BRIEF REPORT

REVISED Apolipoprotein E expression pattern in human induced pluripotent stem cells during *in vitro* neural induction [version 2; peer review: 2 approved, 1 approved with reservations]

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Abstract
Apolipoprotein E (APOE) is a multifunctional protein that plays significant roles in important cellular mechanisms in peripheral tissues and is as well expressed in the central nervous system, notably by adult neural stem cells (NSCs) in the hippocampus. Evidence from animal studies suggest that APOE is critical for adult NSC maintenance. However, whether APOE has the potential to play a similar role in human NSCs has not been directly investigated. To address this question, we conducted a focused study characterising *APOE* gene and protein expression in an *in vitro* model of neural differentiation utilising human induced pluripotent stem cells. We found that *APOE* gene expression was dramatically decreased as the cells became more differentiated, indicating that *APOE* expression levels reflect the degree of cellular differentiation during neural induction. Furthermore, qualitative analysis results of immunocytochemistry showed that intracellular localisation of APOE protein becomes more pronounced as neural differentiation progresses. Taken together, our findings suggest a potential role for APOE in human NSC maintenance and justify further investigations being carried out to understand whether changes in APOE levels can directly impact the neurogenic capacity of human stem cells.

Keywords
Induced pluripotent stem cells, Neural stem cells, Directed differentiation, Apolipoprotein E

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Abbreviations

AD (Alzheimer's disease); APOE (Apolipoprotein E); iPSCs (induced pluripotent stem cells); NSCs (neural stem cells)

Introduction

Apolipoprotein E (APOE) is a pleiotropic protein that plays an important role in lipid metabolism (Mahley & Rall, 2000) and is highly expressed in the brain mainly by glial cells (Boyles *et al.*, 1985; Elshourbagy *et al.*, 1985). Although the primary function of APOE is lipid transport, its expression is also found in other cell types outside the context of lipid metabolism in the brain (Liao *et al.*, 2017). For example, a recent single-cell RNA sequencing study on human post-mortem Alzheimer's disease (AD) brains showed that activated microglia (relevant to the disease state) express high levels of APOE unlike naïve microglia (relevant to healthy/homeostatic state) in the prefrontal cortex, indicating that APOE expression is associated with immune function (Mathys *et al.*, 2019). Furthermore, neuronal APOE can also be expressed at high levels under stress conditions such as brain injury although APOE expression is normally low in healthy neurons (Mahley & Huang, 2012; Xu *et al.*, 2006). Interestingly, APOE is highly expressed in nestin/glial fibrillary acidic protein (GFAP) double-positive neural stem cells (NSCs) in the adult hippocampus of mice, and one of the phenotypes characterised in APOE-null mice is the premature depletion of NSC pool in the hippocampus, suggesting that NSC maintenance requires APOE expression (Yang *et al.*, 2011).

Although the existing literature suggest that APOE plays an important role in stem cell maintenance, one should note that the majority of these findings were generated from rodent models. Since NSCs obtained from different species have been shown to behave in fundamentally different ways (Mertens *et al.*, 2013; Otani *et al.*, 2016; Ray & Gage, 2006), characterisation of *APOE*

expression in 'human' NSCs should be done prior to investigating its exact function. However, such evidence has not been reported to this date. To reduce this knowledge gap, we conducted a short study examining the expression pattern of APOE gene and protein in human induced pluripotent stem cells (iPSCs) undergoing neural induction *in vitro*. We found that gene expression is the highest in cells at the earliest stage of neural induction, whereas protein expression becomes more localised intracellularly, indicating that APOE expression pattern changes according to the differentiation state of cells.

Methods

A list of materials used in this study is presented in Table 1.

Cell line

CTR_M3_36S human induced pluripotent stem cell (iPSC) line was reprogrammed from keratinocytes obtained from a neurotypical male. Keratinocytes were reprogrammed by introducing a set of Sendai virus encoding human OCT4, SOX2, KLF4, and C-MYC (Yamanaka factors) using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher) according to the manufacturer's instructions. The virus was a gift from Dr. Mahito Nakanishi (AIST, Japan).

Stem cell maintenance

Cells were regularly tested for mycoplasma and certified mycoplasma-free. iPSCs were maintained in Essential 8™ medium (Thermo Fisher) without antibiotics at 37°C, 5% CO_2, 5% O_2 in 6-well NUNC™ plates (Thermo Fisher) coated with Geltrex™ (Thermo Fisher). Passaging of iPSCs lines were done with Versene (EDTA) solution (Lonza) according to the manufacturer's instructions. Passaging ratio for iPSC maintenance was kept between 1:6 and 1:18.

Directed differentiation

iPSC colonies approaching 80% confluence were passaged at 3:2 ratio on 6-well NUNC™ plates coated with Geltrex™ on D-2/-1 and maintained at 37°C, 5% CO_2, 5% O_2 for 24–48 hrs until they approached 100% confluence. Directed differentiation began on D0 by changing Essential 8™ medium to neural induction medium and incubating the cells at 37°C, 5% CO_2, 20% O_2. Neural induction lasted for 7 days. To prepare neural induction medium, N2:B27 was first prepared by mixing the N2 medium (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (Sigma Aldrich) supplemented with 1X GlutaMAX™ (Thermo Fisher) and 1X N-2 supplement (Thermo Fisher)) and the B27 medium (Neurobasal® medium (Thermo Fisher) supplemented with 1X GlutaMAX™ and 1X B-27 supplement (Thermo Fisher) or 1X B-27 without vitamin A supplement (Thermo Fisher)) at 1:1 ratio. The following small molecule inhibitors were added to N2:B27 to make the neural induction medium: 100 nM LDN193189 (Sigma Aldrich) and 10 µM SB431542 (Sigma Aldrich) for dual SMAD inhibition (DSi); 100 nM LDN193189, 10 µM SB431542, and 2 µM XAV939 (Sigma Aldrich) for dual SMAD inhibition plus Wnt/β-catenin inhibition (DS-Wi); and 100 nM LDN193189, 10 µM SB431542, 2 µM XAV939, and 1 µM Cyclopamine (LC Laboratories) for dual SMAD inhibition plus Wnt/β-catenin plus sonic hedgehog inhibition (DS-WHi).

Table 1. List of materials used in this study.

Name			Company	Catalogue Number
CytoTune-iPS 2.0 Sendai Reprogramming Kit			Thermo Fisher	A16517
Essential 8™ medium			Thermo Fisher	A1517001
NUNC™ plates			Thermo Fisher	140675
Geltrex™			Thermo Fisher	A1413302
Versene (EDTA) solution			Lonza	BE17-711E
Hank's Balanced Salt Solution (HBSS)			Thermo Fisher	14170-161
Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham			Sigma Aldrich	D6421
GlutaMAX™			Thermo Fisher	35050-061
N-2 (100X)			Thermo Fisher	17502-048
Neurobasal® medium			Thermo Fisher	21103-049
B-27 minus vitamin A (50X)			Thermo Fisher	12587-010
LDN193189			Sigma Aldrich	SML0559
SB431542			Sigma Aldrich	S4317
XAV939			Sigma Aldrich	X3004
Cyclopamine			LC Laboratories	C-8700
Y-27632			Sigma Aldrich	Y0503
DNeasy Blood & Tissue Kit			QIAGEN	69504
Taq DNA Polymerase			QIAGEN	201203
HhaI digestion enzyme			Thermo Fisher	ER1851
TRIzol® reagent			Thermo Fisher	15596026
SuperScript® III First-Strand Synthesis System			Thermo Fisher	18080051
HOT FIREPol® EvaGreen® qPCR Mix			Solis Biodyne	08-24-00001
Name	Concentration	Host/Clonality	Company	Catalogue Number
Hoechst 33342	1:2000	-	Thermo Fisher	H3570
anti-human SOX2	1:1000	Rabbit monoclonal	Millipore	AB5603
anti-human TBR2	1:250	Rabbit polyclonal	Abcam	AB23345
anti-human ApoE	1:200	Goat polyclonal	Millipore	AB947
anti-Rabbit Alexa Fluor 488	1:500	Donkey polyclonal	Thermo Fisher	A21206
anti-Goat Alexa Fluor 594	1:500	Donkey polyclonal	Thermo Fisher	A11058

Neural induction medium was used from D0 to D7, and N2:B27 was used from D8 onwards. Medium was changed every 24 hrs throughout the entire directed differentiation period.

