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SHORT REPORT

Induced pluripotent stem cells from hair follicles as a cellular model for neurodevelopmental disorders

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Abstract Disease-specific induced pluripotent stem cells (iPSC) allow unprecedented experimental platforms for basic research as well as high-throughput screening. This may be particularly relevant for neuropsychiatric disorders, in which the affected neuronal cells are not accessible. Keratinocytes isolated from hair follicles are an ideal source of patients' cells for reprogramming, due to their non-invasive accessibility and their common neuroectodermal origin with neurons, which can be important for potential epigenetic memory. From a small number of plucked human hair follicles obtained from two healthy donors we reprogrammed keratinocytes to pluripotent iPSC. We further differentiated these hair follicle-derived iPSC to neural progenitors, forebrain neurons and functional dopaminergic neurons.
This study shows that human hair follicle-derived iPSC can be differentiated into various neural lineages, suggesting this experimental system as a promising in vitro model to study normal and pathological neural developments, avoiding the invasiveness of commonly used skin biopsies.

Introduction

Reprogramming of somatic cells into induced pluripotent stem cells (iPSC) and differentiating them to neuronal lineage is a new powerful technology that could offer an attractive tool to model neurodevelopmental disorders. Neuropsychiatric disorders, as many other complex disorders, necessitate cellular models with relevance to their underlying biological mechanisms and pharmacotherapy. The biological basis of psychiatric disorders is still enigmatic, despite tremendous advances in

Abbreviations: HF, hair follicle; iPSC, induced pluripotent stem cells; ORS, outer root sheath; HPLC, high performance liquid chromatography.

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science and medicine. Several lines of evidence support a neurodevelopmental, multifactorial etiology in psychiatric diseases, specifically in schizophrenia (Jaaro-Peled et al., 2009), suggesting embryonic insults that impact brain developmental events. However, in the absence of consistent biological markers for these disorders, diagnosis still relies on subjective assessment of a cluster of signs and symptoms. Currently, peripheral or post-mortem brain human samples are available for the biological research of neuropsychiatric diseases, markedly hampering molecular studies (Stevens et al., 2010). The advantage of iPSC as a tool to study neuropsychatric disorders is that they can be largely expanded, they express the individual genome (and possibly epigenome), and can be differentiated into a specific neuronal lineage, thereby enabling the study of neurodevelopmental processes in vitro (Kiskinis and Eggan, 2010).

One of the main obstacles in human studies is sample acquisition. Human iPSC lines are usually produced from fibroblasts isolated from skin biopsies, a traumatic invasive procedure demanding local anesthesia and medical follow-up. Hair follicle (HF) plucking, however, is simple, painless and non-invasive alternative procedure. Moreover, the HF-keratinocytes, similar to neurons are derived from the same ectodermal embryonic layer, and thus, besides carrying the same genetic load, could retain the same epigenetic alterations during early development. Aasen et al. have shown that skin and HF keratinocytes could be reprogrammed into iPSC (Aasen et al., 2008; Aasen and Belmonte, 2010). In the present study we confirmed the feasibility of this approach and improved it by using 1) a polycistronic lentival vector, 2) exogenous inhibitors and 3) stored hairs. Moreover, we show that iPSC from HF (HF-iPSC) have potential to give neural progenitors and functional neurons. We propose a cellular model of iPSC obtained from few hair follicles plucked from the scalp region, utilizing it as a novel source for in vitro neural commitment to model neurodevelopmental disorders.

