HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2012 June 01.

Published in final edited form as:

J Invest Dermatol. 2011 December ; 131(12): 2458–2466. doi:10.1038/jid.2011.242.

Generation of melanocytes from Induced pluripotent stem cells

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Abstract

Epidermal melanocytes play an important role in protecting skin from ultraviolet (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*, *cMyc* 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 embryoid bodies (EBs) 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, providing iPSCs are more compatible for cell replacement therapies.

Keywords

iPSCs; melanocytes; reprogramming; differentiation

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Conflict of interest

The authors state no conflict of interest.

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 (Okita *et al.*, 2007; Takahashi and Yamanaka, 2006; 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 which can be used to understand the underlying mechanisms for disease initiation and progression or to develop customized cellular therapies to treat certain human diseases (Dimos *et al.*, 2008; Hanna *et al.*, 2007; 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 hES cells. Nevertheless, there are still many practical issues with iPSCs which need to be resolved before this breakthrough technology can be used clinically (Yamanaka, 2010).

The most commonly used reprogramming strategies employ retroviral- or lentiviral-based methods to deliver reprogramming factor transgenes and have been shown to be the 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 (Ding *et al.*, 2005; Fraser *et al.*, 1996; Wang *et al.*, 2008; Woltjen *et al.*, 2009; Wu *et al.*, 2006). 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 (Itzhaki *et al.*, 2011; Lee *et al.*, 2010; Morishima *et al.*, 2011; Sancho-Bru *et al.*, 2011; Zhang *et al.*, 2009), there is still a need to develop the methods and conditions necessary to reproducibly generate homogenous populations of distinct cell lineages from iPSCs.

Melanocytes play a critical role in protecting human skin from harmful ultraviolet (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-marginated 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 like vitiligo as well as melanocyte molecular biology. These cells may also help us to understand the etiology of melanoma, the most deadly form of skin cancer.

In this study, we generated iPSCs from adult mouse TTFs using conventional retroviral as well as 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 (AP) activity is an ES cell marker and we performed AP staining and showed that most of the iPSC clones were AP positive (Figure 1a). RT-PCR was performed to examine whether ES cell marker genes were expressed in iPSCs cells. We employed 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 levels found in authentic mouse ES cells (Figure 1b). The five iPSC clones showed similar gene expression patterns. The expression of Nanog and ESC cell membrane marker SSEA1 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, while the TTF cells stopped growing after 3 weeks (Figure 1d).

Characterization of iPSC and its pluripotency

To investigate the DNA methylation status of the *Oct4* and *Nanog* promoters, we performed bisulphite 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 (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 (SMA) to mark mesoderm differentiation and alpha fetal protein (AFP) 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 pre-treated with retinoic acid, plated onto fibronectin-coated plates containing complete differentiation media containing multiple growth factors including Wnt3a, SCF and 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 RT-PCR was performed to determine if differentiated iPSCs expressed melanocytic markers. The results showed that undifferentiated iPSCs or EBs from iPSCs expressed little melanocytic markers, whereas Mela-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 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–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 bFGF, are important regulators of stem cell proliferation and neural crest-lineage differentiation (Baynash *et al.*, 1994; Imokawa *et al.*, 1998; Nishikawa *et al.*, 1991; Okura *et al.*, 1995). 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 previously described (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 5f). We have preciously shown that the Wnt pathway plays 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 (CT). 12-O-tetradecanoylphorbol-13-acetate (TPA) is a powerful activator of protein kinase C (PKC), and it is extensively used in melanocyte culture (Seger 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. Interestedly, we only found this inhibitory effect at the early stage of melanocyte induction (within the first 2–3 weeks), while cells from later stages and from subsequent passages were resistant to TPA cytotoxicity (data not shown).

