Supporting Information

Nissan et al. 10.1073/pnas.1019070108

Supporting Information Corrected September 27, 2011

SI Methods

Characterization of iPSCs. An immunostaining analysis of the expression of pluripotency markers was assessed on SSEA4, OCT4, TRA1-81, and TRA1-60 (Fig. S1*A*). Flow cytometry analyses confirmed that 98% of these cells coexpress SSEA4 and TRA1-81 (Fig. S1*B*). The genomic integrity of iPSCs was assessed by FISH and G-banding analyses, revealing a normal karyotype (Fig. S1*C*). Multiplex FISH and G-banding karyotypes were analyzed as previously described (1) according to the guidelines of the International Stem Cell Initiative (2). Transgene expression was also examined using quantitative PCR (qPCR) analysis, indicating a transitory increase in the transgenic forms of SOX2, CMYC, KLF4, and OCT4 in the ongoing reprogrammed fibroblasts (4–21 d) and their silencing in established iPSCs (Fig. S1*D*). Furthermore, iPSC differentiation abilities were evaluated in vitro through the formation and analysis of embryoid bodies (EBs) (Fig. S1*E*). qPCR analysis revealed a decrease in undifferentiated markers (DNMT3B, OCT4, LEFTB, NANOG, TERT, and CRIPTO) in 14-d-old EBs associated with an increase in three germ layer markers (T, GATA6, PAX6, NEUROD1, SOX17, and AFP). (Fig. S1*F*). Gene expression profiling was finally performed on iPSC-derived EBs by TaqMan arrays, revealing the upregulation of several genes related to differentiation (Fig. S1*G*).

Keratinocyte and Melanocyte Culture. Primary human keratinocytes (HKs) were grown on mitomycin C-treated 3T3 fibroblasts in FAD medium, and primary HEMs were grown in M254-CF medium (Invitrogen). HKs and HEMs were isolated from neonatal foreskin biopsy from circumcisions.

Melanin transfer was assessed by coculturing HKs with melanocytes from primary culture, or derived from pluripotent stem cells were cocultured at a ratio of 1:3 for 3 d in Epilife medium supplemented with growth factor HKGS (Invitrogen). The number of melanized keratinocytes was quantified after 3 d using ArrayScan (Cellomics) by the detection of the coexpression of Keratin 14 (K14) (Novacastra) and TYRP1 (Abcam) in their cytoplasm.

RT-qPCR. Total RNA from pluripotent stem cells and their derivatives was isolated using an RNeasy Mini extraction kit (Qiagen) according to the manufacturer's protocol. An on-column DNase I digestion was performed to avoid genomic DNA amplification. RNA level and quality were checked using Nanodrop (Agilent Technologies) technology. A total of 500 ng of RNA was used for reverse transcription utilizing the SuperScript III reverse transcription kit (Invitrogen). qPCR analysis was performed using a LightCycler 480 system (Roche) and SYBR Green PCR Master Mix (Roche) following each manufacturer's instructions. Quantification of gene expression was based on the Δ-cycle threshold (ΔCt) method and normalized to 18S expression. PCR primers are listed in Table S2.

TaqMan Array Gene Profiling. TaqMan microfluidic cards (Applied Biosystems) were processed as described by the manufacturer's instructions. In brief, 100 ng of cDNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) before being injected into the microfluidic cards and dispersed into the wells by centrifugation. Microfluidic cards were sealed, and qPCR assays were run on the 7900HT Fast Real-Time System (Life Technologies), using ABI PRISM 7900 Sequence Detection System software (v2.4; Applied Biosystems, Life Technologies). Cts were analyzed by using DataAssist Software (v2.0).

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde (15 min at room temperature) before permeabilization and blocking in PBS supplemented with 0.4% Triton X-100 and 5% (vol/vol) BSA (Sigma–Aldrich). Primary antibodies were incubated overnight at 4 °C in blocking buffer. Antibodies included mouse anti-MITF (DAKO), mouse anti-MITF (Abcam), mouse anti-TYRP1 (Abcam), rabbit anti-TYR (Abcam), mouse anti-Rab27 (BD Pharmingen), S100 (Thermo Scientific), SILV (Abcam), HNK1 (BD Pharmingen), p75 (BD Pharmingen), mouse anti-SSEA 4 (R&D), mouse anti–TRA1-81 (eBioscience), mouse anti–TRA1-60 (Abcam), mouse anti-OCT4 (Abcam), mouse anti-RPE 65 (Abcam), rabbit anti-K14 (Novacastra), rabbit anti-PAX6 (Covance), and mouse anti-PAX3 (Santa Cruz Biotechnology). Cells were stained (1 h at room temperature) with the species specific fluorophore-conjugated secondary antibody (Invitrogen), and nuclei were visualized with DAPI. Three independent experiments were performed using each cell type. Pictures were taken using a Zeiss microscope equipped with epifluorescence illumination.

FACS Analysis. Cells were detached from culture plates using Trypsin 0.05% EDTA (Invitrogen) and fixed in 2% (vol/vol) paraformaldehyde (15 min at room temperature). After a PBS wash, cells were permeabilized with 0.1% Saponin (Sigma–Aldrich). Primary antibodies diluted at 1:100 were incubated (1 h at room temperature) in PBS containing 0.1% FCS. Isotype-specific controls were carried out using no primary antibody. Species-specific secondary antibodies were added (1 h at room temperature), and the cells were analyzed on a FACSCalibur using CellQuest software (BD Biosciences). The number of events analyzed for each experiment was 10,000. For mortality measurements, SYTOX reagent (Invitrogen) was incubated on live cells 15 min before FACS analysis. Three independent experiments were performed for each cell line.

