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

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One Sentence Summary:
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ABSTRACT
Stem cell technology has facilitated the development of human cell culture models of disease that can be used to study pathogenesis and test therapeutic candidates. These models hold particular 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 design and characterization of a novel three-dimensional (3D) culture system that exhibits key events in the pathogenic cascade of AD, including extracellular aggregation of amyloid β peptides and accumulation of hyperphosphorylated/aggregated tau protein. 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 (FAD) mutations, the differentiation of the hNPCs in a 3D Matrigel matrix, and the analysis of AD pathogenesis in this model. The same principles may be applicable to models of other inherited neurodegenerative diseases characterized by the aberrant aggregation of misfolded proteins.
INTRODUCTION

Alzheimer’s disease (AD) is the most common form of age-related dementia, characterized by progressive memory loss and cognitive impairment. The two pathological hallmarks are: (1) extracellular amyloid plaques, mainly composed of amyloid β (Aβ) peptides derived from the amyloid precursor protein (APP) via serial cleavage by β- and γ-secretase, and (2), intracellular neurofibrillary tangles, composed of filamentous aggregation of hyperphosphorylated tau (p-tau) proteins. Familial, early-onset (<60 years), autosomal-dominant forms of AD (FAD) can be caused by mutations in the genes APP, presenilin 1 (PSEN1), and presenilin 2 (PSEN2). Sporadic AD typically presents at a later onset and is due to multifactorial genetic and environmental risk factors.

The “amyloid hypothesis”, as originally proposed by Dr. Glenner, is the most widely posited paradigm for the disease, and postulates that the accumulation and aggregation of Aβ causes the formation of neurofibrillary tangles, widespread inflammation, neuronal dysfunction, and cell death. This was later re-termed the “amyloid cascade hypothesis”. While there is abundant evidence for partial aspects of the theory, concrete mechanistic proof has proven difficult due to the lack of complete disease models. Although postmortem studies have provided a wealth of knowledge on pathological hallmarks of AD, they typically represent the end stage of the disease, making the underlying etiology and molecular mechanisms difficult to ascertain. Transgenic mouse models with single or multiple FAD mutations have been and continue to be critical to understanding the pathogenesis of the disease, and have successfully exhibited Aβ accumulation, the formation of oligomers and fibrils, plaque formation, and Aβ-induced synaptic and memory deficits. However, significant gaps in pathological events remain, most notably that none of the AD animal models exhibit β-amyloid-driven tau tangle formation. Indeed, of the over one hundred mouse models to date, 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.

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\textsuperscript{9-14}. However, it has been challenging to reconstitute the aged AD brain environment in the presence of high levels of soluble and insoluble toxic Aβ species and thereby realize full AD pathology\textsuperscript{15-17}. 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 Aβ and accumulation of hyperphosphorylated/aggregated tau as neurofibrillary tangles\textsuperscript{18}. 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 analytical techniques we applied for their analysis, and discuss their use and applications.

**Development of 3D human neural cell culture models of AD**

We designed our AD model around two central technologies: (1) human neural progenitor cells (hNPCs) that produce high concentrations of pathogenic Aβ species and (2) a Matrigel-based 3D culture system that provided an environment that favored Aβ deposition.

First, we engineered an FAD cell line that could both exhibit significant amyloid pathology and survive through multiple passages. We chose the immortalized hNPC cell line ReNcell VM (ReN) as a base for our platform because they can be maintained up to more than 45 passage numbers, are commercially available, and can differentiate into neurons and glial cells with simple growth-factor deprivation\textsuperscript{19-31}. 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 (APP\textsuperscript{SL}), PSEN1 with ΔE9 mutation (PSEN1(ΔE9)), and APP\textsuperscript{SL}/PSEN1(ΔE9) with eGFP or mCherry as a reporter for viral transduction (Fig. 1 and 2). Fluorescence-activated cell sorting (FACS) was then employed to enrich the population of cells with highest expression (Fig. 2; see Fig. 3 as an example for enriching cells with the higher GFP signal by FACS).

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 Aβ. We posited that, in two-dimensional models, secreted Aβ may diffuse into the cell culture medium, disrupting aggregation. The 3D Matrigel possibly prevented this diffusion of Aβ, allowing for high local Aβ concentrations that were then 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 proteoglycans. Moreover, previous studies suggested that 3D conditions that more closely mimic in vivo environments can accelerate neuronal differentiation and neural network formation. Indeed, we found that 3D differentiated FAD-gene ReN cells expressed higher levels of specific neural markers and especially elevated 4-repeat adult tau isoforms versus 2D.

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

Comparison with other AD iPSC models

Most of iPSC-derived human neurons with either APP or PSEN FAD mutations, exhibited significant increases in Aβ42 levels as compared to the control cells, consistent with previous findings in other model systems. Interestingly, increases in p-tau levels were reported in some of these models. Israel et al. showed that ~2-fold increases of p-tau/total tau ratio in AD neurons. Recently, Muratore et al. showed that AD neurons harboring APPV717I mutation also showed p-tau and total tau levels and they also showed that abnormal elevation in tau levels could be decreased by treating anti-Aβ antibodies. Another recent study showed that differentiated human neurons from Down syndrome (DS) patients generated high levels of Aβ40 and 42 due to an extra copy of the APP gene. Interestingly, DS neuronal cultures revealed intracellular and extracellular aggregates of Aβ42 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 Aβ or the aggregated p-tau pathology, as shown in our 3D culture study. Limitations of 2D culture protocols and/or low Aβ42 levels may be responsible for the lack of robust AD pathology in previously described studies using stem cell-derived neuronal cultures.
Applications and limitations of the protocol

As this model is the first to comprehensively show β-amyloid-driven tauopathy, many other central parts of AD pathogenesis may now be examined in vitro, including the molecular mechanisms underlying the production of high concentrations of Aβ, the accumulation of extracellular Aβ, the deposition of Aβ aggregates, the hyperphosphorylation of tau, and p-tau aggregation. These paths may lead to new diagnostic and prognostic biomarkers of AD. We can also use the model 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 can be applicable to other neurodegenerative diseases with genetic variations, and can be especially suitable for diseases with extracellular 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 Aβ 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 Aβ 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 heterogeneous cell populations expressing markers for GABAergic, glutamatergic and dopaminergic neurons as well as astrocytes and oligodendrocytes.