Results and discussion

We found that as few as ten HF in anagen phase, identified by the presence of the outer root sheath, were sufficient to obtain keratinocytes for reprogramming (Fig. 1A). Notably, HF can be stored for at least 48 h in plain medium at room temperature without a decrease in cell recovery, allowing shipments worldwide. Keratinocytes were detached by trypsin treatment from HF and typical keratinocyte colonies appeared 7–10 days later (Fig. 1B) (Limat and Noser, 1986) for all healthy or diseased donors that we tested. Early passage keratinocytes were infected with lentiviral particles expressing the polycistronic plasmid STEMCCA (OCT4, SOX2, KLF4 and c-MYC), which allows simultaneous delivery of the 4 genes with only one integration site, maximizing reprogramming efficiency (Somers et al., 2010). A GFP control virus confirmed high infection rate of primary keratinocytes (Fig. 1C). We found that reprogramming was increased by 3.5 fold ($p=0.04$) with the use of SB431542, CHIR99021, Parnate and PS48 (Zhu et al., 2010). Several colonies with typical hES morphology (defined border, high nuclei/cytoplasm ratio and prominent nucleoli) were obtained and expanded individually (Fig. 1D).

Pluripotency of HF-derived iPSC from 2 healthy individuals (HF-iPSC-1 and HF-iPSC-2) was confirmed by the expression of the pluripotency markers Pou5F1 (Oct3/4), SOX2, NANOG and DNMT3B (Fig. 2A) as well as by TRA-1-81, SSEA-

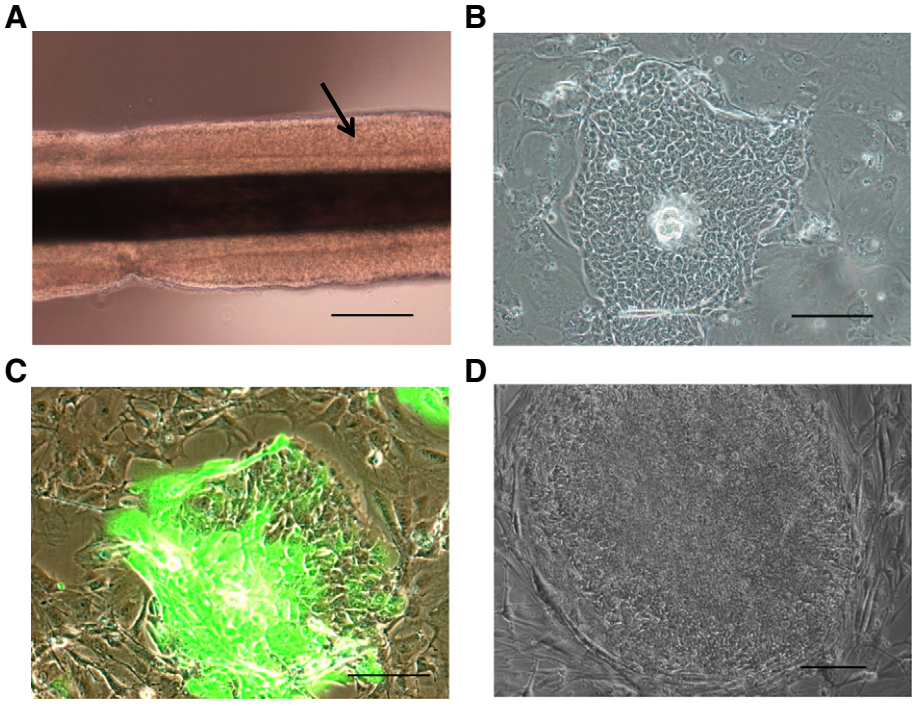

Figure 1 Derivation of iPSC from hair follicles. (A) A hair follicle in anagen phase, the arrow indicates the outer root sheath layer that contains keratinocytes. (B) Keratinocyte colony 10 days after isolation from hair follicles. (C) A GFP-positive keratinocyte colony following infection with GFP-lentivirus. (D) Typical iPSC colony derived from HF keratinocytes. Scale bars: 100 μm.