Neural passaging 1, 2, and 3 were performed with Accutase (Thermo Fisher) on D7, D12, and D15/16, respectively. Briefly, cells were washed with room temperature HBSS and treated with Accutase at 37°C, 5% CO_2, 5% O_2 for 3–4 minutes. Cold Accutase was used for neural passagings 1 and 2, and room temperature Accutase was used for neural passaging 3. Cells in Accutase were then collected with a P1000 pipette. Extra care was taken during neural passagings 1 and 2 where P1000 pipetting was done no more than 5 times when cells in Accutase were collected. Collected cells were then mixed with room temperature DMEM/F12 (twice the volume of Accutase used) so that Accutase could be deactivated, and centrifugation was performed twice to wash off the Accutase from cells. Centrifugation was done at 900 revolutions per minute (RPM) for 2 min during neural passaging 1 and 2, and at 1250 RPM for 2 min during neural passaging 3. After centrifugation, cells were plated on new 6-well NUNC™ plates coated with Geltrex™. Passaging ratios were 1:1 for neural passaging 1 and 2, and 2:3 for neural passaging 3. To ensure cell survival 10 μM Y-27632 (Sigma Aldrich), a Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor, was mixed with the plating medium at each neural passaging and then removed after 24 hrs.

Genotyping
Genomic DNA was extracted from iPSCs using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. The *APOE* locus containing the rs429358 and rs7412 SNPs was amplified with Taq DNA Polymerase (QIAGEN) according to the manufacturer's instructions. Briefly, the reaction mix containing 1X PCR Buffer, 1X Q-Solution, 10 mM dNTP mix (0.2 mM final concentration), primers (forward and reverse each at 0.4 μM final concentration), 1.25 units Taq polymerase, and 1 μg of genomic DNA was incubated at 95°C for 4 mins to activate the Taq polymerase. Then, 35 cycles of 'denaturation at 94°C for 30 secs, annealing at 68°C for 30 secs, elongation at 72°C for 1 min' was performed. Then, final extension was done at 72°C for 10 mins. This polymerase chain reaction (PCR) was done with S1000 Thermal Cycler (Bio-Rad). The primers used for *APOE* genotyping (Table 2) were previously designed by

Henderson and colleagues (Henderson *et al.*, 2002), and they are able to generate PCR products that can be visualised easily by gel electrophoresis after HhaI enzyme (Thermo Fisher) digestion. Amplified PCR products were digested with 1 unit of HhaI digestion enzyme and gel electrophoresis was performed using a 3% agarose gel containing 0.5 μg/mL ethidium bromide. Raw dataset is available as an **underlying data** via Figshare (doi: 10.6084/m9.figshare.12199721.v1).

Gene expression analysis
Total RNA was extracted from D7, D12, D15/16, and D18/19 cells that were not used for neural passaging with TRIzol® reagent (Thermo Fisher) according to manufacturer's instructions and eluted in 25–30 μL of diethyl pyrocarbonate (DEPC)-treated water. Reverse transcription of total RNA into complementary DNA was performed using SuperScript® III First-Strand Synthesis System (Thermo Fisher) according to the manufacturer's instructions. Briefly, the random hexamers were annealed to total RNA at 25°C for 10 mins, then the synthesis was performed at 50°C for 50 mins, and then the reaction was terminated at 85°C for 5 mins. The final product was diluted to 5 ng/μL of total RNA converted to cDNA using DEPC-treated water. S1000 Thermal Cycler (Bio-Rad) was used for reverse transcription.

For gene expression analysis, real-time quantitative polymerase chain reaction (qPCR) was performed using the HOT FIREPol® EvaGreen® qPCR Mix (Solis Biodyne) according to the manufacturer's instructions. Briefly, the reaction mix containing the HOT FIREPol® EvaGreen® qPCR Mix, primers (forward and reverse each at 0.2 μM final concentration), and cDNA was incubated at 95°C for 15 mins to activate the HOT FIREPol® DNA polymerase, then 45 cycles of 'denaturation at 95°C for 30 secs, annealing at 60°C for 30 secs, elongation at 72°C for 30 secs' was performed. Melting curve analysis was done on each gene based on the melting profile generated every 1°C increment between 60°C and 95°C. MJ Research PTC-200 Thermal Cycler (Bio-Rad) was used for qPCR. The sequence of primers are presented in Table 2. C_T values of *APOE* were normalised to that of *GAPDH*, and relative expression of *APOE* across samples were quantified using the $2^{-\Delta\Delta Ct}$ method where D7 sample was used as a reference for each differentiation lineage. Raw dataset is available as an **underlying data** via Figshare (doi: 10.6084/m9.figshare.12136944).

Table 2. Sequence of primers used in this study.

Genotyping	Forward	Reverse
APOE	GAC GCG GGC ACG GCT GTC CAA GGA GCT GCA GGC GAC GCA GGC CCG GCT GGA CGC GGA CAT GGA GGA	AGG CCA CGC TCG ACG CCC TCG CGG GCC CCG GCC TGG TAC ACT

Gene expression	Forward	Reverse
APOE	GTT GCT GGT CAC ATT CCT GG	GCA GGT AAT CCC AAA AGC GAC
GAPDH	AGC CTC AAG ATC ATC AGC AA	CTG TGG TCA TGA GTC CTT CC

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 mins at room temperature, permeabilized with 0.1% Triton™ X-100 in 1X Tris-buffered saline (TBS) for 15–30 minutes, and then blocked with 5% normal donkey serum in TBS for 30 minutes. Primary antibodies were incubated at 4°C overnight followed by 3 washings with TBS. Secondary antibodies conjugated with fluorescent dyes were incubated at room temperature for 1 hours followed by 2 washings with TBS. Nuclei were stained with 5 μg/mL Hoechst® 33342 solution (Thermo Fisher) for 30 seconds immediately prior to imaging, and cells were washed with TBS 2 times after nuclear staining. All primary antibodies were diluted in 5% normal donkey serum in TBS, secondary antibodies in 1% normal donkey serum in TBS, and Hoechst® 33342 solution in TBS. Imaging was done with IX 70 inverted epifluorescence microscope (Olympus) connected to AxioVision imaging software (version 4.4). Scale bars were inserted on the images using ImageJ software (version 1.49c). CellProfiler (version 3.1.9) was used to quantify the percentage of cells immunopositive for SOX2, TBR2, and APOE at the intracellular regions. Raw dataset for the images is available as an **underlying data** via Figshare (doi: 10.6084/m9.figshare.12199745.v1). Raw dataset for the quantification is available as an **underlying data** via Figshare (doi: 10.6084/m9.figshare.12781604.v1).

Statistical analysis

GraphPad Prism (version 8.4.2.679) was used for statistical analysis. The statistical significance of the mean differences between groups were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple testing. The mean, standard error of measurement (SEM), and number of biological replicates are reported. P-value < 0.05 was considered significant to reject the null hypothesis that the differences observed between groups is due to random variation.

Results

To characterise the expression of APOE in human stem cells undergoing neural induction, an iPSC line derived from a neurotypical male with *APOE3* homozygous genotype (CTR_M3_36S cell line) (Figure 1; (Lee, 2020b)) (Deans *et al.*, 2017) were differentiated into neural lineages. Genotyping of CTR_M3_36S was performed using the method developed by Henderson and colleagues (Henderson *et al.*, 2002), and CTR_M3_36S was confirmed to be homozygous for *APOE3* by comparing the data with that of an *APOE3* homozygous cell line (CTR_M1_04) that reported by Henderson and colleagues (Henderson *et al.*, 2002, Figure 1). Neural induction into dorsal forebrain progenitors was performed using modified dual SMAD inhibition protocols (Cocks *et al.*, 2014; Deans *et al.*, 2017; Kathuria *et al.*, 2018; Yu *et al.*, 2014), where combinations of small molecule inhibitors were used to inhibit bone morphogenetic protein (BMP), transforming growth factor (TGF)-β, Wnt/β-catenin, and sonic hedgehog signalling pathways from D0 to D7 of neural induction (Figure 2A).

Gene expression analysis revealed that *APOE* expression was the highest at D7, and the drastic down-regulation of *APOE*

from D7 > to D18/19 was observed regardless of the combination of small molecule inhibitors used from D0 to D7 (p < 0.0001) (Figure 2B, **underlying data** (Lee, 2020a)). Immunocytochemistry showed that D12 and D18/19 cells expressed SRY-box transcription factor 2 (SOX2), a NSC marker, and T-Box Brain Protein 2 (TBR2), a neural progenitor cell (NPC) marker, respectively, for all combinations of small molecule inhibitors used from D0 to D7. Qualitative analysis of immunocytochemistry results revealed that ApoE became more localised to the intracellular region at D18/19 compared to D12 (Figure 3; (Lee, 2020c; Lee, 2020d)).