Regardless, we believe the model and techniques described in this protocol offer an exciting model for the AD field especially given that plaque and tangle formation are the two major hallmarks of the 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.

Experimental design

Establishment of the ReN cells expressing high levels of Aβ (Step 1-17). 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 passagings (>45 passages)\textsuperscript{19-31}. ReN cells were transfected with either CSCW-APP-GFP, CSCW-PSEN1(ΔE9)-mCherry and CSCW-APP-PSEN1(ΔE9)-IRES-mCherry, or CSCW-IRES-GFP or CSCW-IRES-mCherry alone or together to achieve high levels of total Aβ and/or Aβ42/Aβ40 ratio (Fig. 1-4). The FAD mutations \textit{APP} (K670N/M671L (\textit{Swedish}) and V717I (\textit{London})) and \textit{PSEN1}(ΔE9) were chosen specifically because they cause high production of Aβ42 and Aβ40 and a high ratio of Aβ42/Aβ40. 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 may also be utilized\textsuperscript{39-41}.

**Differentiation of control and FAD ReN cells in 3D Matrigel (Step 18-45).** We describe the development of thin-layer (~100–300 µm) 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 and biochemical analyses (Fig. 2). The presence of Aβ 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 Aβ aggregates starting at 6 weeks of differentiation and robust p-tau accumulation after 8 weeks. We also observed that both Aβ aggregates and p-tau pathology were further elevated through 17 weeks, depending on the cell line used (Fig. 5-6).

**Endpoint analysis of Aβ and p-tau pathologies (Step 46).**

For the thin-layer 3D culture, we analyzed Aβ and abnormal p-tau accumulation using immunofluorescence staining and immunohistochemistry, as previously shown\textsuperscript{18}. Since most of our FAD ReN cells (ReN-GA, ReN-mGAP and HReN-mGAP) express high levels of eGFP and mCherry, we used UV-compatible amyloid dye, Amylo-Glo for detecting β-amyloid aggregates in thin layer 3D culture (Fig. 5). For thick-layer 3D culture, we performed biochemical extraction to show accumulation of Aβ and p-tau species in both soluble and insoluble fractions. Specifically, for Aβ aggregation, we used TBS/2%SDS/Formic acid serial extraction; while for tau aggregation, we used a 1% Sarkosyl
extraction method, as shown in Fig. 6. Accumulation of SDS-resistant Aβ oligomers, including dimers, trimers and tetramers, can also be used as indirect markers for Aβ aggregation.

MATERIALS

REAGENTS
· 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)
· KnockOut Serum Replacement (Life Technologies, cat. no. 10828-028)
· D-PBS (Lonza, cat. no. 17-512Q)
· Lentiviral polycistronic CSCW-IRES-eGFP and CSCW-IRES-mCherry vectors (Massachusetts General Hospital (MGH) viral core)
· DAPT (EMD Millipore, cat no. 565770)
· Compound E (EMD Millipore, cat no. 565790)
· BACE inhibitor IV (EMD Millipore, cat no. 565788)
· DMSO (Sigma-Aldrich, cat. No. D2650)
· 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)
· AlexaFluor 350/488/568 secondary antibodies (Life Technologies, cat. no A21068/A11068/A10042/A11011/A1104)
· 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)
· ImmPACT DAB Peroxidase Substrate kit (Vector Laboratories, cat. no. SK-4105)
- Amylo-Glo 100x (Biosensis, cat. no. TR-300-AG)
- 0.9% saline (B. Braun Medical Inc., cat. no. L8001)
- Propidium Iodide (Life Technologies, cat. no. P1304MP)
- NaOH (Sigma, cat. no. 221465)
- β-Mercaptoethanol (Sigma Aldrich, cat. no. M3148)
- HPLC-grade H₂O (Fisher Scientific, cat. no. W5-1)
- 4XLDS sample loading buffer (Life Technologies, NP0007)
- Invitrogen NuPage MES SDS Running Buffer (20X) (Life Technologies, cat. no. NP0002-02)
- Full range molecular weight Marker (Amersham, cat. no. GERPN800E)
- 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)
- 1% Triton X-100 (Fisher Scientific, cat no. 11332481001)
- Formic acid (Sigma-Aldrich, cat no. 399388)
- Human/Rat Aβ40 ELISA Kit (Wako, cat no. 294-62501)
- Human/Rat Aβ42 ELISA Kit (Wako, cat no. 290-62601)
- A multi-array electrochemiluminescence Aβ40/42 ELISA kit (Meso Scale Discovery, cat no. K15200E/K15200G)