Time-Lapse Live-Cell Imaging. For time-lapse microscopy, human melanocytes and HKs were grown on collagen I-coated glass coverslips and transferred to custom-built aluminum microscope slide chambers (Ludin chamber; Life Imaging Services) filled with a culture medium supplemented with 20 mM Hepes. Time-lapse imaging was performed at 37 °C (Life Imaging Services) using a spinning-disk microscope mounted on an inverted motorized microscope (TE2000-U; Nikon).

Measurement of Melanin Content in Reconstructed Epidermis Using the Solvable Extraction Method. Reconstituted epidermal tissues were immersed in 400 μL of Solvable (Perkin–Elmer) and heated at 80 °C for 1 h. Afterward, the optical density of the supernatants was measured at 490 nm and the melanin content was quantified by comparison with the absorbance calibration curve made from synthetic melanin. For histological detection of melanin, epidermal tissues were fixed in 4% (vol/vol) formaldehyde and dehydrated before paraffin inclusion and specific melanin staining (Fontana–Masson staining). Paraffin-embedded sections (6 μm) were deparaffinized and rinsed in distilled water. Thereafter, slides were treated for 1 h in the dark with Fontana silver nitrate solution. After washing with distilled water, the slides were treated with gold chloride solution for 3 min and placed in 5% (vol/vol) sodium thiosulfate solution for 2 min. After washing, the slides were counterstained with eosin.

EM. The cells were seeded on coverslips and fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer for 24 h as described by Raposo et al. (3). In brief, the cells were rinsed with

cacodylate buffer, postfixed in osmium tetroxide, dehydrated in increasing concentrations of ethanol, and embedded in EPON resin (TAAB, Aldermaston, Bershire, UK) while on coverslips. Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed under an electron microscope (CM120; FEI). Micrographs were taken with a KeenView digital camera (Soft Imaging System).

Tissue Culture. The study was carried out on in vitro 3D epidermis reconstituted by StratiCELL. Tissues were reconstructed from normal HKs (Lonza) in the presence of HEMs, mel-hESCs, or mel-iPSCs. Samples of reconstituted epidermis were cultivated for 14 d at the air-liquid interface in StratiCELL proprietary serum-free medium and maintained in a humidified incubator at 37 °C with 5% CO_2 atmosphere. For the functional activity study, samples of epidermis were treated or not treated with 1 μM α-melanocyte stimulating hormone during the 14 d of the in vitro differentiation process.

Statistical Analysis. Statistical analysis was performed by one-way ANOVA, using Dunnett's comparison test. Values of $P < 0.05$ were considered significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

1. Tropel P, et al. (2010) High-efficiency derivation of human embryonic stem cell lines following pre-implantation genetic diagnosis. *In Vitro Cell Dev Biol Anim* 46:376–385.
2. Adewumi O, et al.; International Stem Cell Initiative (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 25: 803–816.
3. Raposo G, Tenza D, Murphy DM, Berson JF, Marks MS (2001) Distinct protein sorting and localization to premelanosomes, melanosomes, and lysosomes in pigmented melanocytic cells. *J Cell Biol* 152:809–824.

