

Generation of Melanocytes from Induced Pluripotent Stem Cells

Ruifeng Yang^{1,4}, Min Jiang^{1,2,4}, Suresh M. Kumar¹, Theodore Xu¹, Fei Wang³, Leihong Xiang² and Xiaowei Xu¹

Epidermal melanocytes have an important role in protecting skin from UV rays, and are implicated in a variety of skin diseases. Here, we developed an efficient method for differentiating induced pluripotent stem cells (iPSCs) into melanocytes. We first generated iPSCs from adult mouse tail-tip fibroblasts (TTFs) using retroviral vectors or virus-free piggyBac transposon vectors carrying murine *Sox2*, *Oct3/4*, *c-Myc*, and *Klf4*. The TTF-derived iPSC clones exhibited similar morphology and growth properties as mouse embryonic stem (ES) cells. The iPSCs expressed ES cell markers, displayed characteristic epigenetic changes, and formed teratomas with all three germ layers. The iPSCs were used to generate embryonic bodies and were then successfully differentiated into melanocytes by treatment with growth factors. The iPSC-derived melanocytes expressed characteristic melanocyte markers and produced melanin pigment. Electron microscopy showed that the melanocytes contained mature melanosomes. We manipulated the conditions used to differentiate iPSCs to melanocytes and discovered that *Wnt3a* is not required for mouse melanocyte differentiation. This report shows that melanocytes can be readily generated from iPSCs, providing a powerful resource for the *in vitro* study of melanocyte developmental biology and diseases. By inducing iPSCs without viruses, the possibility of integration mutagenesis is alleviated, and these iPSCs are more compatible for cell replacement therapies.

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INTRODUCTION

The generation of induced pluripotent stem cells (iPSCs) from adult somatic cells by introducing only four transcription factors, *c-Myc*, *Klf4*, *Oct4*, and *Sox2* or *Lin28*, *Nanog*, *Oct4*, and *Sox2*, represents a remarkable breakthrough in stem cell research (Takahashi and Yamanaka, 2006; Okita *et al.*, 2007; Wernig *et al.*, 2007; Yu *et al.*, 2007). The resulting iPSCs resemble embryonic stem (ES) cells in their properties and potential to differentiate into a variety of adult cell types. iPSC technology is a powerful tool that can be used to understand the underlying mechanisms for disease initiation and progression or to develop customized cellular therapies to treat certain human diseases (Hanna *et al.*, 2007; Dimos *et al.*, 2008; Soldner *et al.*,

2009). The therapeutic use of cells derived from a patient's own iPSCs would avoid the complication of immune rejection, which might occur if cells were derived from allogeneic human ES cells. Nevertheless, there are still many practical issues with iPSCs that need to be resolved before this breakthrough technology can be used clinically (Yamanaka, 2010).

The most commonly used reprogramming strategies use retroviral- or lentiviral-based methods to deliver reprogramming factor transgenes and have been shown to be highly efficient (Okita *et al.*, 2007; Wernig *et al.*, 2007; Yu *et al.*, 2007). Although efficient, these methods also have the potential to induce insertional mutagenesis. Other methods using adenovirus, plasmids, synthetic protein, or RNA minimize the potential for insertional mutagenesis (Kaji *et al.*, 2009; Kim *et al.*, 2009; Yu *et al.*, 2009; Zhou and Freed, 2009), but are less efficient. A newer technique called piggyBac (PB) transposition is host-factor independent and is functional in various human and mouse cell lines (Fraser *et al.*, 1996; Ding *et al.*, 2005; Wu *et al.*, 2006; Wang *et al.*, 2008; Woltjen *et al.*, 2009). The PB transposon-based method has been used successfully to reprogram adult somatic cells to iPSCs and the transgenes can be excised after successful reprogramming (Woltjen *et al.*, 2009).

A concern with using iPSCs in medical and developmental studies is the tendency of these cells to spontaneously differentiate along different lineages. Although substantial progress has been made in differentiating iPSCs into certain cell types (Zhang *et al.*, 2009; Lee *et al.*, 2010; Itzhaki *et al.*, 2011; Morishima *et al.*, 2011; Sancho-Bru *et al.*, 2011), there

¹Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA;

²Department of Dermatology, Huashan Hospital, Fudan University, Shanghai, China and ³Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

⁴These authors contributed equally to this work.

Correspondence: Xiaowei Xu, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 3400 Spruce Street, Philadelphia, Pennsylvania 19104, USA.

E-mail: xug@mail.med.upenn.edu

Abbreviations: bFGF, basic fibroblast growth factor; EB, embryoid body; ES, embryonic stem; ET-3, endothelin-3; iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblast; PB, piggyBac; SCF, stem cell factor; SSEA-1, stage-specific embryonic antigen-1; TPA, 12-O-tetradecanoylphorbol-13-acetate; TTF, tail-tip fibroblast

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is still a need to develop the methods and conditions necessary to reproducibly generate homogenous populations of distinct cell lineages from iPSCs.

Melanocytes have a critical role in protecting human skin from harmful UV rays (Slominski *et al.*, 2004). Their primary function is to produce melanin pigment, which is packaged into vesicles called melanosomes, and transported to the surrounding epidermal keratinocytes (Halaban *et al.*, 1988). Vitiligo is a common melanocyte-related disorder characterized by well-margined milky white spots on the skin that result from the loss of melanocytes (van Geel *et al.*, 2001). The study of iPSC-derived melanocytes may lead to a better understanding of melanocyte-related diseases, such as vitiligo, as well as melanocyte molecular biology. These cells may also help us understand the etiology of melanoma, the most deadly form of skin cancer.

In this study, we generated iPSCs from adult mouse tail-tip fibroblasts (TTFs) using conventional retroviral and virus-free plasmid-based methods. We optimized the differentiation conditions to generate mouse melanocytes from iPSCs and we discovered that mouse and human melanocyte differentiation requires different growth factors.

