



Induction of pluripotent stem cells from autopsy donor-derived somatic cells

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ABSTRACT

Human induced pluripotent stem cells (iPSCs) have become an intriguing approach for neurological disease modeling, because neural lineage-specific cell types that retain the donors' complex genetics can be established *in vitro*. The statistical power of these iPSC-based models, however, is dependent on accurate diagnoses of the somatic cell donors; unfortunately, many neurodegenerative diseases are commonly misdiagnosed in live human subjects. Postmortem histopathological examination of a donor's brain, combined with premortem clinical criteria, is often the most robust approach to correctly classify an individual as a disease-specific case or unaffected control. In this study, we describe iPSCs generated from a skin biopsy collected postmortem during the rapid autopsy of a 75-year-old male, whole body donor, defined as an unaffected neurological control by both clinical and histopathological criteria. These iPSCs were established in a feeder-free system by lentiviral transduction of the Yamanaka factors, Oct3/4, Sox2, Klf4, and c-Myc. Selected iPSC clones expressed both nuclear and surface antigens recognized as pluripotency markers of human embryonic stem cells (hESCs) and were able to differentiate *in vitro* into neurons and glia. Statistical analysis also demonstrated that fibroblast proliferation was significantly affected by biopsy site, but not donor age (within an elderly cohort). These results provide evidence that autopsy donor-derived fibroblasts can be successfully reprogrammed into iPSCs, and may provide an advantageous approach for generating iPSC-based neurological disease models.

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Traditional *in vitro* disease modeling approaches rely on primary or immortalized somatic cells that are either obtained from a diseased individual, or are genetically modified to exhibit a disease phenotype *in vitro* [9]. Unfortunately, these models require a physiologically relevant cell line, which may not be easily available, and genetic modification may not recapitulate the etiology of complex diseases. Human induced pluripotent stem cells (iPSCs) can overcome these shortcomings, because genetically identical, tissue-specific cell types that are disease-applicable can be generated *in vitro* [9,12,18,23]. Donor-specific iPSC models are particularly intriguing for the study of neurological and neurodegenerative diseases, because unlike other tissues of the human body, live brain tissue often cannot be obtained from living subjects without undue risk of cognitive or functional impairment [5].

The statistical power of iPSC-based neurological models is dependent on the diagnostic accuracy of the diseased (case) and unaffected (control) somatic cell donors. For many neurological diseases, premortem clinical criteria do not provide sufficient information for the subject to be given a definite diagnosis, but rather a possible or probable diagnosis [2,4,6–8,14–16,19]. For example, Alzheimer's disease can be identified through clinical evaluations as the possible or probable cause of dementia; however, the definite diagnosis of this pathology cannot currently be confirmed until clinical criteria are combined with postmortem histopathological observations of the subject's brain [2,4,8,14]. Because clinical phenotypes like dementia can be shared by multiple neuropathies, postmortem brain banking programs have become an exceptional resource for providing neuropathy-associated brain tissue that has been subjected to the most robust diagnostic methods. In fact, much of what is known about many neurological and neurodegenerative diseases has been from analyses (histological, biochemical, molecular, etc.) on autopsy donor-derived brain tissue [2,6–8,12,15,16].

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Meske et al. have demonstrated that human dermal fibroblasts (HDFs) can be established in cell culture from autopsy donors up to 48 h postmortem and from individuals up to 99-year-old [13]. To our knowledge, however, there are no published reports of autopsy donor-derived somatic cells being used for human iPSC generation. Because somatic cell senescence has been identified as a potential barrier in iPSC reprogramming, our group sought to investigate whether autopsy donor-derived fibroblasts could be induced to a pluripotent state [1,3,10,20]. In addition, we chose to examine if these iPSCs could be differentiated *in vitro* into derivatives of the neural lineage, since this approach may be particularly valuable for neurological disease research.