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, MEF cells (Figure 6a) were transfected with PB-CAG-rtTA and PB-TET-MKOS (c-Myc, Klf4, Oct4 and Sox2 ORFs 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. AP 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, RT-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 induced 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 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; Li *et al.*, 2010; Yu *et al.*, 2006; Yu *et al.*, 2010). However, in the present study, we found that Wnt3a is dispensable for mouse iPSCs 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 play 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 acts 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 recently showed that melanocytes can be generated from human iPSCs *in vitro* (Ohta *et al.*, 2011). They generated iPSC lines from human dermal fibroblasts using the *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 seven 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, vitiligo, and hair graying (Boissy and Nordlund, 1997). Vitiligo is a common disease affecting approximately 0.1 to 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), embryonic stem cells or iPSCs may 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 transposome 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 ((Wang *et al.*, 2008; Wilson *et al.*, 2007; Woltjen *et al.*, 2009). It has been shown that the PB-based reprogramming system enables the generation of non-genetically 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 albeit 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% FBS, leukemia inhibiting factor (LIF), penicillin/streptomycin, L-glutamine, beta-mercaptoethanol and nonessential amino acids. Mouse tail fibroblast (TTFs) were isolated from C57 mice as previously described (Takahashi *et al.*, 2007), and cultured in DMEM containing 10% FBS.

Retroviral Infection

Oct4, Sox2, c-Myc and Klf4 retroviral vectors were purchased from Addgene Inc (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 Fugene 6 transfection reagent (Roche) according to the manufacturer's recommendations. 30 uL Fugene 6 transfection reagent was diluted in 500 μL DMEM and incubated for 5 min at room temperature. 9 ug retroviral vectors and 9 ug pCL-ECO were added to the mixture, which was incubated for another 15 min at room temperature. After incubation, the DNA/Fugene 6 mixture was added drop by drop onto 293T cells. Cells were then incubated overnight at 37°C with 5% CO2. Twenty-four 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 h, virus-containing supernatants derived from these 293T cultures were filtered through a 0.45 μm cellulose acetate filter (Millipore) and supplemented with 4 μg/mL polybrene (Sigma-Aldrich). Target cells were incubated in the virus/polybrene-containing supernatants for 4–18 h. After infection, the cells were replated in 10 mL fresh medium. Clones appeared 2 weeks later and were selected after 3 weeks.

Generation of iPSC with PB transponse system

MEFs were seeded in DMEM with 10% FBS, on gelatinized (0.1%) 6-well dishes at a density of 1.25×10^5 cells per 10 cm^2. After 24 h culture, FugeneHD (Roche) was used to transfect cells with 1.2 ug 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+) at a Fugene:DNA ratio of 8 μL:2 μg. After 24 h, the media was supplemented with doxycycline (day 0), and changed entirely 48 h after transfection. Cells were fed daily with doxycycline-containing media (1.5 μg/mL). iPSC clones appeared 14 days later and were selected after 30 days.

RT-PCR for ES cell and melanocyte markers

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

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). Monolayer cells were fixed with 4% paraformaldehyde and stained with primary antibodies specific for MITF (Santa cluz), TYRP2 (polyclonal; a gift from Dr. V.J. Hearing, Bethesda, MD), and S100 protein (polyclonal; Dako). After washings, cells were incubated with the appropriate FITC-labeled secondary antibodies (Dako). Formalin-fixed, paraffin-embedded tissues were used for hematoxylin/erosin staining as previously described.