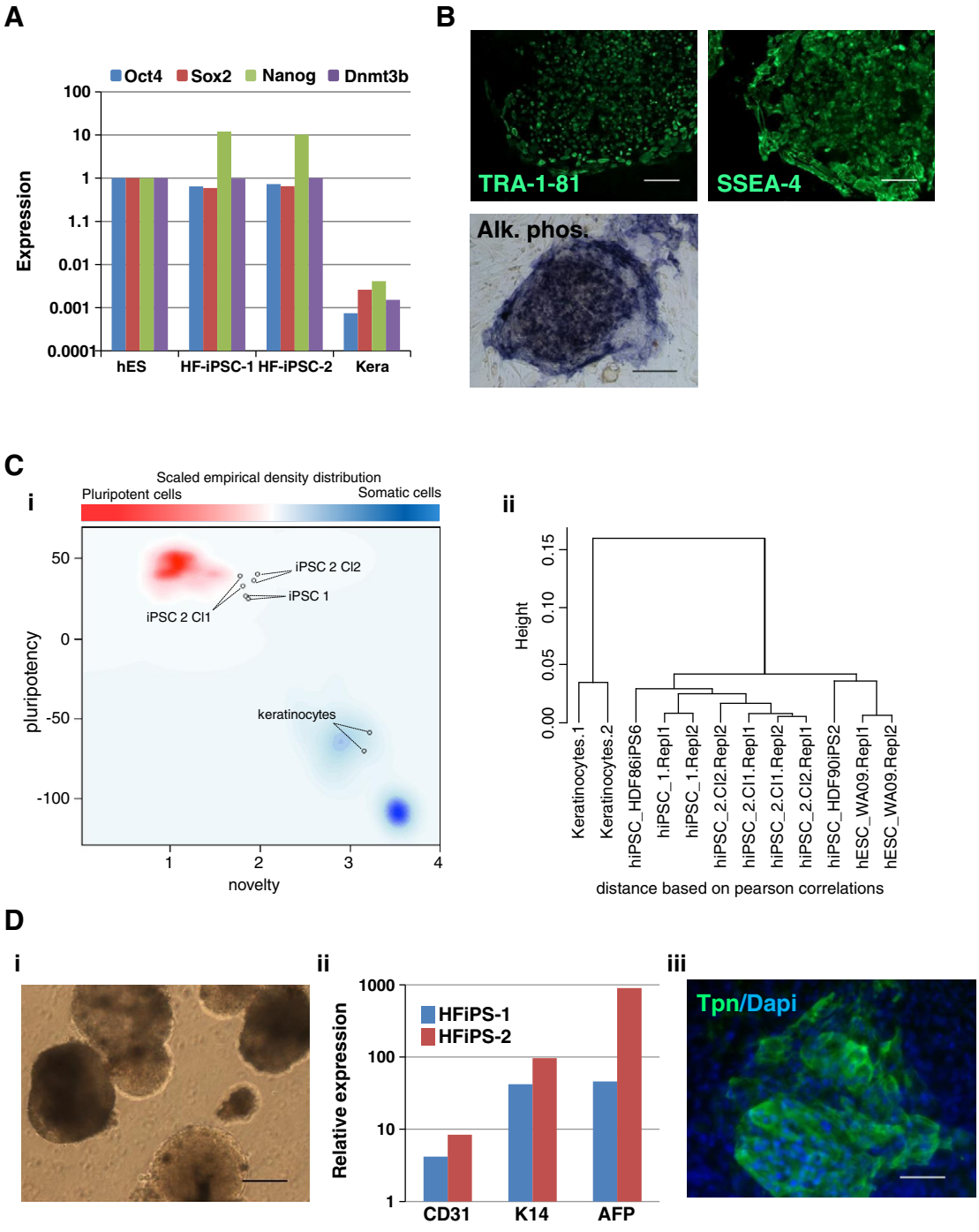

Figure 2 Pluripotency of HF-iPSC. (A) Expression of the pluripotency genes, Oct4. Sox2, Nanog and Dnmt3b determined by qRT-PCR in two iPSC lines (HF-iPSC-1 and-2). Human embryonic stem cells (hES) and HF keratinocytes were used as positive and negative control respectively. Gene expression levels were calculated relatively to their expression in hES. Primers for Oct3/4 and Sox2 recognize endogenous and exogenous forms. (B) HF-iPSC-1 was stained for cell surface expression of TRA-1-81 and SSEA-4 and for alkaline phosphastase activity. Identical results were obtained for HF-iPSC-2 (data not shown). (C) Transcriptional profile of HF-iPSC was analyzed by PluriTest assay. (i) PluriTest results plotted in density distribution for previously referenced pluripotent cells (red cloud) and somatic cells (blue cloud) (Müller et al., 2011), HF-iPSC and keratinocytes. (ii) Hierarchical clustering of HF-iPSC lines HF-iPSC-1 and 2 clones of HF-iPSC-2, keratinocytes, hES (WA09) and iPSC derived from fibroblasts sampled from two individuals (HDF86iPS6 and HDF90iPS2). Samples were run in duplicates. (D) Embryoid bodies from HF-iPSC lines (i). Germ layer markers (CD31-mesoderm, K14-ectoderm and AFP-endoderm) determined by qRT-PCR at day 21. Expression levels were calculated relatively to the basal expression in undifferentiated HF-iPSC (ii). Immunofluorescent staining for cardiac Troponin T (iii). Scale bars: (B and D-i) 100 μm, (D-iii) 50 μm.

4 and alkaline phosphatase (Fig. 2B). Of note, transgene expression was found to be efficiently silenced (Suppl. Fig. 1A). HF-iPSC lines were analyzed in the PluriTest assay, a novel bioinformatic assay for pluripotency, which eliminates the need of animal use for teratoma assay (Müller et al., 2011). Both HF-iPSC lines displayed high Pluripotency Scores, similar to hES and fibroblast-derived iPSC (Fig. 2C). HF-iPSC lines scored relatively high on PluriTest's Novelty Score, a metric that measures the divergence of the global stem cell transcriptome from expected patterns present in well-characterized iPSC and hES. This result suggests that HF-iPSC, while being genetically normal as demonstrated by karyotypic analysis (Suppl. Fig. 1B), might display a distinct signature, possibly due 'epigenetic memory' stemming from their ectodermal origin.

