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TITLE:

Generation of Human Neurons and Oligodendrocytes from Pluripotent Stem Cells for Modeling Neuron-Oligodendrocyte Interactions

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SUMMARY:

The neuron-glial interactions in neurodegeneration are not well understood due to inadequate tools and methods. Here, we describe optimized protocols to obtain induced neurons, oligodendrocyte precursor cells, and oligodendrocytes from human pluripotent stem cells and

provide examples of the values of these methods in understanding cell-type-specific contributions in Alzheimer's disease.

ABSTRACT:

In Alzheimer's disease (AD) and other neurodegenerative disorders, oligodendroglial failure is a common early pathological feature, but how it contributes to disease development and progression, particularly in the gray matter of the brain, remains largely unknown. The dysfunction of oligodendrocyte lineage cells is hallmarked by deficiencies in myelination and impaired self-renewal of oligodendrocyte precursor cells (OPCs). These two defects are caused at least in part by the disruption of interactions between neuron and oligodendrocytes along the buildup of pathology. OPCs give rise to myelinating oligodendrocytes during CNS development. In the mature brain cortex, OPCs are the major proliferative cells (comprising ~5% of total brain cells) and control new myelin formation in a neural activity-dependent manner. Such neuron-to-oligodendrocyte communications are significantly understudied, especially in the context of neurodegenerative conditions such as AD, due to the lack of appropriate tools. In recent years, our group and others have made significant progress to improve currently available protocols to generate functional neurons and oligodendrocytes individually from human pluripotent stem cells. In this manuscript, we describe our optimized procedures, including the establishment of a co-culture system to model the neuron-oligodendrocyte connections. Our illustrative results suggest an unexpected contribution from OPCs/oligodendrocytes to the brain amyloidosis and synapse integrity and highlight the utility of this methodology for AD research. This reductionist approach is a powerful tool to dissect the specific hetero-cellular interactions out of the inherent complexity inside the brain. The protocols we describe here are expected to facilitate future studies on oligodendroglial defects in the pathogenesis of neurodegeneration.

INTRODUCTION:

Oligodendrocyte lineage cells—including oligodendrocyte precursor cells (OPCs), myelinating oligodendrocytes, and transitional types in between—constitute a major group of human brain cells[1] that actively participate in many critical functions for the proper operation and maintenance of our central nervous system throughout neural development and aging[2–4]. While oligodendrocytes are well known for producing myelin to facilitate neuronal activity transmission and support axonal health in white matter, OPCs are abundant (~5%) in gray matter where myelination is scarce and perform activity-dependent signaling functions to govern learning behavior and memory formation[5–8]. How oligodendroglial cells function and dysfunction in the pathogenesis of Alzheimer's disease (AD) and other age-associated neurodegenerative conditions has been understudied[9]. The inadequacies of an appropriate model system and deficiencies in general knowledge to guide an experimental path forward are the major reasons for this gap.

In light of the latest breakthroughs in deriving human brain cells from pluripotent stem cells including embryonic stem (ES) and induced pluripotent stem (iPS) cells, such cellular models in conjunction with modern gene editing tools have emerged as robust tools to handle the intricate nexus of cellular interactions in the brain, and are capable of demonstrating human-specific disease manifestations[10,11]. Considering that individual brain cell types can exhibit distinct and

even conflicting effects in the face of the same AD-promoting conditions[12,13], this stem cell methodology uniquely offers cell type-specific information that has previously been missed using established in vivo or in vitro models that only provide aggregate readouts from collections of brain cell types. In the last decade, a good number of reliable protocols have been developed to generate human neurons from trans-differentiation of ES/iPS cells or direct conversion from other terminally differentiated cell types (e.g., fibroblasts)[14,15]. In particular, the application of key neurogenic transcription factors (e.g., neurogenin 2, Ngn2)[16] to human pluripotent stem cells can generate a homogeneous population of well-characterized neuronal cell types for pure cultures without a need for coculturing with glial cells[12,17,18]. For induced human oligodendrocytes, there are a few published protocols that can generate functional cells highly resembling their primary counterparts, with a wide range of efficiency and demand in time and resources[19–28]. To date, none of these protocols have been applied to investigate how oligodendroglial cells respond to and affect AD pathogenesis.

Here, we describe our improved protocols for single and mixed cultures of human induced neurons (iNs) and OPCs/oligodendrocytes (iOPCs/iOLs). The iN protocol described here is based on the widely used Ngn2 approach[16], and has the additional feature of being glia-free. The resultant iNs are homogenous and highly resemble the cortical layer 2/3 excitatory neurons, with characteristic pyramidal morphology, gene expression pattern and electrophysiological features[17,18] (**Figure 1**). To overcome some of the fundamental barriers in directed differentiation of pluripotent stem cells, we have developed a simple and effective method of low-dose dimethyl sulfoxide (DMSO) pre-treatment[29,30], and reported an enhanced propensity of human ES/iPS cells to transdifferentiate into iOPCs and iOLs[31], based on a widely-adapted protocol by Douvaras and Fossati[32]. We have further simplified the protocol and incorporated a robust differentiation-promoting compound, clemastine[7,33,34], to accelerate the process of oligodendroglial maturation. As a result (**Figure 2**), the iOPCs can be generated in 2 weeks (~95% positive for the marker O4) and iOLs in four weeks (expressing mature markers MBP and PLP1). Interestingly, we found iOPCs alone secrete a remarkable amount of amyloid-β (Aβ), consistent with the independent transcriptomic data showing the abundant expression of the amyloid precursor protein (APP) and the processing protease β-secretase (BACE1) in oligodendrocyte lineage cells[35,36]. Moreover, our iN-iOPC co-culture system promotes the ensheathing of axons by MBP-positive iOL processes and provides significant support for synapse formation (**Figure 3**). Thus, the protocols we have detailed below have technical and biological advantages over previously catalogued neuron-oligodendroglia co-culturing methods, and hold a promise in better modeling the neurodegeneration in AD.

PROTOCOL:

1. Human neuron induction from human pluripotent stem cells

1.1. Lentivirus preparation (~5 days, detailed protocol as described previously[16])

1.1.1. Plate ~1 million HEK293T cells each T75 flask, to have them ~40% confluent when performing transfection. Transfect them with plasmids expressing tetracycline-inducible Ngn2

and puromycin-resistant gene (PuroR; under the same TetO promoter control), rtTA and the three helper plasmids pRSV-REV, pMDLg/pRRE, and VSV-G (12 µg of lentiviral vector DNA and 6 µg of each of the helper plasmid DNA). Prepare at least three flasks per lentivirus preparation. Use PEI for transfection following the manufacturer's instruction. Change the media after 16 h and discard.

1.1.2. Harvest released viral particles by collecting culture media every day and replace with fresh media for 3 days. Pool the collected media containing viral particles for purification. Filter the virus through a 0.22 µm filter and centrifuge at 49,000 x *g* for 90 min. Resuspend the pellet in the appropriate volume of PBS-glucose (~150 µL).

1.2. Neuron Induction (~5 days)

NOTE: This induction protocol (**Figure 1A**; flow diagram) is highly effective for both iPS and ES cells of validated pluripotency (which can be assayed by immunohistochemistry staining of well-characterized pluripotency markers; **Figure 1B**).

1.2.1. Use commercially available H1 human ES cells at the passage of 52 (see **Table of Materials**). Culture the cells on extracellular matrix solution coated 6-well plates (~0.5 mg of matrix solution per 6-well plate; see **Table of Materials**) using ES cell maintenance medium (see **Table of Materials**) media and incubate the plates at 37 °C with 5% CO_2.

1.2.2. On Day -2, detach ES cells (80% confluent) with 1 mL of cell detachment solution (see **Table of Materials**) and incubate at room temperature for 10 min. Transfer the cells to a tube; wash the well with 2 mL of media and combine in the same tube. Centrifuge at 300 x *g* for 5 min, resuspend the pellet in media, and plate the cells onto matrix coated 6-well plates at the seeding density of 1 x 10^5 cells per well.

1.2.3. On Day -1, add lentiviruses expressing Ngn2 plus PuroR and rtTA together with polybrene (8 µg/ml) to the ES cells in fresh ES cell maintenance medium (see **Table of Materials**). The exact amount of viruses should be determined by actual titers or the titration. We typically add 5 µL each virus per well in a 6-well plate.

1.2.4. On Day 0, add Doxycycline (2 µg/mL, to activate Ngn2 expression) in DMEM-F12 medium with N2 supplement without morphogens.