RESULTS

Generation of iPSCs from mouse adult fibroblasts

To generate mouse iPSCs, mouse TTFs were isolated and infected with retroviral vectors encoding four reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*). iPSC clones with ES-like morphology became apparent after 11 days and were harvested by 21 days (Figure 1a). The efficiency of generating established iPSCs from TTFs was 0.5–0.8% (data not shown). Alkaline phosphatase activity is an ES cell marker and we performed alkaline phosphatase staining and showed that most of the iPSC clones were positive for this stain (Figure 1a). Reverse transcriptase-PCR was performed to examine whether ES cell marker genes were expressed in iPSC cells. We used primers designed to amplify transcripts of endogenous genes but not transcripts of transgenes (Takahashi and Yamanaka, 2006). Five different iPSC clones expressed all ES cell markers tested, and the endogenous *Oct3/4*, *Sox2*, and *Nanog* levels were similar to the levels found in authentic mouse ES cells (Figure 1b). The five iPSC clones showed similar gene expression patterns. The expressions of *Nanog* and ES cell membrane marker SSEA-1 (stage-specific embryonic antigen-1) expression were confirmed by immunofluorescence analysis of the iPSC clones (Figure 1c). We compared the growth rates of iPSCs with ES cells and fibroblasts and found that two of the iPSC clones displayed exponential growth rates, similar to ES cells, whereas the TTF cells stopped growing after 3 weeks (Figure 1d).

Characterization of iPSCs and their pluripotency

To investigate the DNA methylation status of the *Oct4* and *Nanog* promoters, we performed bisulfite DNA sequencing analysis of ES cells, iPSCs, and TTFs, respectively. As shown in Figure 2a, both loci were demethylated in ES and iPSCs and fully methylated in TTFs. Meanwhile, chromatin immunoprecipitation analysis was performed to examine

chromatin histone modifications. The results showed that the promoters of *Oct4* and *Nanog* had increased histone H3 acetylation and dimethylation of lysine 4 of histone H3 (Figure 2b). Our results indicate that the epigenetic state of the *Oct4* and *Nanog* genes in the iPSCs is reprogrammed from a transcriptionally repressed state (somatic) to an active (embryonic) state.

To determine the pluripotency of iPSCs, we performed *in vitro* differentiation assays and found that iPSCs have the ability to differentiate into three germ layer-derived cell types. The assay used keratin14 as a marker for ectoderm differentiation, smooth muscle actin to mark mesoderm differentiation, and α -fetal protein for endoderm differentiation (Figure 2c). We then determined the developmental potential of iPSCs *in vivo* using a teratoma formation assay. Histological analysis of iPSC-induced teratomas revealed that the cells had differentiated into cell types representing all three embryonic germ layers. These teratomas contained keratinocytes and nerve tissue (ectoderm), smooth muscle and cartilage (mesoderm), and respiratory epithelium and gut epithelium (endoderm; Figure 3).

Differentiation of iPSCs into melanocytes

The melanocytic differentiation protocol is illustrated in Figure 4a. Briefly, we generated embryonic bodies (EBs) from single iPSCs in suspended culture (Figure 4b). The EBs were pretreated with retinoic acid and plated onto fibronectin-coated plates containing complete differentiation media containing multiple growth factors including Wnt3a, stem cell factor (SCF), and endothelin-3 (ET-3; described in Materials and Methods). After 24 hours, some of the cells migrated out of the EBs. Within 4–6 days, rare, highly pigmented cells were observed near the EBs. After an additional 4–6 days in culture, the migrated cells continued to proliferate and reached 60–70% confluence focally (Figure 4c). These iPSC-derived pigmented cells obtained at day 16 had a melanocyte-like bipolar or tripolar morphology (Figure 4d) and retained their melanin granules after 40 days of culture *in vitro* (Figure 4e).

Characterization of iPSC-derived melanocytes

Although the production of melanin and the formation of melanosomes are the most unique features of melanocytes, there are also several melanocyte-specific gene markers. Therefore, quantitative reverse transcriptase-PCR was performed to determine whether differentiated iPSCs expressed melanocytic markers. The results showed that undifferentiated iPSCs or EBs from iPSCs expressed few melanocytic markers, whereas melan-a melanocytes and iPSC-derived melanocytes obtained at day 40 expressed melanocytic markers and transcription factors associated with melanocyte development, including *Pax3*, *MITF-M*, *TYR*, *TYRP1*, *TYRP2*, *SILV* (*Silver/Pmel17*), *Gpr143* (*Oa1*), *p* (*pink-eyed dilution gene/Oca2*), and *Sox10* (Figure 5a). Immunofluorescence analysis revealed that these cells were also strongly positive for MITF, S-100, and TYRP2 (Figure 5b). As expected, the MITF transcription factor localized in the nucleus, and S100 and TYRP2 were both in the cytoplasm. The DOPA reaction