HF-iPSC pluripotency was further demonstrated by their in vitro ability to form embryoid bodies (EB) and to differentiate into the three embryonic germ layers endoderm (shown by the expression of alpha-fetoprotein (AFP)), mesoderm (CD31) and ectoderm (cytokeratin K14) (Fig. 2D). Spontaneous contraction was observed indicating cardiac commitment (data not shown), which was further confirmed by Troponin T (cardiac marker) positive cells (Fig. 2D).

Characterized pluripotent HF-iPSC were further differentiated into neuronal cells. During the last decade, studies on hES have reported numerous protocols for neuronal differentiation, leading to the production of homogenous and specialized neurons, capable of basic neuronal activities in vitro and upon transplantation in animal models. We evaluated the neural potential of HF-iPSC to form two types of multipotent progenitors, either anterior neuroectodermal $Pax6^+$ cells or floor plate $FoxA2^+$ cells. The two HF-iPSC lines spontaneously formed rosette structures that contained $Pax6^+$/$nestin^+$ neural progenitor cells upon culturing them on MS-5 stroma cells in absence of morphogens (Fig. 3A-a). Moreover, HF-iPSC were also differentiated into floor plate $FOXA2^+$ progenitors, a newly described type of neural precursors (Fasano et al., 2010) (Fig. 3A-b). Quantification by flow cytometry showed that 89% of the cells were $FOXA2^+$ (Fig. 3A-c). In order to fully evaluate the neuronal potential of HF-iPSC, we directed the HF-iPSC to two types of mature neurons, dopaminergic neurons or forebrain neurons, which are involved in various neurological disorders. HF-iPSC were differentiated towards mature βIII-$tubulin^+$ TH^+ dopaminergic neurons by SMAD inhibition protocol (Chambers et al., 2009) (Fig. 3B-a). Quantification by flow cytometry showed that 75% of the cells are βIII-$tubulin^+$ (Fig.3B-b). HPLC analysis of the extracellular medium of these differentiated neural cells revealed the presence of both dopamine and serotonin, and their respective metabolites homovanillic acid (HVA) and 5-hydroxyindolacetic acid (5HIAA) (Fig. 3B-c), while they were undetected in undifferentiated HF-iPSC (data not shown). This further supports the presence of functional dopaminergic and serotonergic neural cells in our differentiated culture. In parallel, HF-iPSC could also be differentiated towards βIII-$tubulin^+$/$TBR1^+$ forebrain glutamatergic neurons by prolonged spontaneous EB differentiation (Fig. 3C).