1.2.5. On Day 1, add Puromycin in fresh medium of DMEM-F12 plus N2 and doxycycline, to the final concentration of 1 µg/mL medium. Select the transduced cells in Puromycin for at least 24 h. Higher Puromycin concentration (up to 5 µg/mL) and longer selection period (up to 48 h) may be required to adequately remove the under-transduced cells if the virus titer is low.

1.2.6. On Day 2, detach differentiating neurons with cell detachment solution (see **Table of Materials**), and re-plate them on 24-well plates (between 80,000–200,000 cells/well) coated with matrix solution (see **Table of Materials**), and maintain them in NBA/B27 medium without

doxycycline. The seeding density is critical.

1.2.7. At this stage, detached neurons can be frozen in specialized commercial freezing medium (see **Table of Materials**) and stored in liquid nitrogen for up to 3 months. Pure neurons can be plated accounting for the typical ~15%–20% cell death post-thaw, cultured alone or co-cultured with other brain cell types (see step 3.2.3. for co-culturing with OPCs).

1.2.8. Culture pure iNs on the plates coated with extracellular matrix-based solutions as instructed by the manufacturer (see **Table of Materials**). The characteristic pyramidal morphology should be apparent by Day 4 (and Day 6; **Figure 1C**). The synapse formation can be detected as early as Day 14 to 16 and is prominent at Day 24 by immunohistochemical staining with standard pre- and post-synaptic markers. (**Figure 1D**; labeled with the pre-synaptic marker Synapsin 1 and the dendritic marker Map2).

2. Human oligodendrocyte precursor cell (OPCs) induction from pluripotent stem cells and oligodendrocyte maturation

2.1. Neural Progenitor Cell (NPC) generation: monolayer protocol (~7 days). See **Figure 2A** for the flow diagram.

2.1.1. Culture H1 human ES cells as described earlier (see step 1.2.1.) and trans-differentiate them into neural progenitor cells (NPCs) by an established approach called dual SMADi, with small molecule inhibitors for multiple signaling pathways. Here we use a widely accepted commercial kit and follow the monolayer protocol provided by the manufacturer (see **Table of Materials**).

2.1.2. On Day -1, plate 0.5–1 x 10^6 cells per well in a 6-well plate coated by a growth factor reduced matrix solution (see **Table of Materials**; ~0.5 mg of matrix solution per 6-well plate) with ES cell maintenance medium (see **Table of Materials**). This growth factor reduced matrix solution is used to coat all the plates that will be used in the following steps.

2.1.3. On Day 0, treat cells for 24 h with ES cell maintenance medium (see **Table of Materials**) supplemented by 2% DMSO.

2.1.4. On Day 1–6, change the full media with warm (37 °C) neural induction medium containing the SMAD inhibitors from the commercial kit (see **Table of Materials**). If cells divide and reach confluence before Day 7, passage them to the seeding density of 0.5–1 x 10^6, as described earlier in step 2.1.2.

2.1.5. On Day 7, passage NPCs using cell detachment solution (see **Table of Materials**) and plate at a seeding density of 1–2 x 10^5 cells/well of a 24-well plate.

2.1.6. Assay the differentiation efficiency by immunohistochemical (IHC) staining for absence of pluripotency marker, OCT4 for example, and presence of NPC markers such as PAX6, Nestin, and

Sox1.

2.1.7. At this stage, detached NPCs can be frozen in the specialized commercial NPC freezing media (see **Table of Materials**) and stored in liquid nitrogen for up to 3 months. After freeze-and-thaw for once, NPCs still retain the multipotency to give rise to neurons, astrocytes, and OPCs with reliable protocols.

2.2. Oligodendrocyte precursor cell (OPC) generation (~7 days). Please see **Figure 2A** for the flow diagram.

2.2.1. On Day 7, passage NPCs using cell detachment solution (see **Table of Materials**) and plate them at a seeding density of 1–2 x 10^5 cells per well in a 24-well plate in warm (37 °C) neural induction medium plus SMAD inhibitors from the commercial kit (see **Table of Materials**).

2.2.2. On Day 8, prepare a solution of 1% DMSO in the OPC differentiation medium and treat the plated NPCs for 24 h. The OPC differentiation medium is composed of: DMEM/F12 medium, 1% N2 supplement, 1% B27 supplement, bFGF at 20 ng/mL, SAG at 1 µM, PDGF-AA at 10 ng/mL (see **Table of Materials**).

2.2.3. On Day 9, replace media with fresh OPC differentiation medium without DMSO. Feed the cells every other day until Day 15. If the cells reach confluence before Day 15, passage them to the seeding density of 1–2 x 10^5 cells per well as described in step 2.2.1.

2.2.4. On Day 14, plate OPCs in OPC differentiation medium at a density of 1–2 x 10^5 cells/well in a 24-well plate.

2.2.5. At this stage (Day 15), test cells for the presence of OPC-specific markers by IHC staining or qPCR (e.g., O4, Olig1/2, CSPG4/Ng2, NKX2.2, PDGFRa; **Figure 2B**) and for the absence of NPC markers (Pax6 or Nestin; **Figure 2D**). We typically detect the O4 immunoreactivity in more than 95% of the cells at Day 15. Of particular relevance to Alzheimer's disease, the expression of *APP* (amyloid precursor protein), *BACE1* (the processing protease β-secreatase 1), and peptide amyloid-β (Aβ) is abundant in OPCs (**Figure 2F**).

2.3. Oligodendrocyte (OL) maturation (~7–20 days)

2.3.1. On Day 15, replace media with OL maturation medium: Neurobasal-A medium, 2% B27 supplement, 1 µM cAMP, 200 ng/mL T3 triiodothyronine, and Clemastine of 1 µM (see **Table of Materials**). Change the medium every other day or every day, if necessary.

2.3.2. When cells reach 90% confluence, split at a 1:3 ratio up to 2 passages or until cell division slows down substantially. If OPCs divide too fast and reach confluency in less than 3 days, add Ara-C (see **Table of Materials**) at a concentration of 2–5 µM for 1–3 days. Active proliferation indicates lowered maturation efficiency.

2.3.3. Examine the efficiency of oligodendroglial maturation by assessing the expression of OL markers, e.g., CLDN11, PLP1, MBP by qPCR, IHC staining or immunoblotting. The characteristic morphology of highly complex structures (**Figure 2C**) and the expression of OL markers (**Figure 2E**) should be readily detected by Day 28.

3. Co-culturing of human induced neurons (iNs) and oligodendrocyte precursor cells (iOPCs)

3.1. iOPC plating (~3 days)

3.1.1. Plate iOPCs at Day 14 at a density of 1 x 10^5 cells per well in a 24-well plate (as described above in step 2.2.4.) in OPC differentiation medium (as described in step 2.2.2.).

3.2. iN-iOPC co-culture set up

3.2.1. On Day 15, detach the induced human neurons at the step of Day 2 after the Puromycin selection (as described in step 1.2.6.) with cell detachment solution (see **Table of Materials**).

3.2.2. Add neurons onto the cultured OPCs, plating at the seeding density of 2 x 10^5 cells per well in the 24-well plate with growing OPCs (from step 3.1.1). Use the co-culture medium containing Neurobasal-A medium, 2% B27 supplement, and 100 ng/mL T3 triiodothyronine. Change the medium on the next day and then every other day afterwards. If OPCs proliferate too fast and reach confluency in less than 3 days, add Ara-C at a concentration of 2–5 µM. A representative image of the iNs and iOPCs grown in co-culture after 7 days neurons is shown in **Figure 3A**.

3.2.3. Use frozen neurons prepared as described above in step 1.2.7 for co-culturing with OPCs. Plate freeze-and-thaw neurons at a higher density of 3 x 10^5 cells per well.

3.2.4. After Day 14–16 in co-cultures, the synapse formation in iNs can be observed by IHC staining of pre- and post-synaptic markers, and by Day 21 the synaptic puncta should be abundant (**Figure 3C**) and neuronal activities can be reliably recorded.

3.2.5. Starting at Day 21, test cells for OL specific markers (for example, MBP and PLP1). By Day 28, we normally observe the phenomenon ensheathing of iN axons by iOL processes, labeled by IHC staining for specific markers (**Figure 3B**; neurofilament NF for iN axons and MBP for iOPC processes).