Discussion

Unlike the existing animal models of *APOE* deficiency and humanised *APOE* expression where genetic modifications were introduced globally (whole body) rather than specifically to NSCs, the *in vitro* model used in this study allowed us to examine *APOE* expression pattern exclusively in stem cells that were pushed towards the neural lineage. Our findings demonstrate that in cells at the earliest stage of neurodevelopment, 1) human *APOE* gene expression is high, and 2) APOE protein is not clearly localised at the intracellular region. Various combinations of small molecule inhibitors did not alter these patterns of expression.

Although further investigations will be needed to understand the exact role of APOE in neurodevelopment, the existing

M1 = CTR_M1_04 (*APOE* E3/E3 iPSC line)
M3 = CTR_M3_36S (iPSC line used in this study)
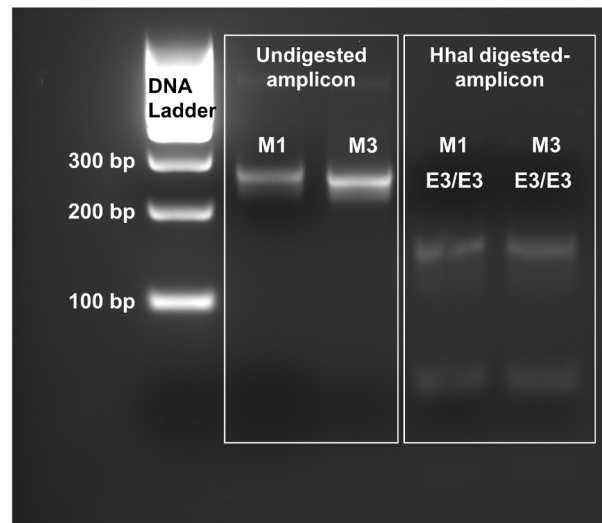
Figure 1. *APOE* genotyping of the cell line used in this study. CTR_M3_36S human iPSC line derived from a neurotypical male is homozygous for *APOE3* (denoted as M3 in this figure). CTR_M1_04 human iPSC line that was known to be homozygous for *APOE3* was used as control (denoted as M1 in this figure). HhaI-digested PCR amplicons were run on a 3% agarose gel, and the band loci were compared with the data previously reported by Henderson and colleagues who developed this genotyping method (Henderson *et al.*, 2002). The band loci for both CTR_M3_36S and CTR_M1_04 lines match with the homozygous *APOE3* data reported by Henderson and colleagues (see Figure 1 of Henderson *et al.*, 2002).

A

Neural Induction   **NPC Expansion**

D7   D12   D15/16   D18/19

→ Stem cell maintenance medium
→ Neural Induction medium
→ N2:B27 medium

Small molecule inhibitors in Neural Induction medium

	DSi	DS-Wi	DS-WHi
100 nM LDN193189	+	+	+
10 µM SB431542	+	+	+
2 µM XAV939		+	+
1 µM Cyclopamine			+

N2:B27 medium (1:1 mixture of N2 and B27 medium)
Media composition

		DSi & DS-Wi	DS-WHi
N2 medium	DMEM/F12	+	+
	1X N-2	+	+
	1X Glutamax	+	+
B27 medium	Neurobasal	+	+
	1X B-27	+	
	1X B-27 without vitamin A		+
	1X Glutamax	+	+

B

DSi
(LDN193189 + SB4313542)

DS-Wi
(LDN193189 + SB431542 + XAV939)

DS-WHi
(LDN193189 + SB431542 + XAV939 + Cyclopamine)