EQUIPMENT
- 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)
- MatTek glass bottomed 35 mm plates (MatTek, cat. no. 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 µm 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 STEP 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.
· Cell strainer filter (70 µm Nylon, Corning/Fisher Scientific, cat. no. 352350)
· 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, TC10)
· Cell counter slide (Bio-Rad, cat. no. 1450011)
· ELISA plate reader (Bio-Tek, Synergy 2)
· Hoefer Semi-Dry Transfer Unit (Hoefer, TE77)
· Molecular imager (Bio-Rad, VersaDoc MP4000)
· Battery-operated spinning homogenizer (MIDSCI, cat. no. A0001)
· BD FACS Aria cell sorter (located at Ragon institute of MGH/MIT/ Harvard FACS core, http://ragoninstitute.org/research/services/flow-cytometry/).
· Water Bath (VWR Scientific cat. no. 1202)
· Microcentrifuge, 4 °C (Beckman Coulter, cat. no. 367160)
· Tissue culture Light microscope (Zeiss)
· BL-2 biosafety culture hoods
· Speed-Vac evaporator
· Vortex
· PTC-200 Thermal Cycler (MJ Research, cat. no. ALD-1244)
· Olympus IX7 fluorescence microscope equipped with spinning disk confocal unit (Olympus)
· Ultracentrifuge tubes (Beckman Coulter, cat no. 343778)
· Ultracentrifuge (Beckman Optima TL Ultracentrifuge)
· SpeedVac (ThermoScientific, cat no. SPD111V)

REAGENT SETUP

**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 STEP 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.

**Cell sorting medium** Add 1 ml of B27 supplement and 1 ml of KnockOut serum replacement to 50 ml of D-PBS.

**BD Matrigel BD Biosciences** Aliquot in 1 ml stocks on ice and keep the stocks at -80 °C. ▲CRITICAL STEP Thaw Matrigel stock overnight at 4 °C before use. Matrigel tends to solidify above 10 °C.

**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 STEP 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 until use.

**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).

**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 STEP** Medium with growth factors can be kept at 4 °C for several months, but use a fresh one if there is any noticeable decrease in cell growth rate.

**TBST** Add 1 ml of Tween-20 (99%, Sigma) to Tris buffered saline containing 50 mM Tris (pH 7.4) and 100 mM NaCl.

**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.

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

**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 1 M NaVO₃, 0.1 ml of 1 M NaF, 0.05 ml of 2 mM PNT, 0.05 ml of 200 mM PMSF and 0.05 ml of phosphatase inhibitor.

**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 1 M NaVO₃, 0.1 ml of 1 M NaF, 0.1 ml of 2 mM PNT, 0.1 ml of 200 mM PMSF and 0.1 ml of phosphatase inhibitor.

**2xSSDS 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 10xGTIP buffer (pH 7.4), 2 tablets of protease inhibitor cocktail, 0.1 ml of 1 M NaVO₃, 0.1 ml of 1 M NaF, 0.1 ml of 2 mM PNT, 0.1 ml of 200 mM PMSF and 0.1 ml of phosphatase inhibitor.

**20 % Sarkosyl solution** Add 0.1 g N-laurylsarcosine with total 0.5 ml of distilled water (HPLC grade).

**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 10XGTIP buffer (pH 7.4), 0.1 ml of 1 M NaVO₃, 0.1 ml of 1 M NaF, 2 tablet of protease inhibitor cocktail, 0.1 ml of 2 mM PNT, 0.1 ml of 200 mM PMSF and 0.1 ml of phosphatase inhibitor.

**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 STEP 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.

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 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/resuspend the cells with 3 ml of pre-warmed ReN proliferation medium and transfer the cells to 15 ml sterile conical tube.

4| Spin down the cells at 800 x g rpm for 3 min, remove the supernatant, mix in 12 ml of ReN proliferation medium.

5| Add 4 ml of the mixed ReN cells into a 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, wait 1 or 2 days.

? TROUBLESHOOTING

Viral transfection of ReNcell VM cells ● TIMING 96 h
6| To prepare ReNcell VM cells for transfection, dislodge and spin down the confluent cells in a T25 flask as described in steps 1-4. Resuspend the cell pellet in 12 ml of pre-warmed ReN cell proliferation medium and add 2 ml of the cell mixture into each well of Matrigel-coated 6-well plate. Gently cross-shake the dish and allow the cells to settle overnight.
Replace the medium with 2 ml of pre-warmed ReN proliferation medium. When cells reach 70–80% confluency, add 6x10^6 TU (Transducing Unit) of viral particles per each well to achieve an approximate M.O.I. of 1. Mix gently and incubate overnight.

On the next day, replace the medium with 2 ml of pre-warmed ReN proliferation medium and wash two times with the same media. Confluence should increase over time. Expression of the transgenes can be expected 48 h after transfection and should be detectable by fluorescence microscopy.

▲ CRITICAL STEP Viral transfection renders the cells vulnerable. Carefully monitor the cells and if there is any sign of abnormal cell death, replace the media immediately.

On day 4, split the cell cultures as described in steps 1-5 and recommence normal culturing. If the cells did not reach 100% confluency, change the medium and incubate until the cells reach the confluence. To generate cells expressing both APPSL/GFP and PSEN1(ΔE9)/mCherry (i.e. ReN-mGAP cells\textsuperscript{18}), the infected cells can be infected again with different lentiviral vectors after splitting the cells.

Validate the transfection by microscopic detection of GFP and mCherry fluorescence and western blot analysis of overexpressed APPSL and PSEN1(ΔE9). Once validated, it’s highly suggested to make some stocks and store it at -80°C before proceeding to the next step.

▲ CRITICAL STEP Western blot analysis of cell extract will show PSEN1(ΔE9) band (~40 kDa) and increased full-length APP (695 isoform, ~110 kDa) and its C-terminal fragment (10-12 kDa) bands. Western blot analysis can also be used to detect the elevated sAPPα levels as shown in Fig. 4.

