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A 3D human neural cell culture system for modeling Alzheimer's disease

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Abstract

Stem cell technologies have facilitated the development of human cellular disease models that can be used to study pathogenesis and test therapeutic candidates. These models hold promise for complex neurological diseases such as Alzheimer's disease (AD) because existing animal models have been unable to fully recapitulate all aspects of pathology. We recently reported the characterization of a novel three-dimensional (3D) culture system that exhibits key events in AD pathogenesis, including extracellular aggregation of β -amyloid and accumulation of hyperphosphorylated tau. Here we provide instructions for the generation and analysis of 3D human neural cell cultures, including the production of genetically modified human neural progenitor cells (hNPCs) with familial AD mutations, the differentiation of the hNPCs in a 3D matrix, and the analysis of AD pathogenesis. The 3D culture generation takes 1–2 days. The aggregation of β -amyloid is observed after 6-weeks of differentiation followed by robust tau pathology after 10–14 weeks.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of age-related dementia and is characterized by progressive memory loss and cognitive impairment. Familial, early-onset (<60 years), autosomal-dominant forms of AD (FAD) can be caused by mutations in the

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DECLARATION OF INTEREST

The authors report no competing financial interest.

AUTHOR CONTRIBUTIONS

D.Y.K. and R.E.T. were equally responsible for experimental design and supervising the whole project. Y.H.K., S.H.C., C.D. and D.Y.K., mainly contributed to writing and revising the manuscript. C.D., E.B., K.J.W., M.H. and D.Y.K. performed the experiments. M.H., O.B., J.B.K. and C.S. contribute to writing the manuscript.

genes amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*)¹. Sporadic AD typically presents at a later onset and is due to multifactorial genetic and environmental risk factors. The two pathological hallmarks of AD are extracellular amyloid plaques, mainly composed of amyloid β ($A\beta$) peptides derived from APP via serial cleavage by β - and γ -secretase, and intracellular neurofibrillary tangles, composed of filamentous aggregation of hyperphosphorylated tau (p-tau) proteins¹. It is hypothesized that the accumulation and aggregation of $A\beta$ causes the formation of neurofibrillary tangles^{2–5}, but this has not been experimentally proven in any animal models. Only the models that overexpress frontotemporal dementia (FTD)-associated P301L *MAPT* (tau) mutant forms in addition to human *APP* and/or *PSEN1* with FAD mutations develop both plaques and tangles, albeit in a disconnected manner^{2–4}. Fundamental species-specific differences in genome and protein composition between humans and mice, such as the difference in number of tau isoforms, have precluded an accurate recapitulation of AD pathology.

Advances in the field of stem cell generation have further advanced the prospect of *in vitro* systems that model AD in human neurons, including the generation of induced pluripotent stem cells (iPSCs) from FAD patient fibroblasts^{5–10}. However, it has been challenging to replicate the aged AD brain environment in the presence of high levels of soluble and insoluble toxic $A\beta$ species and thereby to realize full AD pathology^{11–13}. Recently, we reported that genetically engineered human neural stem cells overexpressing FAD genes combined with a three-dimensional (3D) culture condition induced robust AD pathogenesis, including extracellular aggregation of $A\beta$ and accumulation of hyperphosphorylated/aggregated tau as neurofibrillary tangles¹⁴. In this article, we describe our protocol for the generation of these 3D human neural culture models of AD, detail their technical background, describe the techniques we applied for their analysis, and discuss their use and application.

Development of 3D human neural cell culture models of AD

We designed our AD model around two central technologies: human neural progenitor cells (hNPCs) that produce high concentrations of pathogenic $A\beta$ species and a Matrigel-based 3D culture system that provides an environment that favors $A\beta$ deposition.

First, we engineered a FAD cell line that could exhibit significant amyloid pathology, be easily maintained, and survive through multiple passages. We chose the immortalized hNPC cell line ReNcell VM (ReN) as a base for our platform because the cells can be maintained for more than 45 passages, are commercially available, and can differentiate into neurons and glial cells with simple growth-factor deprivation^{15–27}. The ReN cells were then transfected with IRES-mediated polycistronic lentiviral vectors containing FAD genes encoding human APP with both K670N/M671L (Swedish) and V717I (London) mutations (APPSL), PSEN1 with E9 mutation (PSEN1(E9)), and APPSL/PSEN1(E9) with GFP or mCherry as a reporter for viral infection (Fig. 1 and 2). Fluorescence-activated cell sorting (FACS) was then employed to enrich the population of cells with the highest expression levels (Fig. 2 and 3).

Second, we differentiated and maintained the FACS-sorted ReN cells expressing high levels of FAD genes in a 3D Matrigel culture system to promote extracellular deposition of $A\beta$

(Fig. 4 and Supplementary Fig. 1). We posited that, in two-dimensional models, secreted A β may diffuse into the cell culture medium, disrupting aggregation, whereas the 3D Matrigel may prevent this diffusion of A β , allowing for high local concentrations that are sufficient to initiate aggregation. We chose Matrigel specifically as a 3D culture matrix because it can be easily solidified with ReN cells through moderate thermal change and because it provides a brain-like environment rich in structural proteins such as laminin, entactin, collagen, and heparan sulfate proteoglycans²⁸. We confirmed that the 3D Matrigel serves as an excellent 3D seeding structure for A β aggregation once the aggregation is initiated although it is still permeable to A β species. Moreover, previous studies suggested that 3D conditions that more closely mimic *in vivo* environments can accelerate neuronal differentiation and neural network formation^{23,29–34}. Indeed, we found that 3D differentiated FAD-gene ReN cells expressed higher levels of specific neural markers and elevated 4-repeat adult tau (4R tau) isoforms versus 2D¹⁴.

While the characteristics of the 3D culture system are essential for fully recapitulating AD, the same properties cause several technical difficulties in classical biochemical and imaging analyses. For immunofluorescence (IF) and fluorescence/confocal microscopy, we developed thin layer cultures (~100–300 μ m) that could be imaged at high magnification. For biochemical analyses that required higher concentrations of biomolecules, we differentiated cells into thick layer cultures (~4 mm) (Fig. 2). We confirmed that both thin layer and thick layer 3D cultures robustly differentiate into neurons and glia¹⁴.

Comparison with other AD iPSC models

Most iPSC-derived human neurons with either *APP* or *PSEN1* FAD mutations exhibit significant increases in A β 42 levels as compared to the control cells, consistent with previous findings in other model systems^{5–10}. Interestingly, increases in p-tau levels were reported in some of these models. Israel *et al.* showed a ~2-fold increases of p-tau/total tau ratio in AD neurons⁶. Recently, Muratore *et al.* showed that AD neurons harboring APP^{V717I} mutation also showed increases in p-tau and total tau levels and furthermore, demonstrated that abnormal elevation in tau levels could be decreased by treating with anti-A β antibodies¹⁰. Another recent study determined that differentiated human neurons from Down syndrome (DS) patients generated high levels of A β 40 and 42 due to an extra copy of the *APP* gene⁷. Interestingly, DS neuronal cultures revealed intracellular and extracellular aggregates of A β 42 as well as increases of p-tau levels, even in 2D culture conditions after extended differentiation periods (>90 days). However, these studies could not recapitulate either robust extracellular aggregation of A β or the aggregated p-tau pathology that are evident in our 3D culture study. Limitations of 2D culture protocols and/or low A β 42 levels may be responsible for the lack of robust AD pathology in the previously described studies using stem cell-derived neuronal cultures.

Applications and limitations of the protocol

As this model is the first human cellular model to comprehensively show A β -driven tauopathy this protocol can now be used to examine many other central parts of AD pathogenesis *in vitro*, including the molecular mechanisms underlying the production of high concentrations of A β , the accumulation of extracellular A β , the deposition of A β

aggregates, the hyperphosphorylation of tau, and p-tau aggregation. These paths may lead to new diagnostic and prognostic biomarkers of AD. The model can also be used to test other genetic or environmental factors associated with AD, either in conjunction with the FAD-causing mutations used in this study or in place of them. The flexible scalability of the system also makes it ideal for use in larger scale testing and drug screening. More generally, the following protocols may also be applicable to other neurodegenerative diseases with genetic variations and can be especially suitable for diseases with abnormal aggregation of misfolded proteins.

However, it is important to note that while the 3D-differentiated ReN cells with FAD mutations have reproduced key events in the pathogenic cascade of AD including A β deposition and p-tau accumulation/aggregation, they are not surrogate human brain systems. First, our 3D culture model lacks human microglia cells (*data not shown*) that play a crucial role in A β clearance, brain inflammation, and synaptic and neuronal damage. Our 3D differentiation protocol is also not designed to model the specific brain regions that are most affected in AD, i.e. the hippocampus and specific cortices. Indeed, we found that 3D-differentiated ReN cells are composed of a heterogeneous cell population, expressing markers for GABAergic, glutamatergic and dopaminergic neurons as well as astrocytes and oligodendrocytes¹⁴. The overexpression of AD genes with multiple FAD mutations may generally restrict the use of our model for the study of the early stages of the disease, in which A β gradually accumulates without robust increases in tau phosphorylation. Moreover, our model also characterizes the late stages of the disease, where hyperphosphorylated tau aggregation develops.

Regardless, we believe that the model and techniques described in this protocol offer an exciting model for the AD field, especially given that plaques and tangles formation are the two major hallmarks of this disease. Moreover, the model provides a methodology for the potential development of human neural cell cultures for the study of other neurodegenerative diseases and a robust platform for finding new AD disease targets and therapeutics.

Although the 3D culture described in here is optimized for ReN cells, we have also confirmed that the same 3D culture condition can be applied for differentiating human iPSC-derived neural stem cells including It-NES cells^{35,36} (*data not shown*).

Experimental design

Establishment of the ReN cells expressing high levels of A β (Step 1–16)

This protocol is optimized for a commercially available human neural progenitor cell line (ReNcell VM, EMD Millipore), which is immortalized, well characterized, and genetically stable after prolonged passaging (>45 passages)^{15–27}. ReN cells were transfected with either CSCW-APP-GFP, CSCW-PSEN1(E9)-mCherry or CSCW-APP-IRES-PSEN1(E9)-IRES-mCherry alone or together (Fig. 1–4). The FAD mutations *APP* (K670N/M671L (*Swedish*) and V717I (*London*)) and *PSEN1*(E9) were chosen specifically because they cause high production of A β 42 and A β 40 and a high ratio of A β 42/A β 40. ReN cells with CSCW-IRES-GFP or CSCW-IRES-mCherry alone were also generated as a control. Other FAD mutations may be selected for further characterization or comparison. Other gene

editing and overexpression strategies including electroporation, Zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), or clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas based RNA-guided DNA endonucleases could also be utilized^{37–39}.

Differentiation of control and FAD ReN cells in 3D Matrigel (Step 17–34)

We describe how to develop thin-layer (~100–300 μ m) and thick-layer (~4 mm) 3D cultures by mixing different concentrations of Matrigel with cells. Thin-layer 3D cultures can be used for immunocytochemistry and thick-layer 3D cultures can be used for molecular, biochemical, and ELISA analyses (Fig. 2, 5–9). Also differentiate cells transfected with the GFP or mCherry control lentiviral particles. The presence of A β aggregates and p-tau aggregates described herein may depend on the hNPC line, the passage number, the type of FAD mutations, the number of cells seeded, and the proportion of enriched cells by FACS. Generally, we observed extracellular A β aggregates starting at 6 weeks of differentiation and robust p-tau accumulation after 10 weeks. We also observed that both A β aggregates and p-tau pathology were further elevated through 17 weeks, depending on the cell lines (Fig. 7–9).