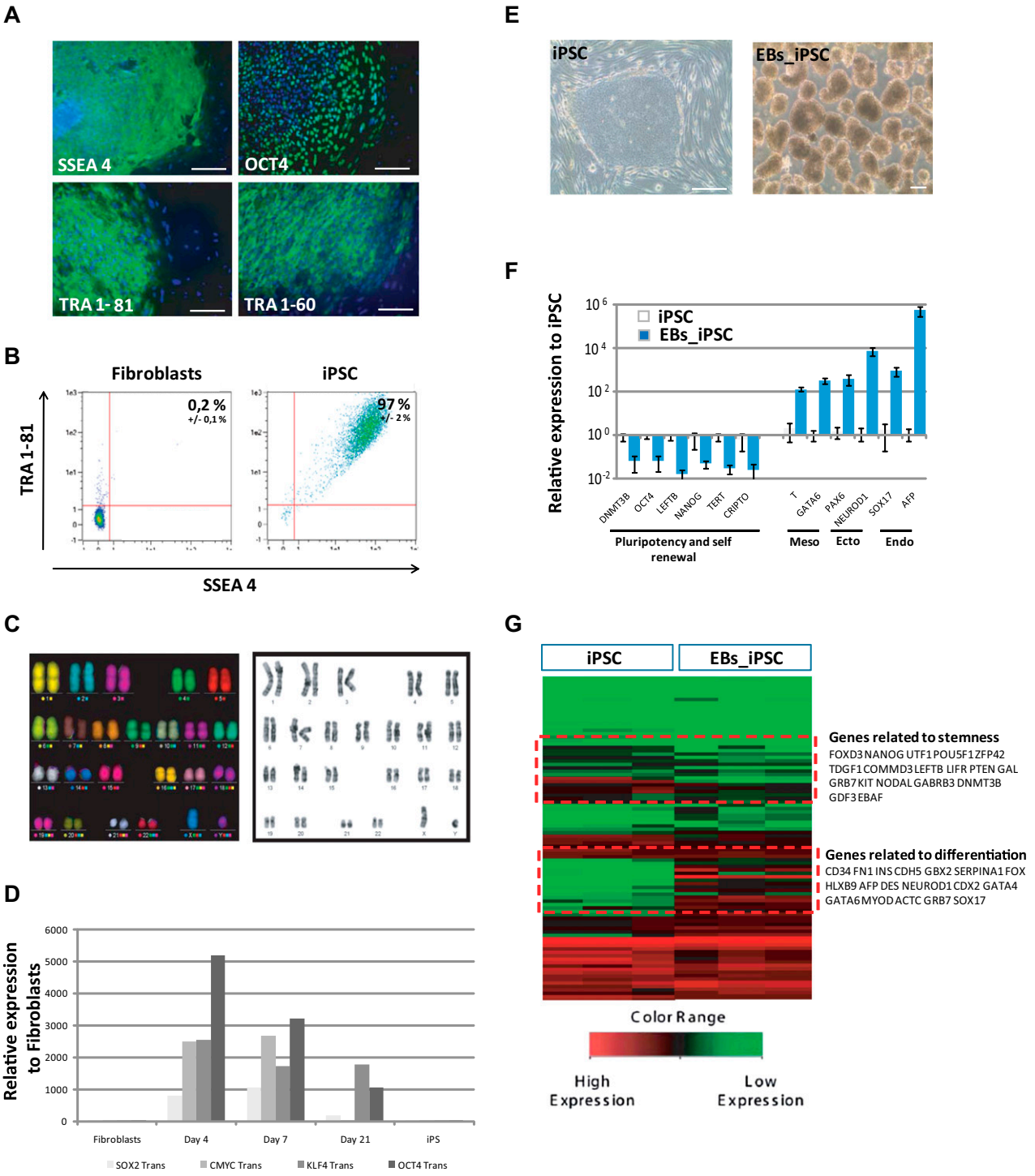


Fig. S1. Derivation and characterization of iPSCs. (*A*) Immunostaining analysis of pluripotency markers (SSEA4, OCT4, TRA1-81, and TRA1-60) in iPSCs. (Scale bar: 50 μm.) (*B*) Flow cytometry analyses of SSEA4 and TRA1-81 in iPSCs. Each value represents the SEM of three independent experiments. (*C*) FISH and G-banding analyses in iPSCs. (*D*) qPCR analysis of transgene expression (*SOX2*, *CMYC*, *KLF4*, and *OCT4*) in the ongoing reprogrammed fibroblasts (4–21 d) and their silencing in established iPSCs. (*E*) Differentiation of iPSCs into EBs. (Scale bar: 50 μm.) (*F*) qPCR analysis of undifferentiated markers (*DNMT3B*, *OCT4*, *LEFTB*, *NANOG*, *TERT*, and *CRIPTO*) and three germ layer markers (*T*, *GATA6*, *PAX6*, *NEUROD1*, *SOX17*, and *AFP*) in 14-d-old EBs derived from iPSCs. Each bar represents the SEM (n = 3). (*G*) TaqMan array gene expression profiling on iPSC-derived EBs. Results are presented as a heat map clustering of $\log_2$ (ΔCt).