Tissue was collected after a postmortem interval (PMI) of approximately 3–7 h during the rapid autopsies of 19 whole-body donors (ages 72–97). iPSCs were generated from one autopsy donor, a 75-year-old male identified by clinical studies and histopathological examination as being suitable as a “control” subject as he was negative for major neurological and neuropathological conditions (non-demented, without clinical parkinsonism and without diagnostic levels of histopathology for any major neuropathological condition). The subjects were enrolled as whole body donors in the Banner Sun Health Research Institute (BSHRI) Brain and Body Donation Program and had previously signed informed consent approved by the BSHRI Institutional Review Board (IRB) [2]. Fibroblast cell lines were established as previously described by Villegas and McPhaul, with minor modifications (see [Supplementary methods](#)) [21].

A One-way Analysis of Variance (ANOVA) and Tukey's HSD Post hoc test was performed on the cell counts at passage 1 (P:1) from 30 primary fibroblast cell lines established from 3 autopsy donors to determine the effect of biopsy site. A One-way ANOVA was performed on the cell counts from 34 primary fibroblast cell lines (all obtained from the same biopsy site (arm)) established from 18 autopsy donors to determine the effect of age (binned into 4-year groups). Data sets met the parametric assumptions of normality and homogeneity of variances. All statistics were performed using IBM SPSS Statistics, Version 19 software. Graphs were generated using Microsoft Excel 2008.

Autopsy donor-derived fibroblast cell line F02AA1 was selected for induced pluripotency experiments. Human iPSCs were generated in a feeder-free culture system by lentiviral transduction of the Yamanaka factors as previously described (see [Supplementary methods](#)) [3,11,18]. iPSC lines (through P:10) were frozen in cryopreservation media (mFreSrR; Stem Cell Tech.) and stored at -130°C prior to characterization and differentiation experiments, which were performed using iPSC clones 2–13 and 2–21 at passage 8 (P:8).

Embryoid bodies (EBs), with approximately 400 cells per EB, were generated using Agrewell 400 Plates (Stem Cell Tech) according to the manufacturer's instructions. EBs were transferred in suspension to low adherence, non-treated 6-well plates (BD Biosciences) with approximately 500 EBs per well.

Inductive loss of pluripotency (LOP) was performed as previously described with minor modifications [17]. Briefly, EBs were cultured in suspension for 5 days in hESC media *without* basic fibroblast growth factor (bFGF) (DMEM/F12 (Invitrogen), 20% Knockout Serum Replacement (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (NEAA; Invitrogen), 0.0007% 2-mercaptoethanol (Sigma Aldrich), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma Aldrich), and 5 $\mu\text{g}/\text{ml}$ Plasmocin prophylactic (Invivogen)), and the media was exchanged every other day. The LOP media was then replaced with NeuroCult NS-A Proliferation media (Stem Cell Tech), supplemented with 20 ng/ml recombinant human epidermal growth factor (rhEGF; Stem Cell Tech), 10 ng/ml bFGF (Stemgent, Cambridge, MA, USA), 2 $\mu\text{g}/\text{ml}$ Heparin (Stem Cell Tech), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma Aldrich), and 5 $\mu\text{g}/\text{ml}$ Plasmocin

prophylactic (Invivogen, San Diego, CA, USA), and the EBs were cultured in suspension for an additional 5 days. EBs were plated in complete NeuroCult NS-A Proliferation media onto 6-well tissue culture-treated plates (BD Biosciences) pre-coated with 1 ml/well hESC-qualified Matrigel matrix (BD Biosciences), diluted 1 aliquot per 10 ml DMEM/F12 (Invitrogen). EBs were allowed to collapse onto the matrix, and adherent cells were further cultured in complete NeuroCult NS-A Proliferation media for 14 days. Neural precursors were collected by bulk enzymatic passage with 1 mg/ml Collagenase IV (Sigma Aldrich) in DMEM/F12 (Invitrogen) as previously described [17]. Cells were seeded for differentiation experiments at 5×10^3 cells/cm² on glass coverslips (VWR, West Chester, PA, USA) pre-coated with 1 ml/coverslip of hESC-qualified Matrigel matrix (BD Biosciences), diluted 1:10 as described above.

Cells plated onto glass coverslips were maintained in NeuroCult NS-A Differentiation media supplemented with 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma Aldrich), and 5 $\mu\text{g}/\text{ml}$ Plasmocin prophylactic (Invivogen), and the media was exchanged every other day. Cells were allowed to differentiate for 14, 21, 28 or 35 days, at which time the coverslips were fixed in 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) in DPBS (Invitrogen) for 15 min at room temperature. The coverslips were then rinsed and stored in DPBS (Invitrogen) at 4°C for later use in immunocytochemistry (ICC) experiments (see below).