Endpoint analysis of A β and p-tau pathologies (Step 35–36)

For the thin-layer 3D culture, we describe how to analyze A β and abnormal p-tau accumulation using immunofluorescence staining and immunohistochemistry (Fig. 7–8), as previously shown¹⁴. Since most of our FAD ReN cells express high levels of GFP and mCherry, the UV-compatible amyloid dye Amylo-Glo can be used for detecting β -amyloid aggregates in the thin layer 3D culture (Supplementary Fig. 2). For thick-layer 3D culture, we describe biochemical extraction to show accumulation of A β and p-tau species in both soluble and insoluble fractions. Specifically, for detecting A β aggregates, we recommend using TBS/2%SDS/Formic acid serial extraction, while for detecting tau aggregation, we recommend using a 1% Sarkosyl extraction method, as shown in Fig. 9. Accumulation of SDS-resistant A β oligomers, including dimers, trimers and tetramers, can also be used as indirect markers for A β aggregation. To monitor the proper 3D differentiation, analyze expression of adult neural markers, including 4-repeat tau, by using RT-PCR, qRT-PCR¹⁴ and immunofluorescence staining (as shown Fig. 6). LDH levels in 3D culture media should also be measured routinely to detect the cell death rate in these cultures.

MATERIALS

REAGENTS

Production of FAD cells

- Lentiviral particles. See Reagent set up.
- KnockOut Serum Replacement (Life Technologies, cat. no. 10828-028)
- Propidium Iodide (Life Technologies, cat. no. P1304MP)

3D Cell Culture

- ReNcell VM cells (EMD Millipore, SCC008)
- DMEM/F12 medium (Gibco/Life Technologies, cat. no. 11320-033)
- Heparin (Stemcell Technology, cat. no. 07980)
- B27 (Gibco/Life Technologies, cat. no. 17504-044)
- bFGF (Stemgent, cat. no. 03-0002)
- EGF (Sigma-Aldrich, cat. no. E9644)
- Penicillin/Streptomycin/Amphotericin B (Lonza, cat. no. 17-745E)
- ReNcell Neural Stem Cell Freezing Medium (EMD Millipore, cat. no. SCM007)
- Accutase solution (Life Technologies, cat. no. A11105-01)
- Matrigel (Corning, cat. no. 356230; cat. no. 356234 also works)
- D-PBS (Lonza, cat. no. 17-512Q)

RNA extraction, RT-PCR and qRT-PCR

- RNaseZap® (Life Technologies, cat. no. AM9780)
- D-PBS (Lonza, cat. no. 17-512Q) **▲CRITICAL** We have not tested DEPC-treated D-PBS for this protocol but opened a fresh bottle keeping it aliquoted only for RNA work.
- Buffer RLT (Qiagen, cat. no. 79216)
- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M3148) **▲CRITICAL** It should be dedicated for RNA work only.
- HPLC-grade H₂O (Fisher Scientific, cat. no. W5-1)
- QIAshredder (Qiagen, cat. no. 79656)
- RNeasy Mini Kit (Qiagen, cat. no. 74104 or 74106, depending on size)
- Ethanol 200 proof (absolute) (Sigma-Aldrich, cat. no. E7023)
- Buffer RW1 (Qiagen, cat. no. 1053394)
- Buffer RPE (Qiagen, cat. no. 1018013)
- RNase-free H₂O (Qiagen, cat. no. 129112)
- Oligo(dT)₂₀ Primer (Life Technologies, cat. no. 18418020)
- 10 mM dNTP Mix (Life Technologies, cat. no. 18427013, 18427088, depending on size)
- 10x RT Buffer (Life Technologies, cat. no. 18067-017)
- 25 mM MgCl₂ (Life Technologies, cat. no. 18080-051)
- 0.1 M DTT (Life Technologies, cat. no. 18080-051)

- RNaseOUT™ (40U/μl) (Life Technologies, cat. no. 10777-019)
- SuperScript® III RT (200U/μl) (Life Technologies, cat. no. 18080-093)
- RNase-H (Life Technologies, cat. no. 18021-071)
- SYBR® Select Master Mix (Life Technologies, cat. no. 4472908)
- 3R4RTau Forward Primer: 5'-AAGTCGCCGTCTCCGCAAG-3'⁴⁰ (synthesized in the MGH DNA core facility)
- 3R4RTau Reverse Primer: 5'-GTCCAGGGACCCAATCTTCGA-3'⁴⁰ (synthesized in the MGH DNA core facility)
- Agarose (Apex, cat. no. 20–102)
- Quick-Load® 2-Log DNA ladder (0.1–10 kb) (New England Biolabs, cat. no. N0469S)
- DNA Loading Dye (6x) (Thermo Scientific, cat. no. R0611)
- Ethidium Bromide (Sigma-Aldrich, cat. no. E1510)
- 3R Tau forward primer (AGGCGGGAAGGTGCAAATAG)⁴¹ (synthesized in the MGH DNA core facility)
- 4R Tau forward primer (GAAGCTGGATCTTAGCAACG)⁴¹ (synthesized in the MGH DNA core facility)
- 3R Tau reverse primer (TCCTGGTTTATGATGGATGTT)⁴¹ (synthesized in the MGH DNA core facility)
- 4R Tau reverse primer (GACGTGTTTGATATTATCCT)⁴¹ (synthesized in the MGH DNA core facility)

Immunofluorescence and Immunohistochemistry

- Paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710)
- Tris (Fischer Scientific, cat. no. BP152)
- Tween-20 (Acros Organics, cat. no. 23360051)
- Bovine Serum Albumin (Sigma-Aldrich, cat. no. A2153)
- Gelatin from Bovine Skin, Type B (Sigma-Aldrich cat. no. G9391)
- Glycine (American Bioanalytical, cat. no. AB00730)
- Donkey serum (Sigma-Aldrich, cat. no. D9663)
- Anti-fade gold (Life Technologies, cat. no. P36930)
- H₂O₂ (Sigma-Aldrich, cat. no. 1001582615)
- ImmPRESS Polymer Detection Kit (Vector Laboratories, cat. no. MP-7402 and MP-7401)

- DAB Peroxidase (HRP) Substrate Kit, 3,3'-diaminobenzidine (Vector Laboratories, cat. no. SK-4100)
- ImmPACT DAB Peroxidase Substrate kit (Vector Laboratories, cat. no. SK-4105)
- Amyloid β -Protein Immunohistostain Kit (Wako, cat. No. 299-56701)
- Chromopure goat IgG (Jackson Immuno Research, cat. No. 005-000-003)
- Amylo-Glo 100x (Biosensis, cat. no. TR-300-AG)
- 0.9% saline (B. Braun Medical Inc., cat. no. L8001)
- 1% Triton X-100 (Fischer Scientific, cat no. 11332481001)
- A summary of antibodies we used is shown in table 1

Biochemical Extraction, Western Blot and Dot Blot

- HPLC-grade H₂O (Fisher Scientific, cat. no. W5-1)
- 4x LDS sample loading buffer (Life Technologies, cat. no. NP0007)
- Invitrogen NuPage MES SDS Running Buffer (20X) (Life Technologies, cat. no. NP0002-02)
- Full range molecular weight Marker (Amersham, cat. no. GERPN800E)
- SuperBlock T20 (TBS) Blocking Buffer (Thermo Scientific cat. no. 37536)
- 50% glutaraldehyde solution (Electron Microscopy Sciences cat. no. 16316)
- Supersignal West Dura Chemiluminescence solution (Thermo Scientific cat. no. 34075AB)
- Supersignal West Femto Chemiluminescence solution (Thermo Scientific, cat. no. 34095AB)
- N-Lauroylsarcosine (Sigma-Aldrich, cat. no. SKU L5777)
- NaVO₃ (Sigma-Aldrich, cat. no. SKU 72060)
- NaF (Sigma-Aldrich, cat. no. 201154)
- Protease inhibitor mixture (Roche Life Science, cat. no. 04693159001)
- Phosphatase inhibitor cocktail (Thermo Scientific, cat no. 88667)
- 1,10-o-phenanthroline (PNT, EMD Millipore, cat. no. M1072250010)
- Phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, cat. no. P7626)
- Formic acid (Sigma-Aldrich, cat. no. 399388)
- Immun-Blot® PVDF Membrane (Bio-Rad, cat. no. 162-0177)
- Nitrocellulose Membranes (0.20 Micron, 7.9 cm x 10.5 cm) (Thermo Scientific, cat. no. PE1846871)
- Bio-Dot SF Filter Paper (Bio-rad, cat. no. 1620161)

- A summary of the used antibodies was shown in table 2

Transmission Electron Microscopy (TEM)

- Mouse-anti-tau46 (Cell Signaling Technology, cat. no. 4019)
- Antibody Diluent (DAKO, cat. no. S3022)
- Goat-anti-mouse IgG 10 nm gold (Ted Pella, cat. no. 15751)
- Phosphotungstic acid (Electron Microscopy Sciences, cat. no. 19500)

ELISA

- Human/Rat A β 40 ELISA Kit (Wako, cat. no. 294-62501)
- Human/Rat A β 42 ELISA Kit (Wako, cat. no. 290-62601)

Lactate dehydrogenase (LDH) assay

- CytoTox-ONE Homogeneous Membrane Integrity Assay Kit (Promega, cat. no. G7890)
- 96-well white, opaque microwell assay plate (Nunc, cat. no. 236105)

EQUIPMENT

Production of FAD cells

- We use a BD SORP FACS Aria2 cell sorter (BD Bioscience) in a Bioprotect IV biosafety cabinet (Baker Company): equipped with 5 lasers (350, 405, 488, 561 and 640), located at the Imaging Core Facility of the Ragon Institute of MGH/MIT/Harvard (<http://ragoninstitute.org/research/services/flow-cytometry/>)
- Cell strainer filter (70 μ m Nylon, Corning/Fisher Scientific, cat. no. 352350)

3D Cell Culture

- Greiner Bio-One 96-well PS uClear black TC plate, flat bottom (Greiner Bio-One, 5665-5090)
- MatTek glass bottomed 96-well plates (MatTek, cat. no. P96GC-0-5-F)
- Corning® Black/Clear bottom 96-well plates (Corning, cat. no. 3340)
- Lab-Tek II 8-well chambered cover glass slides (Thermo Scientific, cat. no. 155409)
- Lab-Tek 16-well glass chamber slides (Thermo Scientific, cat. no. 178599)
- MatTek glass bottomed 35 mm plates (MatTek, cat. no. P35G-0-7-C or P35G-1.5-10-C)
- T25 cell culture flasks (BD Biosciences, cat. no. 353082)
- 6-well cell culture plates (Fisher Scientific/Corning life sciences, cat. no. 353046)
- Cell culture inserts, 0.4 μ m pore size (Fisher Scientific/Falcon, cat. no. 353095)

- 24-well Falcon companion plates for cell culture inserts (Fisher Scientific/Falcon, cat. no. 353504)
- Cell culture inserts, 0.4 μm pore size (Greiner Bio-One, cat. no. 662654)
▲CRITICAL Both cell culture inserts (Falcon and Greiner Bio-One) work well for thick-layer culture for biochemical analysis. Greiner Bio-One has a smaller internal diameter as compared to the ones from Falcon, and therefore works better with a small amount of cells to make a proper thickness suitable for thick-layer 3D culture.
- 0.4 μm disposable cell culture medium filter (Life Technologies, cat. no. 353095)
- Sterile 15 and 50 ml conical tube (Fisher Scientific/Falcon, cat. no. 352099 and 352070)
- Automated cell counter (Bio-rad, cat. no. TC10)
- Cell counter slide (Bio-rad, cat. no. 1450011)
- Water Bath (VWR Scientific, cat. no. 1202)
- Micro-centrifuge, 4 $^{\circ}\text{C}$ (Beckman Coulter, cat. no. 367160)
- Tissue culture Light microscope (Zeiss)
- BL-2 biosafety culture hoods
- Vortex
- Sorvall T6000B Centrifuge (Brochu)