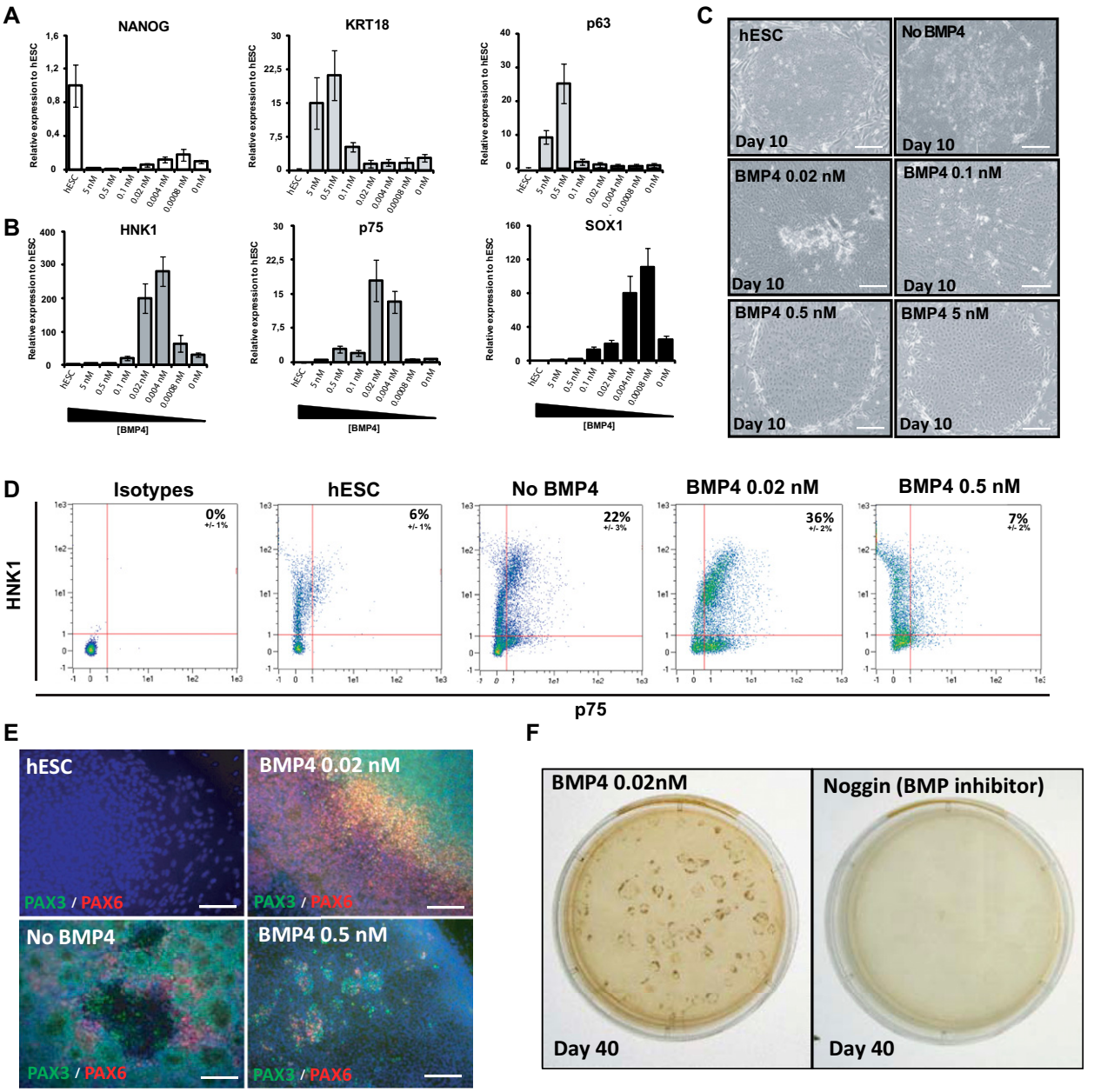


Fig. S2. Role of BMP4 in neural crest commitment of hESCs. (*A*) Quantitative PCR analysis of pluripotency and self-renewal (*NANOG*), epithelial (*KRT18*) and epidermal (*p63*) markers in hESCs treated with different concentrations of BMP4 ranging from 0–5 nM for 10 d. Each bar represents the SEM for (n = 3). (*B*) Quantitative PCR analysis of neural crest (*HNK1* and *p75*) and neural (*SOX1*) markers in hESCs treated with different concentrations of BMP4 ranging from 0–5 nM for 10 d. Each bar represents the SEM (n = 3). (*C*) Microscopy analysis of hESCs at day 0 (hESC) and day 10 of differentiation, with concentrations of BMP4 ranging from 0–5 nM. (Scale bar: 50 nm.) (*D*) FACS analysis of HNK1 and p75 and (*E*) immunofluorescence analysis of PAX3 and PAX6 at day 0 (hESC) and day 10 of differentiation with or without BMP4 at 0.02 nM or 0.5 nM. Each value represents the SEM of three independent experiments. (Scale bar: 100 μm.) (*F*) Morphological observations after 40 d of differentiation with BMP4 at 0.02 nM and one inhibitor of BMP signaling, Noggin.

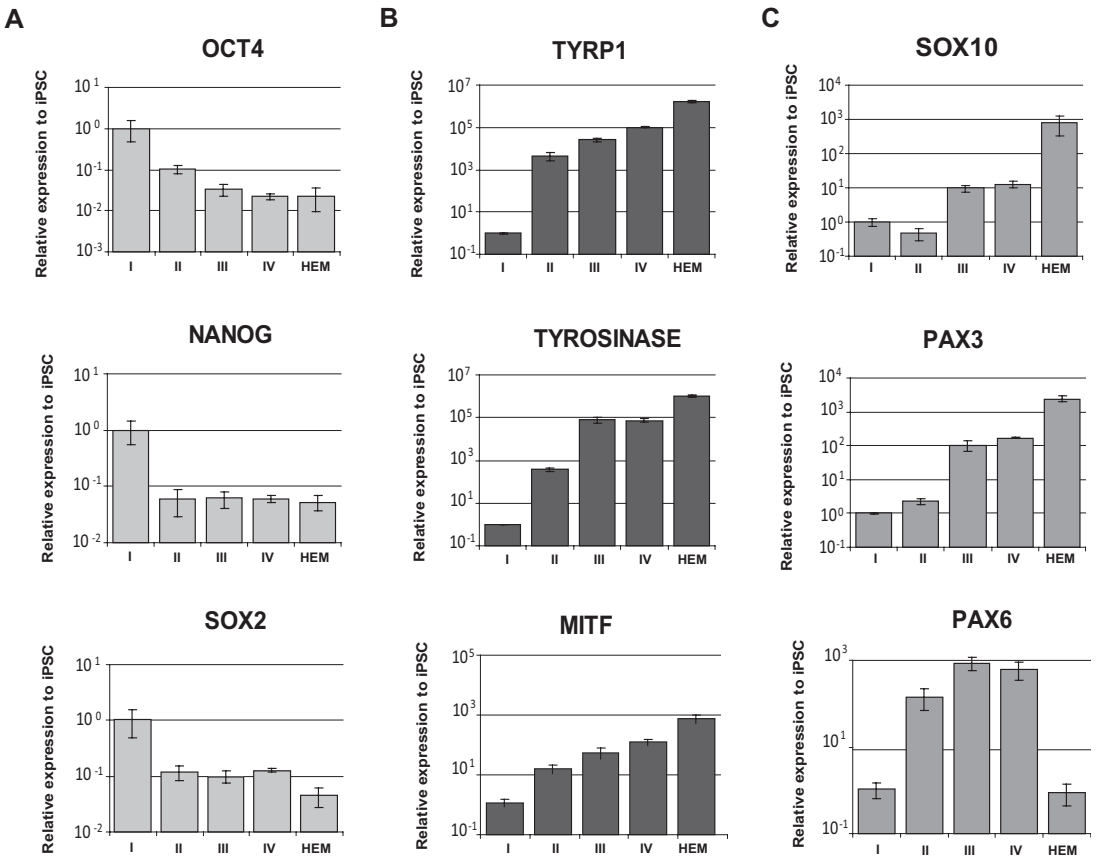


Fig. S3. Molecular characterization of the iPSC pigmentation process. qPCR analysis of pluripotency and self-renewal genes OCT4, NANOG, and SOX2 (*A*); melanogenesis-related genes TYRP1, TYR, and MITF (*B*); and neural crest markers PAX3 and SOX10 and neural lineage marker PAX6 (*C*) during the iPSC differentiation process at different stages of pigmentation: undifferentiated iPSCs (I), iPSC-derived cells before (II) and after (III) pigmentation, and purified pigmented cells derived from iPSCs (IV). Each bar represents the SEM (n = 3).