Cells cultured on Matrigel-coated glass coverslips and fixed with 4% paraformaldehyde were used for all ICC assays. Cells were permeabilized for 10 min with 0.1% Triton X-100 (Thermo Fisher Scientific) in DPBS (Invitrogen), followed by a 30-min incubation with blocking solution (2.5% bovine serum albumin (BSA) in DPBS with 0.1% Tween 20 (Sigma)). All primary antibodies were incubated overnight at 4°C and fluorophore-conjugated secondary antibodies were incubated for 2 h at room temperature. The primary antibodies used for this study were directed against the following antigens: Oct3/4, 1:100 (Stemgent), SSEA4, 1:100 (Stemgent), TRA-1-60, 1:100 (Stemgent), Nanog, 1:100 (Santa Cruz Biotech, Santa Cruz, CA, USA), Neurexin IV (NRXN IV), 1:100 (Santa Cruz Biotech), Neuron-specific Beta III Tubulin (TUBB3), 1:2000 (Abcam, Cambridge, MA, USA), glial fibrillary acidic protein (GFAP), 1:500 (Abcam), and myelin/oligodendrocyte-specific protein (MOG), 1:500 (Millipore). Alexa Fluor-conjugated secondary antibodies (1:2000, excitations 488, 594, and 647; Invitrogen) were used for all experiments. In addition, 0.1 $\mu\text{g}/\text{ml}$ 4'-6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. All brightfield, phase contrast and fluorescence images were obtained using the Leica DM IL inverted microscope with the Leica DFC 290 digital camera and Leica Application Suite software (version 2.8.1). All confocal microscopy images were obtained using an Olympus Fluoview FV1000 confocal microscope and the Olympus FV10-ASW 1.7 imaging analysis software.

Total soluble protein (TSP) was extracted from autopsy donor-derived fibroblasts (F02AA1, P:4), and two iPSC clones (2–13 and 2–21, P:8) using ice-cold RIPA buffer supplemented with a protease inhibitor cocktail and EDTA as per the manufacturer's instructions (Thermo Fisher Scientific). Protein extracts were concentrated approximately 10-fold with Microcon Centrifugal Filter Devices (30,000 nominal molecular weight limit; Millipore, Billerica, MA, USA), and TSP was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Approximately 25 μg TSP/lane was separated using 4–12% Bis-Tris precast gels and NuPage electrophoresis system (Invitrogen). Protein was transferred onto Invitrolon PVDF membranes (Invitrogen) that were subsequently incubated in blocking solution consisting of 5% nonfat dry milk (Safeway, Pleasanton, CA, USA) in tris-buffered saline with 0.1% Tween (TBST; Thermo Fisher Sci-

entific). All primary antibodies were incubated overnight at 4 °C and horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated for 1 h at room temperature. The primary antibodies used for this study were directed against the following antigens: Oct3/4, 1:500 (Stemgent), Sox2, 1:150 (Invitrogen), Klf4, 1:100 (Stemgent), c-Myc, 1:100 (Stemgent), and GAPDH, 1:60,000 (Millipore). The HRP-conjugated secondary antibodies used were as follows: Goat anti-rabbit HRP, 1:5000 (Upstate/Millipore), goat anti-mouse, 1:1000 (Millipore), and rabbit anti-chicken, 1:10,000 (Millipore). Western blots were developed using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and the chemiluminescence was detected on X-ray film (Kodak, Rochester, NY, USA).

