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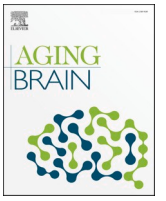

Urine-derived cells from the aged donor for the 2D/3D modeling of neural cells via iPSCs

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ABSTRACT

Human neural cell models derived from induced pluripotent stem cells (iPSCs) have been widely accepted to model various neurodegenerative diseases such as Alzheimer's disease (AD) *in vitro*. Although the most common sources of iPSCs are fibroblasts and peripheral blood mononuclear cells, the collection of these cells is invasive. To reduce the donor's burden, we propose the use of urine-derived cells (UDCs), which can be obtained non-invasively from a urine sample. However, the collection of UDCs from elderly donors suffering from age-related diseases such as AD has not been reported, and it is unknown whether these UDCs from the donor aged over 80 years old can be converted into iPSCs and differentiated into neural cells. In this study, we reported a case of using the UDCs from the urine sample of an 89-year-old AD patient, and the UDCs were successfully reprogrammed into iPSCs and differentiated into neural cells in four different ways: (i) the dual SMAD inhibition with small-molecules via the neural progenitor precursor stage, (ii) the rapid induction method using transient expression of *Ngn2* and microRNAs without going through the neural progenitor stage, (iii) the cortical brain organoids for 3D culture, and (iv) the human astrocytes. The accumulation of phosphorylated Tau proteins, which is a pathological hallmark of AD, was examined in the neuronal models generated from the UDCs of the aged donor. The application of this cell source will broaden the target population for disease modeling using iPS technology.

Introduction

The establishment of the induced pluripotent stem cell (iPSC) technology by the Shinya Yamanaka's group has widely opened the window for disease modeling using human cells [1–3]. Since then, human iPSC-derived cells have been used to study various diseases. The first line of human iPSC (hiPSC) was generated by introducing 4 reprogramming factors (OCT3/4, SOX2, KLF4, c-MYC) into female donor fibroblasts [2]. More recently, peripheral blood mononuclear cells (PBMCs) have been used as a source of human iPSCs [4]. However, obtaining fibroblasts and PBMCs required an invasive approach.

It has been shown that urine-derived cells (UDCs), which are obtained from the urine sample, are also a suitable source for the

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generation of iPSCs [5–7]. Since UDCs can be easily and non-invasively obtained multiple times from the same individual, the use of urine as a source for iPSC generation and the *in vitro* disease modeling will further facilitate the application of human iPSCs. Although there are several sources of somatic cells that are available for iPSC generation, urine-derived UDCs are also another useful source. The iPSCs derived from UDCs have many advantages over iPSCs derived from other cell sources. For example, a high reprogramming efficiency [8], higher generation efficiency compared to fibroblasts [9], and a shorter generation of the iPSCs compared to fibroblasts, lymphocytes, and keratinocytes [6].

Some of the previous studies also suggested that the difference in the origin of the iPSCs might affect the differentiation efficiency into other target cells due to the epigenetic memory [10–12]. Specifically, for the derivation of neuronal cells, one study suggested that there is no influence from the different origin of iPSCs in obtaining the neural progenitor cells (NPCs) [13]. However, another study showed that keratinocytes-derived iPSCs differentiated more efficiently into the 2D and 3D culture models of dopaminergic neurons compared to PBMC-derived iPSCs [14]. Although some previous studies showed the differentiation capabilities of iPSCs derived from other somatic cell sources to several cell types, there was no study that demonstrates the differentiation capabilities of UDC-derived iPSCs of an aged donor. In this study, we showed the successful application of several induction methods of 2D/3D culture models of neural cells to the iPSCs. Furthermore, we examined the accumulation of phosphorylated Tau protein, which is a pathological hallmark of various neurodegenerative diseases including AD, in our hiPSC-derived models.

Materials and methods

Collection of urine sample, isolation, and cell culture of UDCs

The urine sample was collected at room temperature, kept on ice, and the UDCs were isolated within four hours. Isolation of the UDCs from the donor's urine was performed according to the manufacturer's protocol (REPROCELL, Inc.). First, the urine sample was centrifuged (400×*g* for 10 min at room temperature), and after removing the supernatant, the pellet was resuspended in wash buffer (10 mL PBS, 20 µL Primocin (Invivogen ant-pm-1)). The resuspended sample was centrifuged (200×*g* for 10 min at room temperature). After removal of the supernatant, the pellet was resuspended with 2 mL primary medium and plated on a 12-well plate coated with iMatrix-511 silk (Matrixome 892021) and incubated at 37 °C with 5 % CO_2. On days 2 to 4, 1 mL of primary medium was added daily without aspiration. From day 5, the culture medium was gradually changed to the REMC medium (Renal Epithelial (RE) Proliferative Medium + Mesenchymal Cell (MC) Proliferative Medium) by removing 1 mL of the culture medium and adding 1 mL of REMC medium every day. When the colonies of UDCs became large enough, the cells were passaged onto a 10 cm dish without coating. Complete medium replacement with fresh REMC medium was performed every other day. The urine sample was obtained under the ethical approval of the Ethics Committee for Human Research of the Keio University School of Medicine, and informed consent (#20080016).

Generation and culture of iPSCs from Patient's UDCs

UDCs were plated on a pre-coated 6-well plate at a density of $10x10^4$ cells in 2 mL REMC medium per well on day 0, and incubated overnight at 37 °C, 5 % CO_2. On day 1, the cells were infected with Sendai virus using the CytoTune®-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific A16517) [15]. All types of the Sendai virus (KOS, c-Myc, Klf4) were infected into the cells at MOI = 5 in 1 mL REMC medium per well. Starting on day 2, the culture medium was changed from REMC medium to StemFit AK02N medium supplemented with 0.5 % Penicillin/Streptomycin (P/S) (Nacalai 09367-34). Fresh medium was added to the cells every day until the iPSC-like colonies appeared. Cells were incubated in a hypoxic incubator (37 ℃, 5 % O_2) throughout the reprogramming period to enhance the generation of the iPSCs [16]. Once colonies of the iPSCs were formed, the colonies were picked up and cultured with StemFit AK02N medium (Ajinomoto) on the 6-well plated coated with 3.0 µg/mL iMatrix-511 silk (Matrixome 892021) in a 37 °C, 5 % CO_2 incubator. The medium was replaced with fresh medium every other day, and the iPSCs were passaged every 7 days. The cell culture method of the iPSCs followed the protocol provided by the Center for iPS Cell Research and Application (CiRA) [17].

In addition, the control iPSC line, 1210B2, was established from ePBMCs® from the Cellular Technology Limited (OH, USA) at the Center for iPS Cell Research and Application (CiRA: Kyoto, Japan) by an integration-free method [18].

Alkaline phosphatase (ALP) assay

Cells in a 6-well format were fixed with 100 % ethanol for 10 min at room temperature and stained with the alkaline phosphatase reagent (Sigma B5655) diluted in 10 mL of PBS. After washing, cells were imaged using an inverted microscope (Eclipse Ts2; Nikon, Tokyo, Japan).

RNA extraction and quantitative reverse transcription PCR

RNA from the cultured cells was extracted using the Rneasy Mini Kit (Qiagen). From the extracted RNA, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Quantitative reverse transcription PCR (RT-qPCR) was performed using 4 ng/µL cDNA, TB Green II, and Rox Dye II (TaKaRa) with 20 µM of each primer using a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific 4453723) according to the manufacturer's instructions.

Karyotyping

Chromosomes of the generated iPSC line were analyzed by Giemsa staining for the karyotyping of 20 randomized cells of iPSC colonies. Karyotyping was performed by LSI Medience Corporation (Tokyo, Japan).