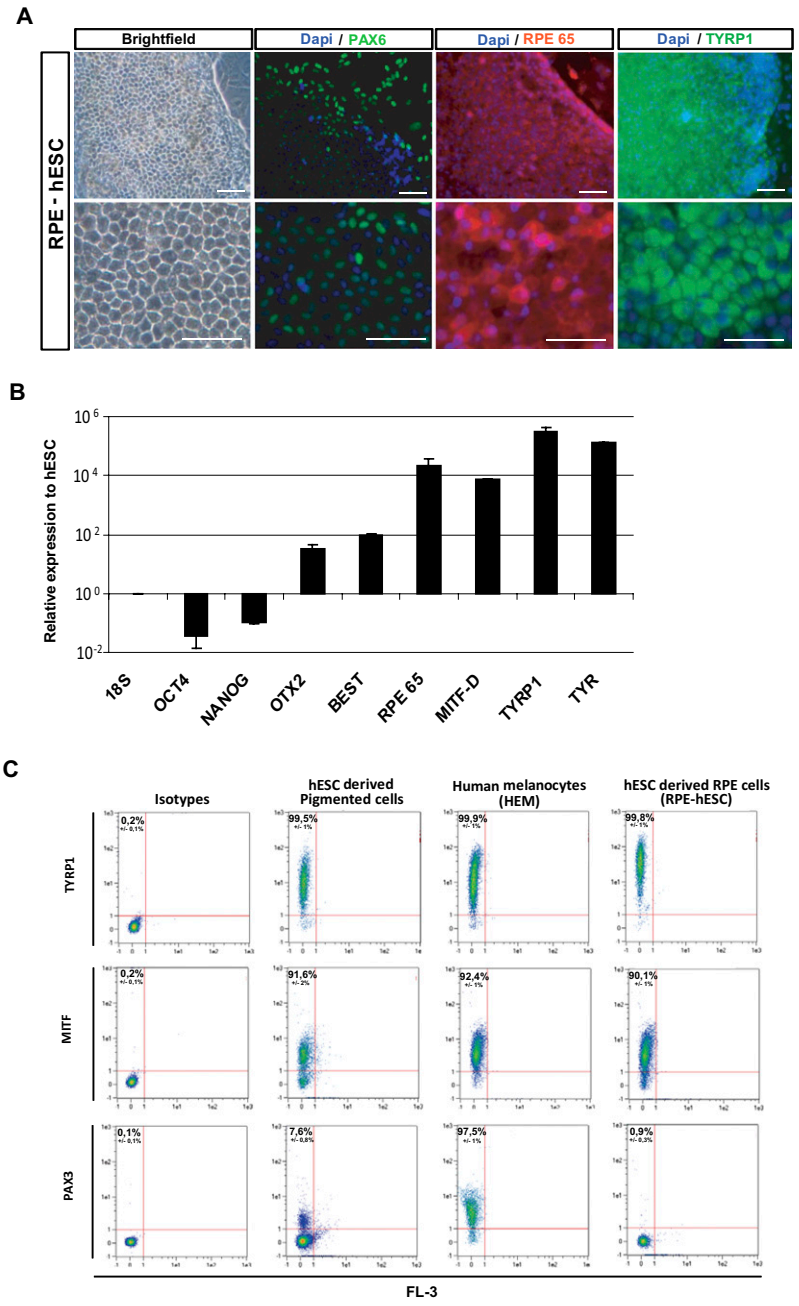


Fig. S4. Molecular characterization of RPEs in the nonmelanocytic population of hESC-derived pigmented cells. (*A*) Microscopy and immunofluorescence analysis of PAX6, RPE 65, and TYRP1 in RPE cells derived from hESCs (RPE-hESCs). (Scale bar: 50 μm.) (*B*) qPCR analysis of *OCT4*, *NANOG*, *OTX2*, *BEST*, *RPE 65*, *MITF-D* isoform, *TYRP1*, and *TYR* in RPEs derived from hESCs (RPE-hESCs). Data are normalized against 18S and expressed as relative expression to undifferentiated hESCs. (*C*) FACS analysis of TYRP1, MITF, and PAX3 in whole hESC-derived pigmented areas, HEMs, and hESC-derived RPEs. Each value represents the SEM of three independent experiments.