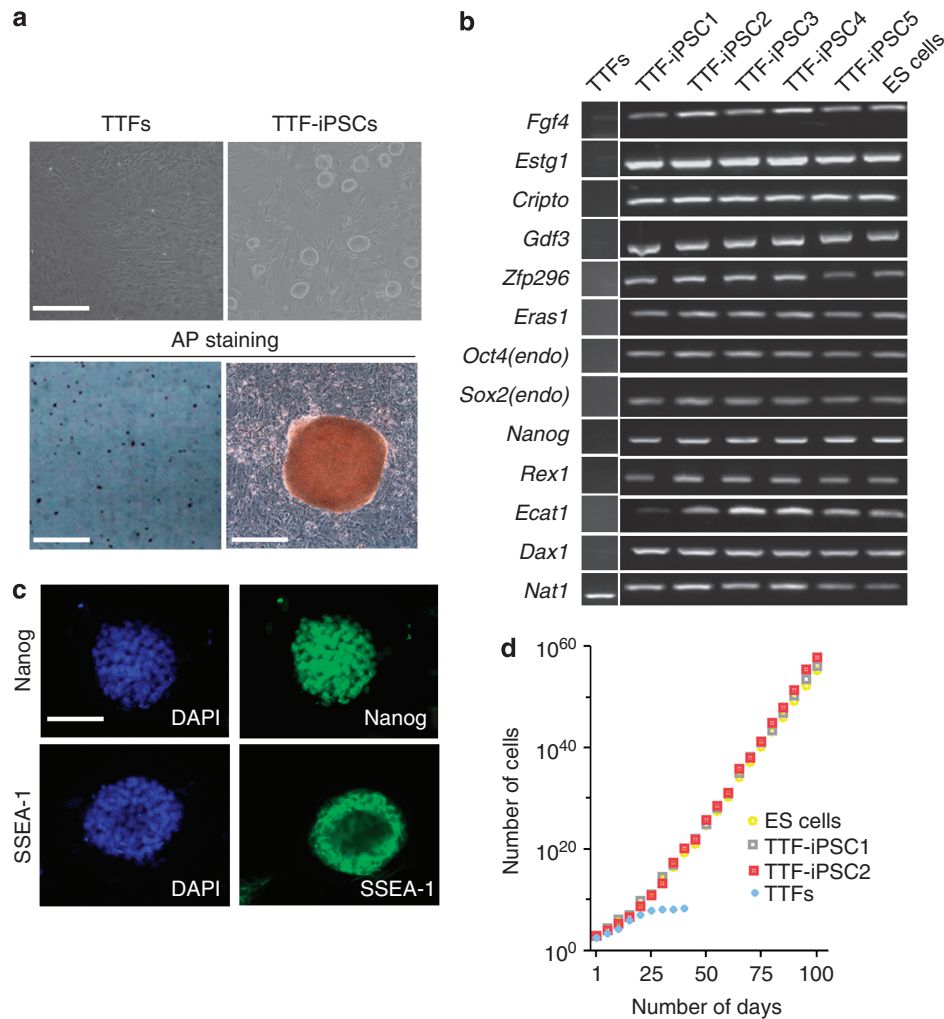


Figure 1. Generation and expression analysis of induced pluripotent stem cells (iPSCs). (a) Morphology of mouse iPSCs and tail-tip fibroblasts (TTFs). (Upper panel) Morphology of mouse TTFs and iPSCs derived from TTFs; bar = 150 μ m. (Lower panel) Alkaline phosphatase (AP) staining results of iPSC clones; left side, bar = 10 mm; right side, bar = 500 μ m. (b) Reverse transcriptase-PCR (RT-PCR) analysis of embryonic stem (ES) cell marker genes in mouse iPSCs, ES cells, and TTFs. *Nat1* was used as a loading control. (c) iPSCs were stained with a mouse mAb against SSEA-1 (stage-specific embryonic antigen-1) or Nanog. As a nuclear marker, 4,6-diamidino-2-phenylindole (DAPI) staining was used. Bar = 500 μ m. (d) Growth curves of TTFs, mouse ES cells, and iPSCs. A total of 3×10^5 cells were passaged every 3 days into each well of a six-well plate.

assay was used to confirm that these differentiated cells did indeed contain tyrosinase activity (Figure 5c). To detect the presence of melanosomes, we performed electron microscopy and found many mature stage III and stage IV melanosomes in iPSC-derived melanocytes (Figure 5d).

To optimize the efficiency of melanocyte differentiation, we tested the effects of different components of the differentiation medium, both individually and in combinations. It has already been shown that growth factors, such as SCF, ET-3, and basic fibroblast growth factor (bFGF), are important regulators of stem cell proliferation and neural crest lineage differentiation (Nishikawa *et al.*, 1991; Baynash *et al.*, 1994; Okura *et al.*, 1995; Imokawa *et al.*, 1998). To test the effect of the different components, we measured the surface area of migrating cells from the EBs, as well as the percentage of pigmented cells in the migrating cells, as described previously (Fang *et al.*, 2006). Both the surface area

of migrating cells from EBs and the efficiency of melanocyte generation were decreased in the absence of ET-3/SCF or bFGF (Figure 5e and f). We have previously shown that the Wnt pathway has a critical role in human melanocyte differentiation from ES cells (Fang *et al.*, 2006). In contrast, in these experiments, we found that Wnt3a was not indispensable in mouse melanocyte induction; no significant inhibition was observed in the absence of Wnt3a. Similarly, no significant inhibition was observed in the absence of cholera toxin. The 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a powerful activator of protein kinase C, and it is extensively used in melanocyte culture (Seeger and Krebs, 1995). Unexpectedly, mouse iPSCs formed smaller EB masses and generated fewer pigmented cells when TPA was added into the medium, suggesting that mouse iPSCs are more sensitive to the cytotoxicity of TPA than human ES/iPSCs. Interestingly, we found this inhibitory effect only at the early

