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Human dental stem cell derived transgene-free iPSCs generate functional neurons via embryoid body-mediated and direct induction methods

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Abstract

Induced pluripotent stem cells (iPSCs) give rise to neural stem/progenitor cells (NSCs), serving as a good source for neural regeneration. Here, we established transgene-free (TF) iPSCs from dental stem cells (DSCs) and determined their capacity to differentiate into functional neurons in vitro.

Generated TF iPSCs from stem cells of apical papilla (SCAP) and dental pulp stem cells (DPSCs) underwent two methods -- embryoid body (EB)-mediated and direct induction, to guide TF-DSC iPSCs along with H9 or H9 Syn-GFP (human embryonic stem cells) into functional neurons in

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Declarations

Ethics approval and consent to participate: To be discarded extracted teeth were collected from Clinics at Boston University (BU) and University of Tennessee Health Science Center (UTHSC) based on exempt protocols approved by the respective Medical Institutional Review Board (BU: #H-28882; and UTHSC:#12-01937-XM).

Consent for publication: This manuscript has been approved by all authors and is solely the work of the authors named.

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Neurogenic genes: See Supplemental Table S1.

vitro. Using the EB-mediated method, early stage neural markers PAX6, SOX1 and nestin, were detected by immunocytofluorescence or RT-qPCR. At late stage of neural induction measured at weeks 7 and 9, the expression levels of neuron-specific markers *Nav1.6*, *Kv1.4*, *Kv4.2*, *synapsin*, *SNAP25*, *PSD95*, *GAD67*, *GAP43* and *NSE* varied between SCAP iPSCs and H9. For direct induction method, iPSCs were directly induced into NSCs and guided to become neuron-like cells. The direct method while simpler, showed cell detachment and death during the differentiation process. At early stage, PAX6, SOX1 and nestin were detected, At late stage of differentiation, all 5 genes tested, nestin, β III-tubulin, NFM, GFAP and Nav were positive in many cells in cultures. Both differentiation methods led to neuron-like cells in cultures exhibiting sodium and potassium currents, action potential or spontaneous excitatory postsynaptic potential. Thus, TF-DSC iPSCs are capable of undergoing guided neurogenic differentiation into functional neurons *in vitro*, thereby may serve as a cell source for neural regeneration.

Keywords

Dental stem cells; DSCs; induced pluripotent stem cells; iPSCs; embryonic stem cells; ESCs; adult stem cells; reprogramming; transgene-free; neurogenesis; DPSCs; SCAP; electrophysiology; patch clamp; in vitro

1. Introduction

Neural regeneration remains challenging. Many neurological disorders await therapeutic strategies including cell-based therapies (Chew, *et al.*, 2012, Chiu and Rao, 2011, Choi, *et al.*, 2014a, Choi, *et al.*, 2014b, Döbrössy and Pruszk, 2013). Neural stem/progenitor cells (NSCs) from neural tissues are the best cell source for neural regeneration (Casarosa, *et al.*, 2014, Okano and Sawamoto, 2008), however, their acquisition is difficult and impractical thereby limiting their clinical applications. Using mesenchymal stromal/stem cells (MSCs) as a source for neurogenesis has been tested rigorously in the hope that non-NSCs may be used as a cell source for neural regeneration. However, the results have been controversial and inconsistent (Aanismaa, *et al.*, 2012, Arthur, *et al.*, 2008, Chen, *et al.*, 2007, Deng, *et al.*, 2006, Gervois, *et al.*, 2015, Jung, *et al.*, 2016, Mung, *et al.*, 2016, Pittenger, *et al.*, 1999, Shiota, *et al.*, 2007, Tondreau, *et al.*, 2008, Wehner, *et al.*, 2003). Many reports attempted to define NSC properties of MSCs only by detecting gene expression but not examining or demonstrating the functional electrophysiology which is the critical characteristic of neurons. Some studies have shown sodium current, intracellular calcium influx, action potential and spontaneous post-synaptic current from neuron-like cells derived from MSCs (Arthur, *et al.*, 2008, Cho, *et al.*, 2005, Greco, *et al.*, 2007, Song, *et al.*, 2007, Tondreau, *et al.*, 2008). For MSCs in dental tissues such as human dental pulp stem cells (hDPSCs), one report showed that human dental pulp stem cells (hDPSCs) cannot mature into functional neurons (Aanismaa, *et al.*, 2012), whereas a more recent study was able to demonstrate electrophysiological characteristics of functional neurons derived from hDPSCs (Gervois, *et al.*, 2015). In general, more consistent findings on MSCs are that they tend to play a supporting role in neurogenesis by preventing cell apoptosis, providing neurotropic factors and differentiating into glial cells instead of becoming neurons (Chen, *et al.*, 2001,

Hofstetter, *et al.*, 2002, Huang, *et al.*, 2008, Kopen, *et al.*, 1999, Lee, *et al.*, 2010, Mantovani, *et al.*, 2012, Mieda, *et al.*, 2016, Sakai, *et al.*, 2012).

Additionally, although there are multiple sources to obtain MSCs, adult stem cells have limited life span in cultures. Their heterogeneity in nature and the isolation of subpopulations for greater potency or for differentiation toward certain lineages render the process more complex (Nichols, *et al.*, 2013, Xu, *et al.*, 2009, Young, *et al.*, 2016). These reasons may make it difficult to reach needed number of cells in most cases for meaningful preclinical testing and eventual clinical use. The idea of partial reprogramming and introducing neural genes or chemicals by direct conversion of cells into specific neural lineages appears to be an alternative for cell-based neural therapy (Biswas and Jiang, 2016, Kim, *et al.*, 2012, Prasad, *et al.*, 2017, Vierbuchen, *et al.*, 2010, Zhang, *et al.*, 2016). However, the key issue is still the limitation of the adult cell life span in vitro for manipulation and for feasible clinical use, especially when managing a case that needs multiple cell transplantation treatments.

The other alternative is resorting to pluripotent stem cells including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) as a source to derive a large number of NSCs (Wernig, *et al.*, 2008, Zhang, *et al.*, 2001). hiPSCs have the advantage over hESCs for their autologous nature if obtained from the patient's own cells. To generate iPSCs, dental stem cells (DSCs) or cells from oral mucosa have been considered one of the most accessible and feasible source to produce iPSCs (Huang, 2010, Tamaoki, *et al.*, 2010, Wada, *et al.*, 2011, Yan, *et al.*, 2010, Zou, *et al.*, 2012). DSCs normally are obtained from discarded wisdom teeth such as dental pulp stem cells (DPSCs) and stem cells from apical papilla (SCAP) (Huang, *et al.*, 2009, Morsczeck, *et al.*, 2013). Previously we have successfully generated iPSCs from various human DSCs (Yan, *et al.*, 2010). Subsequently, new approaches have been developed to remove the introduced reprogramming factors in order to render iPSCs of clinical values. We applied the human STEMCCA-loxP reprogramming system carrying OCT4, SOX2, KLF4, and c-MYC (Somers, *et al.*, 2010), established transgene-free (TF) iPSCs from SCAP, and verified their preliminary neural differentiation capacities (Zou, *et al.*, 2012). However, these DSC derived iPSCs have not been shown for their potential to becoming functional neurons in vitro. In the present study, we attempted to guide TF iPSCs reprogrammed from DSCs into neurons exhibiting electrophysiological capacities using an embryoid body (EB)-mediated method and a direct induction approach.

2. Materials and Methods

2.1. Cell culture

2.1.1. Dental stem cells—SCAP and DPSCs were isolated as described previously (Huang, *et al.*, 2010). Briefly, freshly extracted permanent teeth were collected from healthy donors (aged 16–24 yrs) in the Oral Surgery Clinics or Periodontic Clinics at Boston University (BU) and University of Tennessee Health Science Center (UTHSC) based on exempt protocols approved by the respective Medical Institutional Review Board (BU: #H-28882; and UTHSC: #12–01937-XM); no patient consents were needed. The apical papilla was first removed from the immature teeth and the pulp was obtained after the tooth

was split-opened. Collected tissues were digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 30–60 min at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 µm strainer and seeded into culture plates. The remaining tissue debris was also seeded in the separate wells. The debris also gave rise to DSCs based on our observation. Cells were grown in media containing α -modification of Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin-G, 100 mg/mL streptomycin, and 0.25 mg/mL fungizone (Gemini Bio-Products, Inc., West Sacramento, CA, USA) and incubated at 37°C in 5% CO₂. These DSCs are heterogeneous population of MSCs expressing typical markers as we reported previously (Huang, *et al.*, 2010). We tested potential of SCAP and DPSCs to differentiate into adipogenic, dentinogenic and chondrogenic lineages and the information is shown in Supplemental Materials & Methods and data are shown in Supplemental Fig. S1. Similar to the findings in our previous reports (Huang, *et al.*, 2010, Sonoyama, *et al.*, 2008), DSCs are strong in osteogenesis while weak in adipogenesis and chondrogenesis.