Chromatin immunoprecipitation

10^7 iPSCs, ES cells or TTFs were fixed with 1% formaldehyde for 10 min and then lysed in 1 mL lysis buffer (50mM Tris-HCl, pH 8.0, 10mM EDTA, 1% SDS, and protease inhibitors) for 20 min on ice. The lysate was split into three tubes and sonicated. After 10 min centrifugation, the supernatant was pre-cleared by incubating for 4 h at 4 °C with agarose beads pre-blocked with BSA (1 mg BSA for 10 mL beads) in IP buffer (50mMTris-HCl, pH 8, 150mMNaCl, 2mMEDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors). A total of 100 mL of pre-cleared chromatin per reaction diluted in 1 mL IP buffer in the presence of 20 ug antibody was used for each immunoprecipitation reaction, according to the manufacturer's protocol. The antibodies used for this study were: anti-acH3 (Millipore), anti-dimethyl K4 of H3 (Millipore), and normal rabbit IgG (Sigma). The precipitate was purified and analyzed by quantitive PCR using Bio Rad SYBR Green quantitive 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, embryoid bodies (EBs) were derived from iPSCs by suspending cells in LIF-depleted ES medium. After 4 days, EBs were collected and plated on 10 ng/ml fibronectin(BD)-coated dishes in differentiation