RNA extraction and RT-PCR

- Sorvall Legend T+ centrifuge (Thermo Scientific, cat. no. 75004367)
- RNaseZap®-rinsed, sterile forceps **▲CRITICAL** Forceps should be dedicated for RNA work only and stored in a separate RNA working area
- RNaseZap®-rinsed, disposable razor blades
- RNase-free filter pipette tips (1, 20, 100, 1,000 μl ; USA Scientific, cat. nos. 1121-3810, 1120-1810, 1120-8810 and 1126-7810, respectively)
- RNase-free micro-centrifuge tubes (1.5 ml size; USA Scientific, cat. no. 1615-5500)
- PTC-200 Thermal Cycler (MJ Research, cat. no. ALD-1244)
- iCycler iQTM (Bio-rad, cat. no. 170-8740)
- Bio-rad Tetrad® 2 Thermal Cycler (Bio-rad, cat. no. PTC-0240G with ALS-1296GC)
- NanoDrop 1000 Spectrophotometer (Thermo Scientific, cat. no. ND-1000)
- Bio-rad ChemiDoc XRS System (Bio-rad, cat. no. 170-8070)

Immunofluorescence and Immunohistochemistry

- Olympus IX7 fluorescence microscope equipped with spinning disk confocal unit (Olympus)
- LSM Pascal 5 Carl Zeiss confocal microscope (Zeiss)

Biochemical Extraction and Western Blot

- Hoefer Semi-Dry Transfer Unit (Hoefer, TE77)
- Molecular imager (Bio-rad, VersaDoc MP4000)
- Battery-operated spinning homogenizer (MIDSCI, cat. no. A0001)
- Ultracentrifuge tubes (Beckman Coulter, cat. no. 343778)
- Ultracentrifuge (Beckman, Optima TL Ultracentrifuge)
- SpeedVac (Thermo Scientific, cat. no. SPD111V)
- Savant RVT4104 refrigerated vapor trap with Savant SC110 SpeedVac (Savant)

TEM

- JEOL JEM 1011 transmission electron microscope (JEOL)
- AMT digital imaging system (Advanced Microscopy Techniques)

ELISA

- ELISA plate reader (Bio-Tek Synergy 2 microplate reader)

LDH

- ELISA plate reader (Bio-Tek Synergy 2 microplate reader)

REAGENT SETUP

Lentivirus—We obtained the lentiviral polycistronic vectors (CSCW-IRES-GFP and CSCW-IRES-mCherry) from Massachusetts General Hospital (MGH) viral core⁴², which are available through the MGH viral core (<https://vectorcore.mgh.harvard.edu>). CSCW-APP-GFP, CSCW-PSEN1(E9)-mCherry or CSCW-APP-IRES-PSEN1(E9)-IRES-mCherry vectors (Fig. 1) were constructed by inserting full length *APP* with FAD mutations *APP* (K670N/M671L (*Swedish*) and V717I (*London*)), *PSEN1* with a FAD mutation, or both *APP* and *PSEN1* with the FAD mutations⁴³. We have our lentiviral vectors packaged by our viral core, the by the MGH viral core^{42,43}. To package lentiviral vectors, first generate lentiviral particles by co-transfecting 293T cells with plasmids, the lentivirus packaging genome CMVR 8.91 and envelope coding plasmid (pVSV-G). 48 to 72 h post-transfection, harvest the lentiviral particles, concentrate by ultracentrifugation and titer [transducing units (t.u.)/ml] by real-time PCR using specific primers against WPRE elements as previously described⁴². **▲CRITICAL** The aliquots of packaged viral particles can be stored at −80 °C up to one year without a significant loss of titer.

Human recombinant EGF stock solution (20 µg/ml)—Under a biosafety culture hood, add 2 ml of 0.2 µm-filtered 10 mM acetic acid to 2 mg lyophilized EGF (Sigma) (1 mg/ml), mix, make further dilution with 0.2 µm-filtered 0.1% bovine serum albumin solution to 20 µg/ml final concentration, and store 1 ml aliquots at −80 °C.

Human recombinant bFGF stock solution (25 µg/ml)—Under a biosafety culture hood, add 2 ml of 0.2 µm-filtered 10 mM Tris (pH 7.6) to 50 µg lyophilized bFGF (Stemgent), mix, make 0.2 ml aliquots and store them at −80 °C. **▲CRITICAL** To avoid the activity loss by multiple freezing/thawing cycle, bFGF or EGF stocks should be kept at 4 °C once they are thawed. 4 °C stocks should be used within 2–3 weeks. The bFGF and EGF stock aliquots can be stored up to one year at −80 °C.

Cell sorting medium—Add 2% (v/v) of B27 supplement and 2% (v/v) of KnockOut serum replacement to 50 ml of D-PBS. The cell sorting media can be stored at 4 °C for 6 weeks.

Matrigel—Aliquot in 1 ml stocks on ice and keep the stocks at −80 °C. **▲CRITICAL** Thaw Matrigel stock overnight at 4 °C before use. Matrigel tends to solidify above 10 °C. The frozen Matrigel stock can be stored at −80 °C up to one year.

Matrigel working solution—Mix 0.5 ml of Matrigel and 50 ml cold DMEM/F12 medium (1:100 dilution) on ice under a biosafety culture hood. **▲CRITICAL** Take out a Matrigel stock (1 ml) from −80 °C freezer and place it in 4 °C refrigerator one day before the experiments. Prepare fresh Matrigel working solution.

Matrigel-coated T25 culture flask—Add 3 ml of a cold Matrigel working solution into each T25 flasks, shake gently to cover all the surface area, incubate under 37 °C CO₂ incubator at least for 1 h, and remove the remaining solution. Matrigel-coated culture dishes can be stored at 4 °C for up to 2 months.

ReN differentiation medium—To prepare 500 ml of medium, combine 484.5 ml of DMEM/F12 (Gibco/Life Technologies) with 0.5 ml of Heparin (2 mg/ml stock, StemCell Technology), 10 ml of B27 (Life Technologies) and 5 ml of 100x Penicillin/Streptomycin/Amphotericin (Lonza). The cell sorting media can be stored at 4 °C for 2–3 months.

ReN proliferation medium—To prepare 100 ml of medium, combine 100 ml of ReN differentiation medium with 80 µL bFGF stock and 100 µL EGF stock. Filter the medium after adding all the reagents. **▲CRITICAL** Medium with growth factors can be kept at 4 °C for several weeks, but use a fresh one if there is any noticeable decrease in cell growth rate.

10x Tris-Borate-EDTA (TBE) electrophoresis Buffer—Add 108 g Tris, 55 g Boric Acid and 7.425 g EDTA into a beaker, fill up to 1 L with distilled-deionized H₂O (ddH₂O) and mix to dissolve. The 10x TBE buffer is stable at least for 6 months at room temperature.

10x Tris-buffered saline (TBS) buffer—To prepare 100 ml buffer, dissolve 6.05 g Tris and 8.76 g NaCl in 80 ml of H₂O. Adjust pH to 7.5 with 1 M HCl and make volume up to 100 ml with H₂O. The 10x TBS buffer is stable at least for 6 months at room temperature.

1x TBS/Tween20 (TBST) buffer—To prepare 1 L buffer, add 100 ml **10x TBS buffer** and 2 ml of Tween 20 and make volume up to 1 L with H₂O. The 1x TBST buffer is stable at least for 3 months at room temperature.

Blocking/dilution solution for immune staining—To prepare 250 ml blocking/dilution solution, add 2.5 g bovine serum albumin (Sigma-Aldrich), 5.63g glycine, 0.25 g gelatin in 200 ml TBST, heat at 55 °C for ~10 min to dissolve gelatin, add 10 ml of donkey serum (Sigma-Aldrich) and add TBST to make the final volume to 250 ml. Filter the blocking/dilution solution with 0.4 µm filter unit (Gibco) and store the solution at 4 °C. The Blocking/dilution solution can be stored at 4 °C for 2–3 months.

0.02x Amylo-Glo working solution—Add 2 µl Amylo-Glo (100x) to 10 ml 0.9% (w/v) NaCl. Prepare fresh every time.

0.1x Amylo-Glo working solution—Add 10 µl Amylo-Glo (100x) to 10 ml 0.9% (w/v) NaCl. Prepare fresh every time.

10xTris/EDTA buffer—To prepare 100 ml buffer, dissolve 6.05 g Tris and 8.76 g NaCl in 80 ml of H₂O. Add 4 ml of 0.4 M EDTA (pH 7.4) and adjust pH to 7.6 with 1 M HCl and make volume up to 100 ml with H₂O. The 10x Tris/EDTA buffer is stable at least for 6 months at room temperature.

1xTBS extraction buffer—To prepare 10 ml buffer, mix 8.65 ml of distilled water (HPLC grade), 1 ml of 10xTBS buffer (pH 7.4), 1 tablet of protease inhibitor cocktail, 0.1 ml of NaVO₃ (1 M), 0.1 ml of NaF (1 M), 0.05 ml of PNT (200 mM), 0.05 ml of PMSF (200 mM) and 0.05 ml of phosphatase inhibitor. **▲CRITICAL** Prepare the fresh working solution before use. 1 ml aliquots of the extraction buffer without phosphatase inhibitors can be stored at –80 °C for several months.

2xTBS extraction buffer—To prepare 10 ml buffer, mix 7.5 ml of distilled water (HPLC grade), 2 ml of 10xTBS buffer (pH 7.4), 2 tablets of protease inhibitor cocktail, 0.1 ml of NaVO₃ (1 M), 0.1 ml of NaF (1 M), 0.1 ml of PNT (200 mM), 0.1 ml of PMSF (200 mM) and 0.1 ml of phosphatase inhibitor. **▲CRITICAL** Prepare the fresh working solution before use. 1 ml aliquots of the extraction buffer without phosphatase inhibitors can be stored at –80 °C for several months.

1xSDS extraction buffer—To prepare 10 ml buffer, mix 1 ml of SDS solution (20% stock), 1 ml of 1% Triton X-100 (10% stock), 4.5 ml of distilled water (HPLC grade), 1 ml of 10xTBS/EDTA buffer (pH 7.4), 2 tablets of protease inhibitor cocktail, 0.1 ml of NaVO₃ (1 M), 0.1 ml of NaF (1 M), 0.1 ml of PNT (200 mM), 0.1 ml of PMSF (200 mM) and 0.1 ml of phosphatase inhibitor (100x). **▲CRITICAL** Prepare the fresh working solution before

use. 1 ml aliquots of the extraction buffer without phosphatase inhibitors can be stored at -80°C for several months.

2xSDS extraction buffer—To prepare 10 ml buffer, mix 2 ml of SDS solution (20% stock), 2 ml of 1% Triton X-100 (10% stock), 4.5 ml of distilled water (HPLC grade), 1 ml of 10xTBS/EDTA buffer (pH 7.4), 2 tablets of protease inhibitor cocktail, 0.1 ml of NaVO_3 (1 M), 0.1 ml of NaF (1 M), 0.1 ml of PNT (200 mM), 0.1 ml of PMSF (200 mM) and 0.1 ml of phosphatase inhibitor (100x). **▲CRITICAL** Prepare the fresh working solution before use. 1 ml aliquots of the extraction buffer without phosphatase inhibitors can be stored at -80°C for several months.

20 % Sarkosyl solution—Add 0.1 g N-laurylsarcosine with total 0.5 ml of distilled water (HPLC grade). The 20 % Sarkosyl solution should be made fresh before the experiments.

2xRIPA extraction buffer—To prepare 10 ml buffer, mix 0.5 ml of sodium deoxycholate solution (10% stock), 0.2 ml of 2% NP-40 (ICABAL substitute), 7.8 ml of distilled water (HPLC grade), 1 ml of 10xTBS/EDTA buffer (pH 7.4), 02 tablets of protease inhibitor cocktail, 0.1 ml of NaVO_3 (1 M), 0.1 ml of NaF (1 M), 0.1 ml of PNT (200 mM), 0.1 ml of PMSF (200 mM) and 0.1 ml of phosphatase inhibitor (100x). **▲CRITICAL** Prepare fresh working solution before use. 1 ml aliquots of the extraction buffer without phosphatase inhibitors can be stored at -20°C for several months.