2.1.2. iPSCs and ESCs—iPSCs and human embryonic stem cells (hESCs) H9 (WA09, obtained from WiCell Research Institute, Madison, WI) were grown in conditions following an NIH standard protocol (<http://stemcells.nih.gov/research/NIHresearch/scunit/culture.asp>). Cells were maintained on mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) in gelatin-coated 6-well plates with the hESC medium (80% DMEM/F12, 20% knock-out serum replacement, 1x non-essential amino acid, 1 mM L-glutamine, 0.1 µM β -mercaptoethanol) containing 4 ng/mL basic fibroblast growth factor (bFGF). MEFs were isolated from E13.5 embryos of CF1 or CD1 pregnant mice according to a standard protocol (Jain, *et al.*, 2014) and cultured in DMEM supplemented with 10% FBS. All animal procedures followed a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at BU (protocol #AN15026) and UTHSC (protocol #2082).

2.2. Reprogramming of SCAP and DPSCs into transgene-free (TF) iPSCs

Heterogeneous primary human SCAP and DPSCs at ~p3 were reprogrammed into iPSCs using a lentiviral vector *hSTEMCCA-LoxP*, a polycistronic single vector carrying all four human reprogramming factors *c-MYC*, *KLF4*, *OCT4* and *SOX2*, using a method as described in our previous report (Zou, *et al.*, 2012). Briefly, viral titers of $\sim 1 \times 10^8$ TU (transducing unit)/ml were employed for reprogramming cells in the presence of polybrene (5 µg/mL). Within 6 days, 4×10^4 transduced cells were seeded onto mitomycin C-inactivated MEFs. Within 2 weeks, colonies resembling ESC colonies began to emerge (considered as passage 0 prospective iPSCs). Approximated 28 days post-viral vector transduction, ESC-like colonies were manually isolated/passaged and expanded on MEFs. The expanded DSC iPSCs at p2 were used for further Cre-mediated excision of *hSTEMCCA* as described (Zou, *et al.*, 2012). Briefly, p2 SCAP or DPSC iPSCs were cultured on puromycin resistant MEFs (DR4MEF, GlobalStem, <http://globalstem.com>) and exposed to 2.5 µg of pHAGE2-Cre-IRES-PuroR plasmid DNA, 7.5 µL Trans IT Hela reagent and 5 µL MONSTER reagent (Mirus, <http://www.mirusbio.com>). After 24 h, the medium was changed with fresh hESC medium and incubated for ~6 h. Selection of the transfected SCAP/DPSC iPSCs with puromycin (1.2 µg/mL) began 24 h following the initial transfection and lasted for 48 h. The medium containing puromycin was changed every 24 h. Fresh hESC medium was used after

puromycin treatment and changed daily. New SCAP/DPSC iPSC colonies re-emerged in 2–4 d. On days 11–14, newly emerged colonies from each well were picked and transferred onto new MEF plates and grown/expanded individually. Genomic DNA or cDNA from total RNA from each subclone was extracted and the PCR performed to verify for the excision of the hSTEMCCA. The primers used and the PCR conditions are in the following: *c-MYC* (forward primer): 5' –GGA ACT CTT GTG CGT AAG TCG ATA G-3'; *WPRE* (reverse primer) 5'-GGA GGC GGC CCA AAG GGA GGA GAT CCG-3'; 95°C, 3min; followed by 40 cycles of 94°C, 30s, 60°C, 30s, and 72°C, 5min. The PCR products were examined by electrophoresis on an agarose gel. Verified transgene free clones were named TF-SCAP or DPSC iPSCs. To verify that there is no integration of pHAGE2-Cre-IRES-PuroR plasmid DNA into the genome of TF-SCAP/DPSC iPSCs, these cells were grown on DR4MEFs in the presence of puromycin (1.2 µg/mL). Absence of plasmid integration is indicated by cell death. We reprogrammed SCAP iPSCs from 4 donors (3 of which were used for experiments) and DPSCs iPSCs from 2 donors (1 was used for experiments).

2.3. Neurogenic induction

2.3.1. Embryoid body (EB)-mediated neurogenesis—The experimental process was based on a report (Hu, *et al.*, 2010) with modifications. TF-SCAP iPSC or H9 colonies grown on MEFs were passaged onto feeder-free Matrigel (growth factor reduced, BD Biosciences, San Jose, CA)-coated wells with TESR2 medium (Stemcell Technology. <http://www.stemcell.com>) and expanded until reaching a sufficient number and size. Subsequently, iPSC colonies were detached by collagenase IV (1 mg/mL), resuspended in hESC medium and plated into Ultra Low attachment 6-well plates at a concentration of $\sim 10^5$ cells /CM² (for 6 well, around 10–15 EBs/well) to form EBs in suspension for 7 days. EBs were then collected and plated onto Matrigel-coated wells of 6-well plates and cultured in DMEM/F12 containing N2 supplement, and 1x nonessential amino acid (NEAA) for 14 days to allow formation of neural tube-like rosettes (NRs) containing primitive neuroepithelial (NE) cells. The NRs were then detached mechanically and transferred into Ultra low attachment 24-well plates (~ 5 NRs per well) with the same medium. NRs in suspension were cultivated with a neural precursor medium containing DMEM/F12, 1 x NEAA, 1 x N2 and other elements for the subsequent 25 days in the following order: SB4352 (10 nM, TGFβ inhibitor) was added for the 5 first days and Noggin (500 ng/mL) for 15 days. Primitive NE cultures were treated with retinoic acid (RA) (100 nM) from day 10 and SHH (200 ng/mL) from day 14. On day 25, suspended neural progenitors were allowed attached onto a laminin-coated well with differentiation medium consisting of neurobasal medium, N2 supplement, and cAMP (1 µM) in the presence of a mixture of BDNF (20 ng/mL), glial cell-derived neurotrophic factor (GDNF, 10 ng/mL), and IGF1 (10 ng/mL) for another ~ 5 weeks to guide neural-committed cells toward motor neurons.

2.3.2. Direct induction neurogenesis—TF-SCAP iPSC, TF-DPSC iPSC or H9 colonies grown on MEFs were passaged onto feeder-free Matrigel-coated wells as described above. When reaching ~ 70 – 80% confluency, colonies were passaged onto Matrigel coated wells of 6-well plates as small clumps at 2.5×10^5 cells per well. During splitting, cells were treated with 10 µM ROCK inhibitor to prevent cell death. One day after, cultures were replaced with neural induction medium (NIM, containing Neurobasal Medium and Neural

Induction Supplement, GIBCO® PSC Neural Induction Medium, ThermoFisher Scientific) for 7 days to derive NSCs, termed NIM-derived NSCs or NIM-NSCs. Subsequently NIM-NSCs were passaged into Matrigel-coated wells in neural expansion medium (NEM, containing Neurobasal Medium, Advanced DMEM/F12, Neural Induction Supplement, ThermoFisher) and continue to be passaged several times. ROCK inhibitor at 5 μ M was used during each passaging. Up to this step the procedures were based on manufacturer's instruction (GIBCO, Life Technologies, PSC neural induction medium). NIM-NSCs at passages 2–14 were seeded onto 1% polylysine- or Matrigel-coated wells and incubated in neural differentiation medium (NDM, consisting of Neurobasal medium, SHH 100 ng/ml, RA 100 nM) for ~3 weeks and then changed to neuronal differentiation medium (NronDM, consisting of Neurobasal medium, SHH 50ng/mL, RA 50 nM, BDNF 10 ng/ml, GDNF 10 ng/ml, IGF1 10 ng/ml, cAMP 1 μ M, Ascorbic Acid 200 ng/ml) for ~2 weeks.