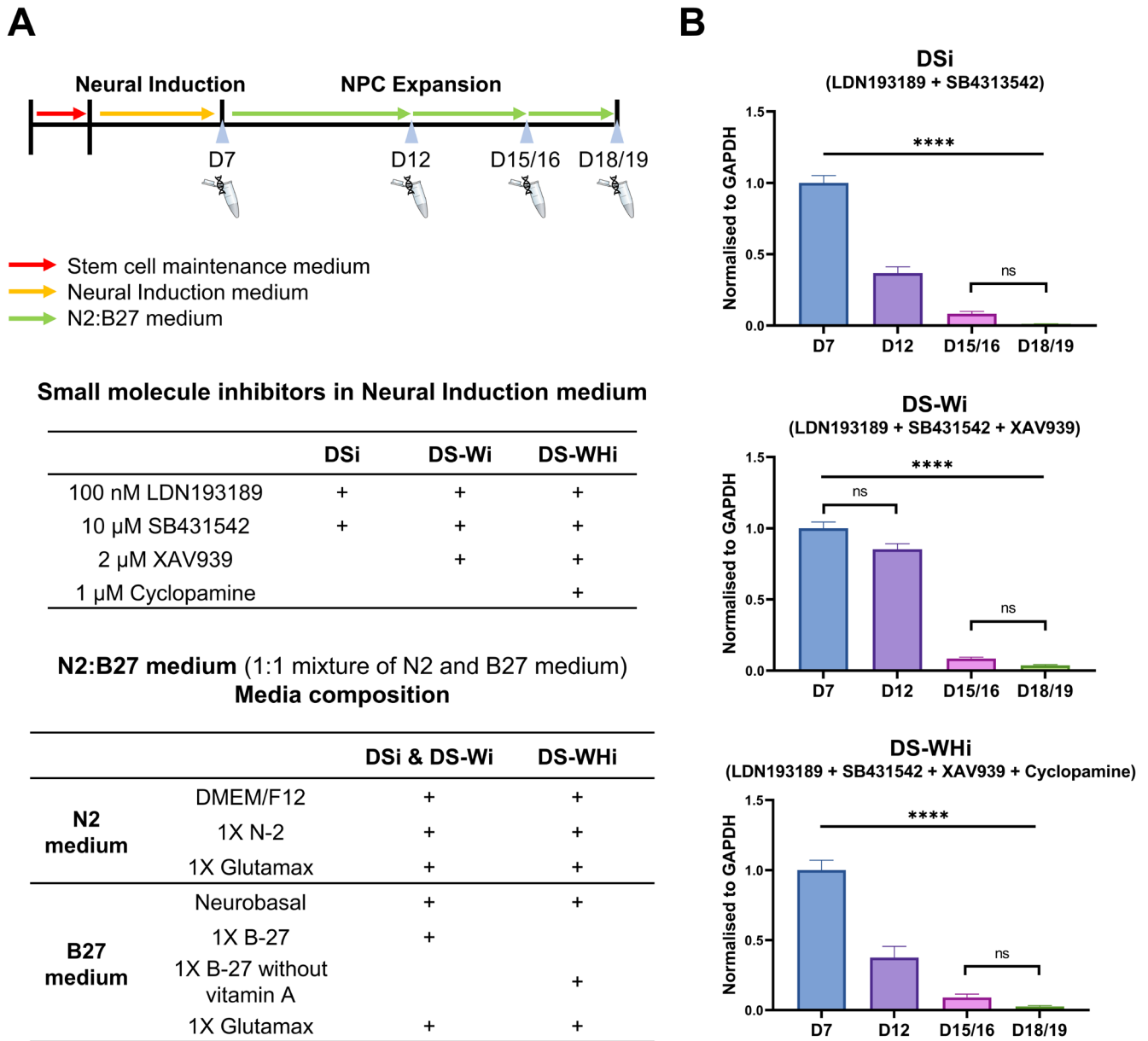**Figure 2.** *APOE* **gene expression changes according to the differentiation state of cells during** *in vitro* **directed differentiation.**
A) Schematic diagram of directed differentiation. CTR_M3_36S iPSCs were maintained in stem cell maintenance medium after replating (D-2/-1). On D0 neural induction began by changing the stem cell maintenance medium to neural induction medium. N2:B27 was used from D8 onwards. Medium was changed every 24 hrs throughout the entire differentiation period. Neural passaging 1, 2, and 3 were carried out on D7, D12, and D15/16, respectively. Total RNA extraction was made on cells that were not used for neural passaging on D7, D12, D15/16, and D18/19. Neural induction medium composition for each differentiation lineage and N2:B27 medium composition are also shown. **B**) *APOE* gene expression is reduced along neural induction regardless of lineage. Real-time qPCR was performed on CTR_M3_36S iPSCs undergoing directed differentiation at D7, D12, D15/16, and D18/19. *APOE* expression was normalised to that of *GAPDH*. D7 samples were used as reference samples for each lineage. One-way ANOVA with Bonferroni correction. n = 3. Mean (bars) with S.E.M. (error bars) shown. **** ANOVA p-value < 0.0001. ns: non-significant after Bonferroni correction. DSi: dual SMAD inhibition. DS-Wi: DSi plus Wnt/β-catenin inhibition. DS-WHi: DS-Wi plus sonic hedgehog inhibition.
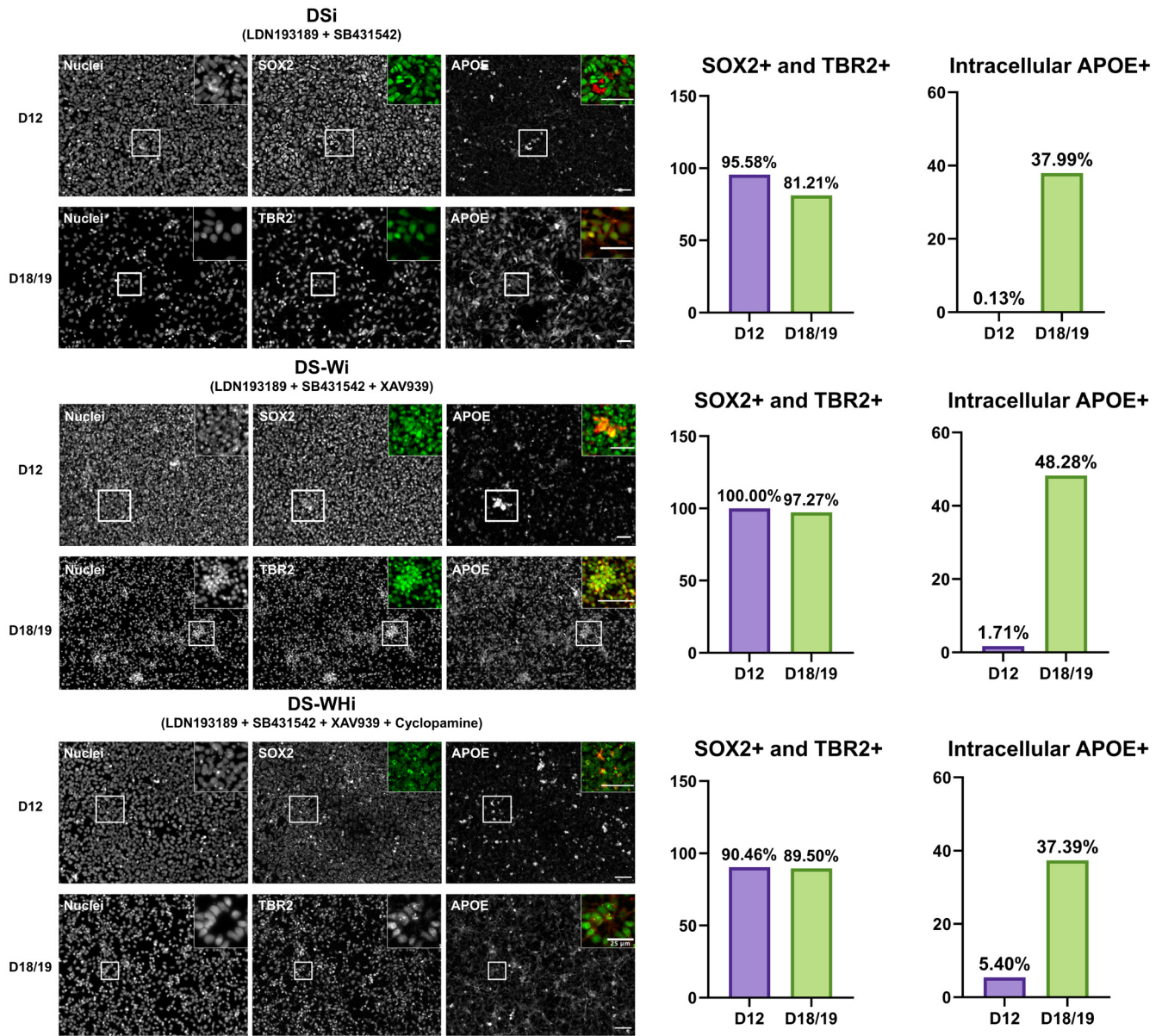
Figure 3. APOE localisation pattern becomes more intracellular with *in vitro* directed differentiation. APOE protein is more localised intracellularly in differentiated cells. (Left) Representative images of cells at D12 and D18/19 expressing SOX2 (NSC marker) and TBR2 (NPC marker), respectively. Insets show images of SOX2/TBR2 in green and APOE in red. Scale bars indicate 50 µm unless stated otherwise. (Right) Quantification of SOX2-/TBR2-positive cells and intracellular APOE-positive cells amongst SOX2-TBR2-positive cells. DSi: dual SMAD inhibition. DS-Wi: DSi plus Wnt/β-catenin inhibition. DS-WHi: DS-Wi plus sonic hedgehog inhibition.

literature seems to suggest that APOE can be both downstream and upstream of stem cell maintenance. For example, several chromatin precipitation studies have shown that POU class 5 homeobox 1 (POU5F1), SOX2, Kruppel like factor 4 (KLF4), MYC proto-oncogene (MYC) and Nanog Homeobox (NANOG) all bind to the promoter region of *APOE*, suggesting that *APOE* expression could be directly regulated by such stem cell maintenance factors (ENCODE Project Consortium, 2004; ENCODE Project Consortium, 2011; Kim *et al.*, 2008; Lachmann *et al.*, 2010; Liu *et al.*, 2008; Marson *et al.*, 2008). However,

other evidence suggests that APOE itself could be a direct regulator of cell fate determination. Meyer and colleagues (Meyer *et al.*, 2019) showed that changing the *APOE* genotype from ε4 (AD risk factor) to ε3 (neutral genotype) in human NPCs can suppress premature neuronal differentiation and maturation via increasing the transcription repressor activity of RE1 silencing transcription factor (REST). Interestingly, *APOE* mRNA levels were lower in ε4 NPCs compared to ε3 NPCs, suggesting higher *APOE* gene expression is indeed likely to be associated with the undifferentiated state of NPCs. As a follow-up to our findings

and the existing literature, we propose that further investigations should be carried out to elucidate the role of APOE in stem cell maintenance. For example, one could examine whether prolonged expression and/or overexpression of *APOE* gene in human NPCs can suppress further differentiation in these cells.

In this study, qualitative analysis was performed on APOE immunocytochemistry results. As the cells became more differentiated from NSCs to NPCs, APOE localisation pattern became more intracellular. To validate this observation, however, additional experiments with a more direct quantitative approach should be conducted. For example, APOE protein levels in various subcellular compartments could be measured and compared by performing Western Blot. Since APOE has been shown to exist in both secreted and intracellular forms (Huang & Mahley, 2014), it will be interesting to see which form of APOE is produced at each differentiation stage. It is possible that more APOE is secreted in undifferentiated cells compared to differentiated cells, which may not be fully captured using immunocytochemistry techniques performed on fixed cells. Interestingly, Gan and colleagues previously reported that APOE is indeed secreted by NSCs as well as NPCs, and secreted APOE was found to play a vital role in regulating NSC survival and neurosphere formation (Gan *et al.*, 2011). Therefore, further investigations on secreted and intracellular APOE using quantitative approaches will be able to clarify whether cells indeed produce different forms and levels of APOE depending on its differentiation state. This will in turn provide more definitive clues to whether APOE plays a stage-dependent role in neurodevelopment.

One limitation of this study is that the time-dependent changes of differentiation markers such as SOX2 and TBR2 were not examined alongside APOE. It is worth noting, however, that TBR2 was shown to be capable of suppressing SOX2 expression during differentiation of NSCs to NPCs (Hodge *et al.*, 2012). Given this information, it is unlikely that TBR2-positive cells observed in this study at D18/19 will simultaneously express high levels of SOX2. However, time-dependent changes of various markers of differentiation would add further validity to our observations and unequivocally clarify whether APOE expression is indeed correlated with the differentiation state of the cells. Another limitation of this study is that the exact locus of APOE expression could not be examined in detail using a standard epifluorescence microscope in this study.High-resolution microscopy techniques (such as confocal microscopy) would have been more ideal to identify the accurate loci of APOE expression and overcome the challenges of imaging densely packed cells at the earliest stages of neural induction (D0–D7). Further investigations with improved imaging capacity will therefore allow us to characterise APOE during the earlier stages of neural induction and hint at potential mechanisms underlying its role in neurodevelopment.