medium, containing 45% DMEM conditioned by L-Wnt3a cells (a kind gift from Dr. Meenhard Herlyn, The Wistar Institute, USA), 45% Medium 254 (Invitrogen), 10% FBS (Invitrogen), 0.5 μM dexamethasone (Sigma-Aldrich), 1× insulin-transferrin-selenium (Invitrogen), 1 mg/ml linoleic acid-bovine serum albumin (Sigma-Aldrich), 10^{-4}M L-ascorbic acid (Sigma-Aldrich), 50 ng/ml SCF (R&D), 100 nM ET-3 (American Peptide Company), 20 pM cholera toxin (CT) (Sigma-Aldrich), 50 nM 12-O-tetradecanoyl-phorbel 13-acetate (TPA) (Sigma-

Aldrich), and 4ng/ml bFGF (Invitrogen). Half of the medium was carefully changed every 2 day.

Electron Microscopy

Cells were fixed with 2.5% glutaraldehyde and 4% PFA in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1% OsO_4. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Dr. Meenhard Herlyn for providing the Wnt3a cell line; the histology laboratory at the Department of Pathology and Laboratory Medicine, University of Pennsylvania for tissue processing; Dr. Kristen Huang for editing the manuscript.

This work was supported by the grants AR-054593 and AR-054593S1 from National Institute of Health to XX.

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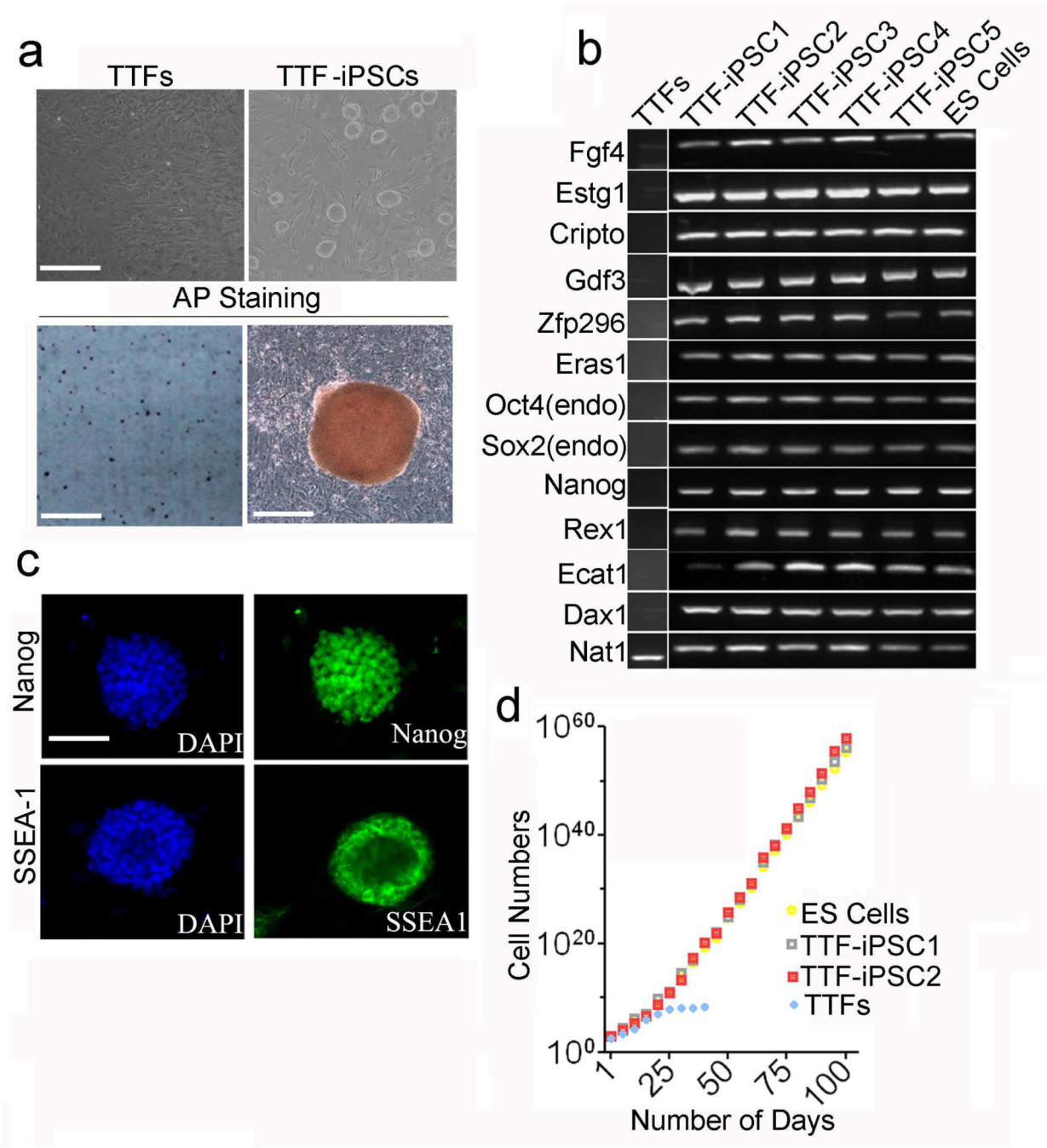