? TROUBLESHOOTING

Prepare transgenic ReN cells for sorting • TIMING 2 h

Culture the transfected ReN cells until confluence is reached (a 95% confluent T25 flask yields approximately 2-3 x 10^6 cells). While the optimal total cell count depends on the transfection efficiency, at least 2-3 x 10^6 successfully transfected cells should be available (assayed by FACS analysis or fluorescence microscopy).
12] Detach the cells as described in steps 1-2 and then resuspend the cell pellets with 4 ml of D-PBS. Count the cell number using automated cell counter.

13] Spin down the cells, remove the supernatant and resuspend the cell pellets in ice-cold sorting medium (1 ml / 10^7 cells) using a 1,000 µl pipette.

▲ CRITICAL STEP Cells have to be resuspended in at least 200 µl.

14] Aspirate the cell suspension with a 1,000 µl pipette, gently press the tip against the strainer mesh at a 90° angle and empty the pipette forcefully. In case of blockage, carefully move the pipette tip across the mesh while upholding pressure on the pipette plunger. Collect the filtered cell mixtures in a separate 15 ml centrifugation tube and store on ice.

15] FACS sorting were performed by MGH/Ragon flow cytometry core facility using a standard cell sorting conditions (BD FACSARia cell sorter, http://ragoninstitute.org/research/services/flow-cytometry/). Both GFP and/or mCherry channels were used to enrich the cells with high expression of APPSL/GFP alone (Fig. 3), APPSL/PSEN1(ΔE9)/mCherry alone, or APPSL/GFP and PSEN1(ΔE9)/mCherry together.18

16] Immediately after sorting, store the collection tubes on ice. If the tube contains two liquid phases, mix carefully by inverting the tube several times to expose the sorted cells to ReN proliferation medium.

? TROUBLESHOOTING

Culturing sorted ReN cells ● TIMING 30 min

17] Spin the cells down at 800 x g for 3 min at 4 °C and remove the supernatant, then resuspend the pellet in pre-warmed ReN proliferation media (1 ml per 2 x 10^6 collected cells).

18] Plate the cells into Matrigel-coated 24-well plates, split serially into Matrigel-coated 6-well plates, and finally Matrigel-coated T25 flasks. Make multiple cell stocks at this stage.

▲ 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 very carefully. We observed that some high expressing cell lines rapidly lost APPSL and PSEN1(ΔE9) expression after 8-10 passages.

**TROUBLESHOOTING**

3D Matrigel cultures ● **TIMING 1 h**

19| Grow ~95% confluent control and FAD ReN cells in 5 Matrigel-coated T25 flasks per each cell line (an approximate number of ReN cells in confluent T25 flask is 3-4 x 10⁶) cells. Follow option A to make thick-layer 3D culture or option B for thin-layer 3D culture. **▲ CRITICAL STEP** Plating high number of cells is important to achieve Aβ aggregates early. The number of cells desirable is 1 x 10⁷ cell/ml in 3D Matrigel mix, 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 1 h**

20| Take out a BD Matrigel stock from -80 °C freezer and place it in 4 °C refrigerator at least one day before.

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 cells differentiation medium and Matrigel stock on ice. **▲ CRITICAL STEP** BD 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, pipette up and down inside the flask at least 3 times.

28| Transfer the cells from all 5 T25 flasks to a 15 ml tube.

29| Centrifuge for 2 min at 4,000 RPM.

30| Take out 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 is about final 2 x 10⁷ cells/ml).

▲ CRITICAL STEP Although it is desirable to achieve high cell concentration at this stage, we have found that 1 x 10⁷ cells/ml cells can show robust Aβ and p-tau accumulation based on biochemical analysis.

34| Add cold Matrigel to cell suspension on ice (1:1 (vol/vol) dilution). Make sure to make the pipette tips cold by pipetting a cold differentiation medium back and forth before transferring Matrigel.

35| Vortex for 30 sec.

36| Plate 300 µl of the mixed Matrigel/cell suspension to each well of 24-well plates using pre-chilled pipettes into tissue culture inserts.
37| Incubate the plates at 37 °C in CO\textsubscript{2} incubator overnight.

38| Next day, add pre-warmed (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.

39| Change half volume of the medium every 3-4 days; at the beginning, changing every 2 days may be needed based on medium color. Never leave differentiated cells drying with no medium.

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

\textbf{\textcolor{red}{\textbullet}} CRITICAL STEP Since it is not easy to monitor the thick-layer culture under optical microscope, it is important to perform a routine LDH release assay to check the status of the cultures. It is also strongly recommended to set up thin-layer 3D cultures from the same Matrigel/cell mix to monitor the culture quality.

**B** Thin-layer 3D cultures (100-300 µm thickness) \textbullet TIMING 1 h

41| For 96-well plates thin-layer cultures, further dilute 1:1 Matrigel/cell mix at step 34 by adding five 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 on 8-well chambered cover glass slides or MatTek glass bottomed 35 mm plates. We also found that a small volume of 1:1 Matrigel/cell mix can be directly loaded onto these glass bottomed dishes and these quasi thin-layer cultures can be used for confocal immunofluorescence studies.

\textbf{\textcolor{red}{\textbullet}} 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 dilution and pick the best dilution rate for each lot.
42] Cool down the 1 ml serological pipettes and plate 1 drop (~80 µl) per each well of 96-well plates. If thicker 3D culture is desirable, use 2 drops per each well. 200 µl volume is recommended for 8-well chambered cover glass slides and 300 µl for glass-bottomed dishes.