REPRESENTATIVE RESULTS:

Direct generation of human induced neurons from human pluripotent stem cells

It is very important that the starting human pluripotent stem cells exhibit a high degree of pluripotency for successful generation of iNs or iOPCs/iOLs. Therefore, cells should be stained for

specific markers, such as Oct4 and SOX2, before starting either of the induction protocols described in the present manuscript (**Figure 1A**). Human H1 cells were used to obtain induced excitatory forebrain neurons following the previously published protocol by Zhang et al. with some modifications (**Figure 1C**)[12,16–18]. Here, we present a protocol in which iNs at Day 2 are re-plated in pure culture on matrix solution (see **Table of Materials**), in the absence of any feeder layer: glia or fibroblasts. In addition to the previously published protocols, we observe that freezing iNs at Day 2 does not significantly affect cell viability (~15%–20% cell death after thawing). Pure neurons in culture will start expressing synapsin1 at Day 14–16 (**Figure 1D**). Establishing a pure neuronal culture is very important because certain factors, for example, the leading AD risk factor ApoE, can be expressed by cells in the feeder layer and this can significantly confound the results.

iOPC generation and iOL maturation is improved by DMSO treatment

Here, we present a fast and efficient protocol that enables the generation of iOPCs in 2 weeks and mature iOLs in 4–5 weeks (**Figure 2A**). We leveraged the method of transient DMSO treatment we previously developed to augment the differentiation efficiency for ES and iPS cells[29–31]. DMSO treatment enriches the number of cells in the early G1 phase for better signaling integration, favoring differentiation. We performed the first treatment before inducing human ES cells to generate NPCs, and the second treatment before differentiating NPCs into iOPCs. We can detect specific OPC markers (Olig2, CSPG4, NKX2.2, and PDGFRA) as early as 2 weeks after plating of ES H1 cells (**Figure 2B,E**). The iOPC population at this stage is fairly homogenous, with >95% of cells positive for O4 staining and a high level of immunoreactivity for other markers (**Figure 2B**). After the start of OL maturation at Day 15, we can typically detect specific OL markers (MBP, O1, CLDN11, and PLP1) starting at Day 28 (**Figure 2C,E**). The expression of these stage-specific markers correlates with the developmental course of oligodendroglial cells and suggests an accelerated pace, with the NPC markers progressively going down, OPC markers peaking around the second week, and OL markers elevating by the third week (**Figure 2D,E**)[37]. Please note that this maturation process diversifies the cell populations. The subpopulations in continuum, comprising multiple intermediate stages between OPCs and mature myelinating oligodendrocytes, can be present and account for varying percentage of total cells, with more mature cells dominating at a later time.

As a comparison, we purchased the highly referenced iOPCs, and matured them in iOL following the manufacturer protocol. We tested the expression of the markers mentioned above in both our iOPC and iOL preparations, and in the cells we purchased. We determined that the cells generated following our protocol had higher expression of all the genes tested (**Figure 2E**). Interestingly, when we tested the secreted levels of two major isoforms of amyloid-β (Aβ40 and Aβ42) in iNs versus iOPCs, we noticed that iOPCs secreted more of both fragments, but the ratio remained the same (**Figure 2F**).

Co-culturing of iNs and iOPCs

This protocol is optimized specifically for co-culturing iNs and iOPCs and allow our real-time

monitoring of the inter-cellular communications between these two cell types along the course of neural development. The ideal plating densities for both cell types need to be decided with a series of cell number titration to achieve proper differentiation (**Figure 3A**). After 4 weeks in co-cultures, the iOPCs are expected to be adequately differentiated into OLs that are positive for specific markers such as MBP and extend processes to ensheath axons (**Figure 3B**). The co-culture system can robustly boost up the number of synapses, indicating that the iOPCs provide a neuronal support through physical contacts or release of trophic factors (**Figure 3C**). We can maintain the co-cultures in acceptable health condition for up to 6 weeks and observe that the synapse number and other neuronal attributes plateau around the fifth week. Of note, astrocytes and microglia are not present in our preparations and their absence can be documented by checking the expression of specific markers (**Figure 3D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Direct Generation of human induced neurons (iNs) from hPSCs. (**A**) Flow diagram of iN generation. (**B**) Representative bright field and immunofluorescence images of the starting culture of human pluripotent stem cells (H1) to confirm the pluripotency. Oct4 is shown in red and Sox2 in green. (**C**) Representative bright field images of iNs at Day 4 and Day 6. (**D**) The characteristic morphology for dendritic arborization and synapse puncta in iNs grown in pure culture for 24 days and stained by immunofluorescence staining for dendritic marker Map2 and pre-synaptic marker Synapsin 1 (Syn1).

Figure 2: iOPC generation and iOL maturation. (**A**) Flow diagram of iOPC and iOL generation. (**B**) Representative bright field and immunofluorescence images of iOPCs at Day 15. Olig2 (pan-oligodendroglia marker) is shown in green, O4 (OPC marker) in red, and DAPI in blue. The imaging revealed that >95% of iOPCs are positive for O4 and 25% for Olig2. (**C**) Representative bright field and immunofluorescence images of iOLs at Day 28. MBP is shown in green, O1 in red, and DAPI in blue. (**D**) The expression of NPC marker PAX6 diminishes dramatically in iOPCs at Day 14 and further lowers to background in OLs at Day 28, indicating a robust NPC trans-differentiation and a high level of homogeneity in the iOPC population. (**E**) The time-course expression profile of common OPC and OL marker genes in cultures generated by the described protocol, without (-DMSO) or with (+DMSO) the step of DMSO incubation (steps 2.1.3 and 2.2.2), assayed at different time points. As a comparison, commercial iOPCs (see **Table of Materials**) were matured according to the manufacturer's instructions, and both iOPCs (iOPC-Tempo) or iOLs (iOL-Tempo) were tested for the same markers. As expected, MBP (a mature oligodendrocyte marker) was not detected (N.D.) at the early stages of differentiation in all the iOPCs tested. The DMSO significantly enhanced the efficiency of OPC differentiation and OL maturation. (**E**) The production and secretion of Aβ40 and Aβ42 in pure iNs and iOPCs cultures, measured by commercial ELISA kits (see **Table of Materials**) on supernatant obtained from pure iNs and iOPCs cultures both at Day 15 and normalized by cell numbers (both at the density of 200,000 cells per well in a 24-well plate).

Data in bar graphs are plotted as mean ± SEM ($n \geq 3$). Statistical significance was evaluated by Student's *t*-test (*, $p < 0.05$; ***, $p < 0.001$); in (**D**), compared to the NPC; in (**E**), compared to the

control iOPC-Tempo; in (**F**), compared to iN.

Figure 3: Co-culture of iNs and iOPCs. (**A**) Representative bright field image of co-cultured iNs and iOPCs at Day 7, showing a proper density for further maturation. (**B**) Representative immunofluorescence image of iNs and iOPCs co-cultured for 28 days. Axonal marker neurofilament NF is shown in green and oligodendrocytic marker MBP in red. Right, a segment of iN axon ensheathed by iOL process (MBP+). (**C**) Synapse formation assayed in 4-week-old co-cultures. Cells were stained for Synapsin 1 (Syn1, green) and MAP2 (red), and synaptic puncta were quantified by confocal analysis of density along the dendritic segments as described[17,18]. (**D**) In our co-cultures of iNs and iOPCs (7 days of co-culturing), the expression of astrocyte markers, ALDHL1 and GFAP, is minimal (top), and the expression of microglia markers, TMEM119, TREM2, and CD33, is not detected (N.D.) by qPCR. The contamination from these two glial cell types is thus excluded.

Data in bar graphs is plotted as mean ± SEM (n ≥ 3). Statistical significance was evaluated by Student's *t*-test (**, $p < 0.005$; ***, $p < 0.001$); in (**C**), compared to the no OPC condition; in (**D**), compared to primary astrocytes in top panel.

DISCUSSION:

In addition to the physical and metabolic support to stabilize the synapse structures and to facilitate the saltatory signal conduction by myelination, oligodendrocyte lineage cells can shape neuronal activity pattern via rapid and dynamic cross-talks with neurons[5–7]. While in AD pathology the oligodendroglial responses were initially regarded as merely secondary to inflammation and oxidative stresses, there is now promising evidence arguing that compromised myelin integrity is an early pathogenic event prior to the appearance of Aβ aggregation and tau hyperphosphorylation[9]. Furthermore, the repair of myelination through self-renewal of OPCs is particularly vulnerable in AD[38], a process that heavily depends on neuronal activities. Understanding the mechanism to support healthy neuron-oligodendrocyte signaling thus represent an excellent opportunity for identifying new therapeutic targets.