DNA sequencing

Genomic DNA from TMGH-1 iPSCs was extracted by the DNeasy Blood and Tissue Kit (Qiagen). The DNA was then amplified using the primers for the *APOE* genotype (Supplementary Table 1) using the Proflex PCR System (Thermo Fisher Scientific) with the following cycle parameters; 95 °C for 2 min, 35 cycles at 95 °C for 30 s/58 °C for 30 s/72 °C for 1 min. After agarose gel electrophoresis of the PCR mixture, the DNA band at the size of the PCR product was cut out. DNA was then extracted and purified using QIAquick Gel Extraction Kit (Qiagen). The PCR product (20 ng) and 1 μL of 10 μM *APOE* primer were submitted to Eurofins Genomics K.K., Japan, for Sanger sequencing.

Trilineage differentiation

Direct trilineage differentiation of iPSCs was performed using the STEMdiff Trilineage differentiation kit (STEMCELL Technologies ST-05230). On day 0, the iPSCs were plated in a 24-well plate on a coverslip pre-coated with 0.1 mg/mL Matrigel (Corning 354277) and 1.6 μg iMatrix-511 (Matrixome 892021) per well for 4 h. The cells were plated at a density of 200,000 cells/cm^2 for ectoderm, 50,000 cells/cm^2 for mesoderm, and 200,000 cells/cm^2 for endoderm assays. Cells were cultured in 0.5 mL StemFit AK02N medium (Ajinomoto) supplemented with 10 μM Y-27632 (Nacalai 08945–42) at day 0 and in 1 mL of lineage-specific medium per well. From day 1, the medium was exchanged to the lineage-specific medium every day for the differentiation of endoderm and mesoderm. The ectoderm-specific medium supplemented with 10 μM Y-27632 (Nacalai 08945-42) was used from day 0. The cells were immunostained for mesoderm and endoderm markers on day 5 and ectoderm markers on day 7.

Immunocytochemistry

For the staining of the iPSCs and the 2D neural models, cells were washed with PBS, fixed with 4 % paraformaldehyde (PFA) solution (Wako 163-20145) for 15 min at room temperature, and washed 3 times with PBS. The cells then were permeabilized and blocked with a solution of 5 % fetal bovine serum (FBS) and 0.3 % Triton X-100 in PBS. Cells were probed with the primary antibodies overnight at 4 °C. The next day, the cells were washed 3 times with PBS and incubated with secondary antibodies for 1 h at room temperature. Cell nuclei were stained with Hoechst 33258 (Dojindo Laboratories) diluted in PBS or DAPI solution (Dojindo Laboratories) diluted in blocking buffer (Thermo Fisher Scientific 37538) for 10 min. The cells were then washed twice with PBS, 1 time with MilliQ water, and mounted with the PermaFlour (Thermo Fisher Scientific TA-030-FM). Images were captured using a fluorescence microscope (BZ-X810; Keyence, Osaka, Japan) or a confocal microscope (Zeiss LSM700; ZEISS Group, Oberkochen, Germany). The expression levels of the markers of the 2D models were evaluated by IN Cell Analyzer 6000 (Cytiva, Tokyo, Japan).

Immunohistochemistry

For the staining of the 3D cortical brain organoids, brain organoids were washed with PBS at 10 weeks or 20 weeks after differentiation and fixed with 4 % PFA solution (Wako 163-20145) for 30 min at room temperature. The organoids were incubated with sucrose solution overnight at 4 °C. Then, the organoids were embedded in Super Cryoembedding Medium (SCEM) (Section-Lab; SECTION-LAB, Yokohama, Japan) and frozen at –80 °C. The embedded samples were sectioned at 12 μm using a cryostat (Leica CM3050 S Cryostat). The frozen sections were first permeabilized with 0.2 % Triton X-100 in PBS for 20 min and washed twice with PBS for 5 min each at room temperature. The sections were stained with primary antibodies diluted in a blocking buffer (Thermo Fisher Scientific 37538) and incubated overnight at 4 °C. The next day, the sections were washed 3 times with PBS for 5 min each. The sections were stained with secondary antibodies diluted in a blocking buffer for 1 h and washed 2 times with PBS for 5 min each. Cell nuclei were stained with the DAPI solution (Dojindo Laboratories) diluted in blocking buffer (Thermo Fisher Scientific 37538) for 20 min. The sections were washed twice with PBS solution, once with MilliQ water, and mounted with the Aqua-Poly/Mount (Polysciences, Inc.). Slides were prepared and imaged using a confocal microscope (Zeiss LSM700; ZEISS Group, Oberkochen, Germany). Primary and secondary antibodies are summarized in Supplementary Table 1 with the dilution rate. The marker expression levels of the organoids were quantified using BZ-X800 Analyzer software (Keyence, Osaka, Japan) after imaging with a fluorescence microscope (BZ-X810; Keyence, Osaka, Japan).

Induction of iPSCs to neurons with the dual SMAD inhibition

The iPSCs used for the neural induction with the dual SMAD inhibition were cultured in a StemFit AK02N medium (Ajinomoto) supplemented with 0.5 % Penicillin/Streptomycin (P/S) (Nacalai 09367-34). First, cells were passaged onto the 6-well plate pre-coated with 3.0 μg/mL iMatrix-511 silk (Matrixome 892021) at 40 x 10^4 cells per well with 1.5 mL StemFit AK02N medium (Ajinomoto) supplemented with 10 μM Y-27632 (Nacalai 08945-42). The 1.5 mL medium was changed daily with fresh 4 mL StemFit AK02N until the cells reached more than 80 % confluence. On day 0, the medium was changed to fresh 4 mL of the neural induction medium (GMEM

medium (Wako 078-05525) supplemented with 8 % KSR (Gibco 10828010), 0.1 mM non-essential amino acid solution (NEAA) (Nacalai 06344-56), 1 mM sodium pyruvate (Nacalai 06977-34), 0.1 mM 2-mercaptoethanol (2-ME) (Gibco 21985023), and 1 % P/S (Nacalai 09367-34)) added with 2 μM DMH1 (Wako 041-33881), 2 μM SB431542 (Sigma), and 10 μM Y-27632 (Nacalai 08945-42). On PID 1–7, the medium was changed with fresh 4 mL of neural induction medium supplemented with 2 μM DMH1 (Wako 041-33881), 2 μM SB431542 (Sigma), and 2 μM IWP-2 (Sigma). On PID 7–15, the medium was exchanged with fresh neural induction medium without DMH1, SB431542, and IWP-2. On PID 15, the induced neural progenitor cells (NPCs) were re-plated with the neural differentiation medium (BrainPhys basal medium/N2-A/SM1 kit (Stem Cell Technologies) supplemented with 10 ng/mL BDNF (Alomone labs B-250), 10 ng/mL GDNF (Alomone labs G-240), 200 μM L-ascorbic acid (Sigma A4544), 0.5 mM dbcAMP (Nacalai 11540–61), 2 μM PD0332991 (Sigma PZ0199-5MG), and 0.5 % P/S (Nacalai 09367–34), 10 μM Y-27632 and 10 μM DAPT (Sigma D5942)) onto the cell-culture plate pre-coated with 1 μg/mL Poly-L-lysine solution (PLL) (Sigma P4832) and 4 ng/mL Laminin (R&D 3400-010-01) from 2 days before the replating day. At PID 18, the medium was exchanged to neural differentiation medium without Y-27632 and DAPT. After PID 21, half of the medium was replaced with a fresh neural differentiation medium without Y-27632 and DAPT every 3 days. Neurons after PID 45 were used for the analysis (Fig. 2A).