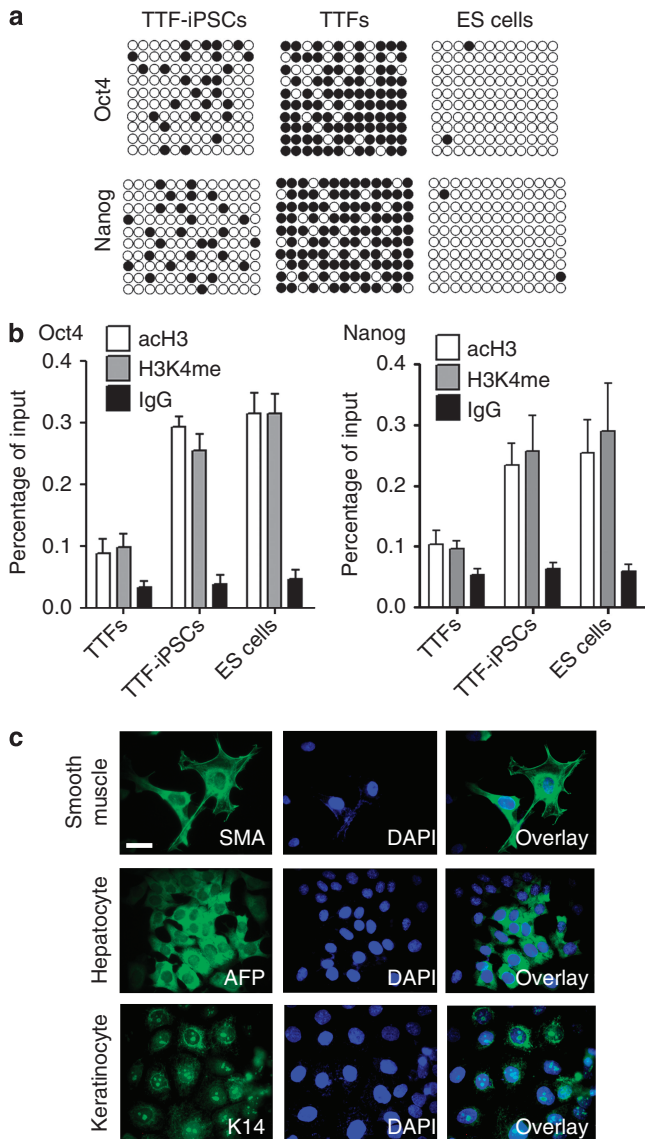


Figure 2. Epigenetic modification and *in vitro* differentiation of induced pluripotent stem cells (iPSCs). (a) Bisulfite genomic sequencing of the promoter regions of *Oct3/4* and *Nanog* in 10 randomly selected iPSC and 10 embryonic stem (ES) cell clones, as well as mouse embryonic fibroblast (MEF) cells. Open circles indicate unmethylated CpG dinucleotides, whereas closed circles indicate methylated CpGs. TTF, tail-tip fibroblast. (b) Chromatin immunoprecipitation was performed using antibodies against dimethylated histone H3K4 (H3K4me2) and H3 acetylation (acH3). *Oct3/4* and *Nanog* promoters showed enrichment for the active (H3K4me2 and acH3) mark in iPSCs, similar to ES cells. In MEFs, *Oct3/4* and *Nanog* promoters appeared in the inactive state. (c) Differentiation of iPSCs into three germ layer-derived cell types. Immunostaining was performed with antibodies against keratin14 (K14) for ectoderm differentiation, smooth muscle actin (SMA) for mesoderm differentiation, and α -fetal protein (AFP) for endoderm differentiation. As a nuclear marker, 4,6-diamidino-2-phenylindole (DAPI) staining was used. Bar = 15 μ m.

stage of melanocyte induction (within the first 2 to 3 weeks), whereas cells from later stages and from subsequent passages were resistant to TPA cytotoxicity (data not shown).

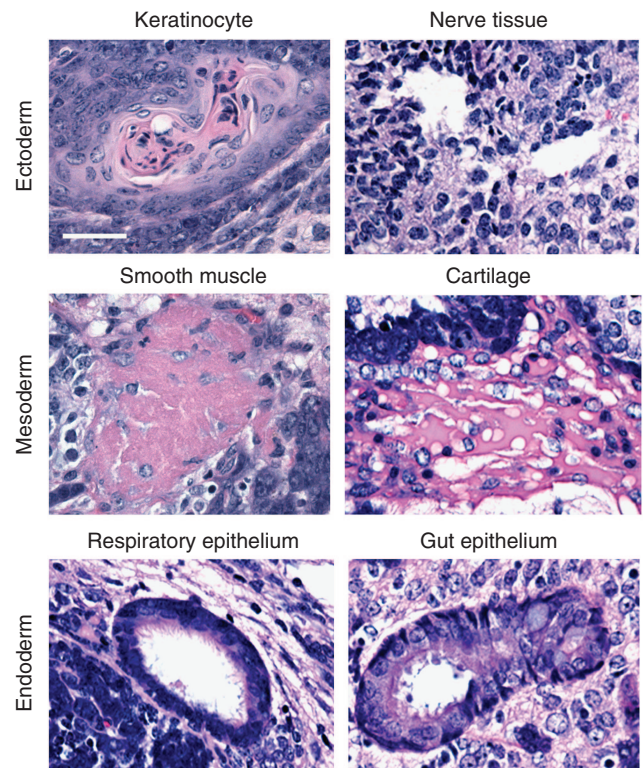


Figure 3. Developmental pluripotency of induced pluripotent stem cells (iPSCs) by teratoma formation. A total of 10^6 iPSCs were injected into dorsal flanks of the nude mice. At 4 weeks after the injection, tumors were removed from the mice. Histology analysis showed that tumors from the iPSCs contained cells derived from three germ layers, keratinocytes and nerve tissue for ectoderm, smooth muscle and cartilage for mesoderm, and respiratory and gut epithelium for endoderm (hematoxylin and eosin (H&E) staining). Bar = 50 μ m.

Generation of melanocytes from iPSCs obtained with virus-free system

Generation of iPSCs with genome-integrating viruses may cause insertional mutagenesis and unpredictable genetic dysfunction, which makes them unstable for clinical trial and regenerative medicine. To obtain virus-free iPSCs, we used a PB transposon/transposase-induced system (Woltjen *et al.*, 2009). In this system, mouse embryonic fibroblast (MEF) cells (Figure 6a) were transfected with PB-CAG-rtTA and PB-TET-MKOS (*c-Myc*, *Klf4*, *Oct4*, and *Sox2* open reading frames linked with 2A peptide sequences). With doxycycline induction, iPSC clones with ES cell-like morphology appeared 14 days later and were picked up by 30 days (Figure 6a). Reprogramming efficiency was roughly 0.01%, which is lower than retrovirus systems. Alkaline phosphatase staining (Figure 6a) and immunostaining with antibodies against Nanog and SSEA-1 (Figure 6b) showed that these cell lines were positive for ES cell markers. Next, we performed melanocyte differentiation assays using these virus-free iPSCs and obtained similar results as with the iPSCs generated using virus. Specifically, reverse transcriptase-PCR results showed that melanocytes generated from virus-free iPSCs expressed melanocyte markers, including *MITF*, *TYR*, *TYRP1*, and

TYRP2 (Figure 6c), indicating that virus-free iPSCs can also be differentiated into melanocytes.