43] Incubate the plates at 37 °C in CO₂ incubator overnight.

44] Next day, add 2 drops of pre-warmed ReN differentiation medium to each well of the 96-well plates.

45] Change half volume of the medium every 3-4 days; at the beginning, changing every 2 days may be needed based on medium color. Never leave differentiated cells drying with no medium. Drug treatments should be done in last two week before the 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 by 4% paraformaldehyde at 2-4 week and tested for neural marker expressions by immunofluorescence or immunohistochemistry.

? TROUBLESHOOTING

Endpoint analysis

46] Differentiated 3D cultures can be either fixed with 4% paraformaldehyde solution for immunofluorescence/histochemical staining or harvested for biochemical analyses. For thin-layer 3D cultures, wash 1 time with D-PBS, add 100 µl of 4% paraformaldehyde solution per each well and incubate at room temperature overnight. For thick-layer cultures, cut ¾ of the membrane at the bottom of tissue culture inserts with a razor blade, put the inserts on top of microcentrifuge and spin down at 800 x g for 30 sec to harvest the Matrigel/cells. These biochemical samples can be stored at -20 or -80 °C freezer until the extraction.

? TROUBLESHOOTING

(A) Immunofluorescence staining of thin-layer 3D culture • TIMING 2 d

(i) Fix thin-layer 3D cultures with 100 µl of 4% paraformaldehyde at room temperature for 24 h.
(ii) Block/permeabilize by incubating with 200 µl of blocking/dilution solution at 4 °C for 12 h.

(iii) Wash with TBST 3 times.

(iv) Incubate with primary antibodies (see Table 1) in blocking/dilution solution at 4 °C for 24 h with gentle rocking.

(v) Wash with TBST 5 times for 10 min per each washing. This step can be extended to 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 of plastic-based materials in these plates. Glass-bottomed dishes will do better for AlexaFluor350 antibody staining. Secondary antibody incubation can also 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 by Olympus DSU confocal microscope; the image analysis and 3D reconstitution can be performed with ImageJ, IPLabs and MetaMorph software.

? TROUBLESHOOTING

(B) Immunohistochemical staining of thin-layer 3D culture ● TIMING 2 d

(i) Permeabilize and block the 4% paraformaldehyde fixed cultures by incubating in the blocking solution at 4 °C for 12 h.

(ii) Incubate the cultures with 0.01% (vol/vol) H₂O₂ solution in TBS for 5 min at room temperature to block endogenous peroxidase activities.

(iii) Wash with TBST 3 times.

(iv) Incubate with primary antibodies (see Table 1) in blocking/dilution solution at 4 °C for 24 h with gentle rocking. ▲CRITICAL STEP We found that BA27-HRP conjugate (Aβ40) and BC07-conjugate (Aβ42) antibodies work well to detect Aβ deposits in 3D culture with minimum background. These antibodies can be developed either with or without secondary antibodies depending on the desired signal strength.

(v) Wash with TBST 5 times.
(vi) Incubate the sections with ImmPRESS anti-mouse and -rabbit Ig HRP polymer conjugates (1:1 diluted with 2.5% (vol/vol)) horse serum for 30 min.
(vii) Wash with TBST 5 times.
(viii) Develop the antibody signal using ImmPACT DAB Peroxidase Substrate kit.
(ix) (optional) Counterstain the DAB-stained cultures with Hematoxylin.

? TROUBLESHOOTING

(C) Amylo-Glo staining of thin-layer 3D culture ● TIMING 2 d
(i) Wash the culture wells three 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, and therefore, reduce the final concentration further if a strong background is detected in the control cells.
(iii) Remove the staining solution.
(iv) Add 200 µl of 0.9% saline and incubate for 5 min.
(v) Wash the wells three times with DDW.
(vi) (optional) Add 200 µl of Propidium Iodide nuclear counter staining solution and incubate for 5 min.
(vii) Wash three 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 40x, 100x, and 400x.

(D) TBS/SDS/Formic acid extraction of thick-layer 3D culture ● TIMING 2 d
(i) Thaw the Matrigel/cell pellet on ice for 10 min.
(ii) Add same volume of 2xTBS extraction buffer (about ~50 µl in general).
(i) Homogenize culture 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.
(iii) Sonicate the sample for 10 min at 4 °C using a sonic cleaning water bath.
(iv) Spin down samples at 100,000 x g for 1 h at 4 °C.
(v) Save supernatant and measure protein levels.
(vi) Separate TBS soluble fractions and save them at -20 °C.
(vii) Save supernatant and measure protein levels.
(viii) Homogenize the pellet with 50 µl of 1xSDS extraction buffer using rotor-driven homogenizer and then sonicate for 10 min at 4 °C.
(ix) Spin down samples at 100,000 x g for 1 h at 4 °C.
(x) Separate TBS-insoluble/2% SDS-insoluble fractions and save them at -20 °C.
(xi) Wash the pellet briefly with 100 µl 1xSDS extraction buffer once.
(xii) Extract the pellet with 10 µl of 70% formic acid on ice.
(xiii) Centrifuge for 1 h at 100,000g to produce TBS-insoluble/2% SDS-insoluble/formic-acid-soluble fractions.
(xiv) Evaporate Formic acid with SpeedVac and neutralize/solubilize the pellet by adding the 1x LDS neutralizing buffer (1 volume of 4xLDS buffer with 8% β-mercaptoethanol + 3 volumes of 1M Tris (PH 8.5)).
(xv) Run on SDS-PAGE.