We encountered cell detachment and cell death situations after NSCs were exposed to NDM and NronDM. Toward the end of neurogenesis, only about 20–50% remained attached with many floating death cells. We made the following modifications to improve the situation. i) Using plastic coverslips: we found the situation did not improve. ii) Coating the glass coverslips with Matrigel: we found that the detachment problem reduced. iii) Using mixture of NEM/NDM of 75/25 or 50/50 (v:v) to initiate differentiation and observed the cell condition carefully. With this approach, the cell death and detachment reduced. However, it took longer time to reach the final step for patch clamp studies. Some took up to >7 weeks as opposed to originally planned ~5 weeks from NSC stage to neurons.

2.4. Real-Time Polymerase Chain Reaction

Expression of neurogenic genes was analyzed by real-time quantitative (q)PCR according to previous reports (Gauthier, *et al.*, 2017, Yu, *et al.*, 2015). Briefly, total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). DNase I (Invitrogen) was used to remove genomic DNA contaminants. The extracted RNA (1 μ g) was used to generate the first strand cDNA with Superscript III (Invitrogen). qPCR reactions were performed using LightCycler® 480 SYBR Green I Master (Roch Diagnostic Corp., Indianapolis, IN) as described by the manufacturer. The reaction mixture was placed in each wells of a 96-well plate, placed in LightCycler® 480 II (Roch Diagnostic Corp.) and run the following thermal cycling conditions: 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 10 s; 1 cycle of melt curve step of 95°C for 30 s, 65°C for 30 s and 95°C continuous before cooling at 40 °C for 1 s. All procedures followed the quality control similar to a published guideline (Bustin, *et al.*, 2009, Fleige and Pfaffl, 2006). Primers shown in Supplemental Table S1 were used at a final concentration of 200 nM, and reactions for each sample were performed in triplicate or duplicate. For qPCR data analysis, a relative quantitative analysis method was performed to quantify the relative gene expression. First, the C_T or C_P values of all samples in the plate were calculated against those of the first reference gene (internal control) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), i.e., $C_P = (C_P \text{ gene of interest} - C_P \text{ internal control})$ through 2^{-C_P} for normalization (Schmittgen and Livak, 2008). After which, another appropriate gene in the experimental group was selected as the second reference gene to derive the relative expression levels of the sample genes through 2^{-C_P} calculations.

2.5. Immunocytofluorescence staining

The staining followed a protocol reported previously (Huang, *et al.*, 2010, Zou, *et al.*, 2012). Cell cultures were fixed in 100% ice cold methanol, incubated in blocking buffer [32.5 mM NaCl, 3.3 mM Na₂HPO₄, 0.76 mM KH₂PO₄, 1.9 mM NaN₃, 0.1% (w/v) bovine serum albumin (BSA), 0.2% (v/v) Triton X-100, 0.05% (v/v) Tween 20, and 5% goat serum] for 30 min followed by addition of the following antibodies for 1 h at room temperature: anti-human OCT4, NANOG, SOX2, PAX6, SOX1, β III-tubulin (TUJ1), nestin, neurofilament medium chain (NFM;), GFAP and CNPase. After washing, cultures were incubated with secondary antibodies for 1 h at room temperature and the cell nuclei stained with DAPI (Invitrogen) of 1:2,000 dilution. All antibodies and their information used for the staining are listed in Supplemental Table S2. Images were analyzed under a fluorescence microscope.

2.6. Electrophysiology

Neurogenic-induced cells via EB-mediated method were grown on glass coverslips (15 mm diam., 0.15 mm thick; Ted Pella, Inc., <http://www.tedpella.com/>), and induced cells via direct induction method were grown on glass or plastic coverslips (15 mm diam., Thermanox, NUNC, Rochester, NY). These coverslips were transferred to a recording chamber in a fixed-stage Olympus BX-WI microscope equipped with an XM-10IR CCD camera (Olympus).

The chamber was constantly perfused (~2 ml/min) with oxygenated artificial cerebrospinal fluid (aCSF, in mM: 119 NaCl, 2.5 KCl, 1 MgSO₄, 2.5 CaCl₂, 1.25 NaH₂PO₄, 23 NaHCO₃, and 10 glucose) for neurons derived using EB-mediated method or NEM for neurons derived using direct induction method. We found cells from the latter method tended to detach once placed in the aCSF. We found that the slight difference in the Na⁺ and K⁺ concentrations did not affect the patch clamp testing. We used whole-cell voltage clamp to determine whether differentiated iPSCs expressed the voltage-dependent conductances characteristic of neuronal cells, using a modified protocol based on our previous report (O'Connell, *et al.*, 2010). If a cell exhibited a large (>1000 pA) fast-activating and rapidly inactivating inward cation current typical of a voltage-gated Na⁺, we determined whether that cell was capable of generating action potentials (AP) using depolarizing current injections while recording membrane potential in current clamp mode. All recordings were performed using a Multiclamp 700B amplifier and Digidata 1440A and controlled using Clampex 10 (Molecular Devices). Data were digitized at 20 kHz and filtered at 5 kHz using the built-in four-pole Bessel filter of the Multiclamp 700B. After the formation of a gigaohm seal, the pipette capacitance was nulled in all experiments.

Recording pipettes were manufactured from filamented thin-wall borosilicate glass (TW150, World Precision Instruments) and had a resistance of 5–7 M Ω when filled with an intracellular solution (in mM: 140 KCl, 0.5 EGTA, 2 MgATP, 0.2 NaGTP, 4 Na-phosphocreatine and 10 HEPES, pH 7.25 with KOH). The liquid junction potential between the pipette and bath solutions was measured to be less than 5 mV and was not corrected.

We encountered an issue for neuron-like cells differentiated via the direct induction method. There was detachment of cells when the cover slip was placed into the recording chamber on

the microscope stage and perfused with oxygenated aCSF. We then utilized the NEM as the substitute buffer during patch clamp with no perfusion, which reduced the number of cells that detached in the recording chamber and allowed electrophysiological recording from these cells. Although the concentration of Na^+ differed slightly between aCSF and NEM, this difference was small and did not impact either the magnitude of the Na^+ currents or the excitability of the neuron-like cells.

2.7. Data Analysis

Two-way ANOVA was used to examine the effects of two independent factors and their interaction effect on the outcome. The two curves were also compared across time. If the main and/or interaction effect found to be statistically significant, post-hoc comparisons were examined by Tukey HSD (Honestly Significant Difference) test. Values are considered statistically significant when $p < 0.01$. Data are reported as mean $\pm$ SEM. All analyses were done using SAS 9.4 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Generation of transgene-free SCAP/DPSC iPSCs

We generated TF SCAP/DPSC iPSCs following the method reported by our group previously (Zou, *et al.*, 2012). Primary human SCAP or DPSCs (Fig. 1Aa) were transduced with lentiviral vectors hSTEMCCA and seeded onto MEFs. ESC-like colonies emerged on MEFs within 2 weeks (Fig. 1Ab). Newly generated iPSCs were subjected to treatment/selection to obtain TF SCAP/DPSC iPSCs (Fig. 1Ac,d). Newly selected colonies were tested for the removal of transgenes by PCR as shown in Fig. 1B that most of the treated/selected iPSCs had the transgene removed. We validated with two approaches. i) Isolation of genomic DNA of the selected iPSCs followed by PCR. ii) Isolation of total RNA, synthesis of cDNA and then by PCR. Both approaches led to the same results. We further validated that there was no integration of pHAGE2-Cre-IRES-PuroR plasmid DNA into the genome of TF-SCAP iPSCs. Treating the cells with puromycin for up to a week showed no survival of any iPSC colonies (Fig. 1C).

We performed immunofluorescence analysis to detect the expression of stemness and neural marker genes. NANOG and OCT4 were expressed in iPSCs and H9. Neural markers SOX1, nestin, β III-tubulin, NFM, and astrocyte marker GFAP were weakly or not detected in iPSCs and H9 (Fig. 1D-F). We performed karyotyping for the generated TF-iPSCs and there was no abnormality detected (Supplemental Materials and Methods, Fig. S2). Representative teratoma formation data are shown in Fig. 1G, with tissues originated from ectoderm (primitive neural tube-like tissue), mesoderm (cartilage- or bone-like) and endoderm (glandular tissue). (Also see Supplemental Materials and Methods).