The single transcription factor Ngn2 protocol is one of the most employed techniques for the generation of stem cell-derived human neurons, and the procedures outlined here are further refinements for obtaining pure neuronal cultures. Our iOPC/iOL protocol has an induction period shorter than the previously published studies (4 to 24 weeks), with a robust yield and purity comparable to other commonly used protocols[19,20,22–28]. Our protocol introduces the stepwise differentiation of ES cells to NPCs, OPCs, and finally oligodendrocytes by characterized patterning cues, and generates functional cells that can be used to study the regulation of myelination homeostasis and repair in vitro or in vivo (e.g., by engraftment into the shiverer mouse model) as described in the previous work. The improvement in our protocol is greatly promoted by the DMSO incubation, which activates the retinoblastoma protein and prolongs the G1 phase of the cell cycle to better integrate the stimuli of directed differentiation, and also enhances terminal differentiation into functional derivatives[29,30]. Finally, the use of clemastine, a muscarinic and antihistaminic compound hit identified through drug screens for remyelination therapeutics[33], additionally shortens the oligodendrocyte maturation, as observed in iPS cell preparation and

living animals[7,28].

The limitations of the technique mainly lie in the intrinsic discrepancy between the simplified in vitro settings and the in vivo milieus in the brain; this discrepancy leads to a discount in full developmental potential at the advanced stages for individual brain cell types. For iNs, recent studies were able to maintain the cultures in good synaptic health for a considerably long period of time, but still revealed some relative immaturity manifested as reduced spine-like structures and impaired spontaneous synaptic transmission in "old" iN cultures (even the 25-month-old ones)[39]. While iOPCs have been reproducibly shown to myelinate axons in vivo after being transplanted into transgenic mouse brains, the in vitro myelination assays with electron microscopic evaluation still represent a technical challenge with unsatisfactory efficiency for almost all published protocols[19,28], including this one. Therefore, our neuron-OPC co-culture system is not anticipated to faithfully mimic the brain-aging process as well as the late stage of AD pathology. Rather, it is uniquely poised to disentangle the elaborate inter-cellular interactions between neurons and OPCs or early-stage oligodendrocyte, which are independent of myelination and yet fundamental for proper neural development and disease pathogenesis.

Each of the three procedures described here has its critical steps within the protocol and may require modifications and troubleshooting. For iN protocol, there are two critical steps: puromycin selection (step 1.2.5) and plating density (step 1.2.6). Incomplete removal of under-transduced cell results in the contamination of poorly differentiated cells and compromises the neuronal survival and functions. The modifications for stronger puromycin selection with higher concentration and longer incubation as described in step 1.2.5 would have to be considered. The suitable plating density should be determined by titration for each pluripotent cell line, as low density leads to collapse of the cultures and high density encourages cell aggregation and impedes neuronal growth. For iOPC/iOL protocol, the two critical steps are the control of cell proliferation in OPC differentiation (step 2.2.3) and plating density for OL maturation (steps 2.2.4 and 2.3.2). The overgrowth of differentiating NPCs signals a poor response to the OPC differentiation stimuli and needs to be dampened by an appropriate dosage of Ara-C treatment (within the indicated range). While plating OPCs for maturation, a lower range of cell density is preferred here as the sparse distribution can facilitate the induction of a physiological morphology of complex structures (as shown in **Figure 2C**). For iN-iOPC co-culturing protocol, we would like to draw attention to the critical step of plating with an appropriate density for both cell types (steps 3.1.1 and 3.2.2). Specifically, the iNs may not attach well to the surface in between growing OPCs and tend to detach first when the culture reaches confluency. The optimal ratio would have to be decided by titrating the cell numbers.

Overall, this reductionist approach residing in our protocols is a powerful tool to dissect the specific hetero-cellular interactions from the inherent complexity of the human brain, and serve to uncover oligodendroglial biology in health and in AD. The significance with respect to existing methods is thus fairly apparent in our opinion. An additional utility of the methods developed here for the future applications is cell-based therapy for demyelinating conditions, such as post-radiotherapy[40] and spinal cord injury[41,42]. Moreover, the high-throughput capacity of this stem cell-based system can also be utilized on a larger scale to screen libraries of small molecules for

compounds that can protect or restore the physiological status of neurons, OPCs, oligodendrocytes and their interactions. Thus, we believe the protocols described here will facilitate future work in developing better modeling tools and effective treatments for AD and other neurodegenerative disorders.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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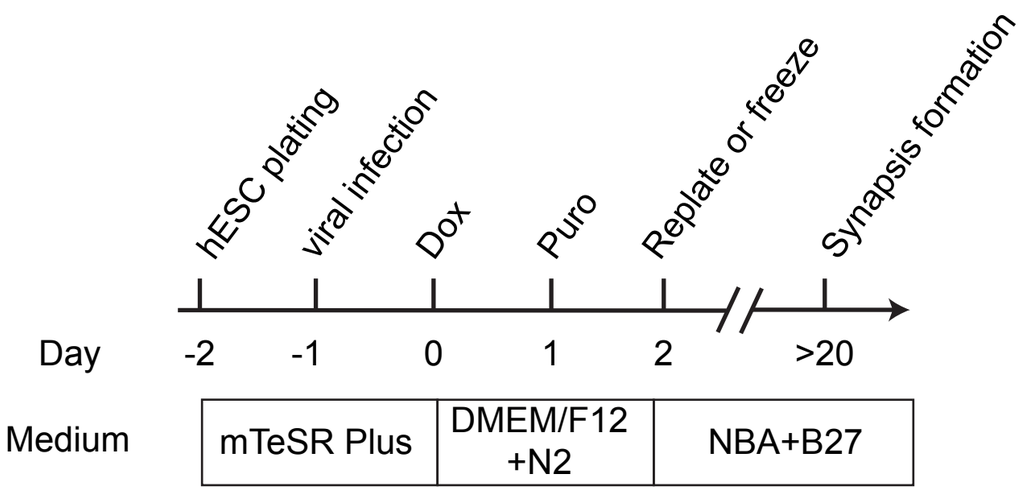

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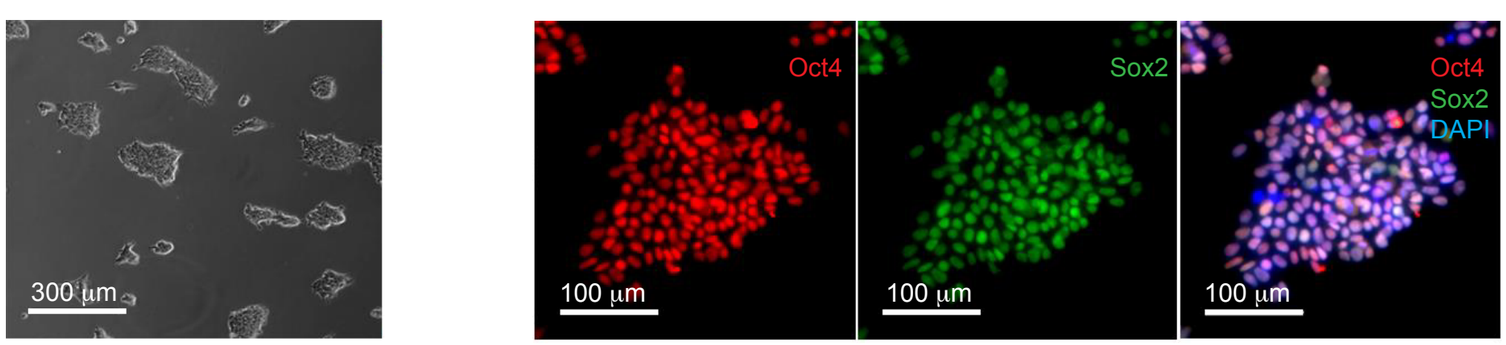

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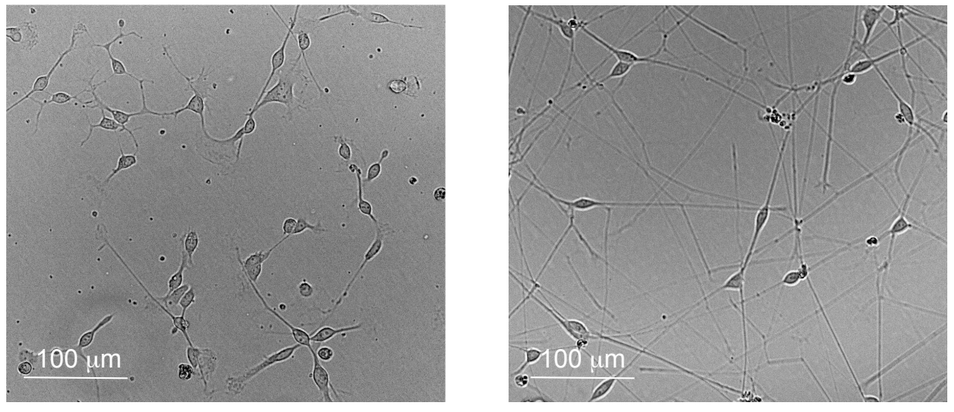