Induction of iPSCs to the neurons using transient expression of Ngn2 and microRNAs

To introduce *Ngn2* into the cells, iPSCs were dissociated on day 0 and transfected with the PiggyBac vectors (PB-TET-PH-lox66FRT-NEUROG2, pCMV-HyPBase_PGK-Puro, and pG-PB-CAG-rtTA3G-IH) using Gene Juice Transfection Reagent (Merck Darmstadt, Land Hessen, Germany) as previously described [19]. After vector transfection, the iPSCs were seeded onto a culture plate coated with 3.0 μg/mL iMatrix-511 silk (Matrixome 892021) with 20 μM Y-27632 in the StemFit AK02N medium. Fresh medium StemFit AK02N containing 20 μM Y-27632 was exchanged 2 h later. On day 2, the culture medium was replaced with StemFit AK02N medium without Y-27632. Next, iPSC colonies that had been successfully introduced with the vectors underwent drug selection using 125 μg/mL Hygromycin B solution (Nacalai 09287–84) and 5 μg/mL Puromycin (solution) (Invivogen ant-pr-1) in the culture medium. The fresh culture medium containing the drug was exchanged daily for 4 to 5 days. After the drug selection, each individual colony was picked up, and seeded onto the new 12-well plate pre-coated with iMatrix-511 silk (Matrixome 892021). Daily media change with StemFit AK02N was continued until the iPSCs with normal morphology were obtained.

Next, to introduce microRNA9/9*/124, the iPSCs containing the *Ngn2* and rtTA3G were infected with the lentiviral particles of CSIV-124-9-BclxL-TRE-EF-BsdT [16]. After 24 h, the culture medium (StemFit AK02N) was exchanged with the fresh culture medium. Then, after the infected iPSCs were amplified enough to form colonies, the iPSCs were exposed to 10 μg/mL Blasticidin S Hydrochloride (Funakoshi KK-400) for the drug selection.

The iPSCs containing rtTA3G, *Ngn2*, and microRNAs were induced into neurons using the following protocol. On day 0, the iPSCs were seeded on a 24-well plate coated at $5x10^4$ cells per well with 1 μg/mL Poly-L-lysine solution (Sigma P4832) and 3.0 μg/mL iMatrix-511 silk (Matrixome 892021) using the induction medium (Neurobasal Plus Medium (Gibco A35829-01) supplemented with 1 % Glutamax (Gibco 35050-061), 2 % B27 Plus supplement (Gibco 17504-001), 1 % CultureOne supplement (Thermo Fisher Scientific A3320201), 0.2 % dbcAMP (Nacalai 11540-61), 0.1 % L-ascorbic acid (Sigma A4544), 2 μg/mL Doxycycline Hydrochloride n-Hydrate (DOX) (Wako 049–31121), 20 μM Y-27632, 20 μM DAPT (Sigma D5942), and 1 % P/S (Nacalai 09367–34)). On day 5, the culture medium was changed to the induction medium without Y-27632 and DAPT supplemented with 20 ng/mL BDNF (Alomone labs B-250). After Day 5, half of the culture medium was exchanged with the fresh medium (half-medium change). The half-medium change was repeated twice a week with the differentiation medium (Neurobasal Plus Medium containing 1 % Glutamax (Gibco 35050–061), 2 % B27 Plus supplement (Gibco 17504-001), 1 % CultureOne supplement (Thermo Fisher Scientific A3320201), 0.2 % dbcAMP (Nacalai 11540-61), 0.1 % L-ascorbic acid (Sigma A4544), and 1 % P/S (Nacalai 09367-34)). Neurons after 14 days after induction were used for the analysis (Fig. 3A, 3B).

Induction of iPSCs to human astrocytes

Human astrocytes were induced from the iPSCs via embryoid bodies (EBs) and neurospheres (NSs) formation. The protocol was modified from the previously reported study [20]. First, the feeder-free iPSCs were passaged and plated onto the mitomycin-C-treated SNL murine fibroblast feeder cells using the 0.1 % gelatin-coated culture dishes. The iPSCs were cultured using human ES medium (D-MEM/Ham's F-12 with Phenol Red, HEPES, and SodiumPyruvate (DMEM/Ham's F-12 medium) (WAKO 042-30795) supplemented with 20 % KSR (Gibco 10828010), 1 % 200 mM L-Glutamine (Gibco), 0.8 % non-essential amino acid solution (NEAA) (Nacalai 06344-56), 0.1 mM 2-mercaptoethanol (2-ME) (Gibco 21985023), and 4 ng/mL fibroblast growth factor 2 (FGF2) (PeproTech) in a 37 ℃, 3 % CO_2 incubator.

On PID 0, the iPSCs were dissociated from the feeder cells using a dissociation solution (0.25 % trypsin, 100 μg/mL collagenase IV (Invitrogen), 1 mM $CaCl_2$, and 20 % KSR (Gibco 10828010)), and cultured in FGF2-free human ES medium supplemented with 10 μM Y-27632. The cells were plated on the ultra-low attachment dish (Falcon) and incubated in a 37 ℃, 5 % CO_2 incubator. On PID 1, cells were cultured using EB medium (D-MEM/Ham's F-12 medium (WAKO 042–30795) containing 5 % KSR (Gibco 10828010), 1 % L-Glutamine (Gibco), 0.8 % NEAA (Nacalai 06344–56), and 0.1 mM 2-ME (Gibco 21985023)) supplemented with 3 μM dorsomorphin (Santa Cruz Biotechnology), 3 μM SB431542 (Sigma), and 3 μM CHIR99021 (Focus Biomolecules) to enhance the differentiation towards neural lineage. On PID 4, the culture medium was changed to EB medium supplemented with 1 μM retinoic acid (RA) (Sigma R2625). On PID 7, the medium was changed to EB medium supplemented with 1 μM RA (Sigma R2625) and 1 μM purmorphamine (PM) (Cayman). The medium was replaced with the fresh EB medium supplemented with 1 μM RA (Sigma R2625) and 1 μM PM

(Cayman) every 3 days.

On PID 16, the culture medium was changed to neurosphere medium (KBM Neural Stem Cell Medium (KOHJIN BIO 16050100) supplemented with 2 % B-27 (GIBCO 17504-001), 0.8 % NEAA (Nacalai 06344-56), 1 μM PM (Cayman), 20 ng/mL FGF2 (PeproTech), and 10 ng/mL epidermal growth factor (EGF) (PeproTech)). The culture medium was changed with the fresh neurosphere medium every 4 days until PID 31 to generate the primary neurospheres. On PID 32, the culture medium was changed to the neurosphere medium without PM. The culture medium was changed with fresh medium every 4 days until PID 47 to generate the secondary neurospheres. On PID 48, to start the astrocyte differentiation, the secondary neurospheres were dissociated into single cells, and the cells were plated at 10 x 10^5 cells per well onto a 6-well plate pre-coated with 0.5 % Matrigel (Corning 354277) diluted in a cold PBS. The cells were cultured using the astrocyte differentiation medium (KBM Neural Stem Cell Medium (KOHJIN BIO 16050100) supplemented with 2 % B-27 (GIBCO 17504-001), 0.8 % NEAA (Nacalai 06344-56), 10 ng/mL brain-derived neurotrophic factor (BDNF) (Alomone labs B-250), and 10 ng/mL glia-derived neurotrophic factor (GDNF) (Alamone labs G-240)). To purify the astrocytic population, the cells were passaged with the fresh medium every 7 days. The cells were detached using Accutase (Nacalai 12679-54) and replated onto a 6-well plate pre-coated with 0.5 % Matrigel (Corning 354277) at 37 ℃, 5 % CO_2 incubator. The astrocytes after PID 62 were used for the analysis (Supplementary Fig. 2).