DISCUSSION

In this study, we developed an *in vitro* method for deriving melanocytes from mouse iPSCs reprogrammed with four

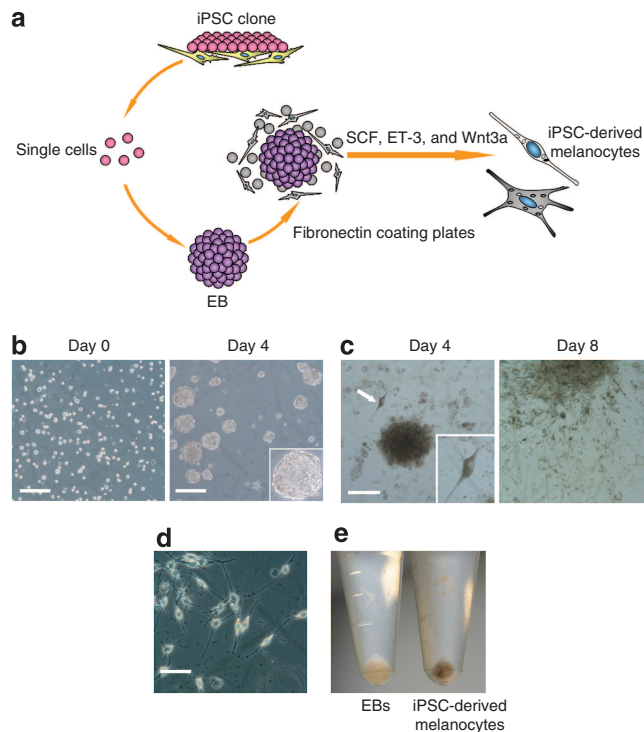


Figure 4. Differentiation of melanocytes from induced pluripotent stem cells (iPSCs). (a) Differentiation overview. Embryonic bodies (EBs) were formed from iPSCs and treated with 1 μ M retinoic acid (RA) after 2 days. After 3 days with RA, the EBs were plated onto fibronectin-coated dishes in melanocyte differentiation medium with endothelin-3 (ET-3), stem cell factor (SCF), basic fibroblast growth factor (bFGF), and Wnt3a. (b) EBs formed in suspension under feeder-free conditions; left side, bar = 10 mm; right side, bar = 500 μ m. (c) Cell migration from EBs on a fibronectin-coated plate. In melanocyte differentiation medium, cells migrated out from EBs at day 4 and differentiated cells appeared with morphology typical of melanocytes at day 8 (bright-field microscopy); bar = 500 μ m. (d) At day 16, differentiated cells displayed more homogenous melanocytic morphology after splitting (phase-contrast microscope). Bar = 25 μ m. (e) Unpigmented EBs before differentiation (left), in contrast to the pigmented cell pellet from iPS-derived melanocytes (right).

factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*). Similar to retroviral vector-mediated reprogramming, the virus-free PB transposon method induced reprogramming of adult TTFs. Mouse melanocytes derived from iPSCs exhibited typical melanocyte morphology, synthesized melanosomes, and expressed typical melanocyte markers. Melanocytes generated from iPSCs could potentially be used to better understand melanocyte biology and reveal the underlying mechanisms of pigmentation disorders and melanoma development.

During embryonic development, melanocytes are derived from neural crest cells (Opdecamp *et al.*, 1997). We previously reported that the factors required for the induction of melanocytes from human ES cells and skin neural crest-like stem cells include bFGF, SCF, ET-3, and Wnt3a (Fang *et al.*, 2006; Yu *et al.*, 2006, 2010; Li *et al.*, 2010). However, in this study, we found that Wnt3a is dispensable for mouse iPSC differentiation into melanocytes. This suggests that different pathways may be required during mouse and human melanocyte development. As the Wnt signaling pathway is known to have a key role during melanocyte induction from neural crest (Jin *et al.*, 2001), our results suggest the presence of an alternative Wnt factor that substitutes for Wnt3a in mouse melanocyte development. We also found that TPA, which functions as a growth enhancer in melanocyte growth medium by activating the protein kinase C pathway (Seger and Krebs, 1995), is unnecessary for melanocyte differentiation from mouse iPSCs. Instead, the presence of TPA inhibits melanocyte differentiation, suggesting that initiating melanocyte differentiation is distinct from promoting melanocyte proliferation in established melanocyte cultures.

