Apolipoprotein E (APOE) expression pattern in human induced pluripotent stem cells during in vitro neural induction.

Hyunah Lee
King's College London
Graham D. Cocks
King's College London
Paulina Nowosiad
King's College London
Lucia M. Dutan Polit
King's College London
Jack Price
King's College London
Deepak P. Srivastava
King's College London
Sandrine Thuret (sandrine.1.thuret@kcl.ac.uk)
King's College London https://orcid.org/0000-0003-1260-8083

Short Report

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

Posted Date: February 15th, 2020

DOI: https://doi.org/10.21203/rs.2.23493/v1

Version of Record: A version of this preprint was published at F1000Research on August 24th, 2020. See the published version at https://doi.org/10.12688/f1000research.23580.2.

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 on APOE gene expression level using an in vitro model of neural differentiation and human induced pluripotent stem cells derived from a neurotypical individual. We found that APOE expression was dramatically decreased as the cells became more differentiated, indicating that APOE expression levels reflect the degree of cellular differentiation during neural induction suggesting a potential role for ApoE in human NSC maintenance. Our findings justify further investigations being carried out to understand whether changes in APOE level can directly impact the neurogenic capacity of human stem cells.

Introduction

Apolipoprotein E (ApoE) is a pleiotropic protein that plays an important role in lipid metabolism (1) and is highly expressed in the brain (2). Although the primary function brain ApoE is lipid transport, its expression is also found in other cell types outside the context of lipid metabolism (3). 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 (4). 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 (5,6). 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 (7).

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 (8–10), 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* in human induced pluripotent stem cells (iPSCs) undergoing neural induction *in vitro.* We found that *APOE* expression is the highest in cells at the earliest stage of neural induction, indicating that its levels were correlated with the undifferentiated state of cells.

Materials And Methods

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). 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. The primers used for *APOE* genotyping were previously designed by Henderson and colleagues (11), 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.

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. 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. 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.

Statistical analysis

GraphPad Prism v8 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,*

Table 1, Table 2) (12) were differentiated into neural lineages. Neural induction into dorsal forebrain progenitors was performed using modified dual SMAD inhibition protocols (12–15) *(Figure 2A, Table 1).* 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. *APOE* expression was measured on cells harvested at D7, D12, D15 (or 16), and D18 (or 19) by performing real-time semi-quantitative polymerase chain reaction on *APOE* mRNA. The relative expression of *APOE* was calculated by 1) normalising the cycle threshold (C_T) values of *APOE* to that of glyceraldehyde–3-phosphate dehydrogenase (*GAPDH)* in each sample, and then 2) calculating the relative expression level compared to that of D7 for each neural induction lineage *(Table 2).* For each lineage, three biological replicates (different passage numbers) were used to analyse the statistical significance of difference between the mean values of each group.

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).* Unlike the existing animal models of *APOE* deficiency and humanised *APOE* expression where genetic modificationswere 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 human *APOE* expression is the highest in cells that are at the earliest stage of neurodevelopment, and various lineages induced by different combination of small molecule inhibitors did not alter this pattern of expression.

Discussion

In summary, we found that *APOE* expression directly reflects the undifferentiated state of cells during *in vitro* directed differentiation which suggests that ApoE plays a vital role in maintaining the multi-/pluripotency of undifferentiated stem cells during neurodevelopment. However, our characterisations studies do not clearly demonstrate whether ApoE is directly in control of stem cell maintenance function or is merely one of many downstream targets regulated by other transcription factors that play a central role in stem cell maintenance.

The existing literature seems to suggest that both scenarios are plausible. Several chromatin precipitation studies have shown that POU class 5 homeobox 1 (POU5F1), SRY-box transcription factor 2 (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 them (16–21). However, other evidence suggests that *APOE* itself could be a direct regulator of cell fate determination. For example, Meyer and colleagues (22) showed that changing the *APOE* genotype from ε4 (AD risk factor) to ε3 in human neural progenitor cells (NPCs) can increase the transcription repressor activity of RE1 silencing transcription factor (REST) in these cells and ultimately rescue their abnormal phenotypes of accelerated neuronal differentiation and maturation. REST is a transcriptional repressor of pro-neural genes and is highly expressed in undifferentiated stem cells (23), and as shown by Meyer and colleagues, *APOE* genotype can directly regulate the activity of REST in human NPCs. Although the relationship

between *APOE* levels and REST activity *per se* was not examined by Meyer and colleagues, *APOE* mRNA expression levels were lower in ε4 NPCs suggesting that lower-than-normal levels of *APOE* expression is likely to be associated with reduced activity of REST nuclear translocation. Taken together, these findings indicate that *APOE* can be an upstream regulator of multipotency in human 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, examining whether prolonged expression and/or overexpression of *APOE* in human NPCs and neurons (where *APOE* expression is lower compared to stem cells) can revert the identity of these cells would be a good starting point.