Induction of iPSCs into the cortical brain organoids

The iPSCs were plated at 72 x 10^4 cells per dish onto a 6-cm culture dish coated with 3.0 μg/mL iMatrix-511 silk (Matrixome 892021). The culture medium was changed daily for 7 days with 4 mL StemFit AK02N (Ajinomoto) supplemented with 0.5 % Penicillin/Streptomycin (P/S) (Gibco 15140122). At PID 0, to generate the cell aggregates, iPSCs were replated onto a round bottom 96-well plate (SUMITOMO BAKELITE CO, Ltd MS-9096 V) at 1x10^4 cells per well using 100 μL aggregation medium (DMEM/F-12 medium (Wako 042–30795) supplemented with 20 % KSR (Gibco 10828010), 0.5 % non-essential amino acids (NEAA) (Gibco 11140050), 0.1 % 2-mercaptoethanol (2-ME) (Gibco 21985023), 1 % Glutamax (Gibco 35050061), 2 μM IWP-2 (Sigma 10536-5MG), 10 μM SB431542 (Sigma S4317-5MG), 50 μM Y-27632 (Wako 034–24024), and 0.5 % P/S (Gibco 15140122)). On PID 3, the medium was exchanged with 200 μL per well of the fresh aggregation medium supplemented with 200 nM LDN193189 (Stemgent). On PID 7, the culture medium was exchanged with induction medium (aggregation medium with 1 μM SB431542, without KSR, 2-ME, IWP-2, and Y-27632, supplemented with 0.5 % N2 supplement (Gibco 17502001), 1 μM CHIR99021 (Axon Medchem), and 50 % Matrigel (Corning)) at 150 μL per well. On PID 14, cell aggregates were washed with the wash medium (DMEM/F-12 medium (Wako 042-30795)), and the cell aggregates were transferred to bioreactors (Able BWV-S03A). From PID 14, 30 mL of differentiation medium consisting of DMEM/F-12 medium (Wako 042-30795) supplemented with 0.5 % N2 supplement (Gibco 17502001), 1 % B27 supplement (Gibco), 0.5 % non-essential amino acids (NEAA) (Gibco 11140050), 0.1 % 2-mercaptoethanol (2-ME) (Gibco 21985023), 2.5 μg/mL Insulin (Wako 093-06351), 1 % Glutamax (Gibco 35050061), 1 % Chemically Defined Lipid Concentrate (CDLC) (Gibco 11905031), and 0.5 % P/S (Gibco 15140122) was exchanged 2 times a week. B27 supplement without vitamin A was used from PID 14 to PID 28, and B27 supplement with vitamin A was used after PID 28. The cortical brain organoids at 10 weeks and 20 weeks after transferring cells to the bioreactor (PID 14) were used for the immunohistochemistry (Fig. 4A).

Results

Derivation of UDCs from a very old donor and generation of iPSCs

Thirty ml of urine sample was collected from an 89-year-old donor who had been diagnosed with Alzheimer's disease (AD) (TMGH-1 (Table 1)) based on the following biomarkers. Cerebrospinal fluid (CSF) collected at 83 years old showed an increased level of total Tau (t-Tau) at 400.60 mg/dL and phosphorylated Tau (p-Tau)-181 at 60 mg/dL [21,22]. In addition, magnetic resonance imaging (MRI) also confirmed the atrophy of the hippocampus and the medial temporal lobes. The clinical course was shown as a change in the Mini-Mental State Examination (MMSE) score and the MRI imaging analysis of the hippocampal atrophy (voxel-based specific regional

Table 1
Characteristics of the donor.

Name of the iPSC line	TMGH-1
Age at sampling	89 y.o.
Sex	Female
Ethnicity	Japanese
Disease	Alzheimer's disease
***APOE* Genotype**	*APOE* ε3/ε3
Family History	No AD patients in the family
CSF (measured at 83 y.o.)	Aβ (1–42) 463.9 pg/mL, t-Tau 400.60 mg/dL, p-Tau 60 mg/dL

CSF: Cerebrospinal Fluid; Aβ (1–42): Amyloid-beta (1–42); t-Tau: total Tau protein level; pTau: phosphorylated Tau level. Facility Standardized Normal Value: Aβ (1–42) <500 pg/mL, t-Tau > 300 mg/dL, p-Tau > 55 mg/dL.

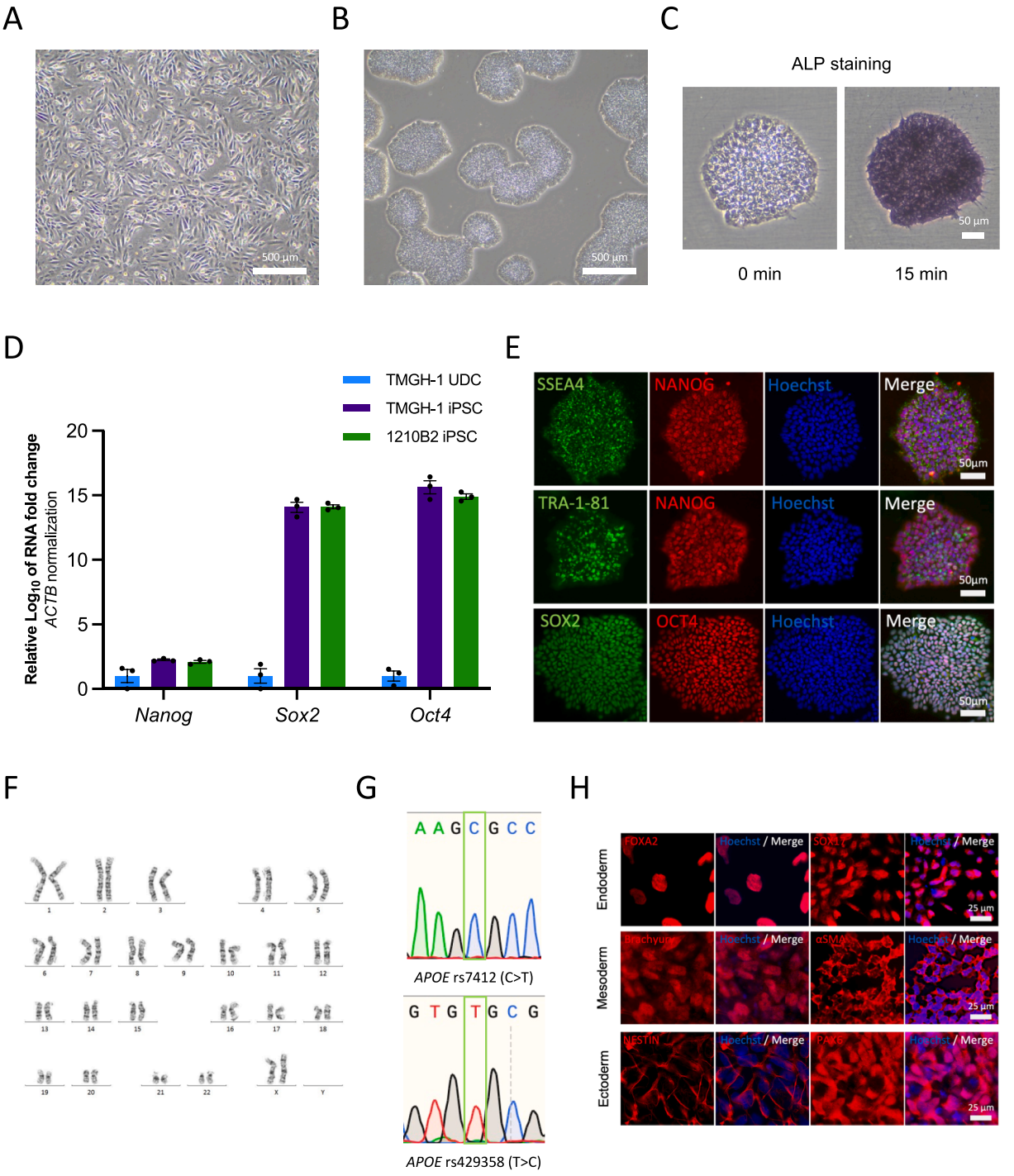

Fig. 1. Derivation of the UDCs from the very old donor's urine sample and generation of the iPSCs. (A) The UDCs. Scale bar, 500 μm.; (B) The generated iPSCs. Scale bar, 500 μm.; (C) ALP staining of the iPSCs. Scale bar, 50 μm.; (D) qPCR results of the pluripotency markers (*Nanog*, *Sox2*, and *Oct4*) from the TMGH-1 UDCs (n = 3), the TMGH-1 iPSCs (n = 3), and the control 1210B2 iPSCs (n = 3). Bars, mean ± SEM; (E) Immunocytochemistry of the pluripotency markers (SSEA4, TRA-1–81, SOX2, NANOG, and OCT4) on TMGH-1 iPSCs. Scale bar, 50 μm; (F) Karyotype result of TMGH-1 iPSCs (Female: 46, XX); (G) *APOE* genotyping results of the generated iPSCs: (H) Trilineage differentiation assay of the iPSCs into the three germ layers (endoderm, mesoderm, and ectoderm). Scale bar, 25 μm.

analysis system for AD (VSRAD)) (Supplementary Fig. S1).