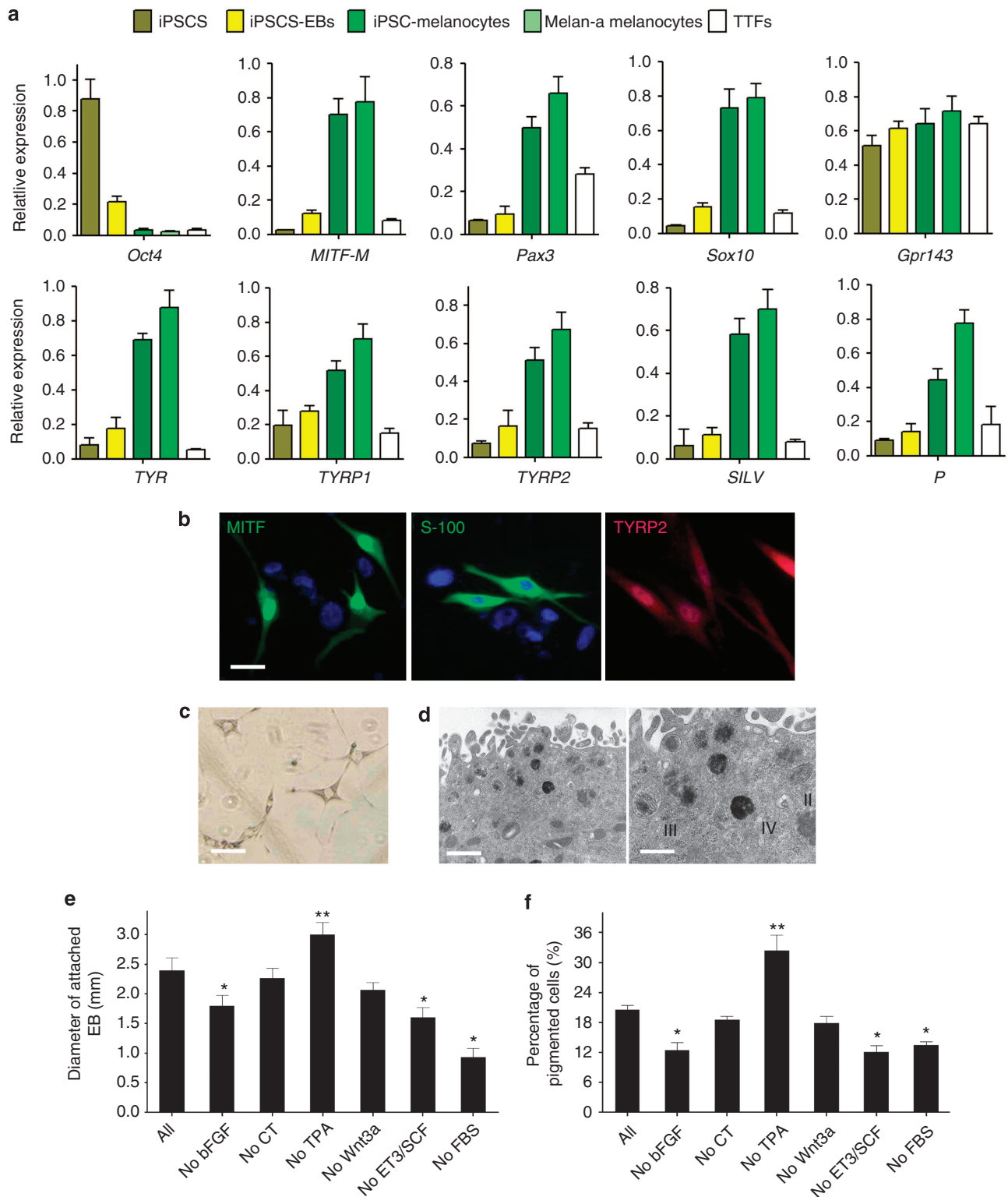
Ohta *et al.* (2011) recently showed that melanocytes can be generated from human iPSCs *in vitro*. They generated iPSC lines from human dermal fibroblasts using *SOX2*, *OCT3/4*, and *KLF4*, with or without *c-MYC* in retroviral vectors. The EBs were then cultured in melanocyte differentiation medium containing Wnt3a, SCF, ET3, FGF-2, and cholera toxin, and mature melanocytes were generated 7 weeks after inducing differentiation. Our findings here are consistent with their results. In addition, we used the viral-free PB transposon method to generate iPSCs and optimized the differentiation protocol for mouse melanocytes.

Defects in melanocytes can lead to a number of pigmentation disorders, such as piebaldism, albinism,

Figure 5. Characterization of melanocytes derived from induced pluripotent stem cells (iPSCs). (a) Quantitative reverse transcriptase-PCR (RT-PCR) analysis of melanocyte markers in iPSCs, embryoid bodies (EBs) from iPSCs (iPSC-EBs), melanocytes from iPSCs (iPSC melanocytes), mouse melanocyte melan-a cells, and tail-tip fibroblasts (TTFs), including *Pax3*, *MITF-M*, *TYR*, *TYRP1*, *TYRP2*, *SILV* (*Silver/Pmel17*), *Gpr143* (*Oa1*), *p* (*pink-eyed dilution gene/Oca2*), and *Sox10*; *GAPDH* was used as a loading control. (b) Immunostaining analysis of iPSC-derived melanocytes with antibodies against MITF, S-100, and TYRP2. Bar = 15 μ m (immunofluorescent microscopy). (c) DOPA (3,4-dihydroxyphenylalanine) reaction of melanocytes derived from iPSCs. The pigmented cells represent DOPA-positive cells. Bar = 30 μ m. (d) Electron microscopy images show an iPSC-derived melanocyte with many mature melanosomes in the cytoplasm. II: Stage II melanosome; III: stage III melanosome; IV: stage IV melanosome. Left side, bar = 1 μ m; right side, bar = 400 nm. (e) Percentage of flask surface area occupied by cells migrated out of EBs. Adherent EBs were cultured in the medium with the various growth factors in different combinations. Without SCF/ET-3 or bFGF, the surface area of migrating cells decreased, whereas no change was observed in the group without Wnt3a. In the absence of TPA, the surface area of migrating cells covered more than the other combinations. * $P < 0.05$ (mean $\pm$ SEM $n = 3$), ** $P < 0.01$ (mean $\pm$ SEM $n = 3$). bFGF, basic fibroblast growth factor; ET-3, endothelin-3; SCF, stem cell factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate. (f) Efficiency of melanocyte generation from iPSCs. Consistent with the increased area covered by migrating cells from EBs, the efficiency of melanocyte generation decreased without SCF/ETs or bFGF. In contrast, more melanocytes were observed without TPA. * $P < 0.05$ (mean $\pm$ SEM $n = 3$), ** $P < 0.01$ (mean $\pm$ SEM $n = 3$).