3.2. EB-mediated neurogenesis

We selected TF-SCAP iPSCs and H9 for the EB-mediated neurogenesis approach which took multiple steps to turn these cells into neuron-like cells as depicted in Fig. 2. The iPSCs and H9 were maintained on MEFs and passaged to Matrigel (feeder-free condition) before generating EBs (Fig. 2 Aa,b; Ba,b). The neural induction began following reattachment of

EBs onto Matrigel-coated well to allow NR formation as shown in Fig. 2Ac, Bc. NRs in suspension were cultivated with a neural precursor medium and the clumped floating cell mass of NRs were observed (Fig. 2Ad, Bd). At around 6th week after the initiation of the neural differentiation at the EB attachment step, suspended NRs containing neural progenitors were attached onto a laminin-coated wells and guided toward neuronal lineages. After cell attachment, the emergence of neuron-like cells gradually occurred. There were heterogeneous population of less differentiated neural stem/progenitor cells or more neuron-like cells showing extending axon-like structures in cultures (Fig. 2Ae, Be). Near the end of 11th week, more and more neuron-like cells appeared (Fig. 2Af, Bf) and were ready for patch-clamp analysis. Schematic timeline of the whole process is depicted in Fig. 2C)

3.3. Neural marker expression in iPSCs and H9 after EB-mediated neurogenic induction

We first detected the expression of neural markers during the NR formation. The immunofluorescence analysis showed that PAX6, nestin, β III-tubulin, NFM were expressed in this stage of cells of both SCAP iPSCs and H9, while OCT4 was very low and difficult to detect (Fig. 3A, B). Between NRs, axon-like structures were observed and they expressed β III-tubulin and NFM. After the cells underwent further neuronogenesis under the stimulus of BDNF, GDNF, IGF1, AA and cAMP, more cells in cultures showed neuron-like and stained positively for β III-tubulin and NFM with numerous axon-like structures. Some cells not showing neuron-like morphology expressed nestin. GFAP was also detected in some cells (Fig. 3C,D).

We also performed qPCR to detect more neural markers at various time points. A preliminary test was first performed to examine gene expression of selected neural markers before and after 14 days of neural differentiation (Supplemental Fig. S3). There were differences of certain neural gene expression levels between SCAP iPSCs and H9. Some genes were more expressed in SCAP iPSCs while others more in H9. The largest difference was ~3-fold between the two cell types in PAX6 expression after differentiation. Other differences in gene expression were less than 2-fold.

Subsequently we examined the gene expression at multiple time points within the 2 week period as well as at later stages of the neural differentiation. In the first 2 weeks of the induction, the early neural markers *PAX6* and *SOX1* were expressed significantly higher in SCAP iPSCs than in H9, while *musashi*, *DCX* and *NCAM* were mostly higher in H9 (Fig. 3E). At late stage of neural induction measured at weeks 7 and 9, different neural markers expressed different levels comparing between SCAP iPSCs and H9. For more general neural markers including glial cell markers shown in Fig. 3F, *DCX*, *NF1* and *NCAM* tended to express higher in SCAP iPSCs whereas glial markers *GFAP*, *OLIG2* and *S100* were higher in H9. The expression levels of neuron-specific markers *Nav1.6*, *Kv1.4*, *Kv4.2*, *synapsin*, *SNAP25*, *PSD95*, *GAD67*, *GAP43* and *NSE* varied between SCAP iPSCs and H9. No specific pattern can be observed except some markers were higher in H9 while others were higher in SCAP iPSCs at week 7. A number of markers appeared lower at week 9 than week 7 (Fig. 3G).

3.4. Direct neurogenic induction

Using the direct neurogenic induction method, we tested SCAP iPSCs, DPSC iPSCs as well as H9. These cells were first seeded onto Matrigel-coated wells followed by digesting the colonies into smaller size and passaging them into new wells (Matrigel coated). After one day of culturing, cells were induced toward NSC lineage under NIM for 1 week. As shown in Fig. 4Aa, Ba, Ca, cell colonies increased in size while being guided into NSC lineages. After which, cell colonies were digested into detached single cells and passaged onto Matrigel-coated wells as passage 0 NIM-NSCs. These cells were cultured under NEM and continuously passaged to at least p4 (Fig. 4Ab, Bb, Cb). Most cells at this stage showed typical NSC morphology with mainly triangular shape and some cells clustered together forming pin-wheel appearances. We found that NSCs generated from different clones of iPSCs, different batch of H9, or at different experiments showed some variations in terms of the progression into typical NSCs. Some batches of NSCs tended to take a longer time during expansion (more passaging) to reach typical NSC morphology, i.e., those tended to form pluripotent stem cell-like colonies than single NSCs in cultures. The overall time for direct induction method is shorter for cells to reach mature neuron-like state compared to that for EB-mediated method. However, we encountered cell detachment and cell death situations after NSCs were exposed to NDM and NronDM. Toward the end of neurogenesis, only about 20–50% remained attached with many floating death cells. As indicated in Fig. 4Ac,d; Bc,d; Cc,d, many dead cells were observed in cultures. Therefore, we made the modifications to improve the situation as describe in Materials and Methods.

We examined the neurogenic gene expression in NSCs during the neural expansion phase using immunocytofluorescence. As shown in Fig. 5A-C, among iPSCs and H9, the pluripotent gene markers NANOG and OCT4 were no longer detectable. As for various neurogenic genes, PAX6, SOX1, nestin, β III-tubulin and NFM were detected, whereas, GFAP were not detected. When cells reached late stage after neural and neurogenic differentiation, we tested neurogenic gene expression of SCAP iPSCs and DPSC iPSCs. We found that all 5 genes tested, nestin, β III-tubulin, NFM, GFAP and Nav were positive in many cells in cultures (Fig. 5D, E).

3.5. Electrophysiological function of TF iPSC-derived neurons

A necessary characteristic to confirm that the iPSCs were successfully differentiated into functional neurons *in vitro* is the expression of the voltage-gated conductances that establish neuronal excitability, in particular voltage-dependent Na⁺ and K⁺ currents. H9 was also treated in parallel and subjected to same measuring process. We selected cells that had a neuron-like morphology (e.g., round, phase-bright cell body with one or more neurite-like processes) and then used whole-cell voltage-clamp to determine whether these cells exhibited a robust inward current with rapid activation and inactivation kinetics typical of voltage-gated Na⁺ currents.

3.5.1. EB-mediated neurogenesis—With the well-established EB-mediated neurogenesis method as shown in Fig. 2, we selected a clone of TF-SCAP iPSCs derived from one donor and H9 for patch clamp studies. At the time of patch clamp study, cells were in good state with most cells showing neuron-like morphology (Fig. 6Aa, Ba). We detected

spontaneous sodium and potassium currents as shown in Fig. 6Ab, Bb,c. Additionally, we noticed spontaneous excitatory postsynaptic current (EPSC) from TF-SCAP iPSCs-derived neurons measured by voltage clamp, indicating the presence functional synapses (Fig. 6Ac). To determine whether these currents were sufficient to promote excitability, we used whole-cell current clamp to record membrane potentials in these cells. None of the cells recorded exhibited spontaneous action potentials (APs), but all cells tested did fire one or more APs in response to depolarizing current injections (Fig. 6Ad,Bd). We were able to pick any healthy looking neuron-like cells in cultures and detect consistent sodium current and AP.