Urine-derived cells (UDCs) were successfully cultured and amplified from the urine sample of the patient (Fig. 1A). The UDCs were reprogrammed into iPSCs using the Sendai virus delivery system [13] (Fig. 1B). The generated iPSCs showed normal iPSC morphology.

The stemness of the cells was evaluated by alkaline phosphatase (ALP) staining (Fig. 1C). RT-qPCR revealed that the TMGH-1 iPSCs at passage 30 increased the expression of pluripotent stem cell markers (*Nanog*, *Sox2*, and *Oct4*) at levels comparable to the control iPSCs which were derived from PBMCs of the 29-year old female donor [18] (Fig. 1D). The immunocytochemistry confirmed the expression of SSEA4, TRA-1–81, SOX2, NANOG, and OCT4 of the TMGH-1 iPSCs (Fig. 1E). Karyotyping revealed the normal chromosomes of TMGH-1 iPSCs (Female: 46, XX) (Fig. 1F). DNA sequencing of the iPSCs confirmed the *APOE* genotype (*APOE* ε3/ε3) of the donor (Fig. 1G). Furthermore, the iPSCs held pluripotency characteristics and were successfully differentiated into three germ layers: Endoderm (FOXA2 and SOX17); Mesoderm (Brachyury and αSMA); and Ectoderm (NESTIN and PAX6) (Fig. 1H). Thus, this suggested that the iPSCs with normal pluripotency and stemness can be generated from the urine sample of the old donor.

Generation of 2D neural models from the iPSCs derived from UDCs

The TMGH-1 iPSCs were induced into neuronal cells. First, the iPSCs were efficiently converted into neurons with neuronal morphology using the small-molecule compounds based on dual SMAD inhibition (Fig. 2A, 2B) [23]. These hiPSC-derived neurons

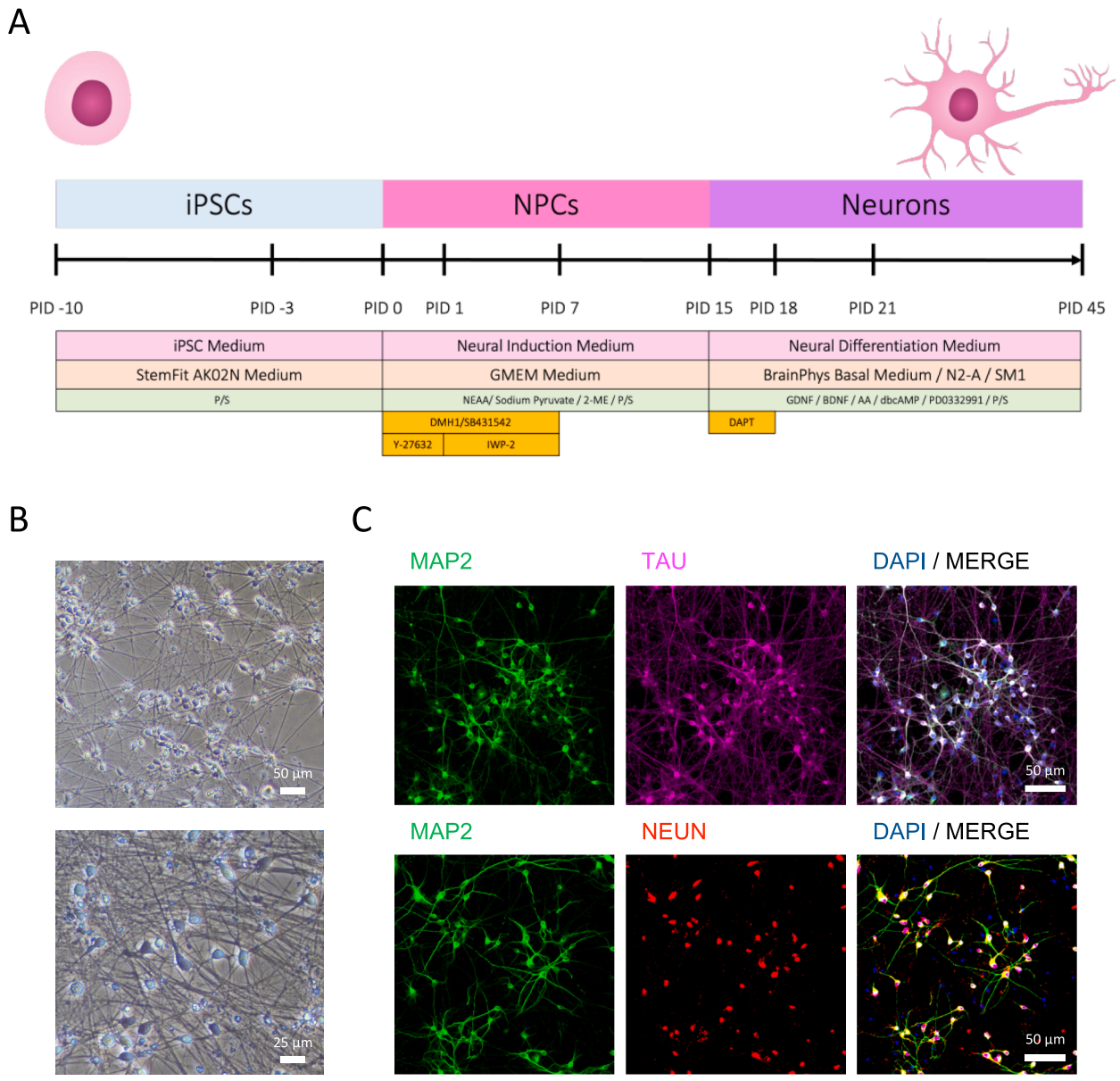

Fig. 2. 2D neuronal models induced from the iPSCs derived from the UDCs using the small-molecule compounds according to the dual SMAD inhibition. (A) Induction method; (B) Induced neurons at PID 45. Scale bar, 50 and 25 µm; (C) Neurons from the dual SMAD inhibition at PID 45 expressed neuronal markers (MAP2, βIII-TUBULIN, TAU, and NEUN). Scale bar, 50 µm.