Genomic DNA was extracted from fibroblast cell line F02AA1 and iPSC clone 2–21 using the Qiagen Blood & Cell Culture DNA Mini Kit as per the manufacturer's instructions (Qiagen, Valencia, CA, USA). Concentration and purity of both DNA extracts were analyzed using a NanoDrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and Quant-iT PicoGreen Kit (Invitrogen). DNA aliquots were prepared at a concentration of 50 ng/μl in a total volume of 10 μl for use in the Affymetrix Genome-Wide Human SNP Array 6.0, performed as per the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA) (see [Supplementary methods](#)). The number of matching and mismatching SNP calls between the two samples were extrapolated in Microsoft Excel 2008 and this was used to calculate the percent concordance. In addition,

Affymetrix Genome-Wide Human SNP Array 6.0 results were used for copy number variation (CNVs) analysis. CNVs were initially detected for both the fibroblast and iPSC samples using the free software tool PennCNV (www.openbioinformatics.org/penncnv) [22]. The log R Ratio (LRR) and B Allele Frequency (BAF) plots were manually inspected using the visualize.cnv.pl program available in the PennCNV package, and any identified CNVs that appeared as potential false-positives were systematically removed.

The primary aim of this study was to examine if autopsy donor-derived dermal fibroblasts could be reprogrammed to a pluripotent state, for potential use in iPSC-based disease modeling. A visual summary of the various cell types used and generated in this proof-of-principle study are illustrated ([Fig. 1](#)). iPSC clones from several additional autopsy donors have been generated using the same procedures as described, demonstrating this approach to iPSC generation is reproducible (data not shown).

The primary dermal fibroblast cell counts were analyzed to determine if biopsy site or age affected proliferation. Three biopsy sites (arm, leg, and torso) were evaluated, from which all cell lines were established using the same amount of tissue and procedures. A One-Way ANOVA demonstrated a significant difference in fibroblast proliferation between biopsy sites (ANOVA: $F=9.616$, $\nu_1=2$, $\nu_2=27$, $P=0.001$). A Tukey's HSD Post hoc test subsequently revealed the arm biopsy site had statistically greater fibroblast proliferation than either the leg ($P=0.003$) or torso ($P=0.004$) sites, while there was no significant difference between the leg and