Human iPSC lines are usually produced from fibroblasts isolated from skin biopsies, a traumatic invasive procedure demanding local anesthesia and medical follow-up. Hair follicle (HF) plucking, however, is simple, painless and a non-invasive alternative procedure. In the genuine study demonstrating that skin keratinocytes can produce iPSC, HF has been suggested as an alternative source for cell reprogramming (Aasen et al., 2008) and our study confirms that as few as ten HF in anagen phase can be used to successfully reprogram keratinocytes into iPSC and shows that HF-iPSC can serve as an efficient model to obtain various types of donor-specific neuronal lineages. An additional advantage of HF, demonstrated in the present study, is the ability to store plucked HF in medium for at least 48 h at room temperature, allowing exchange of materials between clinical centers and research laboratories. Taken together, these advantages suggest hair follicles as a material of choice for deriving of iPSC lines from a relatively large sample size of patients suffering from neuropsychiatric disorders. Moreover, the HF-keratinocytes, similar to neurons are derived from the same ectodermal embryonic layer, and thus, besides carrying an identical genetic load, could retain the same epigenetic alterations during early development. Indeed, it has been demonstrated that iPSC can retain epigenetic memory of the tissue of origin (Kim et al., 2010). It is therefore conceivable that iPSC derived from HF keratinocytes would retain conserved epigenetic insults that occurred during embryonic neuroectodermal fate in patients, which will enable studying epigenetic factors during normal and pathological neurodevelopment in vitro.

One can argue that multifactorial pathologies are difficult to model with iPSC due to the heterogeneity of the genetic background and environmental factors involved in their etiology. Nevertheless, a recent study revealed abnormal neuronal connectivity in schizophrenic patient-derived iPSC, demonstrating the relevance of this approach (Brennand et al., 2011). In their study, commercially available skin-derived fibroblasts were used. However, the compliance of patients to skin biopsy is rather low and therefore hair follicles are an attractive and painless alternative to obtain large number of iPSC lines-and their derived neurons. Indeed, we have already produced iPSC lines from four schizophrenic patients with similar efficiency to that of healthy donors (data not shown). We believe that HF-iPSC-based models will facilitate the biological research of complex neurodevelopmental disorders and may provide new insights into neuron development in health and disease. In addition HF-iPSC may serve as a tool for the design of alternative therapeutics and for personalized pharmacogenetic studies to optimize drug therapy for the benefit of the patients.

In summary, this study illustrates the use of hair follicles as an easy source of reprogrammable cells to produce patient-specific neurons. HF-iPSC can differentiate into different types of functional neuronal cells and therefore could be a material of choice to model psychiatric, neurodevelopmental disorders.

Materials and methods

Hair follicle keratinocyte culture

Samples collection from two healthy individuals was approved by The Rambam Medical Center Institutional Review Board Hair follicles in anagen phase (5–15) were plucked and washed twice in DMEM supplemented with penicillin,