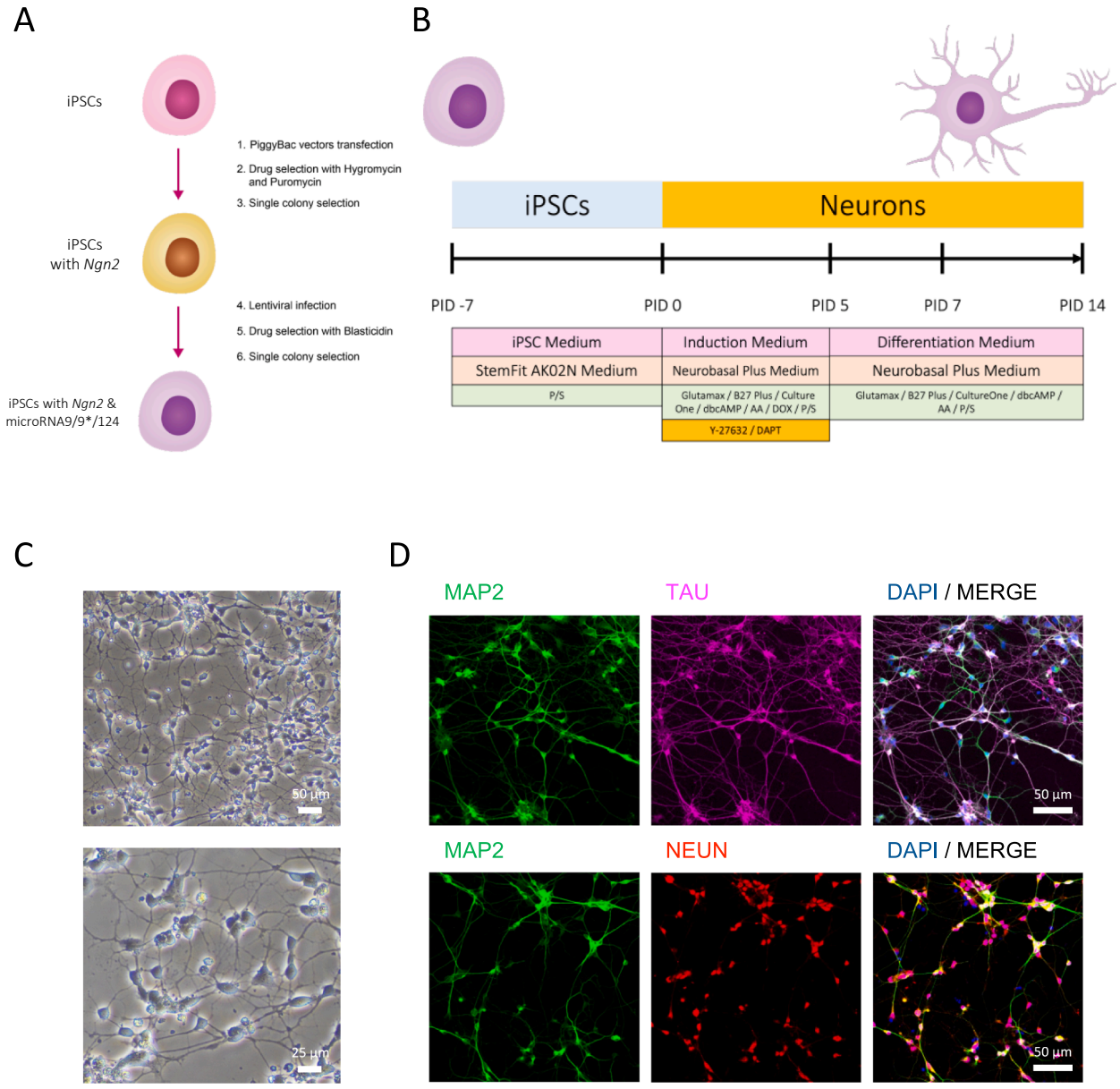

Fig. 3. 2D neuronal models induced from the iPSCs derived from the UDCs using the transient expression of *Ngn2* and microRNAs. (A) Establishment of the iPSCs containing *Ngn2* and microRNAs; (B) Neuronal induction method; (C) Induced neurons at PID 14. Scale bar, 50 and 25 µm; (D) Neurons from the transient expression of *Ngn2* and microRNAs at PID 14 expressed neuronal markers (MAP2, βIII-TUBULIN, TAU, and NEUN). Scale bar, 50 µm.

expressed neuronal markers (MAP2, Tau, and Neun) (Fig. 2C). Next, the rapid neuronal induction by the transient expression of *Ngn2* and microRNAs was applied to the TMGH-1 iPSCs [19]. The iPSCs introduced with transcription factor and microRNAs were efficiently induced into neurons after 14 days of neuronal induction (exposure to DOX for 5 days) (Fig. 3A, 3B, 3C). The induced neurons had normal neuronal morphology and expressed neuronal markers (Fig. 3D).

We also demonstrated that the urine-derived TMGH-1 iPSCs from the urine of a very old donor can also be used to generate embryoid bodies (EBs), neurospheres (NSs), and finally differentiate into human astrocytes. Following the previously reported protocol (Supplementary Fig. 2A) [20], the TMGH-1 iPSCs can be differentiated into human astrocytes (Supplementary Fig. 2B). The induced astrocytes expressed astrocytic markers, including S100β, GFAP, CD44, and Vimentin (Supplementary Fig. 2C).

Induction of cortical brain organoids from the iPSCs derived from UDCs

Finally, 3D cortical brain organoid induction was applied to the TMGH-1 iPSCs (Fig. 4A). The induction in the round bottom well at