Since NSCs derived from iPSCs *in vitro* may not fully resemble the developmental and postnatal NSCs found *in vivo*, APOE expression should be further investigated in animal models of brain development as well. The most direct evidence of

in vivo APOE expression in NSCs to this date comes from a study by Yang and colleagues, where Nestin-positive NSCs in the mouse developing dentate gyrus was isolated using fluorescence-activated cell sorting, and APOE expression was examined from as early as postnatal day 7 (P7) (Yang *et al.*, 2011). NSCs at P7 had low expression of APOE which increased with the age of mice, and the deletion of APOE had detrimental effects on the maintenance of stem cells in the dentate gyrus. Although these findings clearly demonstrate the importance of APOE in brain development, the study had limitations in that prenatal NSCs were not examined, and functional studies of APOE were based on global rather than conditional knockouts. Furthermore, Yang and colleagues' data cannot be directly compared with our dataset due to species difference and the lack of detailed characterisation of NSCs in this study. To address this knowledge gap, more data from both *in vitro* and *in vivo* samples derived from various species should be generated and compared against each other. We hope that our focused study has laid a strong foundation to such collaborative investigations that may be conducted in the future.

In conclusion, we report that human *APOE* gene expression levels are highly correlated with the undifferentiated state of cells during directed differentiation *in vitro*, and ApoE protein is localised more in the intracellular region in cells at later stages of differentiation. Combining our observations and previous evidence reported in the literature, we speculate that APOE has an important role in stem cell maintenance and propose that further investigations should be carried out to validate our findings including methods that were not employed in this study. Moreover, it would be interesting to examine the exact underlying mechanisms such as 1) whether APOE is an upstream or downstream factor of stem cell maintenance, and 2) whether *APOE4* genotype and APOE loss-of-function would produce similar phenotypes.

Data availability

Underlying data

Figshare: raw data for qPCR. https://doi.org/10.6084/m9.figshare.12136944.v1 (Lee, 2020a)

This project contains the following underlying data:

- Lee *et al.* raw data for qPCR.csv (C(t) values, efficiency of amplification, and values calculated for normalised gene expression analysis for APOE.)

Figshare: raw data for Genotyping. https://doi.org/10.6084/m9.figshare.12199721.v1 (Lee, 2020b)

This project contains the following underlying data:

- Lee *et al.* raw data for Genotyping.TIF (Gel image used in Figure 1. Genomic DNA from human iPSCs amplified for the APOE locus, then digested with HhaI enzyme. Run on 3% agarose gel.)

Figshare: raw data for D12 and D18/19 immunocytochemistry. https://doi.org/10.6084/m9.figshare.12199745.v1 (Lee, 2020c)

This project contains the following underlying data:

- 01 Lee *et al.* raw data for DS D12 Hoechst.tif

- 02 Lee *et al.* raw data for DS D12 SOX2.tif

- 03 Lee *et al.* raw data for DS D12 APOE.tif

- 04 Lee *et al.* raw data for DS D18-19 Hoechst.tif

- 05 Lee *et al.* raw data for DS D18-19 TBR2.tif

- 06 Lee *et al.* raw data for DS D18-19 APOE.tif

- 07 Lee *et al.* raw data for DS+Wi D12 Hoechst.tif

- 08 Lee *et al.* raw data for DS+Wi D12 SOX2.tif

- 09 Lee *et al.* raw data for DS+Wi D12 APOE.tif

- 10 Lee *et al.* raw data for DS+Wi D18-19 Hoechst.tif

- 11 Lee *et al.* raw data for DS+Wi D18-19 TBR2.tif

- 12 Lee *et al.* raw data for DS+Wi D18-19 APOE.tif

- 13 Lee *et al.* raw data for DS+WHi D12 Hoechst.tif

- 14 Lee *et al.* raw data for DS+WHi D12 SOX2.tif

- 15 Lee *et al.* raw data for DS+WHi D12 APOE.tif

- 16 Lee *et al.* raw data for DS+WHi D18-19 Hoechst.tif

- 17 Lee *et al.* raw data for DS+WHi D18-19 TBR2.tif

- 18 Lee *et al.* raw data for DS+WHi D18-19 APOE.tif

(Images were taken with IX70 inverted epifluorescence microscope (Olympus) connected to AxioVision imaging software 4.4. ImageJ 1.49c was used to generate TIFF files.)

Figshare: raw data for quantification of D12 and D18/19 immunocytochemistry. https://doi.org/10.6084/m9.figshare.12781064.v1 (Lee, 2020d)

This project contains the following underlying data:

- pipeline_01_DSi.cppipe

- pipeline_02_DS-Wi.cppipe

- pipeline_03_DS-WHi.cppipe

- quantification.xlsx

(cppipe files: CellProfiler v.3.1.9 was used to generate the pipelines for each image set (one differentiation protocol, 3 channels, 2 timepoints. quantification.xlsx: The raw data generated after running the pipelines were copied to a spreadsheet. 'Cells / Nuclei (%)' column shows the percentage of cells positive for SOX2 at D12 and TBR2 at D18/19. 'Intracellular APOE / Cells (%)' column shows the percentage of intracellular APOE-positive cells amongst SOX2- and TBR2-positive cells. These 2 columns are presented as bar graphs on Figure 3 of the manuscript.)

Acknowledgements

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Open Peer Review

Current Peer Review Status: ✓ ✓ ?

Version 2

Reviewer Report 07 September 2020

https://doi.org/10.5256/f1000research.28981.r70145

✓ **Noelia Urbán** (ID)

Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna Biocenter Campus (VBC), Vienna, Austria

The authors have either addressed my concerns or clearly discussed the limitations of the study. I have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Adult neurogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 10 July 2020

https://doi.org/10.5256/f1000research.26021.r65479

? **Jade Marsh** (ID)

University of Oxford, Oxford, UK

Summary:
- Previous studies in mice have shown that APOE is highly expressed by neural stem cells

(NSCs) and that it has an isoform-dependent regulatory role in their maintenance. Whether this function extends to human NSCs is not known. Here, the authors begin to address this knowledge gap by characterising the expression of APOE in APOE3/APOE3 human induced pluripotent stem cell (iPSC)-derived NSCs undergoing differentiation. Through qPCR and immunocytochemistry (ICC) techniques, the authors show a reduction in APOE gene expression during the differentiation of NSCs to NPCs and observe a change in the cellular distribution of APOE.

General comments:

○ This short study is concise and provides preliminary but important data to contribute to our understanding of the role of APOE in NSCs of human origin. Although the qPCR data is robust and clearly demonstrates a significant decrease in APOE gene expression as NSCs differentiate, the qualitative assessment of the ICC data is not as convincing.

Specific comments:

○ Fig 2 C: APOE appears to be more widely expressed at D18/19 for all three NSC lineages (DSi, DS-Wi and DS-WHi). Quantification of the signal intensities of APOE and the differentiation markers should be carried out to validate the conclusion that APOE expression decreases as NSCs differentiate.

○ Fig 2 C: It may be more helpful and striking to compare D18/19 images with D7 images instead of D12 since APOE mRNA levels are highest at this time point. Notably, D7 is also used as the baseline for the qPCR data.

○ The authors describe an increase in intracellular localisation of APOE following NSC differentiation – providing higher magnification images may reveal changes in APOE distribution more clearly.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular and cellular neuroscience

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Aug 2020
Sandrine Thuret, King's College London, London, UK

Comment 1. Fig 2 C: APOE appears to be more widely expressed at D18/19 for all three NSC lineages (DSi, DS-Wi and DS-WHi). Quantification of the signal intensities of APOE and the differentiation markers should be carried out to validate the conclusion that APOE expression decreases as NSCs differentiate.