Figure 1. Generation and expression analysis of iPSCs

(a). Morphology of mouse iPSCs and TTFs. Upper panel: Morphology of mouse TTFs and iPSCs derived from TTFs, bar indicates 150 μm. Lower panel: AP staining results of iPSC clones; left side, bar indicated 10 mm; right side, bar indicates 500 μm. (b). RT-PCR analysis of 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 monoclonal antibody against SSEA-1 or Nanog. DAPI staining was used as a nuclear marker. Bar indicates 500 μm. (d). Growth

curves of TTFs, mouse ES cells and iPSCs. 3×10^5 cells were passaged every 3 days into each well of a six-well plate.

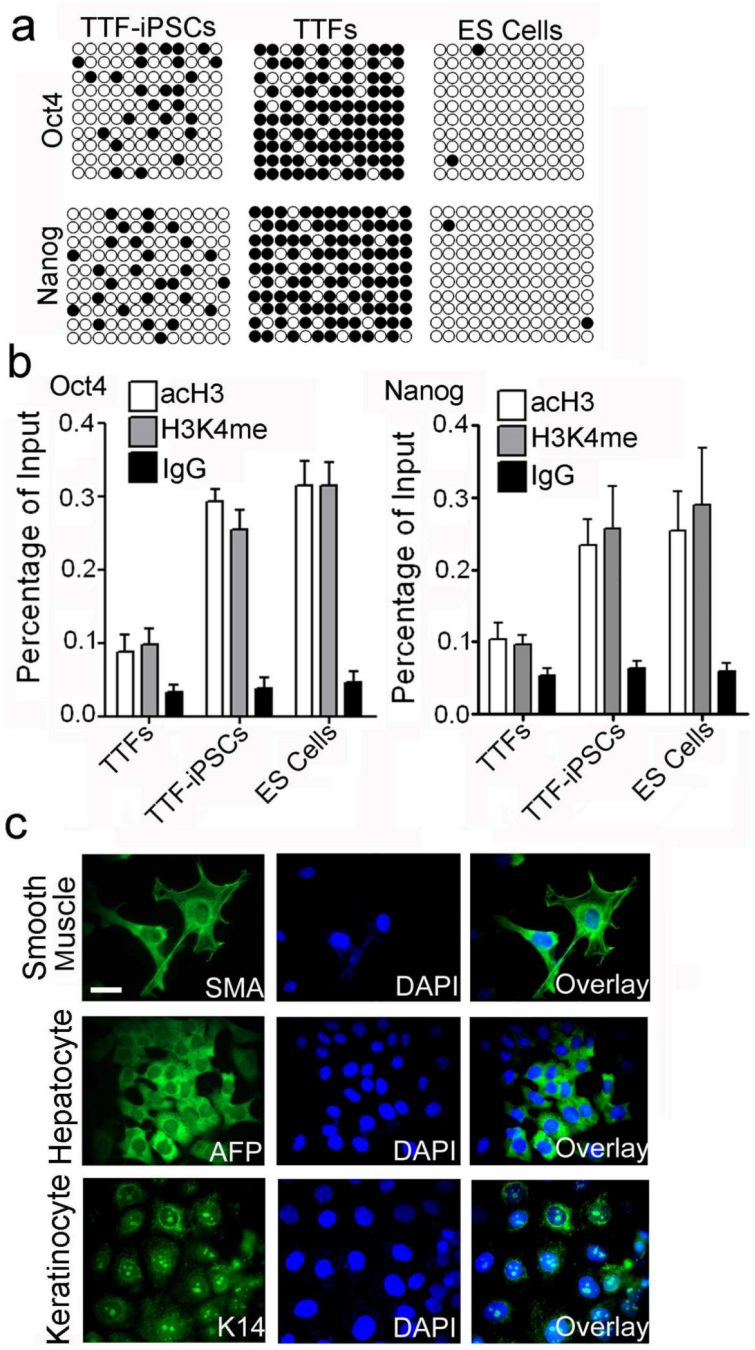

Figure 2. Epigenetic modification and *in vitro* differentiation of iPSCs

(a). Bisulfite genomic sequencing of the promoter regions of Oct3/4 and Nanog in 10 randomly selected iPSC and 10 ES cell clones as well as MEF cells. Open circles indicate unmethylated CpG dinucleotides, while closed circles indicate methylated CpGs. (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 (H3K4 me2 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 for ectoderm differentiation, smooth muscle actin (SMA) for mesoderm differentiation and alpha fetal protein (AFP) for endoderm differentiation. DAPI staining was used as a nuclear marker. Bar indicates 15 μm.