High salt extraction buffer—To prepare 10 ml buffer, mix 1 ml of 10xTBS, 0.47g NaCl, 0.01 ml EGTA (1 M), 1 g sucrose, 1 tablet of protease inhibitor cocktail, 0.1 ml of NaVO_3 (1 M), 0.1 ml of NaF (1 M), 0.1 ml of PNT (200 mM), 0.1 ml of PMSF (200 mM) and 0.1 ml of phosphatase inhibitor (100x). Make 10 ml with distilled water (HPLC grade). **▲CRITICAL** Prepare fresh working solution before use. 1 ml aliquots of the extraction buffer without phosphatase inhibitors can be stored at -20°C for several months.

LDH assay reagent preparation—Using a CytoTox-ONE Assay Kit, add 11 ml of Assay Buffer to each vial of Substrate Mix to prepare CytoTox-ONE Reagent. The CytoTox-One reagent is stable for 6–8 weeks at -20°C .

EQUIPMENT SETUP

Placing tissue culture inserts on 24-well plates for 3D thick layer culture—Sterilize the forceps under the biosafety hood using ethanol and UV radiations. Place 24-well plates (Falcon), open the lids and carefully place cell culture inserts into each well with the sterilized forceps. **▲CRITICAL** Always use the companion plates (Falcon) for setting tissue culture inserts (Falcon).

3D thin-layer culture with chambered coverglass slide plates or glass-bottomed 35 mm dishes—Place the coverglass slide plates (Nunc) or the glass-bottomed 35 mm dishes (MatTek) inside 150 ml culture dishes to reduce the chance of contamination.

RNA extraction from 3D differentiated cells—RNA work should be done in a dedicated area or bench. Keep a separate set of equipment only used in this area. Wipe the work surfaces with RNaseZap® or other RNase removal reagents before RNA extraction. Solutions and reagents should be stored in small aliquots to minimize contaminations with RNases. Gloves should be worn when handling RNA and should be changed frequently. The temperature in the benchtop centrifuge should be set-up to 4 °C or placed in a fridge.

PROCEDURE

ReN cell maintenance and passaging ● TIMING 30 min

- 1| Warm the ReN proliferation medium and Accutase solution in 37 °C water bath for at least 10 min.
- 2| Wash ~95% confluent ReN cells in T25 flask with 3 ml of D-PBS, aspirate the D-PBS, and add 0.5 ml of Accutase under a biosafety culture hood.
- 3| Incubate the cells in 37 °C CO₂ incubator for 3–5 min, add 3 ml of pre-warmed ReN proliferation medium and transfer the cell suspensions to 15 ml sterile conical tube.
- 4| Spin down the cells at 2,000 g (Sorval T6000B) for 3 min, remove the supernatant, and resuspend the cell pellet with 12 ml of ReN proliferation medium.
- 5| Dispense 4 ml of the ReN cell suspension into each Matrigel-coated T25 flask and incubate in 37 °C CO₂ incubator.

▲**CRITICAL STEP** After subculture (1:3 rate), ReN cells are generally confluent again in 3–4 days. If cells are not confluent at that time, replace the medium with a fresh ReN proliferation medium and wait 1 or 2 days.

? TROUBLESHOOTING

Viral infection of ReNcell VM cells ● TIMING 96 h

- 6| To prepare ReN cells for infection, dislodge and spin down cells from a confluent T25 flask as described in steps 1–4. Resuspend the cell pellet in 12 ml of pre-warmed ReN cell proliferation medium and dispense 2 ml of the cell suspension into each well of a Matrigel-coated 6-well plate. Gently cross-shake the dish and allow the cells to settle overnight.
- 7| Replace the medium with 2 ml of pre-warmed ReN proliferation medium. When cells reach 70–80% confluence, add 6x10⁶ TU (Transducing Units) of viral particles per well to achieve an approximate M.O.I. of 1. Mix gently and incubate overnight.
- 8| Wash twice with 2 ml of pre-warmed ReN proliferation medium, then apply 2 ml of the medium. Confluence should increase over time. Expression of the transgenes can be expected 48 h after infection and should be detectable by fluorescence microscopy.

▲CRITICAL STEP Viral infection renders the cells vulnerable. Carefully monitor the culture. If abnormal cell death is observed, replace the medium immediately.

- 9| On day 4, if the cells are confluent, passage the cells as described in steps 1–5 and recommence normal culturing. If the cells did not reach complete confluence by this point, replace the medium instead of passaging and incubate until the cells reach confluence. To generate cells expressing both APPSL/GFP and PSEN1(E9)/mCherry (i.e. ReN-mGAP cells¹⁴), the transduced cells can be infected again with different lentiviral vectors after splitting, as described in steps 6–8.
- 10| Validate the infection by microscopic detection of GFP and mCherry fluorescence and western blot analysis of overexpressed APPSL and PSEN1(E9). Typically, more than 50% of infected cells visibly express the fluorescent marker.

▲CRITICAL STEP Western blot analysis of successfully transduced cell extracts will show a PSEN1(E9) band (~40 kDa) and increased bands of full-length APP (695 isoform, ~110 kDa) and its C-terminal fragment (10–12 kDa). Western blot analysis can also be used to detect the elevated sAPP α levels in the supernatant as shown in Fig. 5.

■ PAUSE POINT The infected/validated cells can be frozen and stored at liquid LN2 tank at least for 1 year before FACS analyses.

? TROUBLESHOOTING

Enrich high-expressing transgenic ReN cells ● TIMING 2 h

- 11| Culture the transduced ReN cells until confluence is reached (a 95% confluent T25 flask yields approximately $2\text{--}3 \times 10^6$ cells). While the optimal total cell count depends on the infection efficiency, at least $2\text{--}3 \times 10^6$ successfully transduced cells should be available (when assayed by FACS analysis or fluorescence microscopy).
- 12| Detach the cells as described in steps 1–4 but resuspend the cell pellets with 4 ml of D-PBS. Determine the cell number using an automated cell counter.
- 13| Spin down the cells, remove the supernatant and resuspend the cell pellets in ice-cold sorting medium ($1 \text{ ml}/10^7$ cells) using a 1,000 μl pipette.

▲CRITICAL STEP Cells have to be resuspended in at least 200 μl of Cell sorting medium.

- 14| To singularize the cells, aspirate the cell suspension with a 1,000 μl pipette, gently press the tip against a cell-strainer mesh at a 90° angle and empty the pipette forcefully. In case of blockage, carefully move the pipette tip across the mesh while maintaining pressure. Collect the filtered suspension in a 15 ml centrifugation tube.

■ **PAUSE POINT** The suspended cells can be stored on ice at least for 1 hour before FACS sorting.

- 15| Sort cells: We perform FACS cell sorting at the Ragon Institute Imaging Core on a BD SORP FACSAria 2 cell sorter (“Special Order Research Product”) using the 100 μ m nozzle and a sheath pressure of 20 psi. BD FACSDiva Software is used to acquire and analyze samples, which are viewed on a dot plot of GFP (488 nm, 200 mW excitation, 530/30 emission) vs. mCherry (561 nm, 150 mW excitation, 610/20 emission) to define sort populations for the final bulk sorting. Gates are set to select cells with high expression of APPSL/GFP alone (as shown in Fig. 5), APPSL/PSEN1(E9)/mCherry alone, or APPSL/GFP and PSEN1(E9)/mCherry together¹⁸.

- 16| Immediately after sorting, plate the cells into Matrigel-coated dishes.

■ **PAUSE POINT** The suspended cells can be stored in a cell collection tube on ice up to 1 hour before plating.

Culturing sorted ReN cells ● TIMING 30 min

- 17| Spin the cells down at 2,000 g for 3 min at 4 °C, then remove the supernatant and resuspend the pellet in pre-warmed ReN proliferation medium (1 ml per 2×10^6 collected cells).

▲**CRITICAL STEP** Determine the cell concentration after resuspension and adjust the volume accordingly. Even counts from sorting can lead to substantial overestimation.

- 18| Seed the cells on Matrigel-coated 24-well plates at an initial density of 2×10^5 cells/cm² and culture. Expand serially into Matrigel-coated 6-well plates, and finally into Matrigel-coated T25 flasks. Make multiple cell stocks at this stage. Passage as described in steps 1–5. When sufficient cells have been grown up, proceed to next step.

▲**CRITICAL STEP** High seeding density after sorting is pivotal to promote cell proliferation. At this stage, it is very important to make multiple frozen cell culture stocks and to record passage numbers. We observed that some high expressing cell lines rapidly lost APPSL and PSEN1(E9) expression after 4 passages.

? TROUBLESHOOTING

3D Matrigel cultures ● TIMING 1 h

- 19| Take out a Matrigel stock from –80 °C freezer and place it in 4 °C refrigerator 1 day before use.
- 20| Grow ~95% confluent control and FAD ReN cells in 5 Matrigel-coated T25 flasks. An approximate number of ReN cells in confluent T25 flask is $3\text{--}4 \times 10^6$. It takes generally 2 days after passaging the cells.

- 21| Spray down ice bucket and ice with ethanol and leave them in the hood under UV radiation for about 20 min.
- 22| Place ReN cell differentiation medium and Matrigel stock on ice
▲CRITICAL STEP Matrigel tends to solidify above 10 °C. Thaw it at 4 °C overnight and then keep it in the ice until it is plated with the cells.
- 23| Remove medium from the T25 flasks with aspirating pipettes. Be careful not to touch the cells with the pipettes; always use the vacuum on the non-cell side.
- 24| Wash once with 3 ml D-PBS, then carefully remove it with the aspirating pipettes.
- 25| Add 0.5 ml Accutase into each flask and incubate at 37 °C for 3–5 min.
- 26| Hit the side of the container 3–5 times to help cells to detach from the coated plates; do not shake since shaking can significantly lower cell count.
- 27| Resuspend the cells with 3 ml ReN differentiation medium and pipette up and down inside the flask at least 3 times.
- 28| Transfer the cell suspensions from all 5 T25 flasks into a 15 ml tube.
- 29| Centrifuge for 2 min at 2,000 g (Sorvall T6000B).
- 30| Remove the medium with aspirating pipettes.
- 31| Resuspend the cell pellet in 2 ml cold ReN differentiation medium and vortex for 10 sec. Set the 15 ml tubes on ice.
- 32| Take a small aliquot of suspended cells and dilute 1:10 for cell count (10 µl suspension: 90 µl differentiation medium).
- 33| Count the cells using a cell counter slide; dilute cells if needed (optimum concentration is about 2×10^7 cells/ml before adding Matrigel at 1:1 ratio in the next steps).
▲CRITICAL STEP It is desirable to achieve high cell concentration at this stage. We have found that 1×10^7 cells/ml cells (after adding Matrigel at 1:1 ratio; see the step 34) shows robust A β and p-tau accumulation based on biochemical analysis.

3D Culture cell seeding

- 34| Follow option **A** to make thick-layer 3D cultures or option **B** for thin-layer 3D cultures (See supplementary Fig. 1 for the pictures of each 3D culture steps).
▲CRITICAL STEP Plating a high number of cells is important in order to achieve A β aggregates early. The desired number of cells is 1×10^7 cells/ml in 3D Matrigel mixture. Therefore, enough cells need to be grown at this stage. The passage number and the condition of the cells are also important.

(A) Thick-layer 3D culture (3–4 mm thickness) ● TIMING 2 d

- i. Add cold Matrigel to cell suspension on ice (1:1 (vol/vol) dilution). Make sure to chill the pipette tips first by pipetting cold differentiation medium back and forth before transferring Matrigel.
- ii. Vortex for 30 sec.
- iii. Dispense 300 µl of the Matrigel/cell suspension mixture into each tissue culture insert in 24-well plates using pre-chilled pipettes.
- iv. Incubate the plates at 37 °C in CO₂ incubator overnight.
- v. Add pre-warmed (in 37 °C water bath) differentiation medium to the plates (1 ml: 500 µl to the top of the inserts and 500 µl to the bottom) and place them back in the incubator.
- vi. Change half volume of the medium every 3–4 days; at the beginning, medium changing may be needed every 2 days depending on the medium color. Never leave differentiated cells drying with no medium.