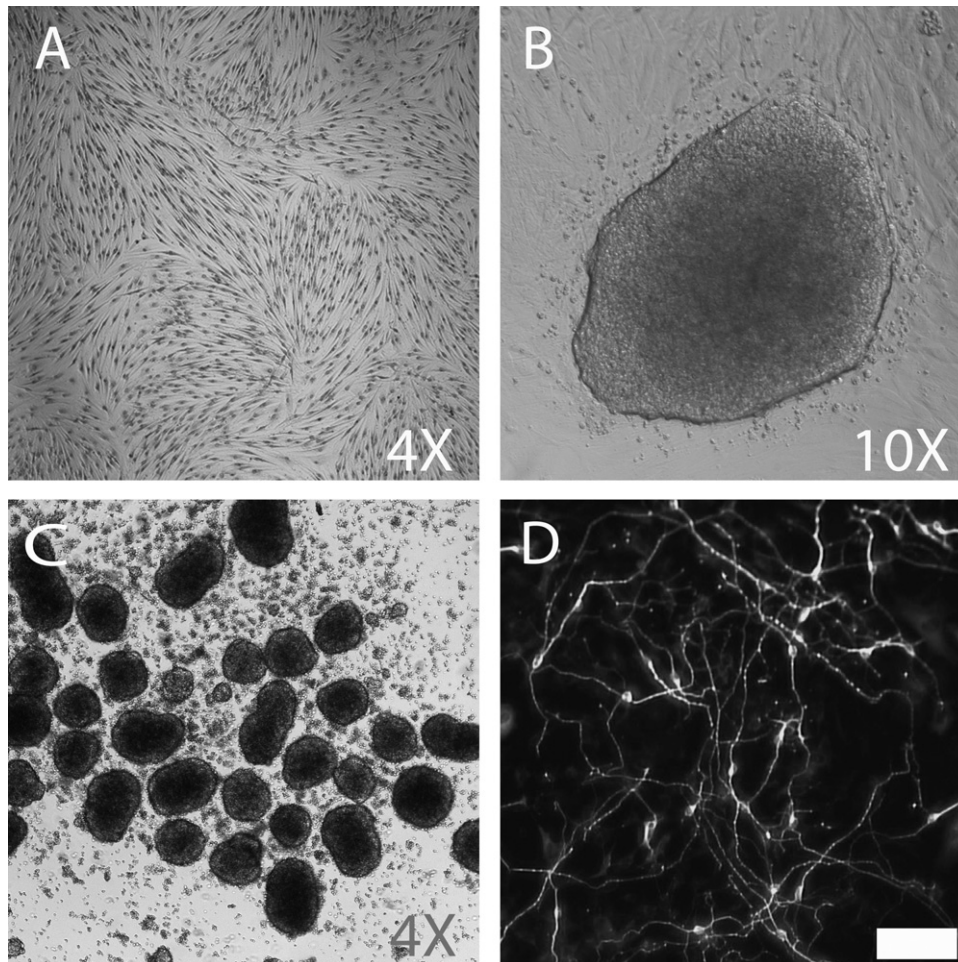


Fig. 1. iPSC induction and neural differentiation from autopsy donor-derived fibroblasts. Brightfield, phase contrast, and immunofluorescence images of (A) autopsy donor-derived dermal fibroblasts (F02AA1; Wright-Giemsa contrast stain), (B) iPSC colony arising from feeder-free conditions 21 days post transduction, (C) EBs generated from iPSC clone 2–13, and (D) neurons after 14 days of *in vitro* differentiation (neuron-specific beta III tubulin antibody stained). Scalebar = 50 μm.

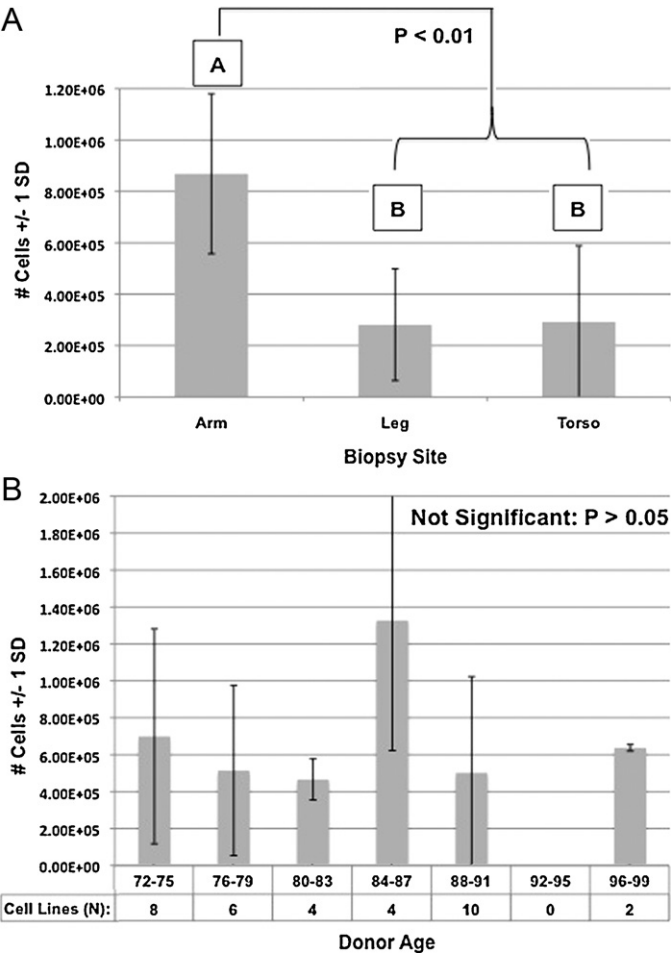


Fig. 2. Fibroblast proliferation is affected by biopsy site but not autopsy donor age (within an elderly cohort). Number of cells counted (y-axis) in both graphs was recorded 18 days post biopsy (Passage 1). (A) Graphical representation of One-Way ANOVA and Tukey's HSD Post hoc test results, demonstrating biopsies obtained from the arm result in a significant increase in fibroblast proliferation as compared to biopsies obtained from the leg or torso. (B) Graphical representation of One-Way ANOVA results, demonstrating fibroblast proliferation is not significantly affected by autopsy donor age.

torso ($P = 0.998$) (Fig. 2A). Donor age, within an elderly cohort (ages 72–97), was also analyzed statistically and the One-Way ANOVA showed there was no significant difference in fibroblast prolifera-

tion based on donor age (ANOVA: $F = 1.756$, $\nu_1 = 5$, $\nu_2 = 28$, $P = 0.155$) (Fig. 2B). While these biopsies were collected within a short PMI of 3–7 h, Meske et al. additionally demonstrated that (in a rat model) fibroblast viability/proliferation was negatively affected by longer PMIs [13]. Collectively, these results suggest that researchers aiming to successfully establish fibroblast cell lines from non-optimal biopsy sites or after extended PMIs should collect and use more tissue per cm^2 of tissue culture surface.