3.5.2. Direct induction neurogenesis—The patch clamp studies for neuron-like cells derived via direct induction method were more problematic as mentioned above. When the conditions were less optimal, the surviving neuron-like cells did not exhibit sodium currents or APs and were less stable and more difficult to obtain high-resistance seals. As mentioned in Materials and Methods, we replaced oxygenated aCSF with NEM as buffer during patch clamp with no perfusion. We also noticed that iPSC or H9 derived NIM-NSCs performed better between passages 5–10. We measured a large inward current sensitive to block by the voltage-gated Na^+ channel blocker tetrodotoxin (TTX) (Fig. 7Aa-d, Ba-d, Ca-d), consistent with the expression of functional voltage-gated Na^+ channels; only cells displaying this large transient current were able to fire APs. Similarly, cells exhibited a large non-inactivating current consistent with voltage-dependent K^+ channels. Although none of the cells recorded exhibited spontaneous APs, they did fire one or more APs in response to depolarizing current injections, consistent with a neuronal phenotype. Some of the tested neuron-like cells from SCAP iPSCs showed multiple AP peaks and others showed one peak (Fig. 7Ae), whereas those from DPSC iPSCs or H9 showed mainly one peak (Fig. Be, Ce) (Supplemental Fig. 4). Some cells were able to continuously fire APs during a current injection, while others remained persistently depolarized following the initial spike. These cells were able to repolarize to a resting membrane potential similar to that pre-injection, suggesting that although these iPSC-derived neurons are able to fire APs, consistent with a neuron-like physiology, they are not capable of sustained, high-frequency output. From the experiments where differentiation process was more optimal, we counted the tested cells having AP to be the following: SCAP iPSC-neurons, 7/21 (33%); DPSC iPSC-neurons, 6/13 (46%); and H9, 2/10 (20%). Percentage of tested cells exhibiting Na^+ or K^+ currents were higher ranging from 57–100% (See Supplemental Table S3).

In all experiments using direct induction method for electrophysiology, only one clone from each donor were used. The experimental conditions varied from experiment to experiment. For the DPSC iPSCs, we have tested neurogenesis total of 9 times, some failed due to technical issues as mentioned above. Out of 7 times that we were able to patch the cells, only 2 experiments successfully recorded Na^+ current and action potential. For SCAP iPSCs, we tested 3 clones each from one donor. Two clones tested twice and in only one experiment we obtained testable cells but no action potential; the 3rd clone was tested 4 times and was successful twice with action potential.

3.5.3. Direct induction neurogenesis using H9 Syn-GFP—We also tested direct neurogenesis for H9 Syn-GFP which carries GFP reporter gene driven by the Synapsin

promoter (Supplemental Materials & Methods). H9 Syn-GFP were cultured in feeder-free condition and induced in the same process as H9 and iPSCs (Fig. 8A-F). Under the fluorescence detection, we were able to select GFP⁺ cell for patch clamp studies (Fig. 8G-L). We verified that cells with neuron morphology and were GFP⁺ were able to show strong Na⁺ currents and demonstrate AP similar to H9 and iPSC derived-neurons (Fig. 8M-N). Tested H9 Syn-GFP-neurons exhibited 11/27 (41%) having AP with only one peak detected. Also see Supplemental Table S3 for percentage of cells having Na⁺ or K⁺ currents.

4. Discussion

The present study demonstrated that iPSCs derived from DSCs can be guided to becoming functional neurons in vitro exhibiting AP and spontaneous EPSC measured by voltage clamp indicating functional synapses. Both SCAP and DPSC derived iPSCs possess such abilities similar to hESCs, indicating that these DSCs may be served as a good cell source for neural tissue regeneration.

The safety issues of using hESCs or hiPSCs for clinical applications have been rigorously tested. The most tested clinical trial cases using hESCs have been to treat diseases of the retinal pigment epithelium (RPE) -- macular dystrophy or degeneration, and reports so far have shown no adverse effects or tumor formation (Peterson and Loring, 2014, Schwartz, *et al.*, 2012, Schwartz, *et al.*, 2015). Clinical trials of using hiPSCs have been also under taken to test if iPSCs are also safe. A recent case report by Manai et al demonstrated the first evidence that with careful and extensive inspection of the iPSCs generated from cells of patients in their 60s-70s, transplanted RPE derived from autologous iPSCs may be safe and feasible for clinical applications (Mandai, *et al.*, 2017). Patient did not receive immunosuppressants and had no signs of rejection, nor was there local or systemic malignant disease at 25 months of follow-up.

The studies of hESC neurogenesis have established a foundation to guide these cells into various neural lineages (Chambers, *et al.*, 2009, Eiraku, *et al.*, 2008, Kim, *et al.*, 2014, Zhang, *et al.*, 2001). This foundation has been utilized to guide iPSCs to differentiate into neural cells because iPSCs have been considered a good replacement of ESCs which need to be harvested from embryos or dealt with nuclear transfer (Huang, 2010, Huang, *et al.*, 2017). Our studies tested EB-mediated neurogenesis method which allowed us to confirm that DSC-derived iPSCs, similar to hESCs H9, can generate functional neurons in vitro. The process, while long, generated good quality functional neurons from DSC iPSCs. We had no difficulty selecting a neuro-like cell to patch and detect AP. There was little or no cell detachment or cell death issues during the lengthy process. In contrast, the direct induction method (Yan, *et al.*, 2013), which was considered a faster way to direct hESCs to become functional neurons in vitro encountered technical difficulties. If there were no such problems, the total time needed to obtain functional neurons in vitro via direct induction method is approximately 9 weeks (Fig. 4) as opposed to 11 weeks for the EB-mediated method (Fig. 2). The difference mainly lies in the production of NIM-NSCs which was straightforward and can be prepared in large amount and cryopreserved.

However, the key problem we encountered was the cell detachment and massive death during the neuro/neuronogenesis. In order to ameliorate the problem, we utilized Matrigel coating and tried using the plastic coverslips. We found that plastic coverslips did not enhance the attachment while coating with Matrigel appeared to help cells attach better than using polylysine or laminin. Additionally, we needed to modify the timing of the NDM addition depending on the conditions of the cells. NIM-derived NSCs normally attached well with minimal cell death and loss after the initial seeding onto glass coverslips under the NEM. After the addition of NDM, cell death and detachment began to occur. We then changed to adding the NEM/NDM mixture (1:1 or 4:3 then 1:1) which reduced the cell death/detachment while lengthened the time of the entire process. More cells would undergo cell death during the final phase of neuronal differentiation under NDM. At the time of 6–7 weeks after the initiation NDM induction, functional electrophysiology of cells could be detected. If counting the first phase of generating NIM-NSCs of 3–4 weeks, the total time for the entire process could need up to 11 weeks which is approximately the same amount of time needed for EB-mediated method. Nonetheless, if starting the process by using previously preserved NIM-NSCs, neuronal induction process alone takes 6–7 weeks only. The issue of cell detachment and death requires further investigation. During the accelerated process of guiding the iPSCs/H9 into NSCs and then neurons in vitro, it involves a myriad of intracellular signaling triggered by the neurogenic stimuli which may lead to the activation of cell death pathways. This phenomenon was observed in other process such as direct conversion of cell lineages (Prasad, *et al.*, 2017). Addition of antiapoptotic agents may be needed and helpful to minimize such situations.

In terms of generating different subpopulations of neurons in the brain, protocols that guide hPSCs into telencephalic neural progenitors before further guidance to produce dorsal telencephalic or ventral telencephalic neural progenitors are well described in the literature (Kim, *et al.*, 2014). The EB-mediated method contains FGF2, SB431542 (to block TGF β) and noggin (to block BMP) that guide cells toward telencephalic lineages. Using the direct induction method, however, is not clear whether the NIM-NSCs have the same maturation phase as those by EB-mediated method. Since no significant cell detachment/death occurred during the EB-mediated neuro/neuronogenic process, and the involved factors are similar during that differentiation phase in both methods, it is likely that the generated NSCs from EB-mediated method or by NIM inherited unknown differences that may play a role in the occurrence of cell detachment/death during neuro/neuronogenesis.

Our in vitro findings indicate that TF-DSC iPSCs are as potent as H9 to become neuron-like cells possessing neuron physiological activities. The expression of GFP by H9 Syn-GFP-derived neuron-like cells suggests that using a synapsin driven reporter gene is suited for future in vivo studies by tracing the GFP expression. Although the in vitro neuron-like cells derived from iPSCs or H9 were not as potent as the isolated neurons from neural tissues, these NSCs may become potent neurons after transplantation into the central nervous system.

Conclusion

Taken together, our in vitro results confirmed that functional neurons can be derived from TF-DSC iPSCs either by using EB-mediated or the direct induction method, which set a stage for further in vivo functional studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations:

bFGF	basic Fibroblast Growth Factor
BSA	bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DPSCs	dental pulp stem cells
EB	embryoid body
hESC	human embryonic stem cell
hSTEMCCA-loxP	(human) A single lentiviral “stem cell cassette” flanked by loxP site
iPSC	Induced pluripotent stem cell
MEF	Mouse embryonic fibroblast
MSC	mesenchymal stromal/stem cell
NSC	neural stem/progenitor cell
SCAP	stem cell of apical papilla
TF-iPSC	transgene-free iPSC

Reference

Aanismaa R, Hautala J, Vuorinen A, et al. 2012, Human dental pulp stem cells differentiate into neural precursors but not into mature functional neurons, *Stem Cell Discovery*, 2: 85–91.