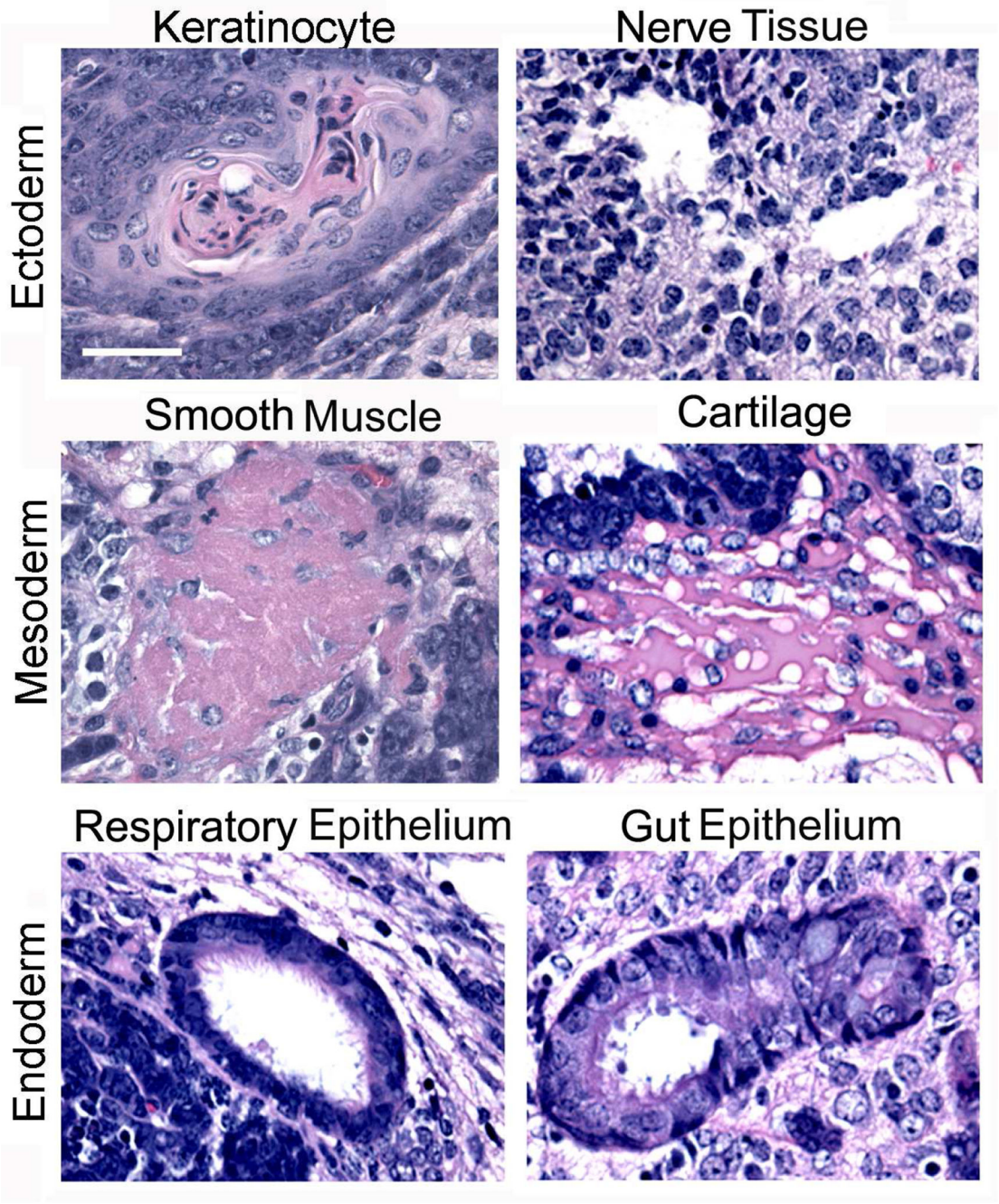

Figure 3. Developmental pluripotency of iPSCs by teratoma formation

10^6 iPSCs were injected into nude mice dorsal flanks. Four 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 (H&E staining). Bar indicates 50 μm.