Conclusion

In conclusion, we report that human *APOE* expression levels are highly correlated with the undifferentiated state of cells during directed differentiation in vitro, and this was in line with publicly available gene expression array datasets. 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 investigate the exact underlying mechanisms such as 1) whether *APOE* is an upstream or downstream factor of stem cell maintenance, and 2) whether *APOE4* genotype would produce similar phenotypes as loss-of-function.

Abbreviations

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

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: PhD studentship of HL is sponsored by the King's College London Graduate School PGR (postgraduate research) International Research Studentship. The lab of ST (Thuret Lab) is partly funded by MRC research grants MR/N030087/1 and MR/S00484X/1.

Authors' contributions: HL, JP, DS and ST contributed to the conception and design of the work. PN and JP provided the study material. HL, GP and LP acquired the data. HL analyzed and interpreted the data.

HL and ST drafted the manuscript. ST revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements: The authors would like to thank Rupert Faraway, Matthew J. Reid, and James Williams, past members of the iPSC technicians' team of Jack Price group, for reprogramming, performing quality-control, and providing guidance for maintaining the CTR_M3_36S iPSC line used in this study. We also thank Dr. Mahito Nakanishi (AIST, Japan) for providing the Yamanaka factors Sendai virus that was used for reprogramming.

References

1. Mahley RW, Rall SC. Apolipoprotein E: far more than a lipid transport protein. Annu Rev Genomics Hum Genet. 2000;1:507–37.
2. Elshourbagy NA, Liao WS, Mahley RW, Taylor JM. Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. Proc Natl Acad Sci USA. 1985 Jan;82(1):203–7.
3. Liao F, Yoon H, Kim J. Apolipoprotein E metabolism and functions in brain and its role in Alzheimer's disease. Curr Opin Lipidol. 2017;28(1):60–7.
4. Mathys H, Davila-Velderrain J, Peng Z, Gao F, Mohammadi S, Young JZ, et al. Single-cell transcriptomic analysis of Alzheimer's disease. Nature. 2019 Jun;570(7761):332–7.
5. Mahley RW, Huang Y. Apolipoprotein e sets the stage: response to injury triggers neuropathology. Neuron. 2012 Dec 6;76(5):871–85.
6. Xu Q, Bernardo A, Walker D, Kanegawa T, Mahley RW, Huang Y. Profile and regulation of apolipoprotein E (ApoE) expression in the CNS in mice with targeting of green fluorescent protein gene to the ApoE locus. J Neurosci. 2006 May 10;26(19):4985–94.
7. Yang C-P, Gilley JA, Zhang G, Kernie SG. ApoE is required for maintenance of the dentate gyrus neural progenitor pool. Development. 2011 Oct;138(20):4351–62.
8. Otani T, Marchetto MC, Gage FH, Simons BD, Livesey FJ. 2D and 3D Stem Cell Models of Primate Cortical Development Identify Species-Specific Differences in Progenitor Behavior Contributing to Brain Size. Cell Stem Cell. 2016 Apr 7;18(4):467–80.
9. Ray J, Gage FH. Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. Mol Cell Neurosci. 2006 Mar;31(3):560–73.
10. Mertens J, Stüber K, Wunderlich P, Ladewig J, Kesavan JC, Vandenberghe R, et al. APP processing in human pluripotent stem cell-derived neurons is resistant to NSAID-based γ-secretase modulation. Stem Cell Rep. 2013 Dec 5;1(6):491–8.
11. Henderson JN, Crook R, Crook J, Hardy J, Onstead L, Carson-Henderson L, et al. Apolipoprotein E4 and tau allele frequencies among Choctaw Indians. Neurosci Lett. 2002 May 10;324(1):77–9.
12. Deans PJM, Raval P, Sellers KJ, Gatford NJF, Halai S, Duarte RRR, et al. Psychosis risk candidate ZNF804A localizes to synapses and regulates neurite formation and dendritic spine structure. Biol