? TROUBLESHOOTING

- vii. Differentiate 3D-plated cells for 4–17 weeks, depending on the experiments. Drug treatments should be done in the last two weeks before endpoint analyses.

▲CRITICAL STEP Since it is not easy to monitor the thick-layer culture under optical microscope, it is important to perform a routine lactate dehydrogenase (LDH) release assay to check the status of the cultures (see step 36 option N). It is also strongly recommended to set up thin-layer 3D cultures from the same Matrigel/cell mixture to monitor the culture quality.

(B) Thin-layer 3D cultures (100–300 µm thickness) ● TIMING 1 h

- i. Add cold Matrigel to cell suspension on ice (1:1 (vol/vol) dilution). Make sure to chill the pipette tips first by pipetting cold differentiation medium back and forth before transferring Matrigel. For 96-well plate thin-layer cultures, further dilute 1:1 Matrigel/cell mixture by adding 5 volume of a cold ReN differentiation medium (1:11 dilution final) and vortex for 30 sec. The same dilution rate can be used for thin-layer 3D cultures in 8-well/16-well chambered cover glass slides or MatTek glass bottomed dishes. We also found that a small volume of 1:1 Matrigel/cell mixture can be directly loaded onto these glass bottomed dishes and these quasi thin-layer cultures can be used for confocal immunofluorescence analysis.

▲CRITICAL STEP Each lot of Matrigel has a different protein concentration and therefore needs to be pre-tested. We generally test 1:10, 1:15, and 1:20 dilutions and pick the best dilution rate for each lot.

? TROUBLESHOOTING

- ii. Plate 100 µl of the Matrigel/cell suspension mixture per each well of 96-well plates using pre-chilled pipettes. If a thicker 3D culture is desired, use 2 drops per each

well (160 μ l using a multichannel pipette). A volume of 200 μ l is recommended for 8-well chambered cover glass slides and 300 μ l for glass-bottomed dishes.

- iii. Incubate the plates at 37 °C in CO₂ incubator overnight.
- iv. Next day, add 2 drops of pre-warmed ReN differentiation medium to each well of the 96-well plates (160 μ l using a multichannel pipette), 200 μ l for 8-well chambered cover glass slides and 300 μ l for glass-bottomed dishes.
- v. Change half volume of the medium every 3–4 days; at the beginning, medium changing may be needed every 2 days depending on the medium color. Never leave differentiated cells drying with no medium. Drug treatments should be done in the last two weeks before endpoint analyses.

▲CRITICAL STEP ReN cell differentiation in thin-layer 3D cultures can be closely monitored by optical and fluorescence microscopy. Some of the cultures can be fixed with 4% paraformaldehyde at 2–4 weeks and tested for neural marker expressions by immunofluorescence staining or immunohistochemistry.

Endpoint analysis

- 35| The following options describe processing of the 3D culture to produce DNA, RNA, protein samples or fixed cultures, as required for further analysis. To extract RNA from a thick layer culture, follow option A. To extract RNA from a thin layer culture, follow option B. To fix thin layer 3D cultures for immunofluorescence/histochemical/Amylo-Glo staining, follow option C. To quantitate A β and p-tau levels using biochemical analyses, initially follow option D to prepare a matrigel cell pellet. Alternatively, proceed directly to step 36 for further analyses of the collected 3D culture media with A β ELISA and LDH release assay..

(A) RNA Extraction from thick layer 3D cultures ● TIMING 1–2 h—▲CRITICAL

Extraction from one thick layer culture insert usually yields enough RNA for quantitative RT-PCR analyses.

- i. Place the cell culture dish on ice. Using forceps, carefully take out the inserts and place them on top of a RNase-free micro-centrifuge tube.
- ii. Release the 3D Matrigel culture from the insert by cutting a circle around the outermost edge of the bottom of the insert. Place the insert back on top of the micro-centrifuge tube and centrifuge at 3,200 g (Beckman Coulter cat. no. 367160) for 30 sec at room temperature (~24 °C).

▲CRITICAL STEP Do not cut a full circle around bottom. Leave an overhang of ~1 mm, thereby keeping the membrane of the insert attached to the insert. Be sure that the resulting opening is large enough to release the Matrigel into the micro-centrifuge tube. Be aware that the insert is not placed loosely as it might fall down during centrifugation.

- iii. Gently remove the media supernatant by pipetting carefully as to not disrupt or remove parts of the Matrigel.
- iv. Resuspend the Matrigel with one half volume of cold RNase-free D-PBS.
▲CRITICAL STEP Cut a 1,000 μ L RNase-free pipette tip with RNaseZap®-rinsed scissors to obtain an opening of 2mm in diameter.
- v. Centrifuge the resuspended 3D culture at 12,900 g (Beckman Coulter cat. no. 367160) for 5 min at 4 °C. Remove the supernatant.
■ PAUSE POINT The resulting pellet can be directly processed or stored at –80 °C for several months.
- vi. Add 350 μ L Buffer RLT to the pellet and mix thoroughly by repeatedly pipetting up and down. Transfer the lysate into a QIAshredder spin column and centrifuge at 12,900 g (Beckman Coulter cat. no. 367160) for 1 min at room temperature.
▲CRITICAL STEP Add 10 μ L 2-Mercaptoethanol per ml Buffer RLT before use. Make sure that the Matrigel/cell mixture is thoroughly disrupted and homogenized. The viscosity should be greatly reduced to achieve a homogenous lysate as failure in doing so may result in the clogging of the QIAshredder spin column and reduced RNA concentration and purity. The steps using the RNeasy Mini spin column protocol should be done at room temperature.
- vii. Perform RNA extraction according to the manufacture's protocol using the RNeasy Mini Kit. Measuring the RNA concentration using the NanoDrop 1,000 Spectrophotometer.
■ PAUSE POINT The isolated RNA can be directly processed or stored at –80 °C for 6 months.

(B) RNA extraction from thin layer 3D cultures—▲CRITICAL While extraction from one thick layer culture insert usually yields enough RNA for quantitative RT-PCR analyses. If needed, pool together 2–3 wells from thin layer cultures (96-well format).

- i. Place the cell culture dish on ice. Do not remove the media but resuspend the thin layer 3D culture with the cell culture media. Transfer the mixture into an RNase-free micro-centrifuge tube and place on ice.
- ii. Clean each well by adding 100–400 μ L cold RNase-free D-PBS. Repeatedly pipette up and down and transfer the resulting lysate into the same micro-centrifuge tube.
▲CRITICAL STEP Observe the cell culture plate under the tissue culture microscope for any remaining sample and repeat the D-PBS step if necessary. Centrifuge at 12,900 g (Beckman Coulter cat. no. 367160) for 5 min at 4 °C.
- iii. Gently remove the supernatant by pipetting. Resuspend the pellet in one half volume cold RNase-free D-PBS of the resulting pellet. Centrifuge at 12,900 g (Beckman Coulter cat. no. 367160) for 5 min at 4 °C. Remove the supernatant.

■ **PAUSE POINT** The resulting pellet can be directly processed or stored at -80°C for more than several months.

- iv. Add 350 μL Buffer RLT to the pellet and mix thoroughly by repeatedly pipetting up and down. Transfer the lysate into a QIAshredder Spin Column and centrifuge at 12,900 g (Beckman Coulter cat. no. 367160) for 1 min at room temperature.
- v. Perform RNA extraction according to the manufacture's protocol using the RNeasy
- vi. Mini Kit. Measure the RNA concentration using the NanoDrop 1000 Spectrophotometer.

■ **PAUSE POINT** The resulting pellet can be directly processed or stored at -80°C for 6 months.

(C) Fixation of thin-layer 3D cultures

- i. Wash cell layer once with D-PBS.
- ii. Add 100 μL of 4% paraformaldehyde solution per each well and incubate at room temperature overnight.
- iii. Remove the paraformaldehyde solution and wash three times with D-PBS.

■ **PAUSE POINT** The fixed 3D culture plate can be stored at 4°C for 6 months. Make sure to tightly wrap the plates to avoid evaporation of the D-PBS in each well.

(D) Preparation of Matrigel/cell pellet from thick layer 3D cultures ● TIMING 1 h

- i. Place the cell culture dish on ice. Using forceps, carefully take out the inserts and place them on top of a micro-centrifuge tube.
- ii. Release the 3D Matrigel culture from the insert by cutting a circle around at the outermost edge of the bottom of the insert. Place the insert back on top of the micro-centrifuge tube and centrifuge at 3,200 g (Beckman Coulter cat. no. 367160) for 30 sec at room temperature ($\sim 24^{\circ}\text{C}$).

▲ **CRITICAL STEP** Do not cut a full circle around bottom. Leave an overhang of ~ 1 mm, thereby keeping the membrane of the insert attached to the insert. Be sure that the resulting opening is large enough to release the Matrigel into the micro-centrifuge tube. Be aware that the insert is not placed loosely as it might fall down during centrifugation.

- iii. Remove the insert and centrifuge again at 8,000 g for 5 min at 4°C .
- iv. Retain the pellet (Matrigel/cell pellet).

■ **PAUSE POINT** The Matrigel/cell pellet can be frozen and stored at -80°C for several months

- 36| If you have extracted RNA, proceed if desired to RT-PCR (option A), qRT-PCR (option B) or 3R/4R Tau analysis (option C). If you have fixed sections, proceed

to immunofluorescence (option D), immunohistochemistry (option E) or Amylo-Glo staining (option F). For biochemical analyses of A β peptide and p-tau, follow options G-K. For EM analysis of Sarkosyl insoluble tau fibrils, first perform Sarkosyl extraction (option I) and proceed to immuno EM (option L). The protocols for A β ELISA and LDH release assay from 3D culture media are described in option M and N.

? TROUBLESHOOTING

(A) RT-PCR ● TIMING 8 h

- i. Perform cDNA synthesis according to the manufacture's protocol using the SuperScript[®]III first-strand synthesis system and the Oligo(dT)₂₀ primer. Use the same total amount of RNA for each sample.

■ **PAUSE POINT** Synthesized cDNA can be directly processed in downstream application or stored at −20 °C for later use.

- ii. Perform a PCR amplification step for the relative 3R/4R tau expression analysis in a final volume of 20 μ L per reaction. To prepare a Master Mix (1 Reaction), place the following components into an RNase-free micro-centrifuge tube and mix by pipetting. Scale-up the volume of the reaction mixture according to the number of reaction to be performed.

Component	Amount	Final concentration
3R4R tau forward primer (10 μ M)	1 μ L	500 nM
3R4R tau reverse primer (10 μ M)	1 μ L	500 nM
SYBR [®] Select Master Mix (2X)	10 μ L	1X
ddH ₂ O	7 μ L	

▲ **CRITICAL STEP** Mix and briefly spin the reaction tubes to ensure components are at the bottom of the tubes without bubbles.

- iii. Add 1 μ L cDNA per reaction. Quickly centrifuge the samples and perform the PCR amplification step under the following conditions using the Bio-rad Tetrad[®] 2 thermal cycler:

Cycle number	Denature	Anneal	Extend	Final
1	95 °C, 15 min			
2–31	94 °C, 30 s	60 °C, 30s	74 °C, 90 s	
32			74 °C, 10 min	
33				4 °C

- iv. Analyze RT-PCR products by agarose-gel electrophoresis using a 2% w/v agarose gel in 1x TBE buffer. To prepare samples, mix 15 μ L of the RT-PCR product with

3 μ L DNA loading dye (6x). Load 18 μ L into the gel pockets. For size estimation of the products, load 5 μ L Quick-Load[®] 2-Log DNA ladder.

- v. Using a 150 mL agarose gel, run the electrophoresis at 150 V for 50 min to separate the RT-PCR products.
- vi. Image RT-PCR product using Biorad ChemiDoc XRS System.

(B) qRT-PCR for neuronal markers ● TIMING 8 h

- i. To prepare a Master Mix (1 Reaction), place the following components into an RNase-free micro-centrifuge tube and mix by pipetting. Scale-up the volume of the Master Mix according to the number of reactions to be performed.