The authors would like to thank the reviewer for the comment on the quantification of ICC images. We now include a quantification of the images in the updated manuscript. The percentage of SOX2+ cells and TBR2+ cells at D12 and D18/19, as well as the percentage of SOX2+ and TBR2+ cells that showed immunostaining patterns for intracellular APOE were quantified using CellProfiler v3.1.9. **The quantified data presented as bar graphs can be found in Figure 3; the analysis procedure can be found in the 'Methods (Immunocytochemistry)' section; and the analysis pipeline as well as the raw data for quantification can be found on Figshare which can be accessed by the following DOI:** https://doi.org/10.6084/m9.figshare.12781064.v1**.** The results indicate a higher percentage of intracellular APOE/TBR2+ cells at D18/19 compared to intracellular APOE/SOX2+ cells at D12, supporting our conclusions made in the manuscript prior to the inclusion of these quantification data.

Comment 2. Fig 2 C: It may be more helpful and striking to compare D18/19 images with D7 images instead of D12 since APOE mRNA levels are highest at this time point. Notably, D7 is also used as the baseline for the qPCR data.

The authors would like to thank the reviewer for mentioning this important aspect of the ICC experiment reported in our manuscript. **While the authors confirm that the ICC experiments were conducted for APOE on D7 cells, the data were not included in the manuscript due to the following reasons.** According to the differentiation protocol, the cells were maintained at high density approaching near 100% confluence from D0 to D7. We observed that this inadvertently diminishes the quality of immunocytochemistry images for D7 cells, since clear boundaries of nuclei could not be easily identified with epifluorescence microscopy and further complicated the downstream quantification process. The possibility of dissociating D7 cells and plating them on to a different surface for better image quality and quantification was considered briefly. However, such additional handling was not done to the cells so that any potential source of artefacts that could mask the true state of D7 cells can be ruled out in our experiments. While the use of epifluorescence microscopy in our study can be seen as a clear limitation, APOE immunostaining patterns of D7 cells was not qualitatively different from that of D12 cells in our observations. Further investigations using three-dimensional imaging techniques such as confocal microscopy will enable better imaging and quantification of densely packed cells on D7. **The 'Discussion' section in the**

updated manuscript now includes a new paragraph regarding this aspect.
"One limitation of this study is that the time-dependent changes of differentiation markers such as SOX2 and TBR2 were not examined alongside APOE. It is worth noting, however, that TBR2 was shown to be capable of suppressing SOX2 expression during differentiation of NSCs to NPCs (Hodge et al., 2012). Given this information, it is unlikely that TBR2-positive cells observed in this study at D18/19 will simultaneously express high levels of SOX2. However, time-dependent changes of various markers of differentiation would add further validity to our observations and unequivocally clarify whether APOE expression is indeed correlated with the differentiation state of the cells. **Another limitation of this study is that the exact locus of APOE expression could not be examined in detail using a standard epifluorescence microscope in this study. High-resolution microscopy techniques (such as confocal microscopy) would have been more ideal to identify the accurate loci of APOE expression and overcome the challenges of imaging densely packed cells at the earliest stages of neural induction (D0-D7). Further investigations with improved imaging capacity will therefore allow us to characterise APOE during the earlier stages of neural induction and hint at potential mechanisms underlying its role in neurodevelopment.**"

Comment 3. The authors describe an increase in intracellular localisation of APOE following NSC differentiation – providing higher magnification images may reveal changes in APOE distribution more clearly.

The authors would like to thank the reviewer for this comment. We agree that the resolution of the images in Figure 2 is not as high as the original data when the manuscript is viewed/downloaded online. **In the updated manuscript, these images are shown in a separate figure (Figure 3).** The PDF version should enable sufficient magnification to view the composite panels and clearly demonstrate more intracellular localisation on D18/19 cells. **In addition, as indicated in the 'Data availability' section of the submitted manuscript, the original data with higher resolutions for all images can be found on Figshare, a public repository for scientific data, which can be accessed via the following DOI:** https://doi.org/10.6084/m9.figshare.12199745.v1. We are confident that the patterns of APOE immunocytochemistry (ICC) reported in the manuscript can be confirmed with greater detail from the raw data available on Figshare.

Competing Interests: No competing interests to disclose.

Reviewer Report 06 July 2020

https://doi.org/10.5256/f1000research.26021.r65480

? **Noelia Urbán** iD

Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna Biocenter Campus (VBC), Vienna, Austria

Summary:

APOE is highly expressed by neural stem cells (NSCs) in the hippocampus of adult mice, particularly in the quiescent state, and has been associated with stem cell maintenance. However, no data is available on the expression pattern of APOE in human neural stem cells. The authors monitor APOE expression by QPCR and immunocytochemistry during the neuronal differentiation of iPSCs using three different types of neural induction media. They report a dramatic reduction in APOE mRNA levels during differentiation, as well as a change in the cellular distribution of APOE protein.

Comments:

Although preliminary, the changes in APOE expression are an interesting and important observation. However, while the QPCR data is convincing and very robust, the immunocytochemistry studies should be further analysed/improved in order to draw any strong conclusions. The images presented are not of very good quality, and if judging by them, APOE expression rather seems to increase globally during differentiation, with few cells expressing high levels at D12 and most cells expressing moderate levels at D18/19. My main specific recommendations are:
   ○ If possible, provide higher magnification/higher quality images of APOE stainings, including also the other time points during differentiation. Day 7 would be particularly important to include, since it displays the highest levels of expression by QPCR.

   ○ APOE stainings should be quantified to support the conclusions that its expression decreases during differentiation and there is a change in the protein localization. If this is not possible, the conclusions should be toned down and further experiments suggested in the discussion (for example, protein quantification by WB and cellular fractionation and quantification of protein in the media to assess intracellular protein localization and secretion, respectively).

   ○ In the discussion, it should be noted that iPSC-derived NSCs might not fully resemble adult NSCs. A brief discussion of what is known about the expression of APOE in NSCs during development would be very useful.

Other comments/suggestions:
   ○ Sox2 (neural stem and progenitor marker) and Tbr2 (neuronal progenitor marker) stainings are presented only at one stage each. It would be very informative to see if/how these two markers change over the course of the differentiation protocol. If not possible by immuno, a QPCR for these genes would also be enough to show the trends of expression during differentiation.

   ○ Please specify in the methods how long the cells are fixed in PFA.

   ○ Either in the introduction or the discussion, it could be noted that astrocytes express very high levels of APOE in the brain. This is important because it links APOE expression to the

astroglial nature of adult NSCs.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Adult neurogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Aug 2020
Sandrine Thuret, King's College London, London, UK

<>

Comment 1. If possible, provide higher magnification/higher quality images of APOE stainings, including also the other time points during differentiation. Day 7 would be particularly important to include, since it displays the highest levels of expression by QPCR.

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patterns of APOE immunocytochemistry (ICC) reported in the manuscript can be confirmed with greater detail from the raw data available on Figshare.

While the authors confirm that the ICC experiments were conducted for APOE on D7 cells, the data were not included in the manuscript due to the following reasons. According to the differentiation protocol, the cells were maintained at high density approaching near 100% confluence from D0 to D7. We observed that this inadvertently diminishes the quality of immunocytochemistry images for D7 cells, since clear boundaries of nuclei could not be easily identified with epifluorescence microscopy and further complicated the downstream quantification process. The possibility of dissociating D7 cells and plating them on to a different surface for better image quality and quantification was considered briefly. However, such additional handling was not done to the cells so that any potential source of artefacts that could mask the true state of D7 cells can be ruled out in our experiments. While the use of epifluorescence microscopy in our study can be seen as a clear limitation, APOE immunostaining patterns of D7 cells was not qualitatively different from that of D12 cells in our observations. Further investigations using three-dimensional imaging techniques such as confocal microscopy will enable better imaging and quantification of densely packed cells on D7. **The 'Discussion' section in the updated manuscript now includes a new paragraph regarding this aspect.**
"One limitation of this study is that the time-dependent changes of differentiation markers such as SOX2 and TBR2 were not examined alongside APOE. It is worth noting, however, that TBR2 was shown to be capable of suppressing SOX2 expression during differentiation of NSCs to NPCs (Hodge et al., 2012). Given this information, it is unlikely that TBR2-positive cells observed in this study at D18/19 will simultaneously express high levels of SOX2. However, time-dependent changes of various markers of differentiation would add further validity to our observations and unequivocally clarify whether APOE expression is indeed correlated with the differentiation state of the cells. **Another limitation of this study is that the exact locus of APOE expression could not be examined in detail using a standard epifluorescence microscope in this study. High-resolution microscopy techniques (such as confocal microscopy) would have been more ideal to identify the accurate loci of APOE expression and overcome the challenges of imaging densely packed cells at the earliest stages of neural induction (D0-D7). Further investigations with improved imaging capacity will therefore allow us to characterise APOE during the earlier stages of neural induction and hint at potential mechanisms underlying its role in neurodevelopment.**"

We do not have the data for other time points during differentiation yet, and we agree with the reviewer that it would be highly informative to examine more time points. It would be particularly interesting to examine the time-course of APOE expression changes from the stem cell stage to the mature neuronal/glial stage. We hope that the data reported in our manuscript can serve as a foundation to such experiments to be conducted in the future.