Efficient translation of the Yamanaka factor proteins in the described iPSCs was confirmed by western blot (Fig. 3A). All four factors were strongly expressed in the two iPSC clones evaluated as compared to the autopsy donor-derived fibroblasts. Expression of pluripotency markers was also evaluated by ICC (Fig. 3B). The Yamanaka factor Oct3/4 and the hESC antigens SSEA4, TRA-1-60, and NANOG were all expressed and appropriately localized in the iPSCs.

DNA extracted from fibroblast cell line F02AA1 and iPSC clone 2–21 was used for analyzing genotype concordance and CNVs (see Supplementary data). A percent concordance of 99.9625% verified that the iPSC clone had originated from the autopsy donor cell line. In addition, no iPSC-specific CNVs spanned regions greater than 100 kilobases, suggesting that no large chromosomal segments or whole chromosomes had become aneuploid during the reprogramming process.

The two iPSC clones (2–13 and 2–21) characterized in this study were differentiated into derivatives of the neural lineage. ICC assays identified neurons after 14 (Figs. 1D and 4A and B) and 28 (Fig. 4C and D) days of differentiation. TUBB3 and NRXN IV were utilized as terminal neuronal markers; both antigens co-localized specifically to cells exhibiting a neuronal phenotype (Fig. 4A–D). ICC assays also identified glial subtypes, including astrocytes and oligodendrocytes, after 35 days of differentiation (Fig. 4E and F). GFAP was expressed in the majority of the differentiated, non-neuronal cells, while a small subset of this population expressed MOG (Fig. 4E and F). This data suggests that the majority of the glia were astrocytic in nature, while a small minority was becoming oligodendrocytic.

These results provide evidence that postmortem human tissue can be successfully reprogrammed to a pluripotent state. This approach may be significantly useful for studies investigating diseases and/or drugs that may cause sudden death and for neurodegenerative disease research. The ability to combine both clinical diagnostic criteria and postmortem histopathological observations can greatly increase neurodegenerative diagnostic accuracy; this may subsequently increase the statistical power for donor-specific *in vitro* disease models. Human iPSC-based disease models generated from postmortem tissue may provide additional confidence for researchers investigating these conditions, and pro-

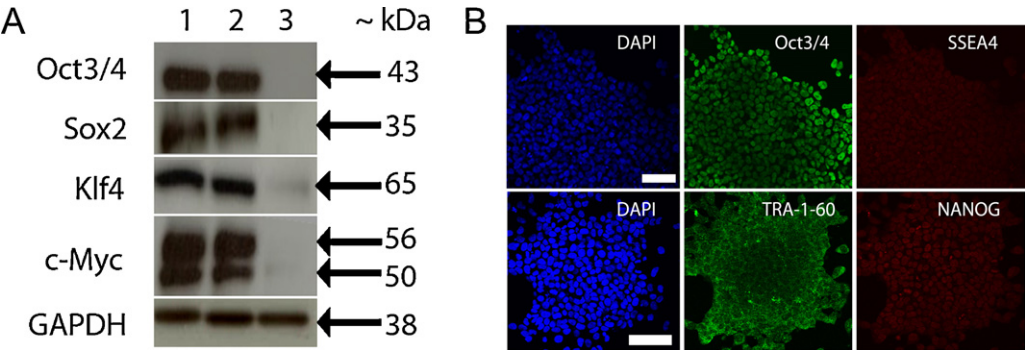


Fig. 3. Western blot analysis and immunocytochemistry of hESC markers. (A) iPSCs generated from autopsy donor fibroblasts express the Yamanka factor proteins. Lanes: (1) iPSC clone 2–13, (2) iPSC clone 2–21, and (3) autopsy donor-derived dermal fibroblast (F02AA1). (B) Confocal microscopy images of iPSCs show expression of nuclear and surface pluripotency antigens. Each row of images displays localization of DAPI and two hESC markers within the same field of view. Top panel: 1 μm optical section; DAPI, Oct3/4, SSEA4. Bottom panel: 3D reconstruction of 16 $\mu\text{m} \times 1 \mu\text{m}$ optical sections through the Z-axis; DAPI, TRA-1-60, and NANOG. Scalebars = 50 μm .