Psychiatry. 2017 Jul 1;82(1):49–61.
13. Cocks G, Curran S, Gami P, Uwanogho D, Jeffries AR, Kathuria A, et al. The utility of patient specific induced pluripotent stem cells for the modelling of Autistic Spectrum Disorders. Psychopharmacology. 2014 Mar;231(6):1079–88.
14. Kathuria A, Nowosiad P, Jagasia R, Aigner S, Taylor RD, Andreae LC, et al. Stem cell-derived neurons from autistic individuals with SHANK3 mutation show morphogenetic abnormalities during early development. Mol Psychiatry. 2018;23(3):735–46.
15. Yu DX, Di Giorgio FP, Yao J, Marchetto MC, Brennand K, Wright R, et al. Modeling hippocampal neurogenesis using human pluripotent stem cells. Stem Cell Rep. 2014 Mar 11;2(3):295–310.
16. Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. Cell. 2008 Mar 21;132(6):1049–61.
17. Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell. 2008 Aug 8;134(3):521–33.
18. Liu X, Huang J, Chen T, Wang Y, Xin S, Li J, et al. Yamanaka factors critically regulate the developmental signaling network in mouse embryonic stem cells. Cell Res. 2008 Dec;18(12):1177–89.
19. Lachmann A, Xu H, Krishnan J, Berger SI, Mazloom AR, Ma'ayan A. ChEA: transcription factor regulation inferred from integrating genome-wide ChIP-X experiments. Bioinformatics. 2010 Oct 1;26(19):2438–44.
20. ENCODE Project Consortium. A user's guide to the encyclopedia of DNA elements (ENCODE). PLoS Biol. 2011 Apr 19;9(4):e1001046.
21. ENCODE Project Consortium. The ENCODE (encyclopedia of DNA elements) project. Science. 2004 Oct 22;306(5696):636–40.
22. Meyer K, Feldman HM, Lu T, Drake D, Lim ET, Ling K-H, et al. REST and Neural Gene Network Dysregulation in iPSC Models of Alzheimer's Disease. Cell Rep. 2019 Jan 29;26(5):1112–1127.e9.
23. Sun Y-M, Cooper M, Finch S, Lin H-H, Chen Z-F, Williams BP, et al. Rest-mediated regulation of extracellular matrix is crucial for neural development. PLoS One. 2008 Nov 6;3(11):e3656.

Tables

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

Table 2 Genotyping primers for *APOE*

Gene	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

Table 3 Primers for *APOE* and *GAPDH*

Gene	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

Figures

M1 = CTR_M1_04 (*APOE* E3/E3 iPSC line)
M3 = CTR_M3_36S (iPSC line used in this study)