- Arthur A, Rychkov G, Shi S, et al. 2008, Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues, *Stem Cells*, 26 (7): 1787–1795. [PubMed: 18499892]
- Biswas D, Jiang P. 2016, Chemically Induced Reprogramming of Somatic Cells to Pluripotent Stem Cells and Neural Cells, *Int J Mol Sci*, 17 (2): 226. [PubMed: 26861316]
- Bustin SA, Benes V, Garson JA, et al. 2009, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, *Clin Chem*, 55 (4): 611–622. [PubMed: 19246619]
- Casarosa S, Bozzi Y, Conti L. 2014, Neural stem cells: ready for therapeutic applications?, *Mol Cell Ther*, 2: 31. [PubMed: 26056597]
- Chambers SM, Fasano CA, Papapetrou EP, et al. 2009, Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling, *Nat Biotechnol*, 27 (3): 275–280. [PubMed: 19252484]
- Chen FG, Zhang WJ, Bi D, et al. 2007, Clonal analysis of nestin(–) vimentin(+) multipotent fibroblasts isolated from human dermis, *J Cell Sci*, 120 (Pt 16): 2875–2883. [PubMed: 17652163]
- Chen J, Li Y, Wang L, et al. 2001, Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats, *J Neurol Sci*, 189 (1–2): 49–57. [PubMed: 11535233]
- Chew DJ, Fawcett JW, Andrews MR. 2012, ‘Chapter 14 - The challenges of long-distance axon regeneration in the injured CNS’ in *Progress in Brain Research*, eds. Stephen BD, Anders B, Elsevier, 253–294.
- Chiu AY, Rao MS. 2011, Cell-based therapy for neural disorders--anticipating challenges, *Neurotherapeutics*, 8 (4): 744–752. [PubMed: 21901585]
- Cho KJ, Trzaska KA, Greco SJ, et al. 2005, Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1 alpha, *Stem Cells*, 23 (3): 383–391. [PubMed: 15749933]
- Choi K-A, Hwang I, Park H-s, et al. 2014a, Stem cell therapy and cellular engineering for treatment of neuronal dysfunction in Huntington’s disease, *Biotechnology Journal*, 9 (7): 882–894. [PubMed: 24827816]
- Choi SS, Lee S-R, Kim SU, et al. 2014b, Alzheimer’s Disease and Stem Cell Therapy, *Exp Neurobiol*, 23 (1): 45–52. [PubMed: 24737939]
- Deng J, Petersen BE, Steindler DA, et al. 2006, Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation, *Stem Cells*, 24 (4): 1054–1064. [PubMed: 16322639]
- Döbrössy M, Pruszk J. 2013, ‘Neural Repair with Pluripotent Stem Cells’ in *Regeneration Wound and Repair*, eds. Gourdie RG, Myers TA, Humana Press, 117–144.
- Eiraku M, Watanabe K, Matsuo-Takasaki M, et al. 2008, Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals, *Cell stem cell*, 3 (5): 519–532. [PubMed: 18983967]
- Fleige S, Pfaffl MW. 2006, RNA integrity and the effect on the real-time qRT-PCR performance, *Mol Aspects Med*, 27 (2–3): 126–139. [PubMed: 16469371]
- Gauthier P, Yu Z, Tran QT, et al. 2017, Cementogenic genes in human periodontal ligament stem cells are downregulated in response to osteogenic stimulation while upregulated by vitamin C treatment, *Cell Tissue Res*, 368 (1): 79–92. [PubMed: 27757536]
- Gervois P, Struys T, Hilkens P, et al. 2015, Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons, *Stem cells and development*, 24 (3): 296–311. [PubMed: 25203005]
- Greco SJ, Zhou C, Ye JH, et al. 2007, An interdisciplinary approach and characterization of neuronal cells transdifferentiated from human mesenchymal stem cells, *Stem cells and development*, 16 (5): 811–826. [PubMed: 17999602]
- Hofstetter CP, Schwarz EJ, Hess D, et al. 2002, Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery, *Proceedings of the National Academy of Sciences*, 99 (4): 2199–2204.

- Hu BY, Weick JP, Yu J, et al. 2010, Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency, *Proc Natl Acad Sci U S A*, 107 (9): 4335–4340. [PubMed: 20160098]
- Huang AH, Snyder BR, Cheng PH, et al. 2008, Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice, *Stem Cells*, 26 (10): 2654–2663. [PubMed: 18687995]
- Huang GT-J. 2010, Induced Pluripotent Stem Cells—A New Foundation in Medicine, *J Exp Clin Med* 2(5): 202–217. [PubMed: 22408700]
- Huang GT, Gronthos S, Shi S. 2009, Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine, *J Dent Res*, 88 (9): 792–806. [PubMed: 19767575]
- Huang GT, Yamaza T, Shea LD, et al. 2010, Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model, *Tissue engineering Part A*, 16 (2): 605–615. [PubMed: 19737072]
- Huang GTJ, El Ayachi I, Zou X-Y. 2017, 'Induced pluripotent stem cell technologies for tissue engineering' in *Tissue engineering and regeneration in dentistry-- current strategies*, eds. Waddington RJ, Sloan AJ, John Wiley & Sons, Ltd, West Sussex, UK.
- Jain K, Verma PJ, Liu J. 2014, Isolation and handling of mouse embryonic fibroblasts, *Methods Mol Biol*, 1194: 247–252. [PubMed: 25064107]
- Jung J, Kim JW, Moon HJ, et al. 2016, Characterization of Neurogenic Potential of Dental Pulp Stem Cells Cultured in Xeno/Serum-Free Condition: In Vitro and In Vivo Assessment, *Stem Cells Int*, 2016: 6921097. [PubMed: 27688776]
- Kim DS, Ross PJ, Zaslavsky K, et al. 2014, Optimizing neuronal differentiation from induced pluripotent stem cells to model ASD, *Front Cell Neurosci*, 8: 109. [PubMed: 24782713]
- Kim J, Ambasudhan R, Ding S. 2012, Direct lineage reprogramming to neural cells, *Curr Opin Neurobiol*, 22 (5): 778–784. [PubMed: 22652035]
- Kopen GC, Prockop DJ, Phinney DG. 1999, Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains, *Proceedings of the National Academy of Sciences*, 96 (19): 10711–10716.
- Lee JS, Hong JM, Moon GJ, et al. 2010, A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke, *Stem Cells*, 28 (6): 1099–1106. [PubMed: 20506226]
- Mandai M, Watanabe A, Kurimoto Y, et al. 2017, Autologous Induced Stem-Cell-Derived Retinal Cells for Macular Degeneration, *N Engl J Med*, 376 (11): 1038–1046. [PubMed: 28296613]
- Mantovani C, Terenghi G, Shawcross SG. 2012, Isolation of adult stem cells and their differentiation to Schwann cells, *Methods Mol Biol*, 916: 47–57. [PubMed: 22914932]
- Mieda T, Suto N, Iizuka A, et al. 2016, Mesenchymal stem cells attenuate peripheral neuronal degeneration in spinocerebellar ataxia type 1 knockin mice, *J Neurosci Res*, 94 (3): 246–252. [PubMed: 26707550]
- Morszeck C, Huang GT-J, Shi S. 2013, 'Stem and progenitor cells of dental and gingival tissue origin' in *Stem Cells Craniofacial Development and Regeneration*, eds. Huang GT-J, Thesleff I, Wiley-Blackwell, Hoboken, New Jersey, 285–302.
- Mung KL, Tsui YP, Tai EW, et al. 2016, Rapid and efficient generation of neural progenitors from adult bone marrow stromal cells by hypoxic preconditioning, *Stem Cell Res Ther*, 7 (1): 146. [PubMed: 27717376]
- Nichols JE, Niles JA, DeWitt D, et al. 2013, Neurogenic and neuro-protective potential of a novel subpopulation of peripheral blood-derived CD133+ ABCG2+CXCR4+ mesenchymal stem cells: development of autologous cell-based therapeutics for traumatic brain injury, *Stem Cell Res Ther*, 4 (1): 3. [PubMed: 23290300]
- O'Connell KM, Loftus R, Tamkun MM. 2010, Localization-dependent activity of the Kv2.1 delayed-rectifier K+ channel, *Proc Natl Acad Sci U S A*, 107 (27): 12351–12356. [PubMed: 20566856]
- Okano H, Sawamoto K. 2008, Neural stem cells: involvement in adult neurogenesis and CNS repair, *Philos Trans R Soc Lond B Biol Sci*, 363 (1500): 2111–2122. [PubMed: 18339601]