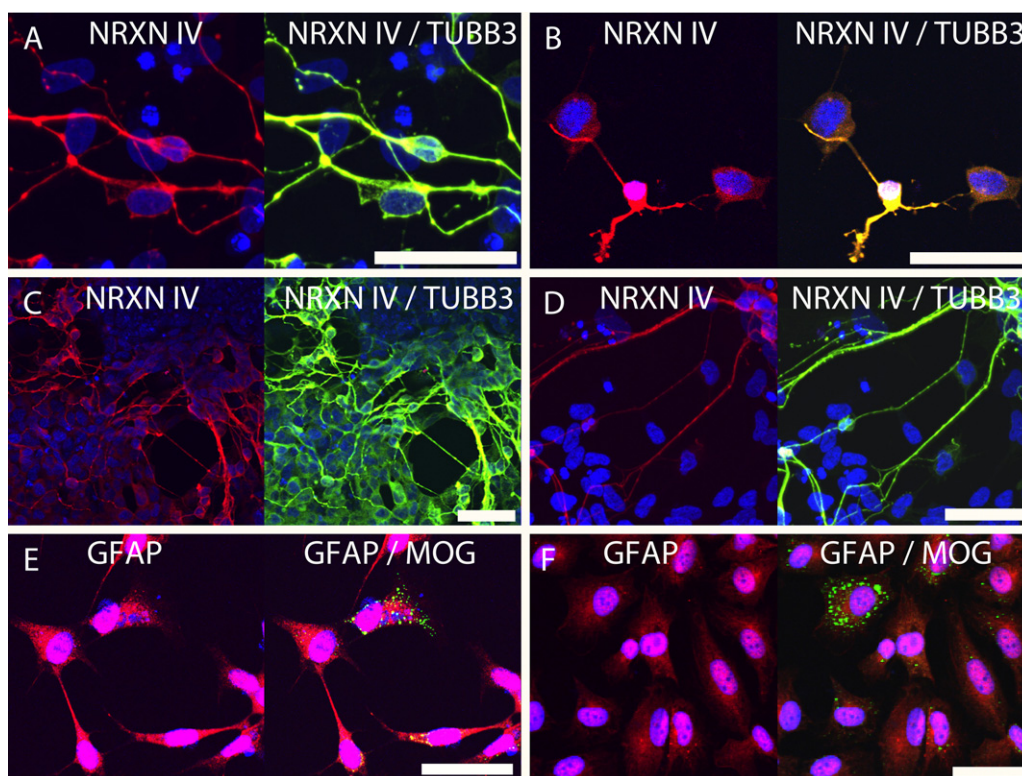


Fig. 4. iPSCs generated from autopsy donor fibroblasts can be differentiated into neurons and glia. Confocal microscopy images shown are from 1 μ m optical sections through the Z-axis of cells fixed after 14 (A and B), 28 (C and D) and 35 (E and F) days of differentiation. Left panel: iPSC clone 2-13; Right panel: iPSC clone 2-21. (A, B, C, D) Neurexin IV (NRXN IV) (red), co-localization of Neurexin IV (NRXN IV) and neuron-specific beta III tubulin (TUBB3) (yellow), and the nuclear counterstain DAPI (blue). (E and F) Glial fibrillary acidic protein (GFAP) (red), myelin/oligodendrocyte-specific protein (MOG) (green), and the nuclear counterstain DAPI (blue). Scalebars = 50 μ m.

vides an avenue for comparing differentiated *in vitro* cell types to endogenous adult tissue from the same individual.

Conflict of interest

The authors have no conflicting financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neulet.2011.07.048](https://doi.org/10.1016/j.neulet.2011.07.048).

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