DAY 4

DAY 6

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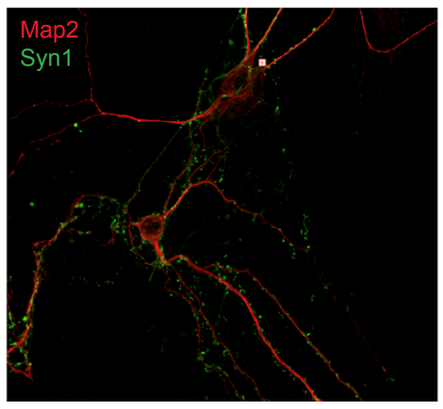

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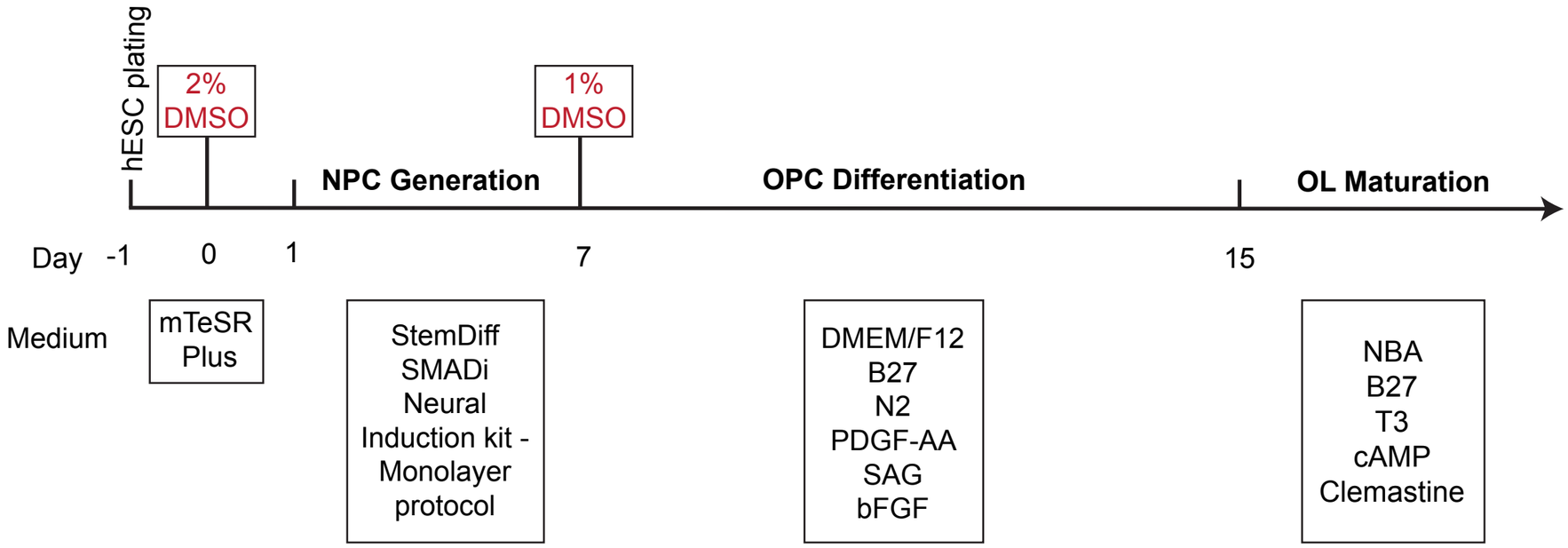

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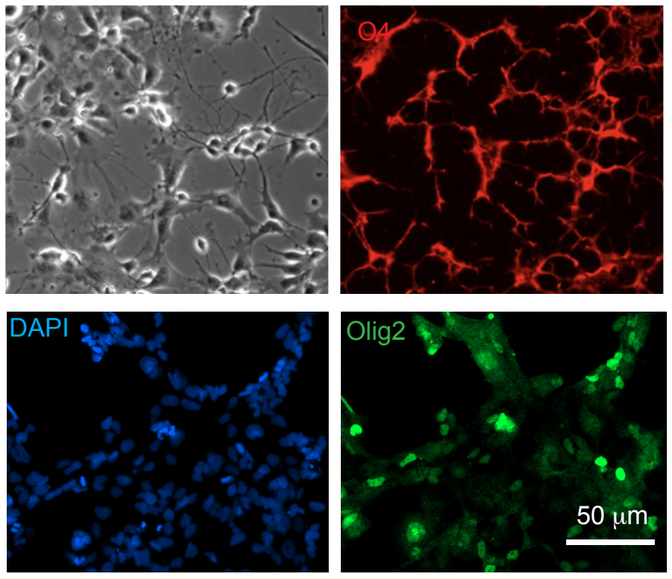

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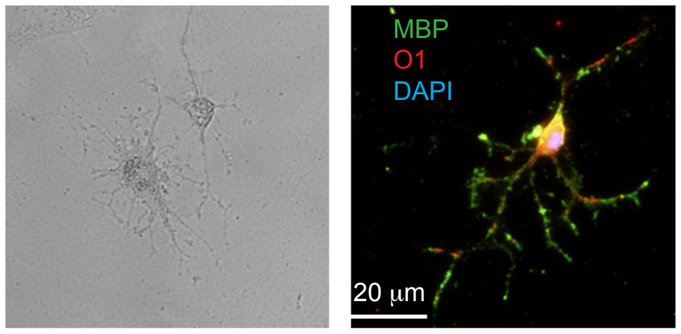

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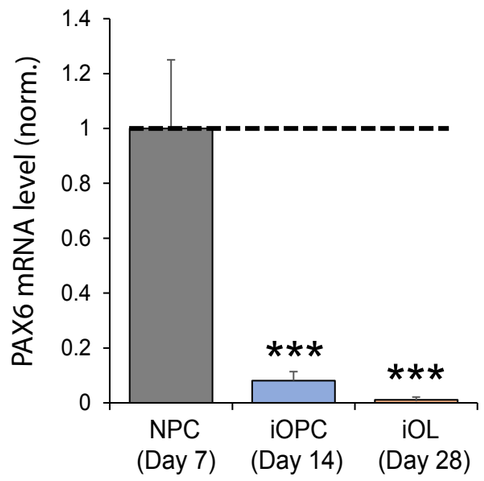

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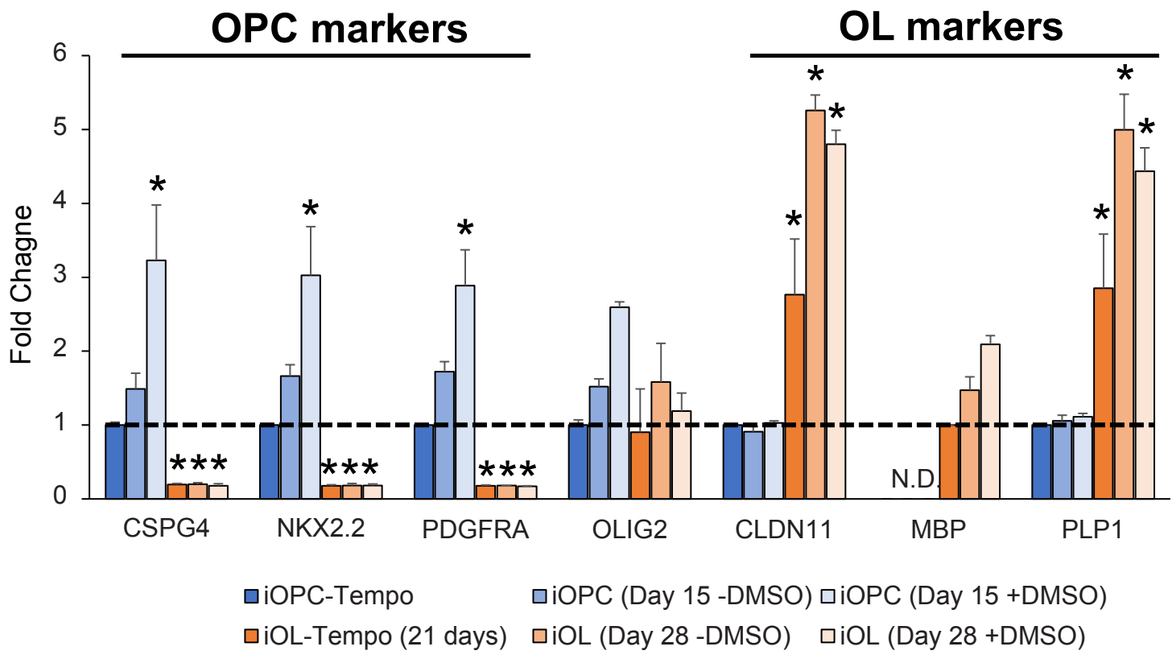