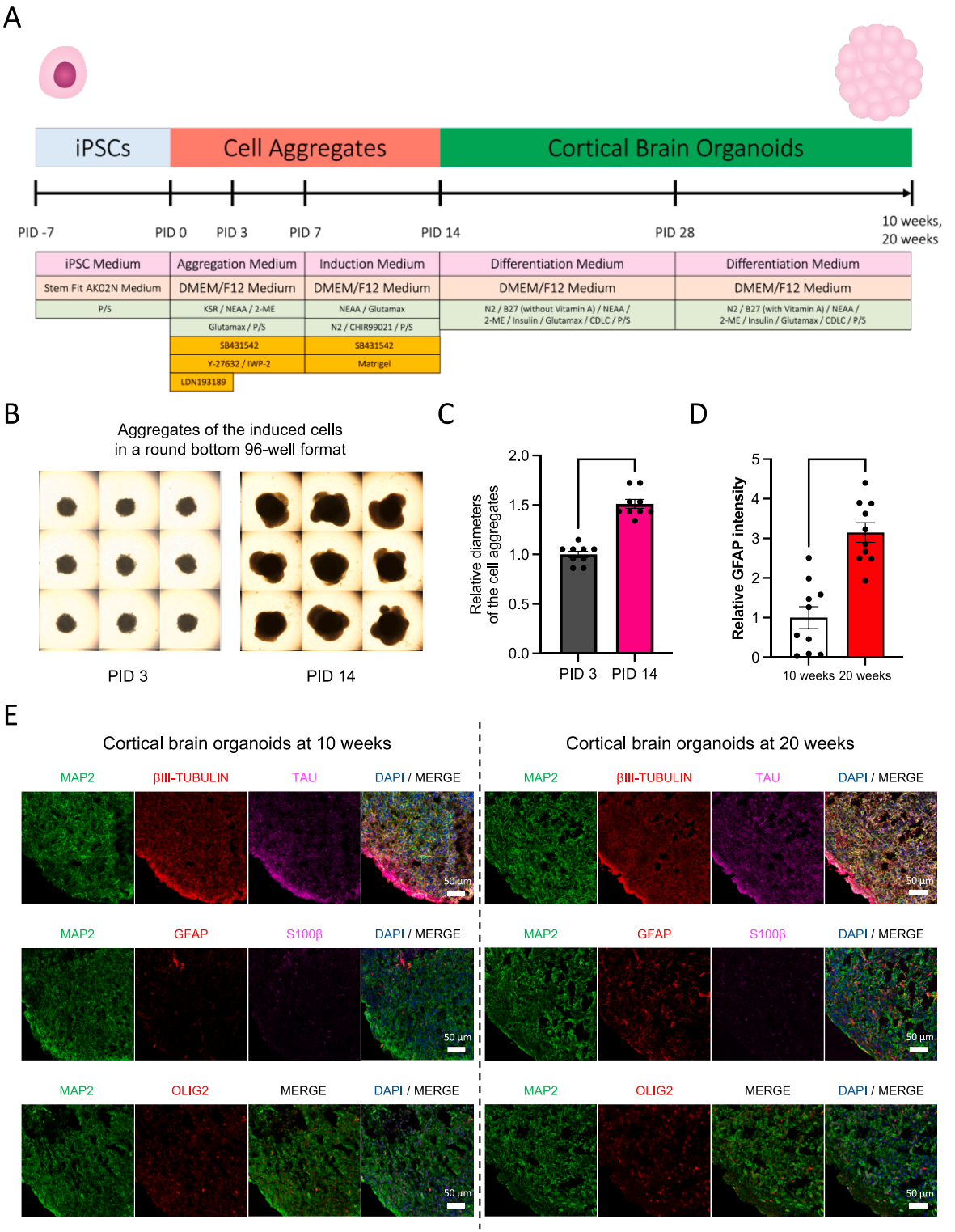

Fig. 4. Cortical brain organoids induced from the iPSCs derived from the UDCs. (A) Induction method of brain organoids from the iPSCs; (B) Cell aggregates in the 96-well format at PID 3 and 14; (C) Quantification of the diameter of the cell aggregates at PID 3 (n = 9) and 14 (n = 9). Bars, mean ± SEM. ****$p < 0.0001$ (Mann-Whitney test); (D) Relative GFAP intensity of TMGH-1 cortical brain organoids at 10 weeks and 20 weeks. Bars, mean ± SEM. ****$p < 0.0001$ (Unpaired *t*-test); (E) Immunohistochemistry of the neuronal markers (MAP2, βIII-Tubulin, and Tau), astrocyte marker (GFAP and S100β), and oligodendrocyte marker (Olig2) in TMGH-1 cortical brain organoids at 10 weeks and 20 weeks after the differentiation. Scale bar, 50 µm.

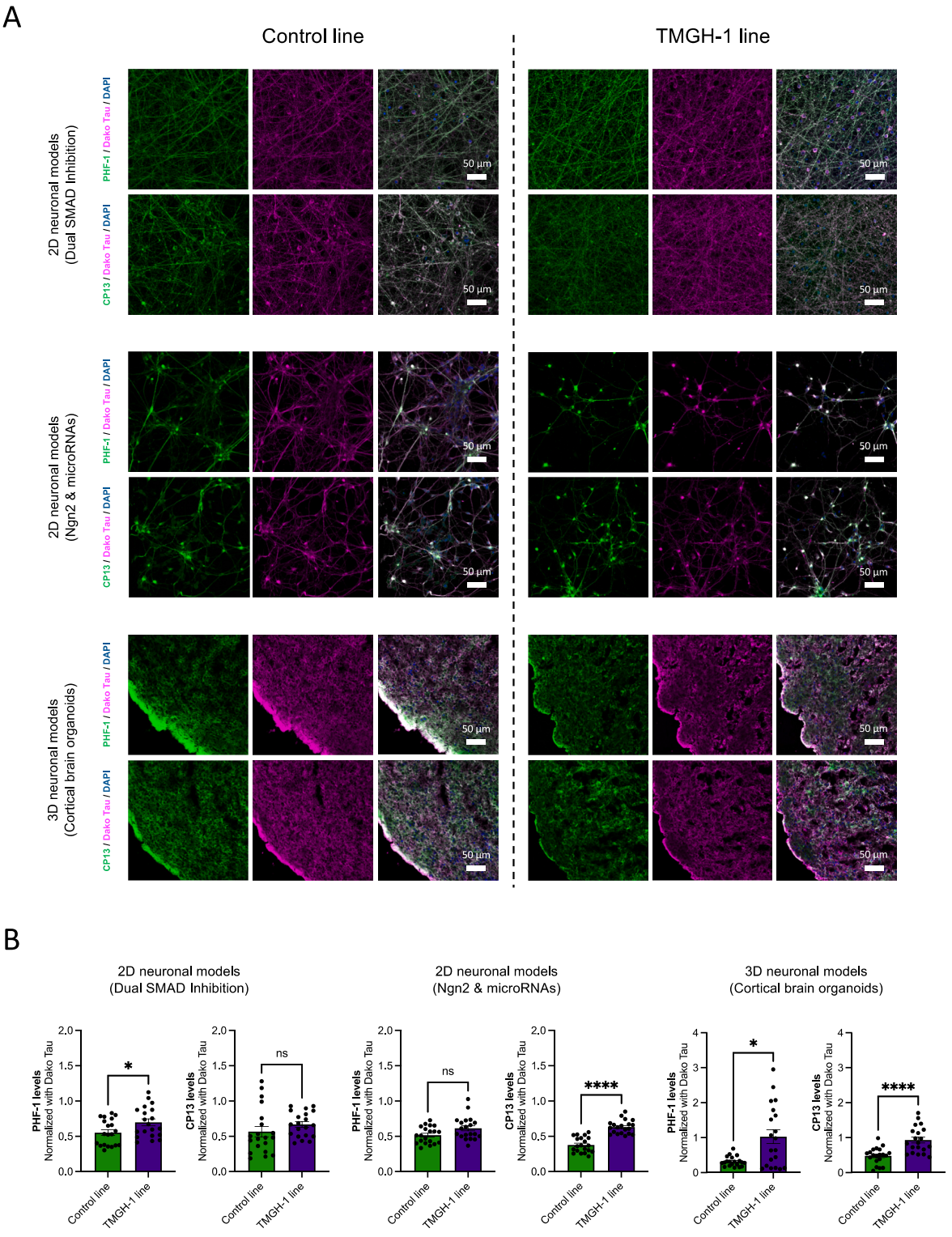

Fig. 5. Alzheimer's Disease (AD)-like phenotypic analysis of the neuronal models generated from TMGH-1 iPSCs. (A) Staining of phosphorylated Tau (pTau) (PHF-1 and CP13) and neuronal markers (MAP2 and Dako Tau) in the 2D neuronal models (Dual SMAD inhibition), 2D neuronal models (Transient expression of *Ngn2* and microRNAs), and 3D cortical brain organoids generated from iPSCs of TMGH-1 line and control line. Scale bar, 50 μm; (B) Quantification of pTau expression levels of each neuronal models (n = 20 each) generated from iPSCs of TMGH-1 line and control line. Bars, mean ± SEM. PHF-1 levels of 2D neuronal model (dual SMAD Inhibition): $^{*}p = 0.0350$; PHF-1 levels of 3D neuronal model (Cortical brain organoid): $^{*}p = 0.0460$; $^{****}p < 0.0001$ (Mann-Whitney test).

two weeks resulted in aggregates of the cells (Fig. 4B). Measurement of the diameter of cell aggregates showed that cell aggregates became larger at PID 14 compared to PID 3. To investigate the changes in organoid characteristics after the culture period, we harvested organoids at 10 weeks and 20 weeks for immunostaining. We found that GFAP expression was increased in the organoids at 20 weeks compared to that at 10 weeks (Fig. 4D, 4E). The increased GFAP signals may be due to non-astrocyte cells such as radial glia because another astrocytic marker (S100β) was not increased at 20 weeks, and the colocalization of GFAP and S100β was also limited at both time points. The organoids also expressed the neuronal markers (MAP2, βIII-Tubulin, and Tau) and the marker for oligodendrocytes (Olig2) at both time points (Fig. 4E).

AD-like phenotypic analysis of the neuronal models generated from iPSCs derived from UDCs

Since iPSCs-derived neuronal models are widely used to recapitulate disease pathogenesis in a dish [3], we stained the iPSC-derive cells in our TMGH-1 iPSC-derived neuronal models with anti-pTau (PHF-1 and CP13) antibodies and compared them with those from the healthy control donor (line 1210B2) (Fig. 5A). In the mixed population of excitatory and inhibitory neurons induced by dual SMAD inhibition, the PHF-1 signal was significantly increased in the TMGH-1 line, while CP13 also showed a trend towards an increase (Fig. 5A, B). In the pure excitatory neuronal culture induced by Ngn2 and miRNAs, the CP13 signal was significantly increased in the TMGH-1 line, while PHF-1 signal showed a trend towards an increase. The 20-week-old brain organoids, which contain more diverse cell types than the other two cultures showed higher levels of both PHF-1 and CP13 in the TMGH-1 line compared to the 1210B2 line (Fig. 5B). These results suggest that the neuronal models generated from TMGH-1 iPSCs can be used for to recapitulate elevated pTau levels.