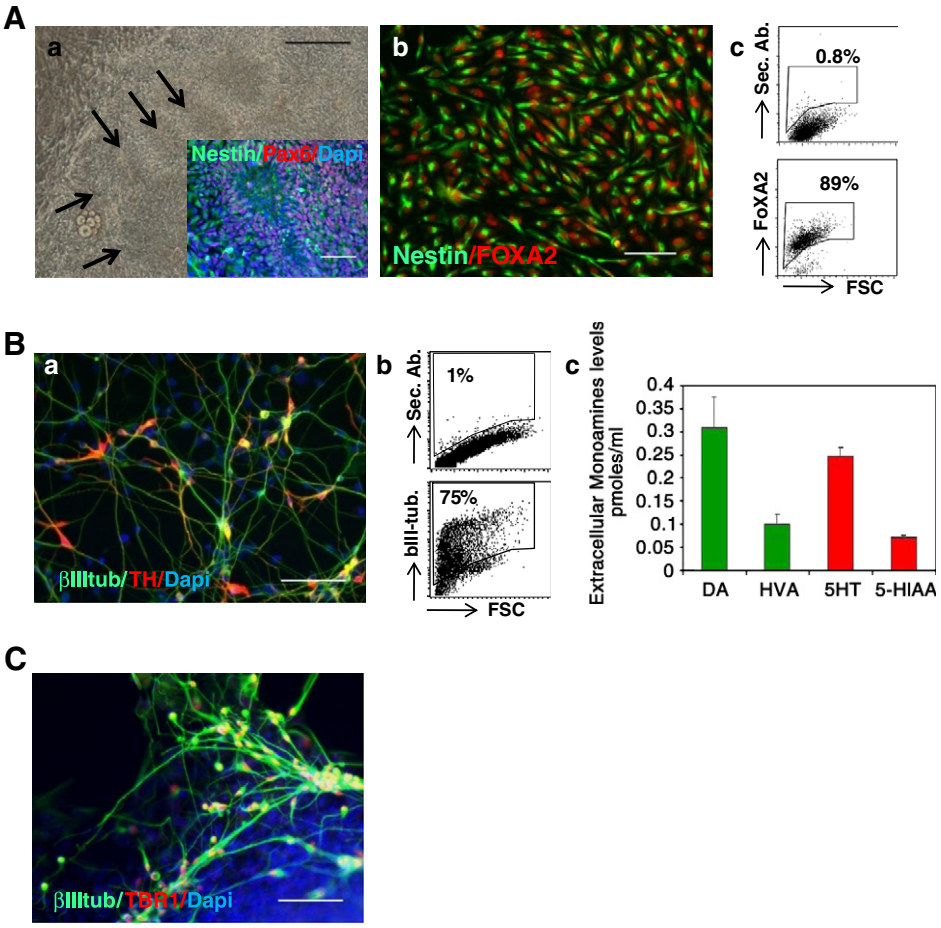

Figure 3 Differentiation of HF-iPSC into neuronal cells. (A) HF-iPSC lines differentiated into neural progenitors: (a) Pax6$^+$nestin$^+$ progenitors on MS-5 feeders (b) FoxA2$^+$nestin$^+$floor plate progenitors by direct differentiation. (c) Quantification of FoxA2$^+$ cells by flow cytometry. Cells incubated with secondary antibody only were used for gating positive cells. (B) HF-iPSC lines differentiated into mature dopaminergic neurons: (a) Dopaminergic βIIItubulin$^+$TH$^+$neurons obtained by SMAD inhibition protocol (b) Quantification of βIIItubulin$^+$neurons by flow cytometry. Cells incubated with secondary antibody only were used for gating positive cells. (c) Extracellular dopamine (DA) and serotonin (5-HT) and their metabolites HVA and 5HIAA, respectively, in differentiated neurons analyzed by HPLC. Plain medium and undifferentiated HF-iPSC were also tested as controls and none of these molecules could be detected. (C) HF-iPSC lines differentiated into mature forebrain glutamatergic βIIItubulin$^+$TBR1$^+$ neurons by spontaneous differentiation of embryoid bodies. Scale bars: (A-phase) 100 μm, (A-inset, A-b, B-a, and C) 50 μm.

streptomycin, anti-fungal nystatin and anti-mycoplasma Biomyc (Biological Industries, Bet Haemek, Israel). The extremities containing outer root sheath (ORS) were cut and placed in 0.25% trypsin/EDTA 3–4 times for 20 min each round. Between each round of trypsin, follicles were vigorously triturated by up and down aspiration. Complete dissociation of outer root sheath cells was monitored under microscope. Cells were washed and seeded on mitomycin C-inactivated 3T3-G2 fibroblast feeders in keratinocyte medium (DMEM:F12 3:1 supplemented with 10% FCS (FcII, Hyclone, Thermo, Waltham, MA, USA), 1 mM glutamine (Biological Industries), 1 mM NaPyruvate (Biological Industries), 0.2 mM adenine (Sigma-Aldrich, St Louis, MO, USA), 5 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 2 nM triiodothyronine (Sigma), 0.1 nM cholera toxin (sigma) and 10 ng/ml EGF (Peprotech, Rocky Hill, NJ, USA)). HF keratinocytes could be amplified for 4–5 passages and frozen down at early passages.