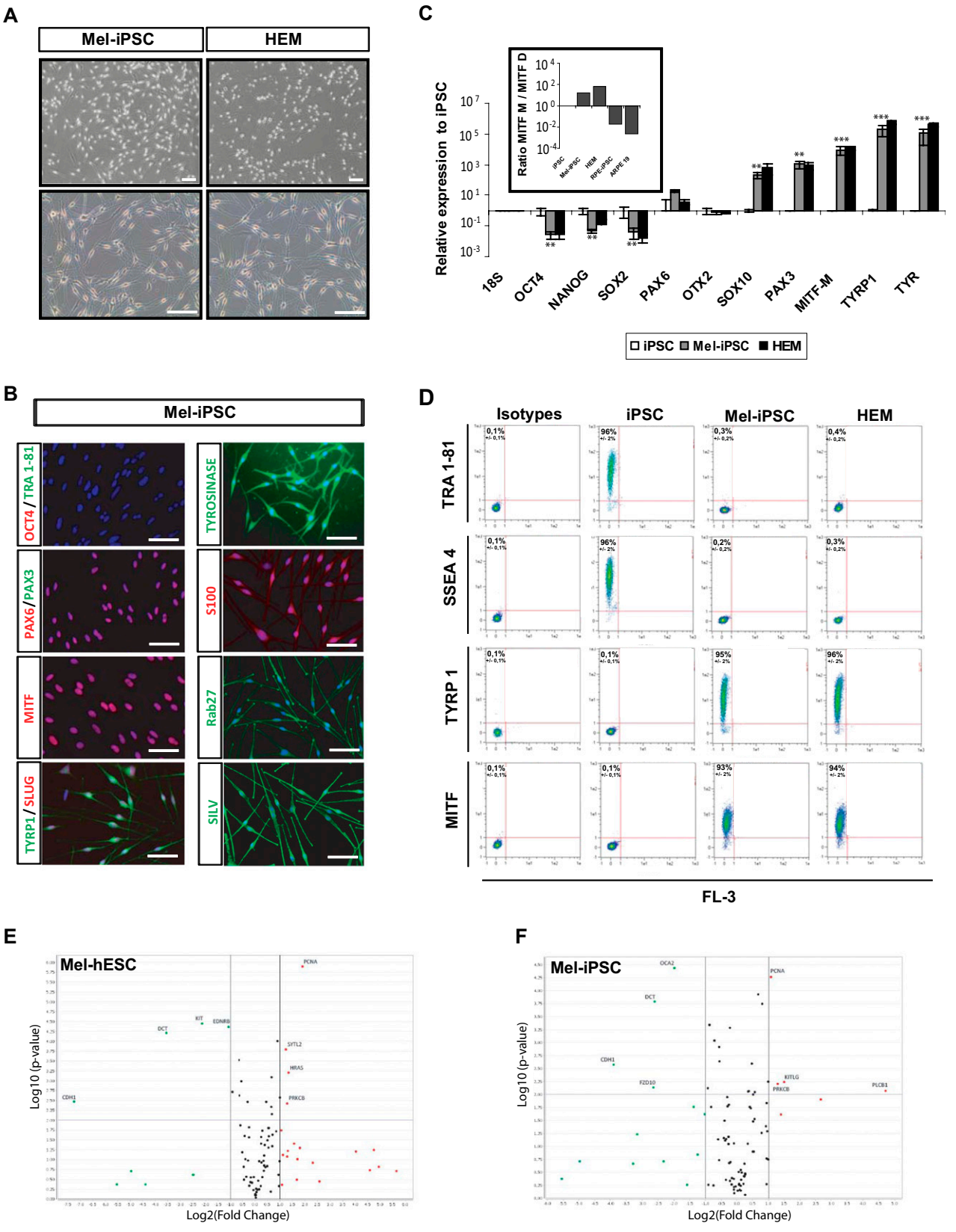


Fig. S5. Establishment of a pure and functional population of melanocytes derived from iPSCs (mel-iPSCs) and hESCs (mel-hESCs) (*A*) Microscopic analysis of melanocytes derived from iPSCs (mel-iPSCs) and HEMs. (Scale bars: 50 μm.) (*B*) Immunofluorescence analysis of the pluripotency markers OCT4 and TRA1-81; the neural lineage marker PAX6; and the melanocyte markers PAX3, total MITF, TYRP1, SLUG, TYR, S100, RAB27, and SILV in mel-iPSCs. (Scale bar: 50 μm.) (*C*) qPCR analysis of *OCT4*, *NANOG*, *SOX2*, *PAX6*, *OTX2*, *SOX10*, *PAX3*, *MITF-M* isoform, *TYRP1*, and *TYR* in mel-iPSCs and HEMs. Boxed histograms show the ratio of *MITF-M* and *MITF-D* isoforms in iPSCs, mel-iPSCs, HEMs, RPE-iPSCs, and ARPE-19 (adult RPE cell line). Data are normalized against 18S and expressed as relative expression to undifferentiated iPSCs. Each bar represents the SEM ($n = 3$) (**$P < 0.01$; ***$P < 0.001$). (*D*) FACS analysis of SSEA4, TRA1-81, TYRP1, and MITF in

Legend continued on following page

undifferentiated iPSCs, mel-iPSCs, and HEMs. Each value represents the SEM of three independent experiments. (*E* and *F*) Molecular characterization of 96 genes related to pigmentation in primary melanocytes (HEMs), mel-hESCs, and mel-iPSCs. A volcano plot compares gene expression between HEM and mel-hESCs (*E*) and mel-iPSCs (*F*), respectively (fold change limits = 2.0, *P* value limits = 0.01). The blue horizontal line represents a *P* value = 0.01, and the dark left and right vertical lines indicate a relative quantification (RQ) = 0.5 (fold decrease >2) and an RQ = 2 (fold increase >2). All data presented in this figure were obtained in melanocytes during four passages (approximately 50 d) after their isolation.