? TROUBLESHOOTING

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

(ii) Thaw the Matrigel/cell pellet on ice for 10 min.
(iii) Add same volume of 2x TBS extraction buffer (about ~50 µl in general).
(iv) Homogenize culture using disposable-tip rotor-driven homogenizer 10 times up and down.
(v) Sonicate the sample for 10 min at 4 °C using a sonic cleaning water bath.
(vi) Mix TBS-extracted samples with equal volume of 2xRIPA buffer.
(vii) Homogenize with rotor-driven homogenizer on ice.
(viii) Incubate on ice for 15 min.
(ix) Sonicate 5 min 2 times.
(x) Centrifuge at max with tabletop centrifuge (about 18,000 x g) for 15 min at 4 °C.
(xi) Transfer the supernatant into a new tube.
(xii) Add 1/20th volume of 20% Sarkosyl solution to the supernatant; incubate at room temperature for 60 min in rotary mixer.
(xiii) Add 80 µl of 1x RIPA with 1% Sarkosyl to pellets in the tubes, resuspend by pipetting, and incubate at 37 °C for 30 min in rotary mixer.
(xiv) Mix the two supernatants (xi and xiii)
(xv) Spin down samples at 150,000 x g for 1 hr at 4°C.
(xvi) Transfer the supernatant into new tubes and measure protein levels (Sarkosyl-soluble fractions)
(xvii) Wash the pellet briefly with 100 µl 2x RIPA buffer once.
(xviii) Wash the pellet with PBS 3 times.
(xix) Add 1x LDS sample buffer with 10 M Urea and 2% β-mercaptoethanol.
(xx) Heat at 95°C for 5 min.
(xxi) Run on SDS-PAGE.

(F) Western Blot Analysis ● TIMING 2 d
(i) Run on 12% NuPage gel for Aβ analysis and 4-12% gel for p-tau analysis.
(ii) Transfer the SDS-PAGE gel into PVDF membrane.
(iii) (Optional) For Aβ detection, incubate the membrane with 0.5% Glutaraldehyde solution for 10 min before blocking. ▲ CRITICAL STEP Do not use skim milk for blocking p-tau blot since it contains phosphatase.
(iv) Blocking the membrane with either 4% skim milk (for Aβ blot) or 4% BSA (for p-tau) for 1 h at room temperature.
(v) Incubate primary antibody solution at 4°C overnight with rocking (for primary antibody concentration, please see table 2).
(vi) Wash with TBST 3 times.
(vii) Incubate secondary antibody solution for 1 h at room temperature with gentle rocking.
(viii) Wash with TBST 3 times.
(ix) Develop the blot with SuperSignal Dura or Femto.

? TROUBLESHOOTING

? 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 to achieve Aβ aggregation early. In addition, the passage number and the condition of the cells are also important. We observed that ReN cell lines with high Aβ42/40 ratio made more tight
amyloid deposits in 3D culture while the cell lines with mid/low Aβ42/40 ratio developed mostly diffuse deposits despite these cells having secreted extremely high levels of total Aβ. In case of p-tau pathology, in general, we observed that the ReN cell secreting high levels of total Aβ develops more robust tau pathology.

To enhance Aβ and p-tau pathologies, we used FACS sorting to enrich ReN cells expressing high levels of Aβ. As shown in Fig. 3 and 4, FACS sorting of top 2, 10 or 50% levels of GFP signals (Fig. 3a, left panel) lead to generate 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 was also prepared (ReN-G2, -G10 and -G50; Fig. 3a and b). As expected, ReN-GA2 and the control ReN-G2 cells comparably differentiated in the thin-layer 3D culture condition (Fig. 3c). Western blot analysis (Fig. 4a) and Aβ ELISA (Fig. 4b) showed robustly elevated Aβ levels in ReN-GA2 cells as compared to the control ReN-G2 cells. Aβ ELISA also detected a robustly elevated level of Aβ in a conditioned medium collected from 1-week differentiated thick-layer 3D cultures of ReN-GA2 cells (Fig. 4c). As we have shown in our recent paper, Aβ42/40 ratio can also be dramatically elevated by selectively enriching ReN cells with high levels of PS1ΔE9/mCherry cells.

With the proper cell density and passage number, extracellular β-amyloid deposits started developing after 6-week 3D cultures in FAD ReN cells and the number and size of β-amyloid aggregations were increased up to 17 weeks as shown in Fig 5. Robust accumulation of p-tau were observed in high Aβ expressing cells after 8 weeks, and p-tau aggregation were further increased up to 17 weeks. We have also observed typical PHF tau species in 3D-cultured FAD cells only after 14 weeks (data not shown).

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**AUTHOR CONTRIBUTIONS**

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

**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, PS1 with ΔE9 FAD mutation (PS1ΔE9) mCherry tagged, or both together APPSL and PS1ΔE9 FAD mutations with mCherry. CMV, cytomegalovirus.

**Figure 2 | Overview of ReN VM cell 3D culture protocol.** The experimental procedure begins with the generation of human neural progenitor cell lines viral transfected with APP and/or PS1 FAD mutations, and enriched based on GFP and/or mCherry signals by fluorescence-activated cell sorting (FACS). The right-hand column shows in details the timing for each steps of the 3D culture method. The day on which cells are mixed with Matrigel is day 0.

**Figure 3 | FACS enrichment of ReN-G and -GA cells for higher expressions of APP with FAD mutations.**

- **a.** FACS sorting of ReN VM cells that were stably transfected with polycistronic GFP lentiviral Vector. The cells were then enriched based on GFP signal by FACS (rectangular boxes, the selected ranges of cells for the experiments).
- **b.** ReN cells stably expressing GFP alone (ReN-G), APPSL/GFP (ReN-GA) with top 2, 10 and 50% GFP signal. Green, GFP; Scale bar, 25 µm.
- **c.** The representative fluorescence microscope images of enriched ReN-G2, -GA2 cells that were differentiated by growth-factor deprivation for 4 weeks (green, GFP; scale bar, 25 µm).