Reprogramming of keratinocytes into iPSC

HF keratinocytes at early passages (1–2) were seeded in 6-well on 3T3-G2 feeders 2–3 days before infection. Lentiviral preparation was made freshly for each experiment, by transfection of 293FT cells (Invitrogen, Carlsbad, CA, USA) with the human STEMCCA plasmid (Somers et al., 2010) using Jet-PEI reagent (PolyPlus-transfection, Illkirch, France). Cells were infected with lentiviral supernatant for 7 h and replaced with fresh medium (Day 0). Infection was repeated on the next day. Three days later (Day 4) keratinocytes were seeded on mitomycin-treated MEFs in keratinocyte medium. On day 7 medium was switched to 50% Green medium/50% iPSC medium (DMEM/F12, 20% knockout serum replacement (Invitrogen), 1 mM glutamine, 100 μM non-essential amino-acids (Biological Industries), 100 μM beta-mercaptoethanol (Invitrogen), 10 ng/ml bFGF (Peprotech)). On day 10 medium

was switched to 100% iPSC medium. The following inhibitors were added from days 8–9 to day 20: 2 μM SB431542 (Sigma), 3 μM CHIR99021 (AxonMedchem, Groningen, the Netherlands), 3 μM Parnate (P8511, Sigma) and 5 μM PS48 (Stemgent, Cambridge, MA, USA). Colonies with hES morphology were picked between days 20–40 and expanded individually.

Pluripotency characterization

Expression of pluripotency markers was determined by immunofluorescence using TRA-1-81 (Santa Cruz Biotechnology, Santa Cruz, CA) and SSEA-4 (R&D Systems, Minneapolis, MN) antibodies and by quantitative real-time PCR (qRT-PCR) using Aurum RNAkit (Biorad, Hercules, CA, USA) for RNA isolation, iScript kit (Biorad) for single strand cDNA synthesis and SYBR SensiMix (Bioline, London, UK) for qRT-PCR. Alkaline phosphatase activity was revealed by incubation with 0.01% Naphtol (Sigma) and 0.5 mg/ml Fast Blue (Sigma) in 100 mM Tris–HCl and 20 mM $MgCl_2$.

For whole genome microarray profiling, RNA was isolated from two biological replicates per cell line (1×10^6 cells per sample) with the mirVana RNA isolation kit (Ambion, Carlsbad, USA). Illumina HT12v3 microarrays were hybridized following the manufacturers instructions and as previously described (Müller et al., 2011). The resulting raw data was processed with the PluriTest algorithm (Müller et al., 2011) for testing pluripotent features in keratinocytes and iPSC lines. Genetic integrity was evaluated by G-banded karyotype analysis.

Spontaneous differentiation

iPSC colonies were cultured in suspension in petri dish in differentiation medium (DMEM supplemented with 10% FBS, 1 mM glutamine,100 μM non-essential amino-acids, 100 μM beta-mercaptoethanol) to allow the formation of embryoid bodies (EBs). EBs were plated on days 7–8 on gelatin-coated plates and further cultured until day 21. Paraformaldehyde-fixed cells were stained for Troponin T (Thermo Ficher scientific, Waltham, MA). The expression of CD31, K14 and AFP was analyzed by quantitative real-time PCR (qRT-PCR) as detailed above.