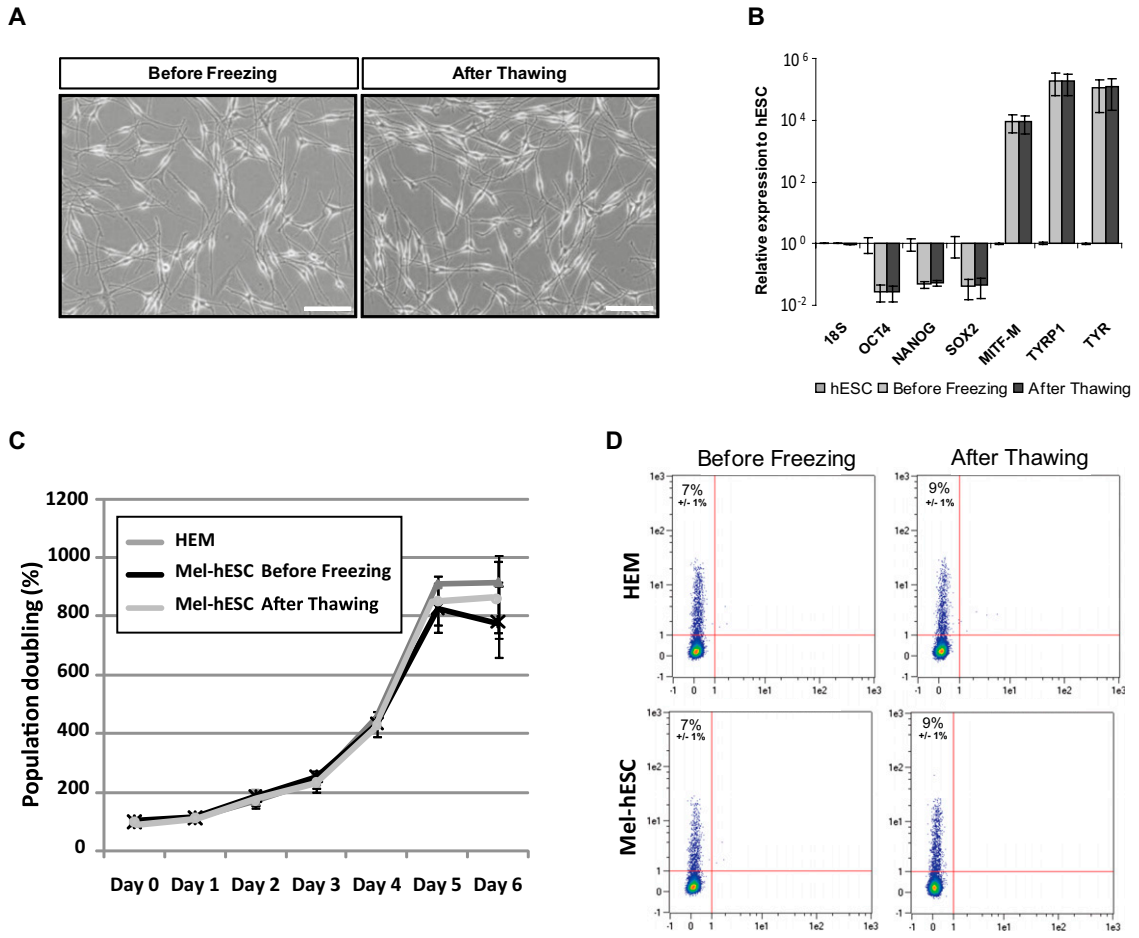


Fig. S6. Maintenance of mel-hESC phenotype after cryopreservation (*A*) Microscopic analysis of melanocytes derived from hESCs (mel-hESCs) before and after freezing. (Scale bar: 50 μm.) (*B*) qPCR analysis of *OCT4*, *NANOG*, *SOX2*, *MITF-M* isoform, *TYRP1*, and *TYR* in undifferentiated hESCs, mel-hESCs before freezing, and mel-hESCs after thawing. Data are normalized against 18S and expressed as relative expression to undifferentiated hESCs. Bars show SEM for three independent experiments. (*C*) Proliferation curve of HEMs and mel-hESCs before freezing and after thawing. (*D*) FACS analysis of cell mortality (SYTOX immunostaining) of mel-hESCs and HEMs before freezing and after thawing. Each value represents the SEM of three independent experiments. All data presented in this figure were obtained in melanocytes during four passages (approximately 50 d) after their isolation.