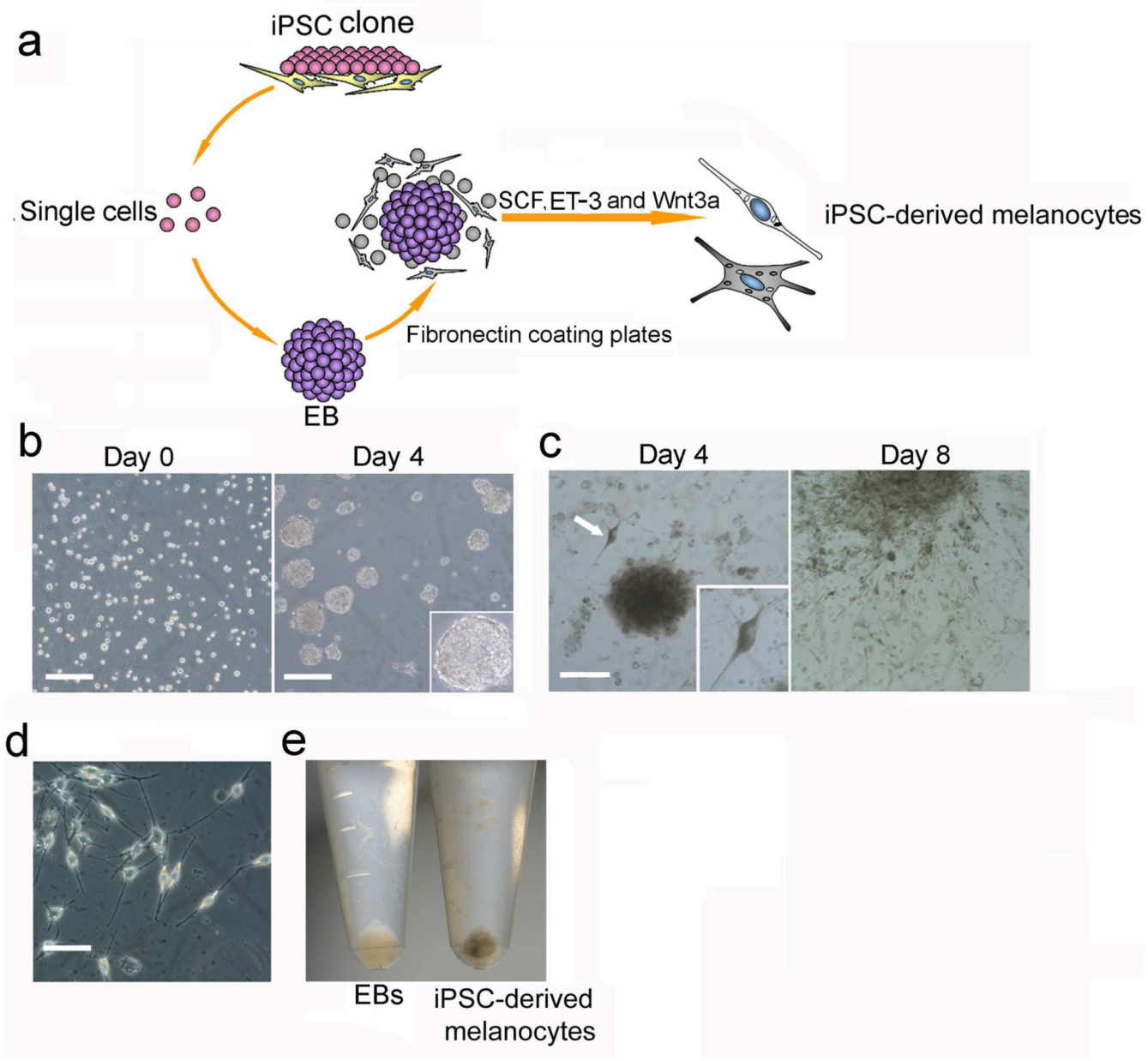

Figure 4. Differentiation of melanocytes from iPSCs

(a). Differentiation overview. Embryonic bodies (EBs) were formed from iPSCs and treated with 1μM RA after two days. After three days with RA, the EBs plated into fibronectin coated dishes in melanocyte differentiation medium with ET-3, SCFa, bFGF, and Wnt3a. (b). EBs formed in suspension under feeder-free conditions; left side, bar indicated 10 mm; right side, bar indicates 500 μm. (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 indicates 500 μm. (d). At day 16, differentiated cells displayed more homogenous melanocytic morphology after splitting (phase contract microscope). Bar

indicates 25 μm. (e). Unpigmented EBs prior to differentiation (left), in contrast to the pigmented cell pellet from iPSC-derived melanocytes (right).