Component	Amount	Final concentration
Neural marker primers F+R (5 μ M)	0.4 μ L	0.1 μ M
SYBR [®] Select Master Mix (2x)	10 μ L	1X
ddH ₂ O	8.96 μ L	

- ii. Aliquot 19 μ L of Master Mix into each well of a 96-well plate and then add 1 μ L of cDNA.
- iii. Use β -actin or GADPH as the housekeeping gene (same Master Mix as neural marker primer).
- iv. Seal the plate with a film and roll it.
- v. Centrifuge down the plate at 2,000 g (Sorvall Legend T+, Thermo Scientific, cat. no. 75004367) for 2 min.

▲CRITICAL STEP Check if there are bubbles. If present, try to remove them by flicking the tubes and centrifuge again.

- vi. Perform the PCR amplification step under the following conditions using the Bio-rad[®]

Table 7

Cycle number	Denature	Anneal	Extend	Final
1	95 °C, 10 min			
2–62	95 °C, 10 s	58 °C, 45s	72 °C, 30 s	
Melting curve	95 °C, 60s	55 °C, 60s followed by 55 + 0.5 °C, 10s each		
Hold				4 °C

- vii. Determine the threshold cycles of the each gene using the iCycler IQ software (Bio-rad). Normalize gene expression levels against β -actin levels in each sample and calculate the percentage changes using the 2^{-CT} method as previously described⁴⁴.

(C) qRT-PCR for 3R/4R Tau ● TIMING 8 h

- i. To prepare a Master Mix (1 Reaction) for 3R or 4R Tau, add the components in the table into an RNase-free microcentrifuge tube and mix by pipetting. Scale-up the volume of the Master Mixes according to the number of reactions to be performed.

Component	Amount	Final concentration
Tau (3R or 4R) forward primer (10 μ M)	1 μ L	500 nM
Tau (3R or 4R) reverse primer (10 μ M)	1 μ L	500 nM
SYBR [®] Select Master Mix (2x)	10 μ L	1X
ddH ₂ O	7 μ L	

- ii. Aliquot 19 μ L of Master Mix in each well of a 96 well plate and then add 1 μ L of cDNA.
- iii. Use β -actin or GADPH as the housekeeping gene (same Master Mix as neural marker primer).
- iv. Seal the plate with a film and roll it.
- v. Centrifuge the plate at 2,000 g (Sorvall Legend T+, Thermo Scientific, cat. no. 75004367) for 2 min.

▲CRITICAL STEP Check if there are bubbles. If present, try to remove them by flicking the tubes and centrifuge again.

- vi. Perform the PCR amplification step under the following conditions using the Bio-rad[®] iCycler:

Cycle number	Denature	Anneal	Extend	Final
1	95 °C, 10 min			
2–62	95 °C, 10 s	58 °C, 45s	72 °C, 30 s	
Melting curve	95 °C, 60s	55 °C, 60s followed by 55 + 0.5 °C, 10s each		
64				
65				
Hold				4 °C

- vii. Determine the threshold cycles of the each gene using the iCycler IQ software (Bio-rad). Normalize gene expression levels against β -actin levels in each sample and calculate the percentage changes using the 2^{-CT} method as previously described⁴⁴.

(D) Immunofluorescence staining of thin-layer 3D culture ● TIMING 2 d—?
TROUBLESHOOTING

- i. Permeabilize the fixed 3D cultures by incubating with 200 μ l of TBST containing 0.5% Triton X-100 and 4% goat IgG at room temperature for 1 h.
- ii. Block the culture by incubating with 200 μ l of blocking/dilution solution supplemented with 4% goat IgG at 4 °C overnight with gentle rocking.
- iii. Wash with TBST once for 10 min.
- iv. Incubate with primary antibodies (see Table 1) in blocking/dilution solution with 4% goat IgG at 4 °C overnight with gentle rocking.
- v. Wash with TBST 5 times for 10 min per washing.
 ■ **PAUSE POINT** This step can be extended by performing one of the 5 washes as an overnight washing at 4 °C.
- vi. Incubate with AlexaFluor secondary antibodies for 5 h at room temperature with gentle rocking (see Table 1).
 ▲ **CRITICAL STEP** The signals from AlexaFluor350 antibodies are relatively weak, especially in 96-well plates due to the UV absorption by plastic-based materials in these plates. Glass-bottomed dishes will perform better for AlexaFluor350 antibody staining. Use of Cy5-labeled antibodies may be another option.
 ■ **PAUSE POINT** This step can be extended to overnight at 4 °C.
- vii. Add a drop of anti-fade gold on top of the fixed/stained thin-layer 3D culture sections to avoid fluorescence quenching before imaging.
- viii. Capture the fluorescence images using an Olympus DSU confocal microscope; perform image analysis and 3D reconstitution. We used ImageJ (NIH), IPLabs (IP Labs) or MetaMorph (Olympus).

(E) Immunohistochemical staining of thin-layer 3D culture ● TIMING 2 d—? TROUBLESHOOTING

- i. Permeabilize the fixed 3D cultures by incubating with 200 μ l of TBST containing 0.5% Triton X-100 and 4% goat IgG at room temperature for 1 h.
- ii. Block the culture by incubating with 200 μ l of blocking/dilution solution supplemented with 4% goat IgG at 4 °C overnight with gentle rocking.
- iii. Incubate the cultures with 0.3% (vol/vol) H₂O₂ solution in TBS for 5 min at room temperature to block endogenous peroxidase activities.
- iv. Wash with TBST for 10 min 3 times.
- v. Incubate with primary antibodies (see Table 1) in blocking/dilution solution with 4% goat IgG at 4 °C for 1 day with gentle rocking.
 ▲ **CRITICAL STEP** We found that BA27 (A β 40) and BC05 (A β 42) antibodies work well to detect A β deposits in 3D culture with minimal background. For BA27,

we recommend overnight incubation at 4 °C, while for BC05, a 2 h room temperature incubation, to avoid over-staining.

- vi. Wash with TBST for 10 min 5 times.
- vii. Incubate with ImmPRESS anti-mouse or -rabbit Ig HRP polymer conjugates (1:1 diluted with blocking/dilution solution supplemented with 4% goat IgG) at room temperature for 30 min.
- viii. Wash with TBST for 10 min 5 times.
- ix. Develop the antibody signal using DAB peroxidase substrate kits.
- x. (Optional) Counterstain the DAB-stained cultures with Hematoxylin.

(F) Amylo-Glo staining of thin-layer 3D culture ● TIMING 2 d—?

TROUBLESHOOTING

- i. Wash the culture wells 3 times with 0.9% (w/v) NaCl solution.
- ii. Add 100 µl of 0.02x Amylo-Glo working solution and incubate for 5 min at room temperature.

▲ **CRITICAL STEP** Amylo-Glo over-stains easily. Therefore, we recommend using 0.02x Amylo-Glo working solution to avoid the background. If the signal is too weak, try the 0.1x solution instead.
- iii. Remove the staining solution.
- iv. Add 200 µl of 0.9% saline and incubate for 5 min.
- v. Wash the wells 3 times with ddH₂O.
- vi. (Optional) Add 200 µl of propidium iodide nuclear counter staining solution and incubate for 5 min.
- vii. Wash 3 times with 0.9% (w/v) NaCl solution.
- viii. Add 100 µl of Anti-fade gold on top of the wells.
- ix. Image the wells under 4x, 10x, and 40x magnification.

(G) TBS/SDS/Formic acid extraction of thick-layer 3D culture ● TIMING 2 d

- i. Thaw the Matrigel/cell pellet (from step 35, option D) on ice for 10 min.
- ii. Add same volume of 2x TBS extraction buffer (~100–200 µl in general).
- iii. Homogenize using disposable-tip rotor-driven homogenizer 10 times up and down. Gels should become clear/mildly cloudy.

▲ **CRITICAL STEP** Do not lift tip too far up the tube at risk of losing sample.
- iv. Sonicate for 10 min at 4 °C using a sonic cleaning water bath.
- v. Spin down at 100,000 g (Beckman, Optima TL Ultracentrifuge) for 1 h at 4 °C.

- vi. Retain the supernatant as TBS-soluble fractions and measure protein levels. Freeze the TBS-soluble fractions at -20°C .
- vii. Homogenize the pellet with 50 μl of 1x SDS extraction buffer using rotor-driven homogenizer and then sonicate for 10 min at 4°C .
- viii. Spin down at 100,000 g (Beckman, Optima TL Ultracentrifuge) for 1 h at 4°C .
- ix. Save the supernatant as TBS-insoluble/2% SDS-soluble fractions and freeze at -20°C .
- x. Wash the pellet briefly with 100 μl 1x SDS extraction buffer.
- xi. Extract the pellet with 10 μl of 70% formic acid on ice.
- xii. Centrifuge for 1 h at 100,000 g (Beckman, Optima TL Ultracentrifuge) and collect the supernatant (TBS-insoluble/2% SDS-insoluble/formic-acid-soluble fractions).
- xiii. (Optional) Evaporate formic acid in SpeedVac to concentrate the formic acid fractions.
- xiv. Neutralize the formic acid samples by adding 10 volumes of 2M Tris. Mix with a quarter volume of 4x LDS buffer with 8% β -mercaptoethanol.
- xv. Run SDS-PAGE of desired fractions as described in option H.

(H) SDS-PAGE and Western Blot Analysis of A β ● TIMING 2 d—?

TROUBLESHOOTING

- i. Run the extracted samples from option G on 12% NuPAGE gel.
- ii. Transfer proteins from the SDS-PAGE gels to PVDF membranes.
▲CRITICAL STEP For A β detection, incubate the membrane with 0.5% glutaraldehyde solution for 10 min before blocking.
- iii. Block the membrane with SuperBlock blocking solution for 1 h at room temperature.
- iv. Add primary antibody solution (in 4% BSA) and incubate at 4°C overnight with rocking (for primary antibody concentration, please see table 2).
- v. Wash with TBST 3 times.
- vi. Add secondary antibody solution and incubate for 1 h at room temperature with gentle rocking.
- vii. Wash with TBST 3 times.
- viii. Develop the blot with SuperSignal Dura or Femto ECL solutions.

(I) Sarkosyl Extraction of thick-layer 3D culture ● TIMING 2 d

- i. Thaw the Matrigel/cell pellet (from step 35, option D) on ice for 10 min.
- ii. Add same volume of 2x TBS extraction buffer ($\sim 100\ \mu\text{l}$ in general).

- iii. Homogenize using disposable-tip rotor-driven homogenizer 10 times up and down.
- iv. Sonicate for 10 min at 4 °C using a sonic cleaning water bath.
- v. Mix the TBS-extracted samples with equal volume of 2x RIPA buffer.
- vi. Homogenize using rotor-driven homogenizer on ice.
- vii. Incubate on ice for 15 min.
- viii. Sonicate 2 times for 5 min each.
- ix. Centrifuge at 10,000 g with a tabletop centrifuge for 5 min at 4 °C.
- x. Transfer the supernatant into a new tube.
- xi. Add 80 µl of 1x RIPA to the pellets in the tubes, resuspend by pipetting and sonication.
- xii. Centrifuge at 10,000 g with a tabletop centrifuge for 5 min at 4 °C.
- xiii. Mix the two supernatant samples from x and xii.
- xiv. (optional) The RIPA-insoluble pellets can be further extracted with a high-salt extraction buffer. Follow the same protocols (xi–xii) and proceed to xv.
- xv. Add 1/20th volume of 20% Sarkosyl solution to the supernatant and incubate at room temperature for 60 min in rotary mixer.
- xvi. Centrifuge at 150,000 g (Beckman, Optima TL Ultracentrifuge) for 1 h at 4 °C.
- xvii. Transfer the supernatant into new tubes and measure protein levels (Sarkosyl-soluble fractions).
- xviii. Wash the pellet briefly with 100 µl 2x RIPA buffer once.
- xix. Wash the pellet with 0.5 ml PBS 3 times (Sarkosyl-insoluble fractions).
 - ▲ **CRITICAL STEP** Avoid disrupting the pellets during the PBS washing.
- xx. Add 1x LDS sample buffer with 10 M Urea and 2% β-mercaptoethanol.
- xxi. Heat at 95 °C for 5 min.
- xxii. Run SDS-PAGE as described in option J, or dot blot as described in option K on a small sample prior to TEM as described in option L.