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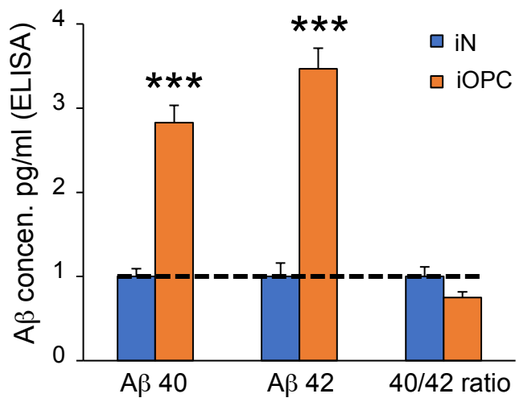

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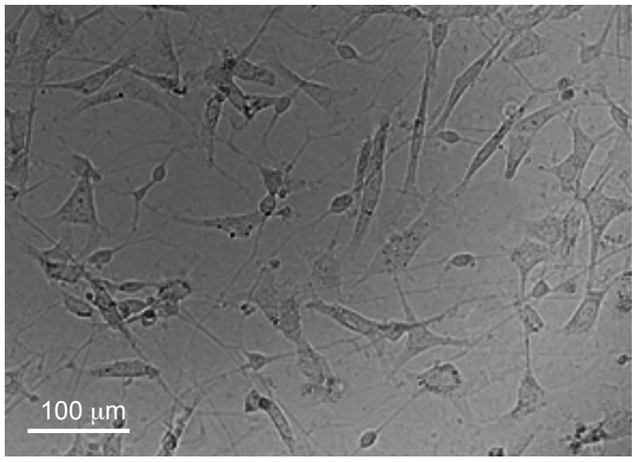

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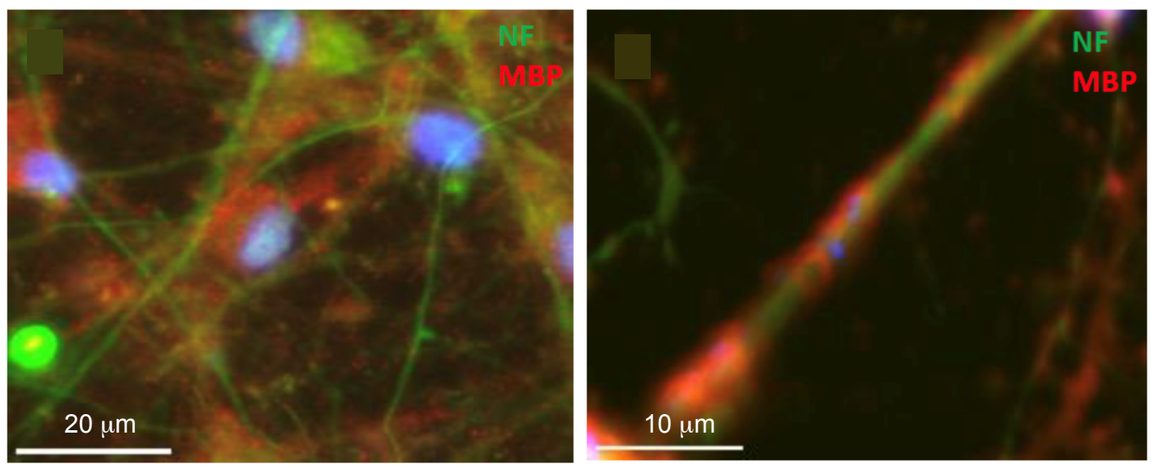

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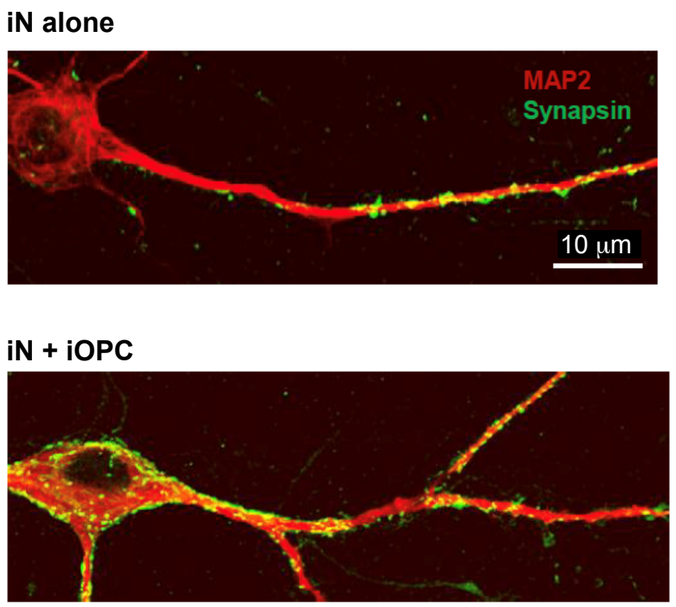

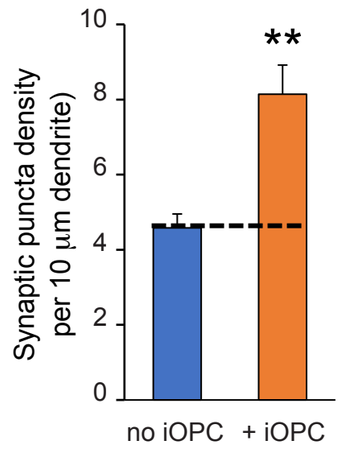

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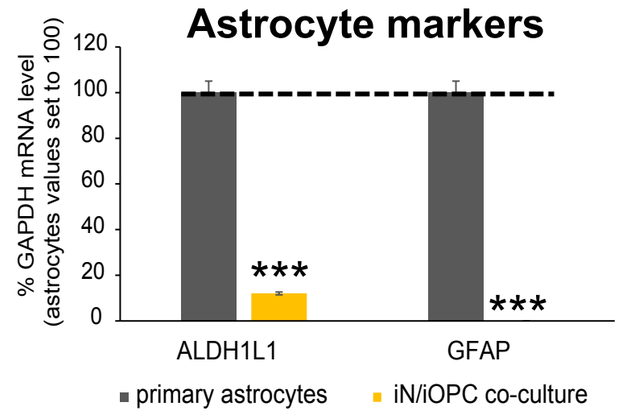

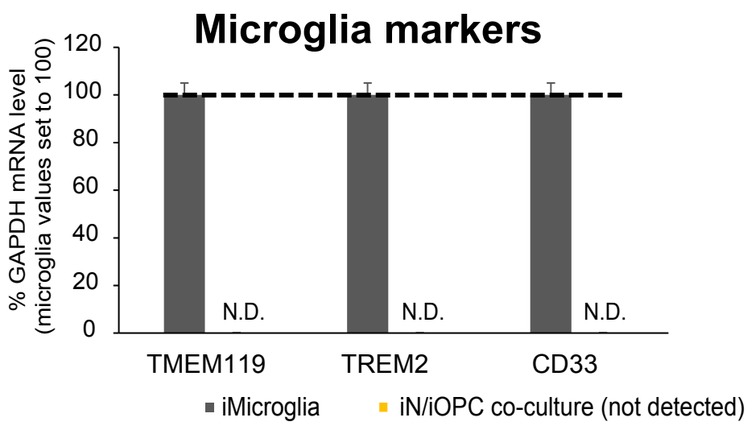

Figure 1 in ai format

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Figure 2 in ai format

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Figure 3.ai

Table of Materials

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Name of Material	Company	Catalog Number
Accutase	STEMCELL Technologies	7920
B27 supplement	ThermoFisher	17504044
bFGF	ThermoFisher	PHG 0266
cAMP	MilliporeSigma	A9501
Clemastine	MilliporeSigma	SML0445
DMEM/F12 medium	STEMCELL Technologies	36254
DMSO	ThermoFisher	D12345
Doxycycline	MilliporeSigma	D3072
Fetal Bovine Serum	ScienCell	10
H1 human ES cells	WiCell	WA01
Matrigel	Corning	354234
mTeSR plus	STEMCELL Technologies	5825
N2 supplement	ThermoFisher	17502001
Neurobasal A medium	ThermoFisher	10888-022
Non Essential Amino Acids	ThermoFisher	11140-050
PDGF-AA	R&D Systems	221-AA-010
PEI	VWR	71002-812
pMDLg/pRRE	Addgene	12251
Polybrene	MilliporeSigma	TR-1003-G
pRSV-REV	Addgene	12253
Puromycin	ThermoFisher	A1113803
ROCK Inhibitor Y-27632	STEMCELL Technologies	72302
SAG	Tocris	4366
STEMdiff Neural Progenitor Freezing Media	STEMCELL Technologies	5838
STEMdiff SMADi Neural Induction Kit	STEMCELL Technologies	8581
T3 triiodothyronine	MilliporeSigma	T6397