**Figure 4 | Analysis of soluble Aβ levels in the enriched ReN-G and -GA 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. 6E10 anti-Aβ detected ~4 kDa Aβ monomer bands in the media collected from FAD enriched ReN cells (ReN-GA10, ReN-GA2).
- **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).
Figure 5 | Increases of extracellular Aβ deposits in 3D-differentiated ReN VM cells with FAD mutations. a. Detection of amyloid plaques with Amylo-Glo, a fluorescent amyloid-specific dye, in control ReN-G and FAD HReN-mGAP cells 3D-differentiated for 7 and 17 weeks. HReN-mGAP cells, ReN-G cells were transfected with APPSL/PSΔE9/mCherry vectors, enriched with FACS to achieve high Aβ secretion; Blue, Amylo-Glo; Red, Propidium Iodide (P.I.); Scale bar, 50 µm. b. The number and size of Amylo-Glo-stained amyloid structures were increased in 17-week differentiated FAD HReN-mGAP cells as compared to 7-week 3D cultures (Blue, Amylo-Glo).

Figure 6 | 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 from 8-week 3D-differentiated HReN-mGAP cells and the control ReN-G cells. b. Dot blot analyses of p-tau and total tau in sarkosyl–insoluble fractions prepared from ReN-G, ReN-mGAP and HReN-mGAP cells (8-week 3D differentiated).

Table 1 | Antibodies used for immunofluorescence and immuno histochemistry.

Table 2 | Antibodies used for Western Blot analysis.

Table 3 | Troubleshooting.
Fig. 1
Human neural progenitor cells

Viral transfection of AD genes with FAD mutations

Enrichment by FACS

3D culture

Neural Differentiation

Characterization

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**3D Culture Procedure**

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**Notes:**
- 3D Thick Culture
- 3D Thin Culture
- Molecular/ Biochemical Analyses
- Immunostaining ELISA

