consisting of DMEM-F12 with N2 supplement (1:200), B27 supplement without vitamin A (1:100), 60 ng/mL T3, 10 ng/mL NT3, 10 ng/mL IGF-I, 200 μM AA, Trace Elements B (1:1,000), biotin (Sigma) and 100 μM cAMP (Sigma). After 7–10 day of differentiation, cells were dissociated with accutase and reseeded at densities of 3 × 10⁴ cells on Matrigel coated glass coverslips of 24-well plates for staining analysis.
Plasmids construction
FUGW plasmid from add gene (#14883) and inserted the gene of short CAG (sCAG) promoter. We amplified human SOX10 and OLIG2 cDNA by PCR, for visualization of vector expression, SOX10 was linked by the mCherry by a self-cleavage sites-P2A and OLIG2 was linked with the EGFP by P2A, finally, we obtained sCAG-Sox10-P2A-mCherry and sCAG-Olig2-P2A-EGFP donor plasmids.

Lentivirous package
Lentiviral particles were produced by co-transfection of 293T cells with individual expression vectors in combination with the packaging plasmids psPAX2 (Addgene #12260) and VSV.G (Addgene Plasmid #14888). Using calcium phosphate/DNA coprecipitation method, lentiviral particles were generated in HEK293 cells. After 3 days of transfection, the supernatant containing the viral particles were collected and concentrated by ultracentrifugation at 4°C. Resuspend the viral particles in DPBS then store at −80°C.

Viral infection and oligodendrocyte spheroids differentiation
The viral infection of the oligodendrocyte spheroids was performed (Lois et al., 2002). In detail, on differentiated day 13, collect the neural spheres into a 1.5 mL microcentrifuge Eppendorf tube containing 500ul NIM supplemented with Rock inhibitor Y27632 and B27 without AA (Gibco), and viral particles. Incubate 24 hours and transferred them into fresh OIM in a six well ultralow attachment plate (Corning), then place them in a 3D shaker, change medium every other day. After 10 days of OIM, change DM until day 60.

Immunofluorescent staining, imaging and quantification
Cells cultured on coverslip were fixed with 4% PFA for 15 min at room temperature and washed with PBS for three times. Then permeabilized by 0.2% Triton X-100 (Sigma) in PBS for 15 min at room temperature. Adding 10% donkey serum in PBS for 1 hour at room temperature before applied primary antibodies overnight at 4°C. After wash three times with PBS, cells were incubated for 1 hour at room temperature with Alexa Fluor-conjugated secondary antibodies (Life Technologies). The nucleus was labeled with Hoechst (Sigma 33324). Primary antibodies were used as detailed: Rabbit anti-PAX6 (1:500; Biolegend 901301); Mouse anti-NKX2.1 (1:500; Millipore MAB5460); Mouse anti-NKX2.1 (1:500; DSHB 74 SAS); Rabbit anti-FOXG1 (1:500; Abcam ab18259); Rabbit anti-Olig2 (1:500; Millipore AB9610); Rabbit anti-Sox10 (1:2000; Abcam ab155279); Mouse anti-O1 (1:500; Millipore 1451014); Chicken anti-mCherry (1:1000; Invitrogen M12121); Mouse anti-O4 (1:500; R&D MAB1326); Rat anti-MBP (1:300; Abcam ab7349); Mouse anti-Tuj-1 (1:5000; Sigma T8660); Mouse anti-MAP-2 (1:500; Sigma M1406); Chicken anti-NF-H (1:1000; Millipore AB5539); Mouse anti-human Nuclei (1:300; Millipore MAB1281); Mouse anti-Stem121 (1:300; TAKARA Y40410). Fluorescent images of cells were obtained using an Olympus FV3000 Zeiss LSM 700 confocal microscope (Olympus, Japan). For quantification of MBP positive cells and neuron oligodendrocyte interaction, we referred to previous paper (Marton et al., 2019). Briefly, for MBP in per mm² in Figures 3E, 3F and S4K, we counted the number of MBP+ cells in each of the OL-spheroid slice from three independent experiments, and the corresponding area of each slice was also calculated with image J software. Then, MBP+ cells per mm2 could be obtained. As for Figure 2M and 3G, MBP/Tuj1 or MBP/MAP-2 double stained axons longer than 1μm were defined as oligodendrocyte interaction with neurons.

Cryopreservation
hOLS were washed in DPBS then fixed in 4% paraformaldehyde (PFA) for 30 minutes. Then washed in DPBS for 3 times and transferred to 30% sucrose for about two days. Subsequently, they were embedded in OCT (Tissue-Tek OCT Compound 4583, Sakura Finetek) and stored at −80°C. For immunofluorescence, 12-14-μm-thick sections were cut using a Thermo Fisher cryostat.

Electron microscopy and immunogold electron microscopy
For Electron Microscopy, day86 hOLS were washed with PBS then fixed in 2.5% glutaraldehyde in PBS overnight, followed with 2h post fixation in 1% osmium tetroxide (Sigma-Aldrich) and wash three times with ddH₂O. Incubate in 2% Uranium acetate overnight at 4°C and washed with ddH₂O. The samples were dehydrated in ethanol (50%, 70% and 90%, 15 min each) at 4°C, and in acetone (90%, 100% three times 100%, 5 min each) at 4°C. After dehydration, samples were embedded in Epon. Ultrathin sections (50–70 nm) and imaged with a FeiTalos L120C electron microscope. For Immunogold Electron Microscopy, grafted mice (n = 3) were perfused with 0.9% NaCl followed by a mixture of 4% paraformaldehyde/0.1% glutaraldehyde in PBS. After 3h post-fixation in the same solution, brains were sectioned in 50-μm-thick and wash three
times in 0.1MPB, then incubated with rabbit anti-GFP IgG with 5%DS/0.03% Triton-100 in PBS overnight at 4°C. The sections were washed by PBS, then incubated with 1.4 nm gold-conjugated Fab’ fragment of anti-rabbit IgG (1:500, Nanoprobes, 2004) with 10% DS in PBS 1h at room temperature, followed with post fixation with 1% glutaraldehyde in PBS. Sections were washed with PBS with 50 mM glycine, PBS-BSA and distilled water successively, and subsequently treated with Gold Enhance EM (Nanoprobes 2114) according to manufacturer’s protocol.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean (SEM) for at least three replicated experiments. Unpaired two-tailed Student’s t test was used to assess differences between two groups. p < 0.05 were considered statistically significant. To quantify the cell populations, at least three randomly selected fields from each of the three replicated independent experiments were analyzed with ImageJ software (Schneider et al., 2012). Each independent experiments initiated with human embryonic stem cells from different generation during passage. Statistical analysis and data performed using the GraphPad Prism 8 software.