Tempo-iOlogo: Human iPSC-derived OPCs	Tempo BioScience	SKU102
TetO-Ng2-Puro	Addgene	52047
VSV-G	Addgene	12259

Re: Authors' response to the reviewers' comments for Assetta et al., "Generation of Human Neurons and Oligodendrocytes from Pluripotent Stem Cells for Modeling Neuron-oligodendrocyte Interactions"

We would like to thank the reviewers and editors for their careful assessment of our manuscript and really appreciate their insightful comments and constructive suggestions. We have addressed every single point raised by the reviewers, major and minor, with new data and further clarification. Specifically, we have performed additional experiments to validate the purity and proper development of our induced cell populations in single and mixed cultures (Fig. 2D and 3D). We have also elaborated and referenced the mentioned parts of our manuscript to adequately inform the readers of the utility and caveats residing in our methods. The text revision is extensive throughout the manuscript. While these changes are made at this turbulent time when our productivity is markedly compromised owing to the pandemic-related delays, they do significantly strengthen our work, which, in our opinion, now is better aligned with the research scope of the *JoVE*.

We hope this rebuttal will meet the editors' and reviewers' full approval and move forward the publication of our article. Below, we cite the editorial and reviewers' comments in full in *italic* typeface and provide our response in **bold** typeface.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have followed this top suggestion and proofread our entire package to our best.

• Protocol Language: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) All steps of your protocol need to be re-written in the imperative voice. For example, "HEK293T cells plated in T75 flasks are transfected with plasmids expressing Ngn2" should be "Plate HEK293T cells in T75 flasks and transfect them with plasmids expressing Ngn2".

We have changed the text in the protocol section exactly as suggested.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add specific details (e.g. button clicks for software actions, numerical values for settings, etc) to all your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 1.1.1: Describe cell plating, cell density. How is transfection performed? Mention culture environmental conditions.

2) 1.1.2: How is collection done? What is the centrifugation speed (in g) and duration.
3) 1.2.1: how is cell culture performed?
4) 1.2.2: describe the detachment steps. How much accutase? How long is it incubated?
5) Please use the above comments as a guide for your entire protocol and add all specific details.

All these mentioned details are incorporated into our manuscript.

• Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

This critical step of Protocol Highlight has been executed and all the listed points have been completely taken into account.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The Discussion section is now reorganized and expanded from three to five paragraphs, with all the listed points underlined.

• Figure/Table Legends: Define all error bars.

All the error bars are now clearly defined in the figure legends.

• References: Please spell out journal names.

The original list of references was generated by using the EndNote with the JoVE style downloaded from the website. Now the journal full names are all spelt out as suggested.

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding

language in your manuscript are WiCell Research Resources (Wicell, Matrigel, PEI, mTeSR, accutase, etc.

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have carried out this editing procedure as suggested.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We do not include any previously published figures in this manuscript; we cite and summarize our prior results to inform the readers.

Reviewers' Comments:

Reviewer #1:
Manuscript Summary:
In the manuscript, Assetta et al. described a new method in inducing neurons and oligodendrocyte lineage cells from iPS cells. The authors also provided a detailed protocol in co-culturing iNeurons and iOligodendrocytes and successfully characterized cell-specific markers for both cell types. The protocol is well-written and the method described in the manuscript will be of great interests for researchers who want to use the iPS cells to study neuron-oligodendrocyte interactions in various neurological diseases, including Alzheimer's disease.

We wholeheartedly appreciate the reviewer's comprehensive comments.

Major Concerns:
None

Minor Concerns:
1. As the protocol describes a co-culture harboring induced neurons and oligodendrocytes, the authors should comment on the purity of the culture - are there any astrocytes (GFAP+) or microglia (Iba1+) present in the co-culture? If so, what are the percentages for these cell types? Since both of these cell types interact with neurons and oligodendrocytes and are involved in

AD, it will be helpful to clarify if they are present in the co-culture system which is intended to model AD.

We are grateful for the reviewer's comment that points to the overall strength of the modern stem cell-based model system, which allows generation of a homogenous, well-characterized population of desired brain cell type. For our co-cultures, we induced the human neurons and OPCs separately along their individual, fate-determining developmental courses before we carefully mixed them to grow together. Microglia and astrocytes thus are not expected to be present in our preparations. To ensure the absence of these two glial cell types, we examine the expression of several marker genes in our neuron-OPC co-cultures and compared that to validated pure human microglia and astrocytes (Fig 3D). We cannot detect any qPCR signal for all the tested microglial markers, *TMEM119*, *TREM2* and *CD33*, and only notice minimal levels of astroglial genes *GFAP* and *ALDHL1*. We did not choose the widely accepted microglial marker IBA1 (AIF1) as this gene is indeed expressed at a low level in OPCs, according to independently published RNA-Seq datasets.[1-3] We hope this additional set of data will further corroborate the purity of our co-culture system. The text changes are at Line 378-380 and 428-431.

2. As AD is a neurodegenerative disease, the authors should discuss the relevance between the length of co-culture and the nature of this disease.

This point indeed constitutes a major limitation for our methodology and we agree with the reviewer that this should be discussed in the manuscript. We have expanded the DICUSSION to include a new paragraph about the limitations of our methods, and addressed this point there (Line 467).

3. In figure 1, "day 6" image is missing.

Our apologies for this error. Due to an unforeseen issue of software compatibility, the image did not appear on all platforms. The error has now been fixed.

Reviewer #2:

Manuscript Summary:

The manuscript by Assetta et al presents a straight-forward and efficient protocol for deriving neurons and oligodendrocyte lineage cells from human pluripotent stem cells for single culture or co-culture. The authors have significantly reduced the time to generate these cells from possibly months in traditional protocols to just a few weeks thus greatly increasing the tractability of this in vitro culture system. However, a satisfactory characterization of the generated cells is lacking, which brings to question its effectiveness. This and other concerns are outlined in more detail below.

We thank the reviewer for his/her concise comments.

Major Concerns:
1) The authors describe the generation of human induced neurons from pluripotent stem cells, but it is not clear what types of neurons are generated. Are they excitatory? Inhibitory? What other characteristics do they exhibit?

We thank the reviewer for this comment which is indeed worthwhile to be addressed in the manuscript. In brief, the induced neuron protocol described here is an improvement of the widely employed method based on the single transcription factor Neurogenin 2 (Ngn2), which has been documented to generate functional excitatory cortical neurons, highly resembling the layer 2/3 counterparts *in vivo*.[4] Our specific method is further enhanced by the unique feature of being glia-free, and has been used in our prior work in which we demonstrated the resultant human neurons exhibit a typical pyramidal morphology and electrophysiological characteristics, quantitatively reproducible and independent of the cell line of origin.[5,6] We have revised the INTRODUCTION (the third paragraph, Line 118) accordingly to provide such information .

2) In figure 1C, the image shown for the "DAY 4" is washed out and the image for "DAY 6" is missing.

Our apologies for this error, which resulted from a software compatibility issue for the image display. This now is fixed and corrected.

3) In figure 2B, only a small subset of the total cells appear to be Olig2 positive. How pure are these OPC cultures?

The reviewer again raised a good point, which is the general limitation for identifying the specific cell type or development stage by using a single marker. Olig2 is a traditional marker for all oligodendrocyte lineage cell types, and its expression level fluctuates along the oligodendroglial differentiation and is not necessarily high at the early OPC stage.[1,2] In fact, as revealed in Fig. 2E, *OLIG2* expression increases with maturation and is the lowest in OPCs. Therefore, researchers in the field of oligodendrocyte biology, particularly those who develop and use stem cell-derived oligodendroglia, prefer the classical surface marker O4 which correlates closely with the functionality.[7-10] Consistent with these notions, we can detect Olig2 immunoreactivity in roughly 25% of our OPCs (Fig. 2B, right bottom panel) and ~95% of the population positive for O4 (Fig. 2B, right top panel). We are thus confident in the efficiency of our protocol and have added the aforementioned quantification as the exemplary information in the figure legends (Line 399).

4) In figure 2D, the authors nicely show expression of OPC and OL markers in the induced human cultures. Can the authors show a corresponding absence of NPC markers such as PAX6 and nestin?