(J) SDS-PAGE and Western Blot Analysis of p-tau ● TIMING 2 d—?

TROUBLESHOOTING

- i. Run on 4–12% gel for p-tau analysis.
- ii. Transfer proteins from the SDS-PAGE gels to PVDF membranes.
- iii. Block the membrane with either 4% BSA or SuperBlock blocking solution for 1 h at room temperature.

▲ **CRITICAL STEP** Do not use skim milk for blocking p-tau blot since it contains phosphatase.

- iv. Add primary antibody solution and incubate at 4 °C overnight with rocking (for primary antibody concentration, please see table 2).
- v. Wash with TBST for 10 min 3 times.
- vi. Add secondary antibody solution and incubate for 1 h at room temperature with gentle rocking.
- vii. Wash with TBST for 10 min 3 times.
- viii. Develop the blot with SuperSignal Dura or Femto ECL solutions.

(K) Dot Blot Analysis ● TIMING 2 d—▲CRITICAL Dot blot analysis can be a useful for quickly testing p-tau levels before EM analysis.

▲CRITICAL Users of vacuum-based systems will need to modify these steps.

- i. Wet the nitrocellulose membrane in 1X TBS buffer for 5 min.
- ii. Locate the membrane on top of a pre wetted Bio-rad blotting paper.
- iii. Load 0.5–1 µl of protein samples.
- iv. (optional) A dot blot kit can be also used for loading the samples.
- v. Incubate the membrane for 20 min until the protein samples are completely absorbed into the membrane.
- vi. Block the membrane with either 4% BSA or SuperBlock blocking solution for 1 h at room temperature.
- vii. Add the primary antibody solution and incubate at 4 °C overnight with rocking (for primary antibody concentration, please see table 2).
- viii. Wash with TBST for 10 min 3 times.
- ix. Add the secondary antibody solution and incubate for 1 h at room temperature with gentle rocking.
- x. Wash with TBST for 10 min 3 times.
- xi. Develop the blot with SuperSignal Dura or Femto ECL solutions.

(L) Immunogold staining of Sarkosyl-insoluble tau for Transmission Electron Microscopy (TEM) analysis ● TIMING 3 h—? TROUBLESHOOTING

- i. Resuspend the Sarkosyl-insoluble fractions in 20–40 µl of PBS (from option I). The volume will depend on the concentration of the fibrils in the fraction obtained).
- ii. Pipet 7–10 µl of the resuspended samples onto formvar-carbon coated 200 mesh Ni grids held in forceps in a dry and dust-free container.
- iii. Allow the sample to adsorb onto grid for 10–20 min. Do not allow grids to dry. Adjust adsorption time for smaller sample volumes.

- iv. Blot or wick away sample droplets and place grids directly on top of 15 μ L drops of primary antibody (mouse-anti-tau46) diluted 1:25 or 1:50 in antibody diluents. Incubate in a humidified chamber for 1 h.
- v. Rinse grids on drops of 1x PBS for 5 min 3 times.
- vi. Blot and place grids on top of 15 μ L drops of secondary antibody (goat-anti-mouse IgG 10 nm gold) diluted 1:15 in antibody diluents. Incubate in the humidified chamber for 1 h.
- vii. Blot and rinse grids on drops of distilled water for 5 min 3 times.
- viii. Blot and place grids on drops of 2% aqueous phosphotungstic acid for 1 min.
- ix. Blot and transfer grids to drops of distilled water briefly to rinse.
- x. Blot and allow to air-dry before proceeding with TEM analysis.
- xi. We examine grids in a JEOL JEM 1011 transmission electron microscope at 80 kV. We collect digital images using an AMT digital imaging system.

(M) Media harvesting protocol for A β peptide quantification

- i. Collect culture media either from the inserts of the thick layer cultures or from the wells of the thin layer cultures.
▲CRITICAL STEP If the cell differentiation is on-going, be careful to collect the media in sterile conditions, using sterile tips and pipettes under the hood. If the differentiation time is at the end and the cell cultures are ready to be used for different analyses, place the plates on ice during the collection of the media.
- ii. Collect half volume of media for each insert/well in a micro centrifuge tube.
- iii. Spin down the media at 4 °C for 5 min at 6,000 g (Beckman Coulter, cat. no. 367160).
- iv. Collect supernatant in a new tube.
- v. Run 30 μ L on SDS-PAGE or load 100 μ L in a WAKO A β 40/42 ELISA kit 96-well plate.
■ PAUSE POINT The remaining media can be stored at –80 °C for several months.

(N) Lactate dehydrogenase (LDH) assay of thick and thin-layer 3D culture media

● **TIMING 2 d**—▲**CRITICAL** In the control ReN cell 3D cultures, we found that levels of LDH release are below 3% of total LDH levels.

- i. Collect culture media from the plastic inserts of thick-layer cultures and/or the wells of thin-layer cultures.
- ii. (Optional) Add 100 μ L of lysis buffer (provided by Cytotox kit) to a couple of wells and incubate 30 min at 37 °C. This is used to determine total LDH levels.

- iii. Transfer 100 μ l of each culture media sample to a 96-well white, opaque assay plate.
- iv. Add 100 μ l CytoTox-ONE reagent to each sample.
- v. Mix by gently pipetting up and down and incubate for 30 min to 1 h at room temperature.
- vi. Measure fluorescence signal (Ex 560 nm / Em 590 nm) using a Biotek Synergy 2 microplate reader powered by Gen5 analysis software.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

ANTICIPATED RESULTS

For reproducible output, we found that it is very important to plate a high number of cells in order to achieve early A β aggregation. In addition, the passage number and the condition of the cells are also important. Especially for the highly enriched cell lines expressing APPSL and PSEN1 E9, we strongly recommend using only cells with very early passage numbers (up to 4) to ensure that a robust A β and tau pathology is consistently observed. FAD ReN cell lines with high A β 42/40 ratio will show denser amyloid deposits in 3D culture (e.g. ReN-mGAP cells that express both GFP:APPSL and mCherry:PSEN1 E9 as shown in Fig. 5d and 7; ReN-mAP cells that express mCherry:APPSL:PSEN1 E9 in Fig. 8; HReN-mGAP (enriched) cells that express both GFP and mCherry:APPSL:PSEN1 E9 in Fig. 9 and supplementary fig. 2) while the cell lines with mid/low A β 42/40 ratio will develop mostly diffuse or no obvious deposits despite these cells having secreted high levels of total A β (e.g. ReN-GA2 cells in Fig. 5b–d). In general, we observed that the ReN cells secreting high levels of A β , preferentially A β 42, develop more robust p-tau pathology.

To enhance A β and p-tau pathologies, we used FACS sorting to enrich ReN cells expressing high levels of transgene. As shown in Fig. 3, FACS sorting of the top 2, 10 or 50% levels of GFP signals (Fig. 3a) lead to the generation of ReN-GA cells (APPSL/GFP) expressing high, mid and low APPSL/GFP (ReN-GA2, -GA10 and -GA50; Fig. 3b). The control ReN-G cells with comparable levels of GFP expression were also prepared (ReN-G2, -G10 and -G50; Fig. 3a–b). As expected, ReN-GA2 and the control ReN-G2 cells differentiated comparably in the thin-layer 3D culture condition into neurons and glial cells (Fig. 6). Western blot analysis (Fig. 5a) and A β ELISA (Fig. 5b–d) showed robustly elevated A β levels in ReN-GA2 cells as compared to the control ReN-G2 cells. A β ELISA also detected an elevated level of A β in conditioned medium collected from 1-week differentiated thick-layer 3D cultures of ReN-GA2 cells (Fig. 5c). However, the A β 42/40 ratio was moderately increased in these cells because they do not express PSEN1 E9, which alters γ -secretase structures to produce more A β 42. As a result, we observed that ReN-mGAP (enriched) cell lines displayed much more robust A β deposits as compared to ReN-GA (enriched) cells (Fig. 5d). Similar results were also observed in ReN-mAP (enriched) cell lines (data not shown).

The quality of the ReN cell 3D cultures can be monitored by LDH releases assay, qRT-PCR/RT-PCR analyses of adult neural marker expression and immunofluorescence staining (as shown in Fig. 6). Based on immunofluorescence staining, we estimate that ~68% ReN cells differentiate into MAP2-positive neurons and ~30% into GFAP-positive astrocytes following 2–4 weeks of 3D differentiation. We also found a robust increase in adult neuronal markers, particularly 4-repeat adult tau isoforms in 3D culture conditions, which are essential for replicating tauopathy in human neuronal cultures¹⁴.

With the proper cell density and passage number, extracellular β -amyloid deposits start developing after 6 weeks in FAD ReN cell 3D cultures with high levels of A β 42 (Fig. 7) and the number and size of β -amyloid aggregations increases with longer culture. Amylo-Glo⁴⁵, a fluorescent amyloid dye can also be used to detect A β deposits, particularly after 8-weeks differentiation (Supplementary Fig. 2). Accumulation of p-tau in cell bodies and neurites can be detected by immunostaining with p-tau antibodies, including AT-8 and PHF-1, after 8 weeks (Fig. 8) while robust accumulation of p-tau aggregates are detected after 10–14 weeks¹⁴. PHF-like tau filaments can be detected after 14 weeks in Sarkosyl-insoluble fractions of FAD ReN cells with highly robust A β levels (preferentially A β 42) by electron microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Polycistronic lentiviral vectors used in this study

Diagrams showing lentiviral internal ribosome entry sites (IRES) constructs that were designed to express human APP with *Swedish/London* FAD mutations (APPSL) along with coexpressed green fluorescent protein (GFP), mCherry, PSEN1 with E9 FAD mutation (PSEN1 E9) mCherry tagged, or both APPSL and PSEN1 E9 FAD mutations together with mCherry. CMV, cytomegalovirus promotor.

Figure 2. Overview of ReN VM cell 3D culture protocol
The experimental procedure begins with the generation of human neural progenitor cell lines virally transfected with APP and/or PSEN1 FAD mutations and enriched based on GFP and/or mCherry signals by fluorescence-activated cell sorting (FACS). The right-hand column shows in detail the timing for each step of the 3D culture method. Day 0 indicates the day on which cells are mixed with Matrigel. MAP2, microtubule associated protein 2; 4R tau, 4-repeat tau isoform; vGluT1, Vesicular glutamate transporter 1; NR2B, N-methyl D- aspartate receptor subtype 2B; TH, Tyrosine Hydroxylase.

Figure 3. FACS (Fluorescence-activated cell sorting) enrichment of ReN-G and -GA cells for higher expressions of APP with FAD mutations

a. The transfected ReN cells stably expressing GFP alone (ReN-G) were enriched by FACS based on the GFP signal intensity. Rectangular boxes indicate the cell populations that were selected/enriched for the experiments. ReN-G50 or 50, ReN-G cells with the top 50% GFP signal intensity; ReN-G10 or 10, ReN-G cells with the top 10%; ReN-G2 or 2, ReN-G cells with the top 2%; fsc, forward scatter which is roughly size/diameter; ssc, side scatter which is granularity. **b.** The transfected ReN cells stably expressing APPSL/GFP (ReN-GA) were

enriched by FACS based on the GFP signal intensity. ReN-GA50 or 50, ReN-GA cells with the top 50% GFP signal intensity; ReN-GA10 or 10, ReN-GA cells with the top 10%; ReN-GA2 or 2, ReN-GA cells with the top 2%. **c.** Representative confocal fluorescence images showing ReN-G and ReN-GA cells with the top 2% (Ren-G2 and Ren-GA2), 10% (Ren-GA10) and 50% (ReN-GA50) GFP signal intensity. Green, GFP; Scale bar, 25 μ m. **d.** The confocal fluorescence microscope images of enriched ReN-G2, -GA2 cells that were differentiated by growth-factor deprivation for 4 weeks (Green, GFP; Scale bar, 25 μ m).