vitiligo, and hair graying (Boissy and Nordlund, 1997). Vitiligo is a common disease affecting ~0.1–2.0% of the world population (Alkhateeb *et al.*, 2003). Autologous melanocyte transplantation has been used to treat persistent vitiligo with some success. However, the method

is limited by the difficulty in generating sufficient numbers of autologous melanocytes (Czajkowski *et al.*, 2007) because adult melanocytes have limited proliferation capacity. The generation of melanocytes from neural crest-like adult stem cells (Yu *et al.*, 2006), ES cells, or iPSCs may



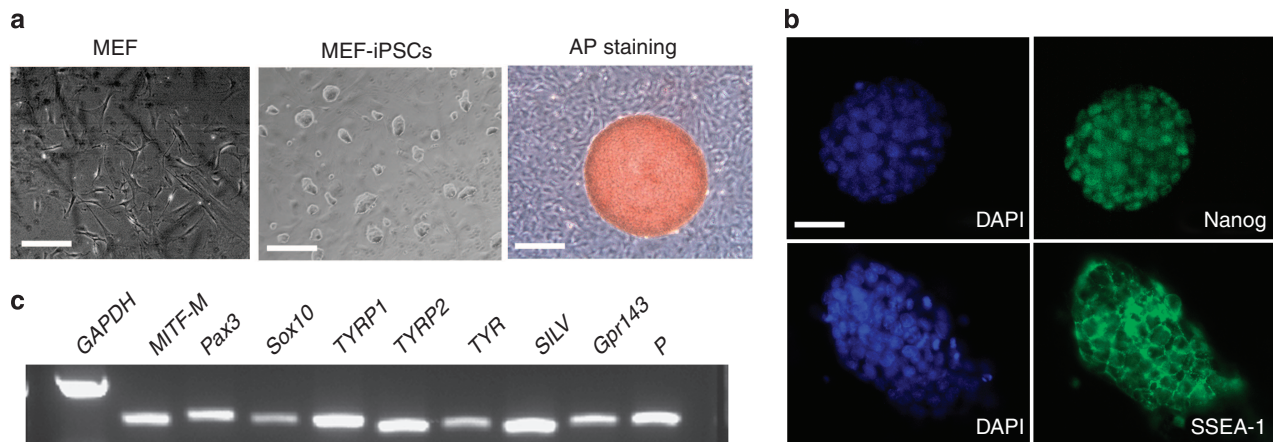


Figure 6. Generation of induced pluripotent stem cells (iPSCs) with virus-free system and characterization of melanocytes derived from virus-free iPSCs. (a) Morphology of mouse embryonic fibroblasts (MEFs) and iPSCs. Left panel represents the morphology of MEFs; middle panel represents the morphology of iPSCs; right panel represents alkaline phosphatase (AP) staining of a representative iPSC colony. In MEF and MEF-iPSC, bar = 150 μ m; in alkaline phosphatase (AP) staining, bar = 500 μ m. (b) Immunostaining of iPSCs with antibodies against Nanog and SSEA-1 (stage-specific embryonic antigen-1). Bar = 200 μ m. DAPI, 4,6-diamidino-2-phenylindole. (c) Reverse transcriptase-PCR (RT-PCR) analysis of melanocytes derived from iPSCs. The iPSC-derived melanocytes are positive for *Pax3*, *Sox10*, *MITF-M*, *TYR*, *TYRP1*, *TYRP2*, *SILV* (*Silver/Pmel17*), *Gpr143* (*Oa1*), and *p* (*pink-eyed dilution gene/Oca2*).

offer a valuable resource for the treatment of vitiligo and other skin disorders.

Melanocytes generated from autologous iPSCs have advantages over ES cells, including a negligible chance of immunological rejection and no concern of ethical issues. However, iPSCs obtained by a retroviral system have the potential for tumor genesis (Nakagawa *et al.*, 2008). Therefore, virus-free systems such as the PB transposon system used in this study are more likely to produce clinical-grade iPSCs because they alleviate the genomic integration concerns of retroviral transgenes. Transposons are sequences of DNA that can move or transpose themselves to new positions within the genome of a single cell. The integration sites in human and mouse cells typically occur within introns. PB excises precisely from the original insertion site (Ding *et al.*, 2005) and the precision is evidenced by the absence of "footprint" mutations at the transposon excision sites (Wilson *et al.*, 2007; Wang *et al.*, 2008; Woltjen *et al.*, 2009). It has been shown that the PB-based reprogramming system enables the generation of nongenetically modified human iPSCs (Woltjen *et al.*, 2009). Here we show that iPSCs induced using this viral-free method can also be differentiated into melanocytes.

In summary, we have developed an efficient method to differentiate mouse melanocytes from iPSCs. Virus-free induction of iPSCs alleviates the concern of integration mutagenesis, although with lower reprogramming efficiencies. In contrast to human ES cell differentiation into melanocytes, *Wnt3a* is dispensable during mouse melanocyte development.

MATERIALS AND METHODS

Cell culture

ES and established iPSCs were cultured on irradiated MEFs in DMEM containing 15% fetal bovine serum, leukemia-inhibiting

factor, penicillin/streptomycin, L-glutamine, β -mercaptoethanol, and nonessential amino acids. TTFs were isolated from C57 mice as described previously (Takahashi *et al.*, 2007), and cultured in DMEM containing 10% fetal bovine serum.

Retroviral infection

Oct4, *Sox2*, *c-Myc*, and *Klf4* retroviral vectors were purchased from (Addgene, Cambridge, CA) (pMXs-Oct3/4: 13366, pMXs-Sox2: 13367, pMXs-Klf4: 13370 and pMXs-c-Myc: 13375). The day before transduction, 293T cells were seeded at 8×10^6 cells per 100 mm dish. The next day, pMXs-based retroviral vectors and pCL-ECO helper plasmids were introduced into 293T cells using the Eugene 6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's recommendations. A volume of 30 μ l Eugene 6 transfection reagent was diluted in 500 μ l DMEM and incubated for 5 minutes at room temperature. Then, 9 μ g retroviral vectors and 9 μ g pCL-ECO were added to the mixture, which was incubated for another 15 minutes at room temperature. After incubation, the DNA/Eugene 6 mixture was added drop by drop onto 293T cells. Cells were then incubated overnight at 37 $^{\circ}$ C with 5% CO₂. At 24 hours after transduction, the medium was replaced. MEFs or TTFs were seeded at 8×10^5 cells per 100 mm dish on mitomycin C-treated MEF feeders. After 24 hours, virus-containing supernatants derived from these 293T cultures were filtered through a 0.45 μ m cellulose acetate filter (Millipore, Billerica, MA) and supplemented with 4 μ g ml⁻¹ polybrene (Sigma-Aldrich, St Louis, MO). Target cells were incubated in the virus/polybrene-containing supernatants for 4–18 hours. After infection, the cells were replated in 10 ml fresh medium. Clones appeared 2 weeks later and were selected after 3 weeks.