Discussion

Our present study demonstrated that UDCs derived from a very old adult could be efficiently reprogrammed into the iPSCs using a typical non-integrating reprogramming method, the Sendai virus delivery system. In addition to the UDCs from healthy individuals in their 20s, UDCs from 65-year-old healthy individuals could also be reprogrammed into iPSC with lower efficiency than 20s [5]. Regarding donor condition, UDCs from disease patients aged 4–41 years could be reprogrammed into iPSCs [24,25]. However, the ability of iPSCs to differentiate into different cell types depends on the epigenetic status of the original cells [12,26–28]. The most important epigenetic feature of cells is DNA methylation, which accumulates with age. Therefore, we aimed to confirm the differentiation capacity of iPSCs from a very old donor. In particular, in this study, UDCs were isolated from a very old AD patient and converted to iPSCs and then differentiated into neural cells using different methods to test their differentiation capacity. This suggests the utility of this cell source for reprogramming to generate iPSCs and for targeted study of diseases in the elderly population. As urine samples can be collected from donors or caregivers at home, our confirmation of the applicability of urine for AD research will broaden the population for the research [29].

To further confirm the applicability of UDC-derived iPSCs for the modeling of neurological diseases, we demonstrated the induction capacity of iPSC into different types of neural cells. The iPSCs could be induced into the 2D neuronal models using both the dual SMAD inhibition with small-molecule compounds via the neural progenitor stage and the rapid induction method using transient expression of *Ngn2* and microRNAs not via the neural progenitor stage. The glial cells like astrocytes can also be differentiated from our urine-derived iPSCs. Furthermore, the iPSCs can be induced into the 3D cortical brain organoids mimicking the 3D structure of the brain. These results demonstrated the usefulness of urine as a source for iPSCs and disease modeling of the old donor.

The modeling of AD using the iPSCs has provided insights into the mechanism of the disease in human cells and the development of new drugs [3,29,30]. The first 2D model of AD using the iPSCs was established from the fibroblasts of familial AD patients with *PSEN1* mutation. The neurons induced from the iPSCs via the formation of EBs and NSs increased the secreted amyloid-beta (Aβ) levels, and the secreted Aβ levels were decreased by the treatment with γ-secretase inhibitor [31]. Another study, using the iPSCs reprogrammed from fibroblasts of sporadic AD patients, investigated the association between the disease pathology and the single nucleotide polymorphisms (SNPs) of *SORL1*; secreted Aβ levels of the neurons with specific *SORL1* genotypes were reduced after the treatment with BDNF [32].

Particularly in the study of sporadic diseases of diverse etiology, such as AD, it is essential to use multiple cases to represent the general characteristics of the disease, especially in the study where patient samples are available. In our previous study, 32 lines of the iPSCs from different patients were used for disease modeling and drug screening in sporadic amyotrophic lateral sclerosis [33]. For sporadic AD, many recent studies have also utilized iPSCs from many patients [34,35]. However, in these studies, the iPSCs were originally established from the patient's PBMCs. The iPSCs were induced into neurons using the rapid differentiation method with *Ngn2* overexpression. In this study, we demonstrated that the iPSCs reprogrammed from the stable and easily obtainable cell source like UDCs, combined with the rapid neuronal induction method that provides mature neuronal cells, could accelerate disease analysis and drug screening. In addition, our study demonstrated that the rapid differentiation method using the *Ngn2* and microRNAs is also applicable to the urine-derived iPSCs derived from the urine sample.

The cortical brain organoids have been used to study familial and sporadic types of AD patients. Brain organoids generated from the iPSCs of the familial AD patients with *PSEN1* mutation, initially derived from fibroblasts, expressed the accumulation of Aβ plaque and increased phosphorylated tau (pTau) levels [36,37]. On the other hand, the 3D model using the iPSCs derived from the sporadic AD case with exposure to human serum resulted in increased Aβ and pTau levels and synaptic loss [38]. In this study, we could demonstrate the possibility of generating the 3D model of cortical brain organoids from urine samples of a sporadic AD case via iPSCs. The organoids generated in our study demonstrated the complex structure in which several cell types including excitatory neurons,

inhibitory neuron, and astrcoytes could be observed.

The phenotypic analyses of AD pathologies of the 2D/3D models generated from the TMGH-1 iPSC line in our study showed an increase in pTau levels (PHF-1 and CP13) compared to the control line. Although some of the markers were not significantly increased in the 2D models, both PHF-1 and CP13 were significantly increased in the 3D cortical brain organoid models when compared with the control line. The pathogenesis of the sporadic AD is milder compared to familial AD in general [39], which resulted in difficulty in the recapitulation of disease phenotypes using iPSC-derived models especially because the TMGH-1 donor had the *APOE*3/3 genotype. However, some of the previous studies also showed an increase in pTau levels even in sporadic cases [32,40].

We demonstrated that the iPSCs generated from the urine of a very old donor is also applicable to the generation of the non-neuronal glial population such as astrocytes. Although there are many studies using the models of iPSCs that showed the recapitulation of AD-like phenotypes in the neurons, only few studies demonstrated the phenotypes of the non-neuronal population [41]. Some of the astrocytic phenotypes found in the brain biopsy of AD patients include astrogliosis or astrocytic hypertrophy [42,43], in which the soma of astrocytes became larger and the proportion of GFAP-positive cells increased. This can be due to the low percentage of astrocytes or the requirement of pathological genetic factors like *APOE*4 and familial AD mutations. Future perspectives in the application of UDC-derived iPSCs to recapitulate these phenotypes include the generation of a co-culture model with enriched astrocytes differentiated from the iPSCs of familial cases like previous study using co-culture of astrocytes and microglias [44]. The co-culturing of several differentiated cells from the iPSCs was shown to enhance the maturation of each cell type. Subsequently, the enhanced maturation from co-culturing better recapitulates the disease phenotypes [44].

Lastly, age-dependent epigenetics are erased in iPSCs [45], and the erasure limits the utility of the iPSC-derived neural model to study age-dependent diseases like AD. In addition, because the iPSC-derived neurons do not express adult tau isoforms, four-repeat tau isoforms, it is not appropriate to mimic the phenotypes by four-repeat tau [46]. Despite such limitations, it is known that approximately 50 % of patients with sporadic AD have some genetic predisposition to the disease, and iPSC technology may be useful to demonstrate the phenotype of these genetically predisposed diseases *in vitro*. In fact, some previous studies demonstrated that the neuronal models derived from the iPSCs could recapitulate the disease pathogenesis in relation to the polygenic risks [34,35]. Thus, iPSCs-derived neural models can be used to model some aspects of age-dependent disease. Then, our confirmation of the applicability of UDC-derived iPSCs, even from an old AD patient, proved that urine is a reliable cell source for the phenotypic analysis of neurological diseases in a dish.

Conclusions

This study demonstrated that urine samples, which can be easily obtained from donors at home, are an efficient source for the generation of the iPSCs, even from a very old donor. Using the widely used establishment method, such as the non-integrating Sendai virus system, the generated iPSCs from the UDCs expressed normal stemness and pluripotency. The iPSCs derived from urine samples were efficiently differentiated into various neural models comprised of both 2D and 3D. The neuronal models generated from the iPSCs in our study also showed differences in disease phenotype compared to the control iPSC line. Since urine, unlike other somatic sources, can be obtained repeatedly with the non-invasive approach, this cellular source is promising for application in modeling diseases that require large sample sizes. In particular, neuronal models derived from urine samples via iPSCs will be a powerful tool for studying disease mechanisms in patients with known mutations, drug screening, and stratification of sporadic diseases where cellular modeling of many patient cases is required.

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Institutional review board statement

The study was conducted per the Declaration of Helsinki, approved by the Ethics Committee for Human Research of the Keio University School of Medicine (#20080016) and the Ethics Committee of the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology (#R20-52).

Informed consent statement

Informed consent was obtained from the patient subject in this study. Written informed consent has been obtained from the patient to publish this paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbas.2023.100101.

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