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Figure 4. Various 3D culture formats

a. A 24-well plate with inserts yields the thick-layer cultures ideal for molecular, biochemical, and ELISA analyses. **b–d.** For thin culture, the 96-well plate (**b**), 35 mm dishes (**c**), and 8- or 16-chambered cover glass compartments (**d**) offer glass-bottom options that facilitate high-resolution immunofluorescence staining.

Figure 5. Analysis of soluble Aβ levels in the enriched FAD ReN cells by Western Blot and Aβ ELISA

a. Western Blot of Aβ levels in the conditioned medium collected from undifferentiated enriched ReN-G and -GA cells. The conditioned medium was collected from T25 flask after culturing the cell for 2 days. 6E10 anti-Aβ detected ~4 kDa Aβ monomer bands in the media collected from the enriched FAD ReN cells (ReN-GA10, ReN-GA2). The 6E10 antibody also detected sAPPα (~100 kDa), which would represent the amount of full-length APPs in these cell lines. **b.** Increased Aβ40 and 42 levels in undifferentiated FAD ReN-GA2 cells, detected through Aβ ELISA (***, $p < 0.001$; Student t-test; $n = 3$ per each sample). **c.** Increased Aβ40 and 42 levels were also detected in a conditioned media collected from 1-week old thick-layer 3D cultures of ReN-GA2 cells (***, $p < 0.001$; Student t-test; $n = 3$ per each sample). The soluble Aβ levels cannot be directly compared to the levels shown in (b) since the total cell numbers of thick-layer cultures are higher than 2D undifferentiated cultures used in (b). **d.** Soluble Aβ levels of the various enriched FAD ReN cells. The FAD cells with PSEN1 E9 showed elevated Aβ42/40 ratio as compared to the enriched ReN-GA cells.

Figure 6. Images of ReN-G2-differentiated neural cell populations in 3D cultures after 2- and 4-week differentiation

Representative images of 2-week (upper panels) or 4-week (lower panels) differentiated ReN-G2 cells (**a** and **d**) that are co-labeled with MAP2 (**b** and **e**). The overlay images (**c** and **f**) demonstrate that most ReN-G2 cells differentiated in 3D cultures show neuronal properties. Scale bar: 25 μm.

Figure 7. Reconstitution of amyloid- β aggregates in 3D-cultured FAD ReN cells
a–f. IHC of amyloid- β deposition, detected by anti-A β 40 BA27 antibodies, in control (ReN-G cells, **a–c**) and FAD ReN (ReN-mGAP (enriched), **d–f**) cells. Arrowheads indicate amyloid- β aggregates. **g–i.** Enlarged images of amyloid- β deposition in ReN-mGAP cell cultures. Scale bars: 50 μ m.

Figure 8. p-Tau accumulation in cell body and neurites of the enriched ReN-mAP cells
a–e. Immunofluorescence of p-tau, detected by PHF-1 antibody, in the ReN-m control (**a**) and the enriched ReN-mAP cells (**b–e**) after 3D differentiation. **b–e.** Select confocal Z-stack images of p-tau in the soma and neuritis of ReN-mAP cells in 3D cultures. Z-sections were capture in 0.4 μm intervals. The ReN-mAP (enriched) and control ReN-m cells were 3D-differentiated for 8 weeks. Scale bars: 25 μm .

Figure 9. Detection of aggregated p-tau in the enriched ReN-G and HReN-mGAP cells

a. Western blot analyses of p-tau and total tau levels in 1% Sarkosyl-soluble and -insoluble fractions prepared from 3D cultures of 8-week 3D-differentiated ReN-mGAP (enriched) cells and control ReN-G cells. ReN-mGAP (enriched) cell line is one of the FACS-enriched cell lines that stably express both GFP:APPSL and mCherry:PSEN1 E9 constructs. **b.** Dot blot analyses of p-tau and total tau in Sarkosyl-insoluble fractions prepared from ReN-G, ReN-mGAP and ReN-mGAP (enriched) cells (8-week 3D differentiated).

Table 1

Antibodies used for immunofluorescence and immunohistochemistry.

Useful markers for	Antibody	Host	Supplier	Cat. No.	Dilution (I.F.)	Dilution (I.H.C.)
Neuron (synaptic)	anti-NR2B	Mouse	NeuroMab	N/59/36	1:100	
Neuron (synaptic)	anti-GluR2	Mouse	NeuroMab	L21/32	1:100	
Neuron (neuritic)	anti-beta-tubulin III	Rabbit	Abcam	ab24629	1:200	1:100
Neuron (dendritic)	anti-MAP2	Rabbit	Millipore	8717	1:400	
Neuron (dendritic)	anti-MAP2	Rabbit	Cell Signaling Technology	4542	1:200	
Neuron (dendritic)	anti-MAP2	Chicken	EMD Millipore	AB5543	1:500	
Neuron (dendritic)	anti-GFAP	Mouse	Antibodies Incorporated	N206A/8	1:200	
Astrocyte	anti-GFAP	Rabbit	DAKO	Z0334	1:2000	
Astrocyte	anti-GFAP	Rabbit	DAKO	Z0334	1:2000	
Neuronal	anti-GABAR2	Rabbit	Cell Signaling Technology	4819	1:100	
Neuron (dopaminergic)	anti-Tyrosine Hydroxylase	Rabbit	Cell Signaling Technology	2792	1:100	
p-Tau	AT8	Mouse	Thermo Scientific	MN1020	1:30	1:20
p-Tau	PHF1	Mouse	A gift from Dr. Davies	N/A	1:2000	1:1500
Total tau	tau46	Mouse	Cell Signaling Technology	4019	1:200	1:200
Aβ	3D6	Mouse	A gift from Lilly	N/A	1:400	
Aβ 42	β-Amyloid (1-42 Specific)	Rabbit	Cell Signaling Technology	D9A3A	1:1600	
Aβ 42	BC05	Mouse	Wako Chemicals USA	299-56701 (A part of Aβ immuno staining kit)		1:1
Aβ 40	BA27	Mouse	Wako Chemicals USA	299-56701 (A part of Aβ immuno staining kit)		1:1
Cy5 secondary antibody	anti-mouse	Goat	Jackson Immunoresearch	715-175-150	1:300	
AlexaFluor350,405, 488, 568 secondary antibodies	anti- mouse,rabbit, chicken	Goat	Life technologies	A-21049/A-21140/A-31553/A-11001/A-11008/A-11004/A-11011/A-11041	1:200-400	

Table 2

Antibodies used for Western Blot analysis.

Useful markers for	Antibody	Host	Supplier	Cat. no.	Dilution
p-tau	AT-8	Mouse	Thermo Scientific	MN1020	1:40
p-tau	PHF-1	Mouse	A gift from Dr. Davis	N/A	1:1000
Total tau	anti-Tau	Rabbit	DAKO	A0024	1:2000
Abeta	6E10	Mouse	Covance	SIG-39300	1:400
Abeta	MOAB-2	Mouse	Biosensis	M-1586-100	1:100
Neuron (dendritic)	anti-MAP2	Rabbit	Cell Signaling Technology	8717	1:200
Neuron (dendritic)	anti-MAP2	Rabbit	EMD Millipore	AB5622	1:500
presenilin	anti-Presenilin 1	Rabbit	Cell Signaling Technology	5643	1:2000
BACE1	anti-BACE1	Rabbit	Cell Signaling Technology	5606	1:1000
Amyloid Precursor Protein	anti-C66 APP C-terminal	Rabbit	A gift from Dr. Kovacs	N/A	1:2000
Neuron	anti-NCAM	Mouse	Cell Signaling Technology	3576	1:1000
Neuron (synaptic)	anti-synapsin I	Rabbit	Cell Signaling Technology	5297	1:500
ER	anti-Calnexin	Rabbit	Cell Signaling Technology	2679	1:1000
Heat shock protein/control	anti-HSP70	Rabbit	Enzo Life Sciences	ADI-SPA-812	1:1000
human Mitochondria	anti-human mitochondrial antigen	Mouse	EMD Millipore	MAB1273	1:500

Table 3

Troubleshooting.

Steps	Problem	Possible reason	Solution
5	Cells do not proliferate and begin to differentiate.	Activity of EGF and/or bFGF is lost.	Avoid multiple freezing/thawing cycle for EGF/bFGF stocks.
10	The transfection rate is low.	Low titer of lentiviral vector, poor cell condition, negative impact on cell proliferation of APPSL or PSEN1(E9).	Increase titer of lentiviral vector. Do multiple transfections with the same lentiviral vector.
18	FACS-sorted cells are not proliferating or apoptotic.	Expression of FAD mutations obtained with the FACS sorting is too high.	1) Plate the FACS-sorted cells in small-scale dishes to increase the cell density (the high cell density helps to accelerate ReN cell proliferation); 2) Enrich cells with lower fluorescence signal intensities.
34 (A) vi	The color of the ReN differentiation medium rapidly changes into yellow in 1–2 days.	The cell density is too high.	1) Change the 3D cell culture medium more frequently in early period (every 2 days); After 2–3 weeks, switch to a normal medium change schedule (every 3–4 days); 2) Reduce the number of cells at step 33.
34 (B) i	ReN cells sink down to the bottom and look like 2D culture.	Matrigel concentration is too low (since each lot of Matrigel has a different protein concentration, the optimal dilution rate should be determined every time).	1) Use different dilution rate to increase the final Matrigel concentration (Even if the cell body seems to be at the bottom, we observed that the Matrigel still formed 3D gels on top of the cells, making 3D neurite outgrowth and displaying A β and tau pathologies. Actually, low Matrigel concentration is desirable for immunohistochemical staining to reduce background). Pre-test 1: 10, 1:15 and 1:20 dilutions and pick the best dilution rate for each lot; 2) Prewarm the plates and move them to 37 °C immediately after plating. This will accelerate the gelation of Matrigel before the cells sink down.
36	A β aggregates/p-tau pathologies are delayed or not evident.	Number of cells is low.	The starting number of cells/ml is between 3–10 million/ml, but number of cells desired is 10 million/ml.
36 (D) (E)	Strong backgrounds	A β 42 levels (A β 42/40 ratio) are not high.	1) Enrich cells further with higher fluorescence signal intensities; 2) Start 3D culture again using high quality culture of cells with earlier passage number or start again from the viral transfection; 3) We also found that additional transfection with PSEN1(E9) vector could further elevate A β levels.
36 (F)	Strong backgrounds (Amylo-Glo)	Non-specific interaction between the primary/secondary antibodies and the fixed Matrigel matrix. Uneven solidification of the Matrigel in thin-layer 3D cultures.	Increase permeabilization, blocking and washing time. Decrease secondary antibody concentrations. Use lower Matrigel concentrations (1:15 or 20) during thin-layer 3D culture generation.
36 (H) (J)	Normalization of total protein levels for Western blot does not fit to control protein levels including GAPDH.	1) The concentration of Amylo-Glo working solution is too high. 2) Presence of autofluorescent aggregates.	Reduce the Amylo-Glo working solution concentration and incubation time. Check the fluorescence background in other fluoropore filters including FITC/Rhodamine.
36 (L)	Poor fibrils imaging	The total protein extracts are contaminated by the solubilized Matrigel components liberated during the extraction.	Use the human specific protein markers such as human-specific mitochondrial antigen (H-mito, see Table 2) to renormalize the samples. Use dot blot analysis.
	Contamination with other non-specific structures.	Tau fibril structures are damaged during the extraction. Tau fibrils are not mature.	Reduce the Sarkosyl extraction time at room temperature. Use 3D culture samples differentiated more than 14 weeks.
		Sarkosyl extraction was not enough.	Increase the Sarkosyl extraction time. Re- centrifuge the samples before Sarkosyl extraction.