Generation of iPSCs with PB transposon system

MEFs were seeded in DMEM with 10% fetal bovine serum, on gelatinized (0.1%) six-well dishes at a density of 1.25×10^5 cells per 10 cm². After 24 hours of culture, Eugene HD (Roche) was used to

transfect cells with 1.2 µg of PB-TET-MKOS, 600 ng PB-CAG-rtTA, and 200 ng of pCyl43 PB transposase plasmid (normalized to 2 µg total DNA with empty pBluescriptKS+) in a Eugene/DNA ratio of 8 µl:2 µg. After 24 hours, the media was supplemented with doxycycline (day 0) and changed entirely 48 hours after transfection. Cells were fed daily with doxycycline-containing media (1.5 µg ml⁻¹). iPSC clones appeared 14 days later and were selected after 30 days.

Reverse transcriptase-PCR for ES cell and melanocyte markers

We performed reverse transcription reactions using SuperScript III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). PCR was performed with PCR Master Mix (Promega, Madison, WI). The ES cell-specific gene primer sequences have been described (Takahashi and Yamanaka, 2006). Primer sequences for detecting melanocyte markers are listed in Supplementary Table online.

Bisulfite genomic sequencing

Bisulfite treatment was performed using the CpGenome modification kit (Millipore) according to the manufacturer's recommendations. The PCR primers used here have been described (Takahashi and Yamanaka, 2006). Amplified products were cloned into pCR2.1-TOPO (Invitrogen). Ten randomly selected clones were sequenced with the M13 forward and M13 reverse primers for each gene. Sequencing was performed at the University of Pennsylvania sequencing facility.

Immunocytochemical and immunohistochemical staining

Mouse ES cell lines and iPSC clones growing on feeder layers were harvested and directly stained with antibodies against stage-specific embryonic protein Nanog (Millipore) and SSEA-1 (The Developmental Studies Hybridoma Bank, Iowa City, IA). Monolayer cells were fixed with 4% paraformaldehyde and stained with primary antibodies specific for MITF (Santa Cruz, Santa Cruz, CA), TYRP2 (polyclonal; a gift from Dr VJ Hearing, Bethesda, MD), and S100 protein (polyclonal; Dako). After washings, cells were incubated with the appropriate FITC-labeled secondary antibodies (Dako, Carpinteria, CA). Formalin-fixed, paraffin-embedded tissues were used for hematoxylin/erosin staining as described previously.

Chromatin immunoprecipitation

A total of 10⁷ iPSCs, ES cells, or TTFs were fixed with 1% formaldehyde for 10 minutes and then lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitors) for 20 minutes on ice. The lysate was split into three tubes and sonicated. After 10 minutes of centrifugation, the supernatant was precleared by incubating for 4 hours at 4 °C with agarose beads preblocked with BSA (1 mg BSA for 10 ml beads) in immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). A total of 100 ml of precleared chromatin per reaction diluted in 1 ml immunoprecipitation buffer in the presence of 20 µg antibody was used for each immunoprecipitation reaction, according to the manufacturer's protocol. The following antibodies were used for this study: anti-acH3 (Millipore), anti-dimethyl K4 of H3 (Millipore), and normal rabbit IgG (Sigma-Aldrich). The precipitate was purified and analyzed by quantitative PCR using Bio-Rad, Hercules, CA SYBR Green quantitative PCR Master Mix. PCR primers were described previously (Takahashi and Yamanaka, 2006).

Differentiation induction

To induce melanocytic differentiation, we developed a modified method based on our previous work (Fang *et al.*, 2006). Briefly, EBs were derived from iPSCs by suspending cells in leukemia-inhibiting factor-depleted ES medium. After 4 days, EBs were collected and plated on 10 ng ml⁻¹ fibronectin (BD, Franklin Lakes, NJ)-coated dishes in differentiation medium, containing 45% DMEM conditioned by L-Wnt3a cells (a kind gift from Dr Meenhard Herlyn, The Wistar Institute, Philadelphia, PA), 45% Medium 254 (Invitrogen), 10% fetal bovine serum (Invitrogen), 0.5 µM dexamethasone (Sigma-Aldrich), 1 × insulin-transferrin-selenium (Invitrogen), 1 mg ml⁻¹ linoleic acid-bovine serum albumin (Sigma-Aldrich), 10⁻⁴ M L-ascorbic acid (Sigma-Aldrich), 50 ng ml⁻¹ SCF (R&D, Minneapolis, MN), 100 nM ET-3 (American Peptide Company, Sunnyvale, CA), 20 µM cholera toxin (Sigma-Aldrich), 50 nM TPA (Sigma-Aldrich), and 4 ng ml⁻¹ bFGF (Invitrogen). Half of the medium was carefully changed every 2 days.

Electron microscopy

Cells were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed with 1% OsO₄. Images were obtained using an electron microscope.

Statistical analysis

Data from three independent experiments, presented in Figure 5, were analyzed. General linear modeling with repeated measures was used to test significant differences among the different media. In this model, total EB numbers or the surface area of adherent EBs differentiated under each medium condition was used as dependent variables, and medium type and time were used as the independent variables.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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