Comment 2. APOE stainings should be quantified to support the conclusions that its expression decreases during differentiation and there is a change in the protein localization. If this is not possible, the conclusions should be toned down and further experiments suggested in the discussion (for example, protein quantification by WB and cellular fractionation and

quantification of protein in the media to assess intracellular protein localization and secretion, respectively).

The authors would like to thank the reviewer for the comment on the quantification of ICC images. We now include a quantification of the images in the updated manuscript. The percentage of SOX2+ cells and TBR2+ cells at D12 and D18/19, as well as the percentage of SOX2+ and TBR2+ cells that showed immunostaining patterns for intracellular APOE were quantified using CellProfiler v3.1.9. **The quantified data presented as bar graphs can be found in Figure 3; the analysis procedure can be found in the 'Methods (Immunocytochemistry)' section; and the analysis pipeline as well as the raw data for quantification can be found on Figshare which can be accessed by the following DOI:** https://doi.org/10.6084/m9.figshare.12781064.v1**.** The results indicate a higher percentage of intracellular APOE/TBR2+ cells at D18/19 compared to intracellular APOE/SOX2+ cells at D12, supporting our conclusions made in the manuscript prior to the inclusion of these quantification data.

To incorporate the reviewer's suggestions, **we have clearly stated in the 'Discussion' section of our updated manuscript that further investigations using other techniques should be done to quantify the levels of APOE protein.** The following changes have been made in the 'Discussion'.
"In this study, qualitative analysis was performed on APOE immunocytochemistry results. As the cells became more differentiated from NSCs to NPCs, APOE localisation pattern became more intracellular. **To validate this observation, however, additional experiments with a more direct quantitative approach should be conducted. For example, APOE protein levels in various subcellular compartments could be measured and compared by performing Western Blot.** Since APOE has been shown to exist in both secreted and intracellular forms (Huang & Mahley, 2014), it will be interesting to see which form of APOE is produced at each differentiation stage. It is possible that more APOE is secreted in undifferentiated cells compared to differentiated cells, which may not be fully captured using immunocytochemistry techniques performed on fixed cells. Interestingly, Gan and colleagues previously reported that APOE is indeed secreted by NSCs as well as NPCs, and secreted APOE was found to play a vital role in regulating NSC survival and neurosphere formation (Gan et al., 2011). **Therefore, further investigations on secreted and intracellular APOE using quantitative approaches will be able to clarify whether cells indeed produce different forms and levels of APOE depending on its differentiation state.** This will in turn provide more definitive clues to whether APOE plays a stage-dependent role in neurodevelopment."

Furthermore, **the conclusions have been amended as follows to reflect the changes in the 'Discussion'.**
"In conclusion, we report that human APOE gene expression levels are highly correlated with the undifferentiated state of cells during directed differentiation in vitro, and **ApoE protein is localised more in the intracellular region in cells at later stages of differentiation**. Combining our observations and previous evidence reported in the literature, we speculate that APOE has an important role in stem cell maintenance **and propose that further investigations should be carried out to validate our findings including methods that were not employed in this study**. Moreover, it would be

interesting to examine the exact underlying mechanisms such as 1) whether APOE is an upstream or downstream factor of stem cell maintenance, and 2) whether APOE4 genotype and APOE loss-of-function would produce similar phenotypes."

Comment 3. In the discussion, it should be noted that iPSC-derived NSCs might not fully resemble adult NSCs. A brief discussion of what is known about the expression of APOE in NSCs during development would be very useful.

The authors would like to thank the reviewer for this comment on highlighting the differences between iPSC-derived NSCs and adult NSCs. To incorporate this aspect into our updated manuscript, **we have now added a new paragraph in the 'Discussion' section as follows**.

"Since NSCs derived from iPSCs in vitro may not fully resemble the developmental and postnatal NSCs found in vivo, APOE expression should be further investigated in animal models of brain development as well. The most direct evidence of in vivo APOE expression in NSCs to this date comes from a study by Yang and colleagues, where Nestin-positive NSCs in the mouse developing dentate gyrus was isolated using fluorescence-activated cell sorting, and APOE expression was examined from as early as postnatal day 7 (P7) (Yang et al., 2011). NSCs at P7 had low expression of APOE which increased with the age of mice, and the deletion of APOE had detrimental effects on the maintenance of stem cells in the dentate gyrus. Although these findings clearly demonstrate the importance of APOE in brain development, the study had limitations in that prenatal NSCs were not examined, and functional studies of APOE were based on global rather than conditional knockouts. Furthermore, Yang and colleagues' data cannot be directly compared with our dataset due to species difference and the lack of detailed characterisation of NSCs in this study. To address this knowledge gap, more data from both in vitro and in vivo samples derived from various species should be generated and compared against each other. We hope that our focused study has laid a strong foundation to such collaborative investigations that may be conducted in the future."

<>

Comment 1. Sox2 (neural stem and progenitor marker) and Tbr2 (neuronal progenitor marker) stainings are presented only at one stage each. It would be very informative to see if/how these two markers change over the course of the differentiation protocol. If not possible by immuno, a QPCR for these genes would also be enough to show the trends of expression during differentiation.

The authors would like to thank the reviewer for pointing out the expression changes of SOX2 and TBR2. While we have not examined the time-dependent changes of these markers in this study, the authors can confirm that SOX2 and TBR2 expression was consistently observed at D12 and D18/19 by other experienced members of the lab using the differentiation protocols reported in this study. These data (although not shown in this study) were generated by qPCR, ICC, and microarray experiments that collectively show the expression of SOX2 and TBR2 similar to the ICC data reported in our manuscript. While we are confident with the SOX2 and TBR2 expression pattern in our study, we agree with the reviewer that APOE expression should be examined alongside the differentiation markers.

To incorporate this into our updated manuscript, **we have now included a new paragraph in the 'Discussion' section** as follows to suggest that further investigations should be conducted to address this aspect.

"**One limitation of this study is that the time-dependent changes of differentiation markers such as SOX2 and TBR2 were not examined alongside APOE. It is worth noting, however, that TBR2 was shown to be capable of suppressing SOX2 expression during differentiation of NSCs to NPCs (Hodge et al., 2012). Given this information, it is unlikely that TBR2-positive cells observed in this study at D18/19 will simultaneously express high levels of SOX2. However, time-dependent changes of various markers of differentiation would add further validity to our observations and unequivocally clarify whether APOE expression is indeed correlated with the differentiation state of the cells.** Another limitation of this study is that the exact locus of APOE expression could not be examined in detail using a standard epifluorescence microscope in this study. High-resolution microscopy techniques (such as confocal microscopy) would have been more ideal to identify the accurate loci of APOE expression and overcome the challenges of imaging densely packed cells at the earliest stages of neural induction (D0-D7). Further investigations with improved imaging capacity will therefore allow us to characterise APOE during the earlier stages of neural induction and hint at potential mechanisms underlying its role in neurodevelopment."

Comment 2. Please specify in the methods how long the cells are fixed in PFA.

The authors can confirm that the cells were fixed for 10 mins at room temperature with 4% PFA, and this has been now clearly stated in the 'Methods' section. We thank the reviewer for pointing this out.