- Peterson SE, Loring JF. 2014, Genomic instability in pluripotent stem cells: implications for clinical applications, *J Biol Chem*, 289 (8): 4578–4584. [PubMed: 24362040]
- Pittenger MF, Mackay AM, Beck SC, et al. 1999, Multilineage potential of adult human mesenchymal stem cells, *Science*, 284 (5411): 143–147. [PubMed: 10102814]
- Prasad A, Teh DB, Shah Jahan FR, et al. 2017, Direct Conversion Through Trans-Differentiation: Efficacy and Safety, *Stem cells and development*, 26 (3): 154–165. [PubMed: 27796171]
- Sakai K, Yamamoto A, Matsubara K, et al. 2012, Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms, *J Clin Invest*, 122 (1): 80–90. [PubMed: 22133879]
- Schmittgen TD, Livak KJ. 2008, Analyzing real-time PCR data by the comparative CT method, *Nat Protocols*, 3 (6): 1101–1108. [PubMed: 18546601]
- Schwartz SD, Hubschman JP, Heilwell G, et al. 2012, Embryonic stem cell trials for macular degeneration: a preliminary report, *Lancet*, 379 (9817): 713–720. [PubMed: 22281388]
- Schwartz SD, Regillo CD, Lam BL, et al. 2015, Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies, *Lancet*, 385 (9967): 509–516. [PubMed: 25458728]
- Shiota M, Heike T, Haruyama M, et al. 2007, Isolation and characterization of bone marrow-derived mesenchymal progenitor cells with myogenic and neuronal properties, *Exp Cell Res*, 313 (5): 1008–1023. [PubMed: 17289022]
- Somers A, Jean JC, Sommer CA, et al. 2010, Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette, *Stem Cells*, 28 (10): 1728–1740. [PubMed: 20715179]
- Song S, Song S, Zhang H, et al. 2007, Comparison of neuron-like cells derived from bone marrow stem cells to those differentiated from adult brain neural stem cells, *Stem cells and development*, 16 (5): 747–756. [PubMed: 17999596]
- Sonoyama W, Liu Y, Yamaza T, et al. 2008, Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study, *J Endod*, 34 (2): 166–171. [PubMed: 18215674]
- Tamaoki N, Takahashi K, Tanaka T, et al. 2010, Dental pulp cells for induced pluripotent stem cell banking, *J Dent Res*, 89 (8): 773–778. [PubMed: 20554890]
- Tondreau T, Dejeneffe M, Meuleman N, et al. 2008, Gene expression pattern of functional neuronal cells derived from human bone marrow mesenchymal stromal cells, *BMC Genomics*, 9: 166. [PubMed: 18405367]
- Vierbuchen T, Ostermeier A, Pang ZP, et al. 2010, Direct conversion of fibroblasts to functional neurons by defined factors, *Nature*, 463 (7284): 1035–1041. [PubMed: 20107439]
- Wada N, Wang B, Lin NH, et al. 2011, Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts, *J Periodontal Res*, 46 (4): 438–447. [PubMed: 21443752]
- Wehner T, Bontert M, Eyupoglu I, et al. 2003, Bone marrow-derived cells expressing green fluorescent protein under the control of the glial fibrillary acidic protein promoter do not differentiate into astrocytes in vitro and in vivo, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23 (12): 5004–5011.
- Wernig M, Zhao JP, Pruszak J, et al. 2008, Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease, *Proc Natl Acad Sci U S A*, 105 (15): 5856–5861. [PubMed: 18391196]
- Xu J, Liao W, Gu D, et al. 2009, Neural ganglioside GD2 identifies a subpopulation of mesenchymal stem cells in umbilical cord, *Cell Physiol Biochem*, 23 (4–6): 415–424. [PubMed: 19471109]
- Yan X, Qin H, Qu C, et al. 2010, iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin, *Stem cells and development*, 19 (4): 469–480. [PubMed: 19795982]
- Yan Y, Shin S, Jha BS, et al. 2013, Efficient and rapid derivation of primitive neural stem cells and generation of brain subtype neurons from human pluripotent stem cells, *Stem cells translational medicine*, 2 (11): 862–870. [PubMed: 24113065]

- Young FI, Telezhkin V, Youde SJ, et al. 2016, Clonal Heterogeneity in the Neuronal and Glial Differentiation of Dental Pulp Stem/Progenitor Cells, *Stem Cells Int*, 2016: 1290561. [PubMed: 27313623]
- Yu Z, Gauthier P, Tran QT, et al. 2015, Differential properties of human ALP+ periodontal ligament stem cells vs their ALP- counterparts, *Journal of Stem Cell Research and Therapy*, 5 (7): 292. [PubMed: 26807329]
- Zhang M, Lin YH, Sun YJ, et al. 2016, Pharmacological Reprogramming of Fibroblasts into Neural Stem Cells by Signaling-Directed Transcriptional Activation, *Cell stem cell*, 18 (5): 653–667. [PubMed: 27133794]
- Zhang SC, Wernig M, Duncan ID, et al. 2001, In vitro differentiation of transplantable neural precursors from human embryonic stem cells, *Nat Biotechnol*, 19 (12): 1129–1133. [PubMed: 11731781]
- Zou XY, Yang HY, Yu Z, et al. 2012, Establishment of transgene-free induced pluripotent stem cells reprogrammed from human stem cells of apical papilla for neural differentiation, *Stem Cell Res Ther*, 3 (5): 43. [PubMed: 23095454]

Fig. 1. Generation and characterization of transgene-free DSC iPSCs. (A) Representative data showing generation of TF SCAP iPSCs. (Aa) SCAP at p3 before transduction. (Ab) SCAP iPSC colony formation after reprogramming. (Ac) SCAP iPSCs colonies after pHAGE2-Cre-IRES-PuroR plasmid transfection and 48 hours of puromycin treatment, SCAP iPSC colonies died out leaving behind an empty space (arrow), and new colonies (arrowhead) emerged after Cre-excision and puromycin selection. (scale bar a-c: 200 µm). (Ad) Newly formed colonies after cre-excision were expanded. (scale bar: 400 µm). (B) Excision of hSTEMCCA was verified by PCR. (Ba,b) RNA from DPSC or SCAP iPSC Clones after Cre-excision underwent RT-PCR analysis showing DPSC iPSC clone 1 and 10 not successful while other clones and all SCAP iPSC clones had no detectable hSTEMCCA. Cre(-) is before Cre-excision. (Bc) Genomic DNA of SCAP iPSCs was analyzed by PCR to detect hSTEMCCA showing successful and non-successful Cre-excision. GAPDH served as a control (C) Representative TF-DSC iPSCs grown on DR4 MEFs was treated with puromycin (1.2 µg/mL) for up to a week. (Ca) Before the addition of puromycin. (Cb) 24 h (Cc) 48 h (Cd) 7 days after the addition of puromycin showing complete iPSC death. (scale bar: 200 µm). (D-F) Immunocytofluorescence analysis of stemness and neural markers. TF-SCAP iPSCs (D), TF-DPSC iPSCs (E), and H9 (F) were grown on feeders MEFs and fixed for immunofluorescence staining. Stemness genes NANOG and OCT4 (top panel) and neural markers PAX6, SOX1, nestin, β III-tubulin, NFM and GFAP. Expressed genes stained in green or red; BF, bright field; IF, immunofluorescence stain; DAPI, nuclear stain. (scale bar: 200 µm for all images). (G) Teratoma formation by TF-SCAP iPSCs with tissues

representing ectoderm (ecto), yellow arrow indicates neural tube-like tissue, mesoderm (meso) with blue arrow pointing at bone-like and endoderm (endo) with blue arrow pointing at glandular tissue. Scale bars, 100 μm (left and right panel images).