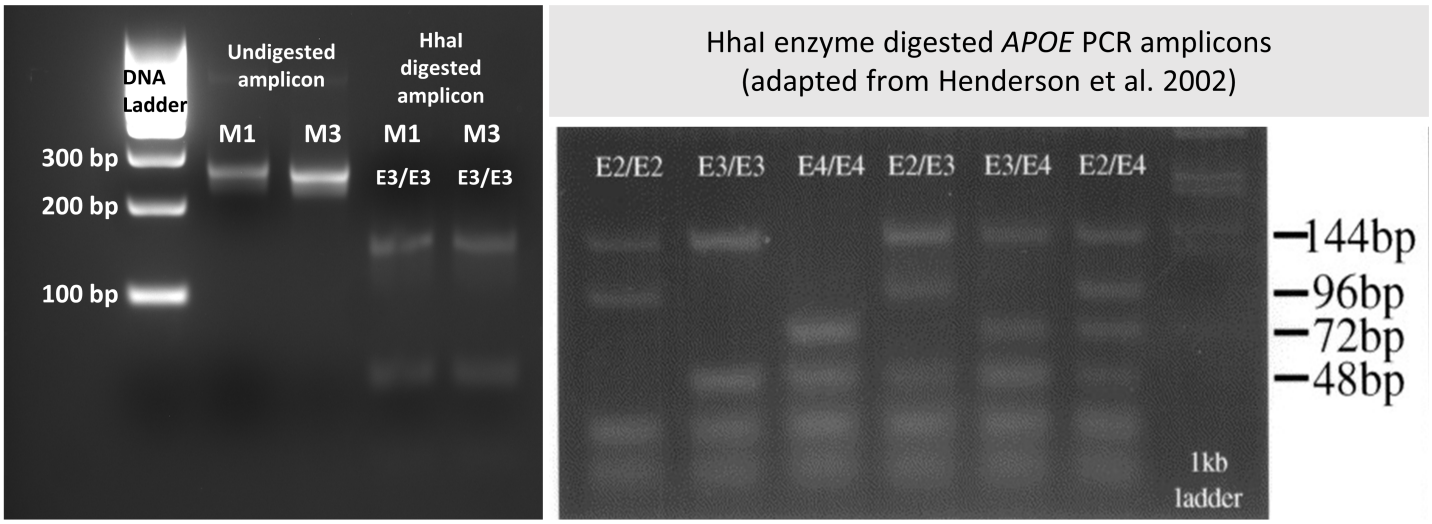
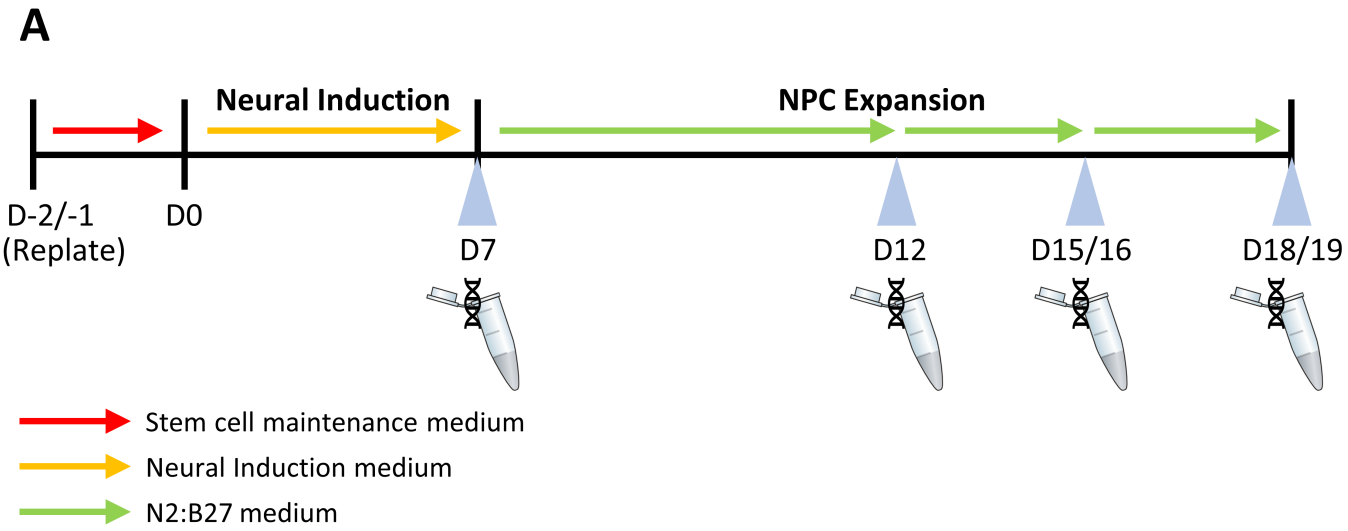


Figure 1

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 (left), and the band loci were compared with the data previously reported by Henderson and colleagues who developed this genotyping method (Henderson et al., 2002) (right). The band loci for both CTR_M3_36S and CTR_M1_04 lines match with the homozygous APOE3 data reported by Henderson and colleagues.



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 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	+	+

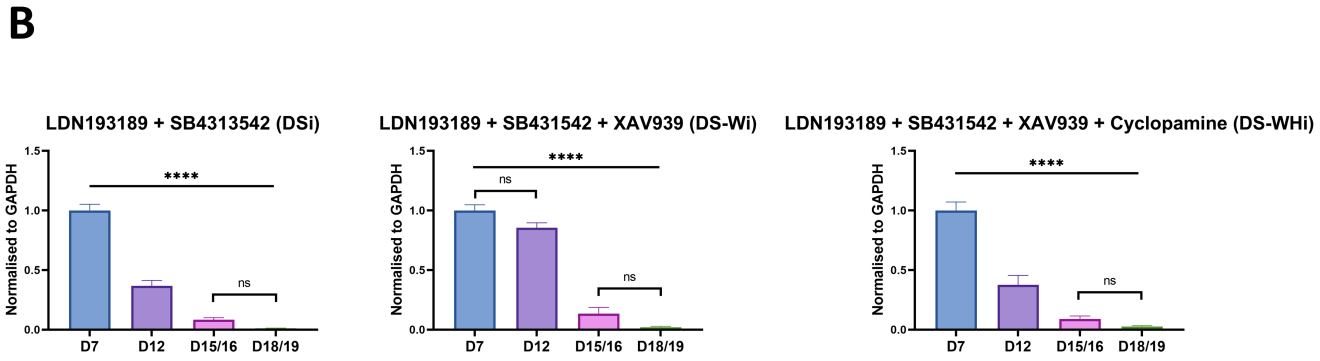


Figure 3

APOE gene expression reflects the undifferentiated 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.