"**Cells were fixed with 4% paraformaldehyde for 10 mins at room temperature**, permeabilized with 0.1% Triton™ X-100 in 1X Tris-buffered saline (TBS) for 15–30 minutes, and then blocked with 5% normal donkey serum in TBS for 30 minutes. Primary antibodies were incubated at 4°C overnight followed by 3 washings with TBS. Secondary antibodies conjugated with fluorescent dyes were incubated at room temperature for 1 hours followed by 2 washings with TBS. Nuclei were stained with 5 µg/mL Hoechst® 33342 solution (Thermo Fisher) for 30 seconds immediately prior to imaging, and cells were washed with TBS 2 times after nuclear staining. All primary antibodies were diluted in 5% normal donkey serum in TBS, secondary antibodies in 1% normal donkey serum in TBS, and Hoechst® 33342 solution in TBS. Imaging was done with IX 70 inverted epifluorescence microscope (Olympus) connected to AxioVision imaging software (version 4.4). Scale bars were inserted on the images using ImageJ software (version 1.49c). CellProfiler (version 3.1.9) was used to quantify the percentage of cells immunopositive for SOX2, TBR2, and APOE at the intracellular regions. Raw dataset for the images is available as an underlying data via Figshare (doi: 10.6084/m9.figshare.12199745.v1). Raw dataset for the quantification is available as an underlying data via Figshare (doi: 10.6084/m9.figshare.12781604.v1)."

Comment 3. Either in the introduction or the discussion, it could be noted that astrocytes express very high levels of APOE in the brain. This is important because it links APOE expression to the astroglial nature of adult NSCs.

The authors would like to thank the reviewer's comment on the existing evidence of APOE

expression in astrocytes. To incorporate this aspect into our updated manuscript**, the 'Introduction' section has been amended as follows**.

"**Apolipoprotein E (APOE) is a pleiotropic protein that plays an important role in lipid metabolism (Mahley & Rall, 2000) and is highly expressed in the brain mainly by glial cells (Elshourbagy et al., 1985, Boyles et al., 1985).** Although the primary function of APOE is lipid transport, its expression is also found in other cell types outside the context of lipid metabolism in the brain (Liao et al., 2017). For example, a recent single-cell RNA sequencing study on human post-mortem Alzheimer's disease (AD) brains showed that activated microglia (relevant to the disease state) express high levels of APOE unlike naïve microglia (relevant to healthy/homeostatic state) in the prefrontal cortex, indicating that APOE expression is associated with immune function (Mathys et al., 2019). Furthermore, neuronal APOE can also be expressed at high levels under stress conditions such as brain injury although APOE expression is normally low in healthy neurons (Mahley & Huang, 2012; Xu et al., 2006). Interestingly, APOE is highly expressed in nestin/glial fibrillary acidic protein (GFAP) double-positive neural stem cells (NSCs) in the adult hippocampus of mice, and one of the phenotypes characterised in APOE-null mice is the premature depletion of NSC pool in the hippocampus, suggesting that NSC maintenance requires APOE expression (Yang et al., 2011)."

Competing Interests: No competing interests to disclose.

Reviewer Report 08 June 2020

✔️ **Robert J. Williams** 🆔

Department of Biology and Biochemistry, University of Bath, Bath, UK

Summary:
It has previously been shown that APOE regulates neural stem cell (NSC) maintenance in rodent models but until now this has not been demonstrated or validated in human cells. The authors address this important question through use of human induced pluripotent stem cells (APOE3 homozygous) and monitor APOE status throughout neural differentiation. APOE gene expression was notably much lower following induction of differentiation and coincident with this was an apparent change in the cellular distribution of APOE with the authors describing a much more pronounced intracellular localisation of APOE.

Major comments:
This is a very focused piece of work and although the findings are quite preliminary they are very interesting and worth reporting particularly because of the human cell context. A key question going forward will be whether the APOE4 genotype influences NSC maintenance or differentiation

as this could have clear implications for neurogenesis in Alzheimer's Disease. The gene expression data presented here is clear and the changes in APOE are quite dramatic but the immunostaining is rather descriptive and is not quite as convincing.

Specific comments:
1. The resolution of the images in the composite cell panels in Fig 2 is not great and it is difficult to clearly see or judge the intracellular expression described. Can these be improved at all?

2. For the gene expression changes, data is provided for D7, D12 and D18, yet for the cell staining only D12 and D18 are shown. Is D7 cell data not available to present here?

3. The most obvious change at the protein level is the apparent increase in cellular APOE staining between D12 and D18 (certainly for DSi and DS-Wi) and as Hoe33342 has been performed I would have thought some quantitative data could be provided here which would help support the conclusions made.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular and Cellular Neuroscience

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 19 Aug 2020
Sandrine Thuret, King's College London, London, UK

Comment 1. The resolution of the images in the composite cell panels in Fig 2 is not great and it is

difficult to clearly see or judge the intracellular expression described. Can these be improved at all?

The authors would like to thank the reviewer for this comment. We agree that the resolution of the images in Figure 2 is not as high as the original data when the manuscript is viewed/downloaded online. **In the updated manuscript, these images are shown in a separate figure (Figure 3).** The PDF version should enable sufficient magnification to view the composite panels and clearly demonstrate more intracellular localisation on D18/19 cells. **In addition, as indicated in the 'Data availability' section of the submitted manuscript, the original data with higher resolutions for all images can be found on Figshare, a public repository for scientific data, which can be accessed via the following DOI:** https://doi.org/10.6084/m9.figshare.12199745.v1**.** We are confident that the patterns of APOE immunocytochemistry (ICC) reported in the manuscript can be confirmed with greater detail from the raw data available on Figshare.

Comment 2. For the gene expression changes, data is provided for D7, D12 and D18, yet for the cell staining only D12 and D18 are shown. Is D7 cell data not available to present here?

The authors would like to thank the reviewer for mentioning this important aspect of the ICC experiment reported in our manuscript. **While the authors confirm that the ICC experiments were conducted for APOE on D7 cells, the data were not included in the manuscript due to the following reasons.** According to the differentiation protocol, the cells were maintained at high density approaching near 100% confluence from D0 to D7. We observed that this inadvertently diminishes the quality of immunocytochemistry images for D7 cells, since clear boundaries of nuclei could not be easily identified with epifluorescence microscopy and further complicated the downstream quantification process. The possibility of dissociating D7 cells and plating them on to a different surface for better image quality and quantification was considered briefly. However, such additional handling was not done to the cells so that any potential source of artefacts that could mask the true state of D7 cells can be ruled out in our experiments. While the use of epifluorescence microscopy in our study can be seen as a clear limitation, APOE immunostaining patterns of D7 cells was not qualitatively different from that of D12 cells in our observations. Further investigations using three-dimensional imaging techniques such as confocal microscopy will enable better imaging and quantification of densely packed cells on D7. **The 'Discussion' section in the updated manuscript now includes a new paragraph regarding this aspect.**
"One limitation of this study is that the time-dependent changes of differentiation markers such as SOX2 and TBR2 were not examined alongside APOE. It is worth noting, however, that TBR2 was shown to be capable of suppressing SOX2 expression during differentiation of NSCs to NPCs (Hodge et al., 2012). Given this information, it is unlikely that TBR2-positive cells observed in this study at D18/19 will simultaneously express high levels of SOX2. However, time-dependent changes of various markers of differentiation would add further validity to our observations and unequivocally clarify whether APOE expression is indeed correlated with the differentiation state of the cells. **Another limitation of this study is that the exact locus of APOE expression could not be examined in detail using a standard epifluorescence microscope in this study. High-resolution microscopy techniques (such as confocal microscopy) would have been more ideal to identify the accurate loci of APOE expression and overcome the challenges of imaging densely**

packed cells at the earliest stages of neural induction (D0-D7). Further investigations with improved imaging capacity will therefore allow us to characterise APOE during the earlier stages of neural induction and hint at potential mechanisms underlying its role in neurodevelopment."

Comment 3. The most obvious change at the protein level is the apparent increase in cellular APOE staining between D12 and D18 (certainly for DSi and DS-Wi) and as Hoe33342 has been performed I would have thought some quantitative data could be provided here which would help support the conclusions made.

The authors would like to thank the reviewer for the comment on the quantification of ICC images. We now include a quantification of the images in the updated manuscript. The percentage of SOX2+ cells and TBR2+ cells at D12 and D18/19, as well as the percentage of SOX2+ and TBR2+ cells that showed immunostaining patterns for intracellular APOE were quantified using CellProfiler v3.1.9. **The quantified data presented as bar graphs can be found in Figure 3; the analysis procedure can be found in the 'Methods (Immunocytochemistry)' section; and the analysis pipeline as well as the raw data for quantification can be found on Figshare which can be accessed by the following DOI:** https://doi.org/10.6084/m9.figshare.12781064.v1**.** The results indicate a higher percentage of intracellular APOE/TBR2+ cells at D18/19 compared to intracellular APOE/SOX2+ cells at D12, supporting our conclusions made in the manuscript prior to the inclusion of these quantification data.

Competing Interests: No competing interests to disclose.

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