We performed the exact experiment as the reviewer suggested and are able to show that the expression of NPC marker PAX6 diminishes dramatically in our induced OPCs and

further decreases to nearly background in differentiated oligodendrocytes. We agree with the reviewer that this piece of data is informative and have incorporated it as our new Fig. 2D with a detailed description in the corresponding figure legend (Line 401).

5) The authors state that myelin wrapping is observed in the induced co-cultures of iNs and iOLs, however the MBP immunostaining shown in figure 2B and figure 3B is not convincing. The gold standard for showing myelination is using TEM. Without TEM images of myelin the most the authors can state with the images they present is that MBP+ OL processes ensheath axons.

We appreciate that the reviewer indicates this inaccurate part in our original manuscript. We have modified our text and figure legends accordingly to avoid any confusion and overstatement (Line 131, 316 and 374).

6) In figure 3C, the authors show a clear increase in synaptic marker staining along neuron cell bodies and dendrites upon co-culture with induced oligodendrocyte lineage cells. Is the increase due to interactions with iOPCs or iOLs? It is not clear.

We are grateful for reviewer's comment on this important issue which was not explained adequately in our previous submission. As demonstrated in the literature[7,9,10] and shown in our time course examination Fig. 2E, the stem cell-derived OPCs, once stimulated by myelinogenic molecules like triiodothyronine (T3), can further differentiate and transition through the intermediate developmental stages toward becoming mature myelinating oligodendrocytes – by an accelerated course. Such stimulated cultures then become heterogenous in population and comprise self-renewing OPCs, pre-oligodendrocyte, pre-myelinating oligodendrocytes and myelinating oligodendrocytes.[11] These subpopulations in continuum would account for different portions at different time points. In this manuscript, we start the co-cultures by mixing the induced neurons and OPCs together and then allow OPCs to mature in the described co-culture medium. The imaging was performed at D21 of co-culturing, at the time when pre-myelinating oligodendrocytes are expected to dominate the oligodendroglial population (Fig. 2E). We have thus revised our description already to clarify this (Line 350-357).

How do early-stage oligodendrocyte lineage cells interact with neurons and influence the synapse formation independent of myelination? This question is currently under active investigation at the Huang lab, by using this particular co-culture system. Our preliminary data suggest OPCs are able to secrete neurotrophins to enhance the neuronal excitability, potentially through promoting synapse formation. We believe the protocol described here will be encouraging and helpful for future studies tackling the neuron-oligodendroglia synaptic interactions.

Minor Concerns:
7) It's not clear what "up to six weeks" (line 117) is referring to in the last paragraph of the introduction

By writing "up to six weeks" we meant that we are able to maintain the co-culture with a proper morphology and gene expression profile for at least six weeks. We have further

clarified by removing this phrase from line 117 and explaining in the REPRESENTATIVE RESULTS section (Line 377).

8) In the protocol the authors state that lentivirus preparation takes ~6 days, but they only describe ~4 days of work.

Our apologies for the confusion. This difference mainly reflects the flexibility in the actual production procedure. We typically have a 5-day procedure: plating (1 day), transfecting (1 day), media change (1 day) and collection (1, but up to 3 consecutive days), ultracentrifugation and suspension (1 day). We have made the changes to make our description consistent (Line 142).

Reviewer #3:
Manuscript Summary:
In Alzheimer's disease (AD) the role of oligodendrocytes is yet to be determined, with a number of evidence to suggest a strong contribution of this cell type to the ongoing neurodegenerative processes in this disease. Authors here describe protocols to generate functional neurons and oligodendrocytes from human pluripotent stem cells, including a co-culture system to model the neuron-oligodendrocyte connections. More specifically, they describe methods for generation induced oligodendrocyte precursor cells (iOPCs) and induced oligodendrocytes (iOLCs) in a relatively short period of time.

We are grateful for the reviewer's concise and positive evaluation.

Major Concerns:
1. In line 172-172 authors say that detached neurons can be frozen without significant cells death. There is always some cell death after thawing, in CS10 can be even up to 20% for neurons, therefore it is beneficial for the reader to specify the expected % of cells death. Please indicate that number based on your experience, especially as the number of neurons to be seeded in the co-culture system is advised to be different if seeding fresh or thawed neurons (protocol 3.2.1, line 252).

We thank the reviewer for drawing attention to this technical point and agree that such information should be included. We do normally notice about 15-20% cell death when we thaw neurons frozen in CS10, and consider this level of loss reasonable for storage and future retrieval. We have added this specific piece of information into the PROTOCOL section and discussed this for the utility (Line 195).

2. Image in Figure 1C day 6 is missing, whereas the day 4 image has poor contrast settings. Please improve these images for better representation for your data.

Our apologies for this error, which resulted from a software compatibility issue for the image display. This now is fixed and corrected.

3. What is specifically meant by "initial seeding density" in line 196?

We have now clarified this by adding extra words to reference this to the same seeding density mentioned in line 187, on day -1 of the protocol.

4. Catalog number is missing for the NPC freezing media (line 205).

This has now been added in the Table of Materials.

5. Add references to all figures in the Protocol section.

We have incorporated the figure references into our PROTOCOL section.

6. As authors are comparing different differentiation conditions to maturation markers, Abeta levels, or synapsin puncta (Figure 3 and 4), it would be most appropriate to present statistical analysis on this data.

Our apologies that the information regarding the statistical significance did not go through in our previous manuscript. We have that presented appropriately in the figures and legends.

7. The data looks promising, but what is missing is the quantification of the percentage on NPCs, OPCs and OLCs obtained compared to the total cellular make up of the differentiated culture. Authors describe the use of IHC differentiation markers, but limited data in presented to give a feel of the efficiency of these differentiation protocols.

We thank the reviewer for again raising a great point. We first validated that the NPCs we generated based on the established approach of SMAD inhibition[12] are homogenous and well-characterized, with more than 95% of cells positive for markers including Nestin, Pax6 and Sox1 but negative for the pluripotential marker Oct4. We have performed additional experiment (Fig. 2D) to show that our protocol efficiently differentiates NPCs into OPCs, with the expression of NPC marker Pax6 considerably reduced in OPCs (>90%) and further diminished in oligodendrocytes (>99%). Hence, we are confident that NPC account for only a minimal fraction of the total cellular make-up in our co-cultures. Moreover, our IHC staining for the standard OPC marker O4 indicated a satisfactory level of purity, with more than 95% of our resultant OPCs being positive (Fig. 2B).

However, the maturation triggered by the myelinogenic compounds (including T3 triiodothyronine and Clemastine) in our described maturation medium diversifies the cell population. Consistent with previous reports on generating functional oligodendrocytes,[7,9,10] we showed in our time course study (Fig. 2E) that our OPCs, upon myelinogenic stimulation for maturation (by T3 triiodothyronine and/or Clemastine), differentiate and transition through intermediate stages (like pro-oligodendrocyte and pre-myelinating oligodendrocytes) toward myelinating oligodendrocytes.[11] Such oligodendroglial subpopulations in continuum constitutes varying percentages of the total cells along the maturation process, with more mature cells dominating at a later

time point and vice versa. We have revised our REPRESENTATIVE RESULTS section to mention this caveat (Line 350-357). Overall, in our opinion, the purity and efficiency of our protocol in generating human OPCs are evident.

8. How long are the authors capable to keep the neuronal oligodendrocyte co-culture healthy in culture? Are there any changes observed on the neuronal activity in this co-culture system? The possibility of a successful long term neuronal co-culture and increased support for neuronal activity through the co-culture system is a very interesting aspect while studding neuronal degeneration, and therefore these concepts should be discussed here as attributes of these protocols.

We completely agree with the reviewer on this critical aspect of the utility of our model system. In one of our attempts we managed to grow the co-cultures for five weeks with a reasonably healthy morphology and appropriate gene expression pattern. We have not yet systemically examined the neuronal activity in our long-term co-cultures, but plan to do so in our follow-up research, in which we intend to study the oligodendroglial influences on synaptic functions, potentially independent of myelination. We have revised our REPRESENTATIVE RESULTS and DISCUSSION as suggested to elaborate this interesting concept for future application (Line 377 and 467-480).

Minor Concerns:
1. In steps 1.2.2 and 2.1.2 please add the % confluence of ES cells at this stage prior to accutase treatment.

We have provided this detail as suggested.

2. In protocol 1.2 and 2.1 please give the concentration of Matrigel and GFR Matrigel used for all iPSC, neurons and OPCs.

We again thank the reviewer for his/her careful assessment. Such information has been added in.

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