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Fig. 2.

EB-mediated neuronal differentiation. (A) TF-SCAP iPSCs, (B) H9. a) Cell colonies grown on MEFs, b) EB formation, c) NR formation after EB attachment, d) suspended NRs, e) early stage of neuronal differentiation showing mixture of neural progenitor cells and more differentiated neuron-like cells with axon-like processes, f) late stage of neuronal differentiation showing more neuron-like cells with ovoid cell body and axon-like processes. (C) Illustration of time-line of EB-mediated neuronal differentiation showing multi-step culturing conditions and addition of neural differentiation factors. Cultures were first guided toward neural lineages followed by guiding toward neuronal lineages. The letters a-f correspond to the images presented in (A and (B). NRs: neural rosettes; IF: immunofluorescence analysis; RA: retinoic acid; AA: ascorbic acid. Scale bars: Aa, Ba, 500 μm; Ab, Ad, Bb, Bc, Bd, 200 μm; Ac, 300 μm; Ae, Af, Be, Bf, 50 μm.

Fig. 3. Expression of stemness and neural markers after EB-mediated neural differentiation. (A-D) Immunocytofluorescence analysis. TF-SCAP iPSCs (A) and H9 (B) were undergone neurogenesis as depicted in Fig. 2. At the stage of NR formation after EB attachment (c step in Fig. 2), cultures were fixed for immunofluorescence staining of neural markers PAX6, nestin, β III-tubulin and NFM; or stemness gene OCT4. Expressed genes stained in green or red; DAPI, nuclear stain. (scale bar: all neural marker genes in A, B: 50 μ m; OCT4, 200 μ m). (C, D) Cells under neurogenesis as depicted in Fig. 2. At the late stage (f step in Fig. 2), cultures were fixed for immunofluorescence staining of neural markers nestin, β III-tubulin, NFM and GFAP. (scale bar: 50 μ m for all images in C, D). (E-G) RT-qPCR analysis of the expression of neural markers. TF-SCAP iPSCs and H9 was analyzed at various time points by qPCR. Neurogenic induction during the first 14 d and the early neural gene markers were examined at days 0, 3, 5, 7, 10 and 14 (E). At the later stage of neurogenesis weeks 7 and 9, general neural markers including glial cell markers (F) and neuron specific markers (G) were examined. (Data represent mean $\pm$ SEM assayed in triplicate in (E) or duplicate in (F, G); significantly different, * p <0.01, ** p <0.001).

Fig. 4.

Direct induction of neuronal differentiation. (A) TF-SCAP iPSCs, (B) TF-DPSC iPSCs, (C) H9. a) Cell colonies grown on Matrigel cultured under NIM to become NSCs. Representing images of 3–5 d of induction. b) NSC expansion on Matrigel under NEM. Representing images of passages 3–9 of expansion. c,d) NSCs under neural and neuronal differentiation conditions showing neuron-like cells with axon-like processes. (D) Illustration of time-line of direction induction for neuronal differentiation showing steps of culturing conditions and use of neural differentiation factors. Cultures were first guided toward neural lineages followed by guiding toward neuronal lineages. The time needed in this phase varied depending on the condition of cells. The letters a-d correspond to the images presented in (A-C). IF: immunofluorescence analysis; RA: retinoic acid; AA: ascorbic acid. Scale bars: Aa, 500 μ m (same for Ba, Ca); Ab, 100 μ m (same for Bb, Cb); Ac, 50 μ m (same for Ad, Bc,d; Cc,d).

Fig. 5. Immunocytofluorescence analysis of stemness and neural markers after direct induction of neural differentiation depicted in Fig. 4. TF-SCAP iPSCs (A), TF-DPSC iPSCs (B), and H9 (C) were grown on feeder free Matrigel and underwent neural induction for 7 d followed by neural expansion, then fixed for immunofluorescence staining. Stemness genes NANOG and OCT4 (top panel) and neural markers PAX6, SOX1, nestin, βIII-tubulin, NFM and GFAP. Expressed genes stained in red; BF, bright field; IF, immunofluorescence stain; DAPI, nuclear stain. (scale bar: 50 μm for all images in A-C). (D, E) At the late stage cultures were fixed for immunofluorescence staining of neural markers nestin, βIII-tubulin, NFM, GFAP and Pan-NaV. Expressed genes stained red; DAPI, nuclear stain (scale bar: 50 μm for all images in D, E).

Fig. 6. Electrophysiology of neurons derived from TF-SCAP iPSCs (A) and H9 (B) after EB-mediated neurogenesis. (Aa) Images of whole cell patch clamp on one neuron-like cell. (Ab) Sodium and potassium currents elicited by 200 ms depolarizing voltage steps from -80 mV to +80 mV. (Ac) Spontaneous excitatory postsynaptic current (EPSC) measured by voltage clamp indicating functional synapses. (Ad) Action potential (AP) evoked by a depolarizing somatic current injection (300 pA). (Ba) Cells showing neuronal morphology at the time of patch clamp. (Bb) Total membrane currents (both putative Na⁺ and K⁺) recorded using 200

ms step depolarization to +20 mV. Immediately upon depolarization, a large inward Na⁺ current develops (indicated by the transient negative current), followed by the activation of a classical delayed rectifier-type K⁺ current (indicated by the persistent positive current). (Bc) Na-IV curve: Current-voltage dependence of the transient Na⁺ current in cells. Average peak inward Na⁺ current from 4 cells at each voltage step was plotted as a function of membrane potential. (Bd) Repetitive APs elicited by a 200 ms depolarizing somatic current injection (solid black line) using current clamp.

Fig. 7.

Electrophysiology of neurons derived from TF-SCAP iPSCs (A), TF-DPSC iPSCs (B) and H9 (C) after direct induction neurogenesis. (Aa, Ba, Ca) Voltage clamp, total membrane currents (both Na^+ and K^+) recorded using 500 ms step depolarization from -70 mV to $+40$ mV in 10 mV steps, from a holding potential of -90 mV. I_{NaT} started to appear at -50 mV. (Ab, Bb, Cb) TTX-insensitive current. I_{NaT} was blocked by $1 \mu\text{M}$ TTX to isolate the outward K^+ current, protocol is the same as middle panel. (Ac, Bc, Cc) The subtracted Na^+ current showing the TTX-sensitive current. (Ad, Bd, Cd) Na^+ -IV curve: Current-voltage

dependence of the transient Na⁺ current in different cells types. Average peak inward Na⁺ current from cells (n=5 for Ad; n=3 for Bd; n=7 for Cd; errors are SEM) at each voltage step was plotted as a function of membrane potential. (Ae, Be, Ce) Action potentials were elicited by a 2 s depolarizing somatic current injection using current clamp mode of the whole-cell patch clamp technique. The stimulus was a depolarizing somatic current injection of 75 pA (Ae), 160 pA (Be), or 120 pA (Ce). The different current injection is because the threshold of Ap was different.

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Fig. 8.

Direct induction of neuronal differentiation of H9 Syn-GFP. (A) Cells were thawed and grown onto Matrigel coated wells forming typical hESC colonies. (B) Cells were passaged with lower density before neural induction. (C) Cells were cultured in NIM for 7 days becoming confluent. (D) Cells were passaged into lower density and cultured in NEM for 3 days. (E, F) Cells were further passaged and expanded in NEM showing NSC morphology. (G-H; I-J; K-L) Paired images showing cells underwent neuronal differentiation and ready for electrophysiology studies. Left images show selected cells patched by the pipette tip for measuring currents and action potentials. Right images are under fluorescence images showing the corresponding cell pointed by the arrowhead to be patched. Scale bars: A-E, 300 μ m; F, 50 μ m; G-L, 30 μ m. (M, N) Electrophysiology of neurons derived from H9 Syn-GFP. (M) Voltage clamp, total membrane currents (both Na^+ and K^+) recorded using 500 ms step depolarization to +40 mV, 10mV step, holding potential was -90 mV. By a test potential ranging from -70mV to 40 mV in 10mV steps. I_{NaT} started to appear at -50 mV. (N) Action

potentials were elicited by a 2 s depolarizing somatic current injection using current clamp mode of the whole-cell patch clamp technique.

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