Neuronal differentiation

For neuronal rosette induction, iPSC colonies were seeded on mitomycin-treated MS-5 feeder in DMEM/F12 supplemented with 15% knockout serum replacement, 1 mM glutamine, 100 μM non-essential amino-acids and 100 μM beta-mercaptoethanol for 15–18 days. Floor plate FOXA2+ progenitors were obtained by direct induction according to Fasano et al. (Fasano et al., 2010). Briefly, iPSC were treated with accutase (Sigma) and plated on matrigel (BD biosciences, Franklin Lakes, NJ) with MEFs-conditioned iPSC medium for 2 days in the presence of Y-27632 (10 μM). bFGF was removed on day 0. KSR was gradually decreased from 20% to 15% on day 5, 10% on day 7, 5% on day 9 while N2 supplement (Invitrogen) was gradually added from 0% to 1% on same days. Neural commitment was induced by Noggin (R&D Systems, 500 ng/ml), SB431542 (Sigma, 10 μM) on days 0–11, SHH-C24-II (R&D Systems, 200 ng/ml) and Wnt1 (Peprotech, 50 ng/ml) on days 1–11, FGF8 (R&D Systems, 100 ng/ml) was added on days 9–11.

Dopaminergic neurons were obtained as previously described (Chambers et al., 2009). Briefly, iPSC were plated as described above for floor plate progenitors and the same KSR/N2 medium was used. Noggin (R&D Systems, 500 ng/ml) and SB431542 (10 μM) were added on days 0–12, SHH (200 ng/ml) was added on days 5–12, FGF8 (100 ng/ml), BDNF (R&D Systems, 20 ng/ml) and ascorbic acid (AA, 0.2 mM) on days 9–12. After splitting, cells were cultured with N2 supplement, BDNF, AA, GDNF (20 ng/ml), TGFβ3 (R&D Systems, 1 ng/ml) and cAMP (Sigma, 1 mM) until day 20.

Forebrain neurons were obtained after plating EBs as previously described (Zeng et al., 2010). Briefly, EBs were cultured in suspension in differentiation medium (as described above) for 4 days then in neural medium consisting of DMEM/F12 (1:1), 20% N2 supplement (Invitrogen), and 2 μg/ml heparin (Sigma) for 2 more days. EBs were seeded on gelatin coated well, and culture prolonged to 5–6 weeks until neural cells were appeared.

PFA-fixed cultures were stained with Pax6, nestin, βIII-tubulin, TH, TBR1 (all from Chemicon (Millipore), Billerica, MA, USA) and FOXA2 (Thermo Ficher scientific) antibodies. Flow cytometry was performed on neural progenitors and dopaminergic neurons. Cells were fixed with 2% PFA and permeabilized with 90% methanol for 30 min on ice. Cells were incubated in 2% donkey serum/2% BSA with FOXA2 or βIII-tubulin primary antibody for 60 min, washed with PBS and incubated with secondary antibody (anti-rabbit or -mouse Alexa Fluor 488, Invitrogen) for 40 min. Cells incubated only with secondary antibody were used as negative control and reference to gate positive cells. Acquisition was performed on FACSCalibur using CellQuest software (BD Biosciences).

Extra-cellular levels of dopamine and its metabolites

Dopamine (DA) and its metabolite homovanillic acid (HVA) as well as serotonin (5-HT) and its metabolite 5-hydroxyindolacetic acid (5-HIAA) were measured in culture medium by HPLC with an electrochemical detector (ESA coulochem II model 5200, ESA Inc. MA, USA) as described previously (Ben-Shachar et al., 1997). Separation of the monoamines and their metabolites was achieved using an Inertsil ODS-2 column (GL Sciences, Japan) with a mobile phase composed of 0.1 M NaH_2PO_4, 0.27 mM Na_2EDTA, 1 mM octane sulfuric acid, 4.5% acetonitrile, 2.5% methanol (pH = 2.75). Column eluates were initially oxidized at a potential of +300 mV using an ESA guard cell placed before the detector, reduced to +100 mV at detector 1 of the analytical cell (ESA model 5010), and measured at −400 mV at detector 2. The limit of detection for was 0.01 pmol. Data were analyzed using Borwin software.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.09.003

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