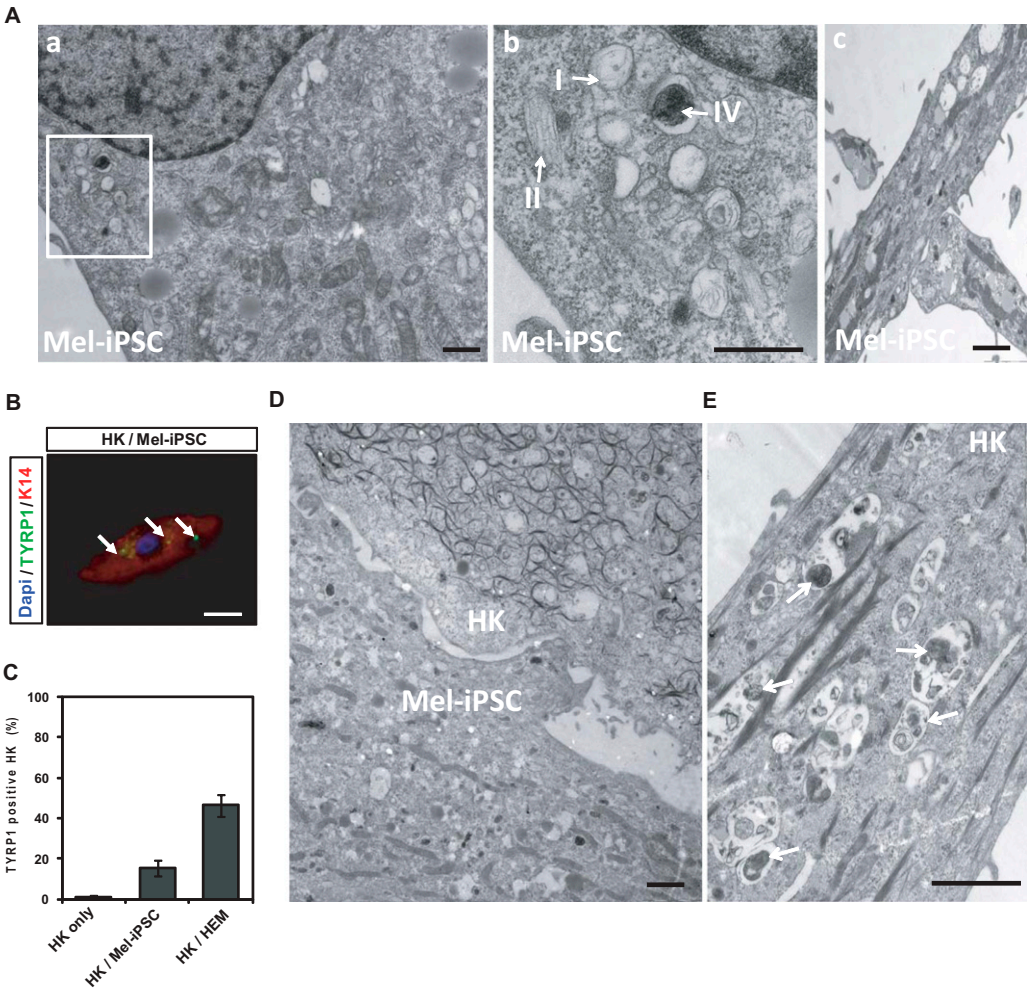


Fig. S7. Establishment of a pure and functional population of melanocytes derived from iPSCs (mel-iPSCs) (*A*) EM showing premature and mature melanosomes in the soma (*a*) and processes in the dendrites (*c*). (*b*) Higher magnification of the box in *a*. Arrows represents the different stages of melanosome maturation. (Scale bar: 400 nm.) (*B*) Immunofluorescence for K14 and TYRP1 in keratinocytes after 3 d of coculture with mel-iPSCs. Arrows represents TYRP1 staining. (Scale bar: 10 μm.) (*C*) ArrayScan quantification of the $TYRP1^+$ keratinocytes after 3 d of coculture with melanocytes (left to right): without melanocytes, with mel-iPSCs, and with HEMs. Each bar represents the SEM for three experiments. For each experiment, 100 cells were analyzed. (*D* and *E*) EM of keratinocytes after 3 d of coculture with mel-iPSCs. HK, human keratinocyte. Arrows represents transferred pigment into keratinocytes. (Scale bar: 400 nm.) All data presented in this figure were obtained in melanocytes during four passages (approximately 50 d) after their isolation.

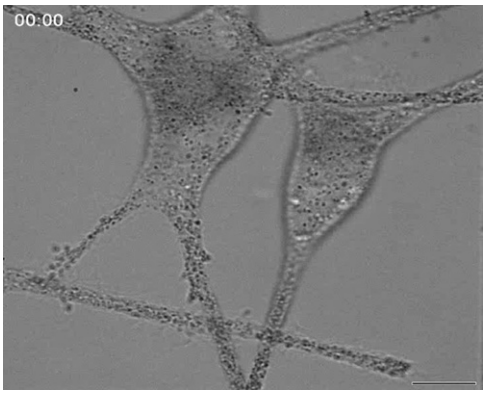
Table S1. List of CT, relative quantification values, and statistical calculation for mel-hESCs, mel-iPSCs, and HEMs

Table S1 (PDF)

$P > 0.01$ is considered nonsignificant.

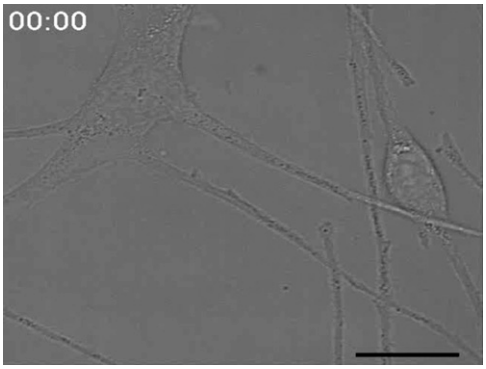
Table S2. Sequence of primers used for qPCR

Table S2 (PDF)



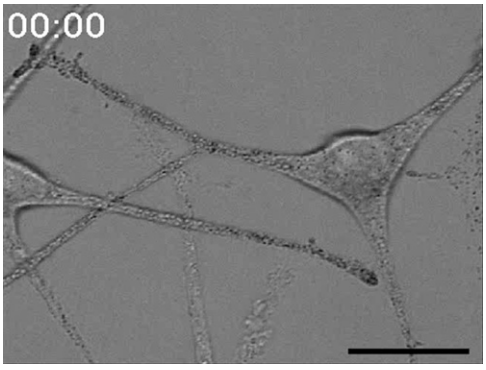
Movie S1. High-resolution time-lapse imaging of melanocytes derived from hESCs. Time-lapse microscopy analysis of mel-hESCs during 2 min. (Scale bar: 5 μm.)

Movie S1



Movie S2. High-resolution time-lapse imaging of melanocytes derived from iPSCs. Time-lapse microscopy analysis of mel-iPSCs during 2 min. (Scale bar: 5 μm.)

Movie S2



Movie S3. High-resolution time-lapse imaging of HEMs. Time-lapse microscopy analysis of HEMs during 2 min. (Scale bar: 5 μm.)

Movie S3