Fig. 2
Fig. 3
Fig. 4
Figure 6
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<td>Mouse</td>
<td>Thermo Scientific</td>
<td>MN1020</td>
<td>1:40</td>
<td></td>
</tr>
<tr>
<td>p-tau</td>
<td>PHF1</td>
<td>Mouse</td>
<td>A gift from Peter Davis</td>
<td>N/A</td>
<td>1:500</td>
<td></td>
</tr>
<tr>
<td>Total tau</td>
<td>anti-Tau</td>
<td>Rabbit</td>
<td>DAKO</td>
<td>A0024</td>
<td>1:2000</td>
<td></td>
</tr>
<tr>
<td>Abeta</td>
<td>6E10</td>
<td>Mouse</td>
<td>Convance</td>
<td>SIG-39300</td>
<td>1:300</td>
<td></td>
</tr>
<tr>
<td>Abeta</td>
<td>MOAB-2</td>
<td>Mouse</td>
<td>Biosensis</td>
<td>M-1586-100</td>
<td>1:100</td>
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<tr>
<td>p-tau</td>
<td>PHF1</td>
<td>Mouse</td>
<td>A gift from Peter Davis</td>
<td>N/A</td>
<td>1:500</td>
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<tr>
<td>Total tau</td>
<td>anti-Tau</td>
<td>Rabbit</td>
<td>DAKO</td>
<td>A0024</td>
<td>1:2000</td>
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<td>Abeta</td>
<td>6E10</td>
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<td>Convance</td>
<td>SIG-39300</td>
<td>1:300</td>
<td></td>
</tr>
<tr>
<td>Abeta</td>
<td>MOAB-2</td>
<td>Mouse</td>
<td>Biosensis</td>
<td>M-1586-100</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>Neuron/dendrite</td>
<td>MAP2</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>8717</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Neuron/dendrite</td>
<td>MAP2</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>8717</td>
<td>1:200</td>
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<td>presenilin</td>
<td>Presenilin 1</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>5643</td>
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<td>beta secretase</td>
<td>BACE1</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>5606</td>
<td>1:1,000</td>
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<td>Amyloid Precursor Protein C66 APP C-terminal antibody</td>
<td>Rabbit</td>
<td>A gift from Dr. Kovacs</td>
<td>N/A</td>
<td>1:2000</td>
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<td>neuronal</td>
<td>NCAM</td>
<td>Mouse</td>
<td>Cell Signaling Technology</td>
<td>3576</td>
<td>1:1,000</td>
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<tr>
<td>Synaptic</td>
<td>synapsin I</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>5297</td>
<td>1:500</td>
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<td>ER</td>
<td>Calnexin</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>2679</td>
<td>1:1000</td>
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<tr>
<td>Heat shock protein/control</td>
<td>HSP70</td>
<td>Rabbit</td>
<td>Enzo Life Sciences</td>
<td>ADI-SPA-812</td>
<td>1:1,000</td>
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</tr>
<tr>
<td>human Mitochondria</td>
<td>human mitochondrial antigen</td>
<td>Mouse</td>
<td>EMD Millipore</td>
<td>MAB1273</td>
<td>1:500</td>
<td></td>
</tr>
<tr>
<td>Steps</td>
<td>Problem</td>
<td>Possible reason</td>
<td>Solution</td>
<td></td>
<td></td>
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<tr>
<td>-------</td>
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<tr>
<td>10</td>
<td>The transfection rate is low</td>
<td>Low titer of lentiviral vector, poor cell condition, negative impact on cell proliferation of APPSli or PS1sE9</td>
<td>Increase titer of lentiviral vector. Do multiple infections with the same lentiviral vector.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>The growth rate of FACS Ren cells expressing high levels of APPSli and PS1sE9 are sometimes extremely low</td>
<td>High level expression of APPSli and especially PS1sE9 may possibly not be desirable for proliferation of the cells</td>
<td>Maintain high-cell densities for slow-growing cell lines, change ReN proliferation media on a regular basis (every three days), even for cells that are not growing fast. Adding naive ReN cell promotes the growth of FACS sorted cells; additional FACS sorting will be required after getting high number of total cells by co-culturing with naive cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>The Ren differentiation medium color is changing yellow in 1-2 days in the early 3D cultures.</td>
<td>The cell density is too high; Some of the Ren cell still expanding in 3D culture early</td>
<td>Change the cell culture medium more frequently in early period of 3D cultures (every 2 days); After 2-3 weeks of 3D culture, switch to a normal medium change schedule (every 3-4 days); Alternatively, reduce the number of cells to 1 x 10^5 cells/ml at step 33.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Ren cells sink down to the bottom and look like 2D culture</td>
<td>Matrigel concentration is too low</td>
<td>Use different dilution rate to increase the final Matrigel concentration. Even if cell body seems to be at the bottom, we observed that still Matrigel formed 3D gels on top of the cells and making 3D neurite outgrowth and displayed Aβ and tau pathologies; Actually low Matrigel concentration is desirable for immunohistochemical staining to reduce background.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>45</td>
<td>Decreases in cell and neurites numbers after 4 weeks of differentiation in FAd cells</td>
<td>A potential neurodegenerative change possibly by high overexpression of AD genes with FAD mutations</td>
<td>We observed a pattern of cell/neurite decreases in some of FAD Ren cells expressing high levels of AD genes with FAD mutations. BACE1 or γ-secretase modulator may be treated at this stage to reduce unwanted cell death.</td>
<td></td>
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<tr>
<td>36, 37</td>
<td>Matrigel is solidified too fast or is not solidified</td>
<td>Matrigel tends to solidify above 10 °C.</td>
<td>Thaw it at 4°C overnight and then keep it in the ice until it is plated with the cells. If Matrigel is not solidified, then start again from the step 19.</td>
<td></td>
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</tr>
<tr>
<td>41</td>
<td>Each lot of Matrigel has a different protein concentration</td>
<td></td>
<td>Pre-test 1:10, 1:15 and 1:20 dilution and pick the best dilution rate for each lot.</td>
<td></td>
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</tr>
<tr>
<td>16</td>
<td>Cells are not proliferating or apoptotic</td>
<td>Too high expression of FAD mutations obtained with the FACS sorting</td>
<td>Make sure the correct dilution was used.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Cells do not survive</td>
<td>Cell numbers are too low.</td>
<td>The starting number of cells/ml is between 3-10 million/ml, but number of cells desirable is 10 million/ml.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Neurons do not proliferate and begin to differentiate</td>
<td>Activity of EGF and/or bFGF is lost</td>
<td>Avoid multiple freezing/thawing cycle for EGF / bFGF stocks.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>Cell growth rate is low</td>
<td></td>
<td>If growth rate is low, use fresh media with growth factors.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Cells differentiate immediately</td>
<td>Using animal serum might cause the immediate differentiation</td>
<td>Do not use animal serum / serum components.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Contamination</td>
<td>Various</td>
<td>Remove the contaminated samples using H2O2.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46(A)</td>
<td>Strong background fluorescence signal</td>
<td>Non-specific interaction of primary and secondary antibodies to the fixed Matrigel. Uneven solidification of Matrigel in thin-layer 3D culture.</td>
<td>Increase blocking and washing time. Decrease secondary antibody concentrations. Use lower Matrigel concentration (1:15 or 20) during thin-layer 3D culture generation.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46(B)</td>
<td>Strong background signals</td>
<td>1) Non-specific interaction of primary and secondary antibodies to the fixed Matrigel. 2) Matrigel thickness is too high</td>
<td>Increase blocking and washing time. Reduce ImmPRESS anti-mouse and -rabbit Ig HRP polymer incubation time to 15 min. We also found that lower Matrigel concentration (1:15 or 20) during thin-layer 3D culture generation particularly worked well with DAB staining due to the low background.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46(D)</td>
<td>Normalization of total protein levels for Western blot does not fit to control protein markers including GAPDH</td>
<td>Presence of high protein contents derived from Matrigel</td>
<td>Use the human specific protein markers such as human-specific mitochondrial antigen (H-mito, see the table 2) to renormalize the sample, increase blocking and washing time. Reduce ImmPRESS anti-mouse and -rabbit Ig HRP polymer incubation time to 15 min. We also found that lower Matrigel concentration (1:15 or 20) during thin-layer 3D culture generation particularly worked well with DAB staining due to the low background.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Aβ aggregates / pTau pathologies are delayed or not evident</td>
<td>Number of cells is low</td>
<td>The starting number of cells/ml is between 3-10 million/ml, but number of cells desirable is 10 million/ml.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ab42 levels (Ab42/40 ratio) is not high</td>
<td></td>
<td>Enrich cells further with higher fluorescence signals by FACS; Start 3D culture again using high quality culture of cells with earlier passage number or start again from the viral transfection.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Too much cell death</td>
<td></td>
<td>Decrease total cell numbers in 3D culture; Use the cell lines with different passage numbers.</td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>