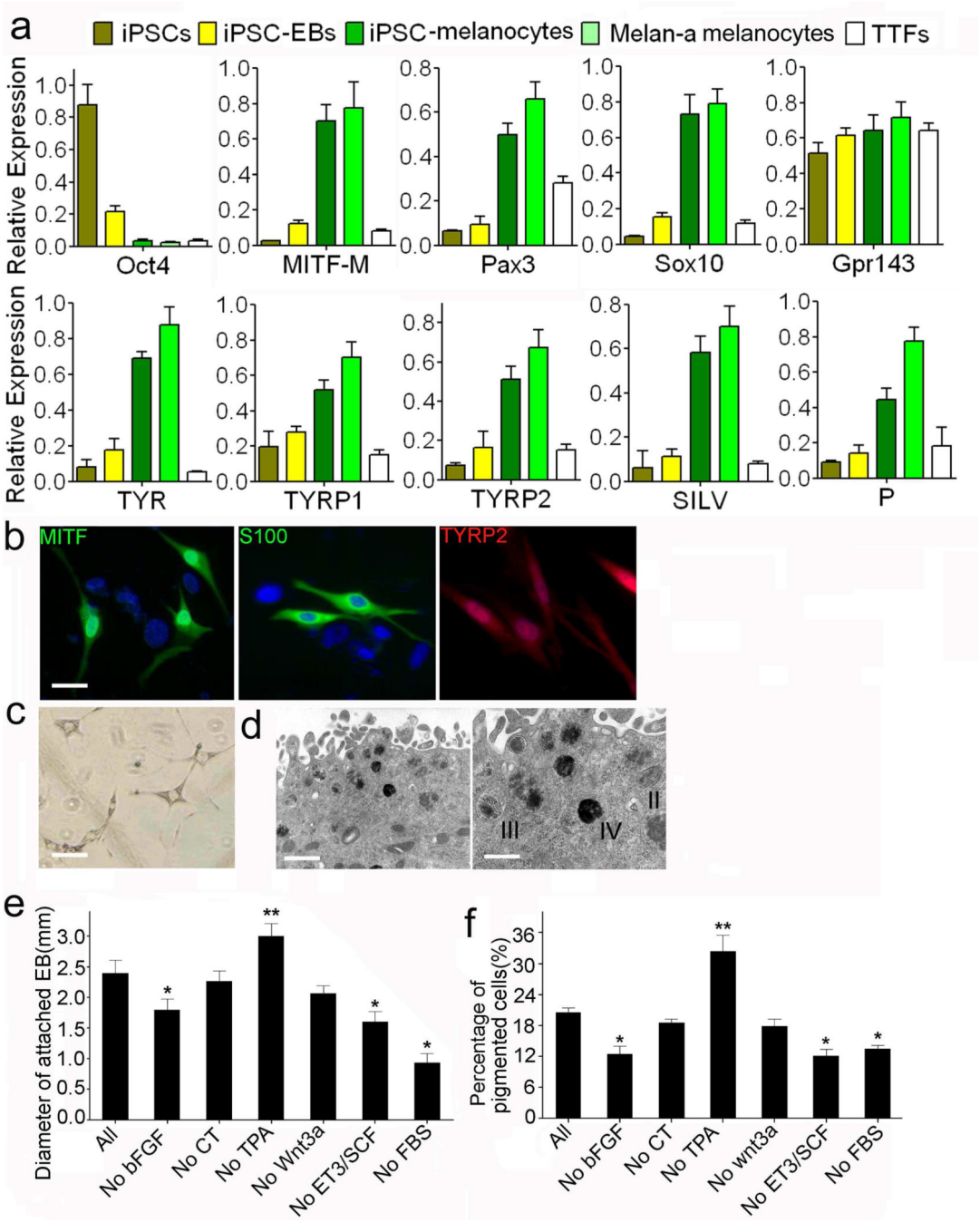

Figure 5. Characterization of melanocytes derived from iPSCs

(a). Quantitative RT-PCR analysis of melanocyte markers in iPSCs, EBs from iPSCs (iPSC-EBs), melanocytes from iPSCs (iPSC-melanocyte), mouse melanocyte melan-a cells, and TTFs, including *Pax3*, *MITF-M*, *TYR*, *TYRP1*, *TYRP2*, *SILV (Silver/Pmel17)*, *Gpr143(Oa1)*, *p (pink-eyed dilution gene/Oca2)* and *Sox 10*; *GAPDH* was used as a loading control. (b). Immunostaining analysis of iPSCs derived melanocyte with antibodies against MITF, S-100 and TYRP2. Bar indicates 15 μm (immunofluorescent microscopy). (c). DOPA reaction of melanocytes derived from iPSCs. The pigmented cells represent DOPA positive cells. Bar

indicates 30 μm. (d). Electron microscopy images show an iPSCs-derived melanocyte with many mature melanosomes in the cytoplasm. II: stage II melanosome; III: stage III melanosome; IV: stage IV melanosome. Bar in the left indicates 1 μm; bar in the right indicates 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, while 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 ±SEM n=3), **P<0.01 (mean±SEM n=3). Abbreviations: ET-3, endothelin-3; SCF, stem cell factor. (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±SEM n=3), **P<0.01 (mean±SEM n=3).

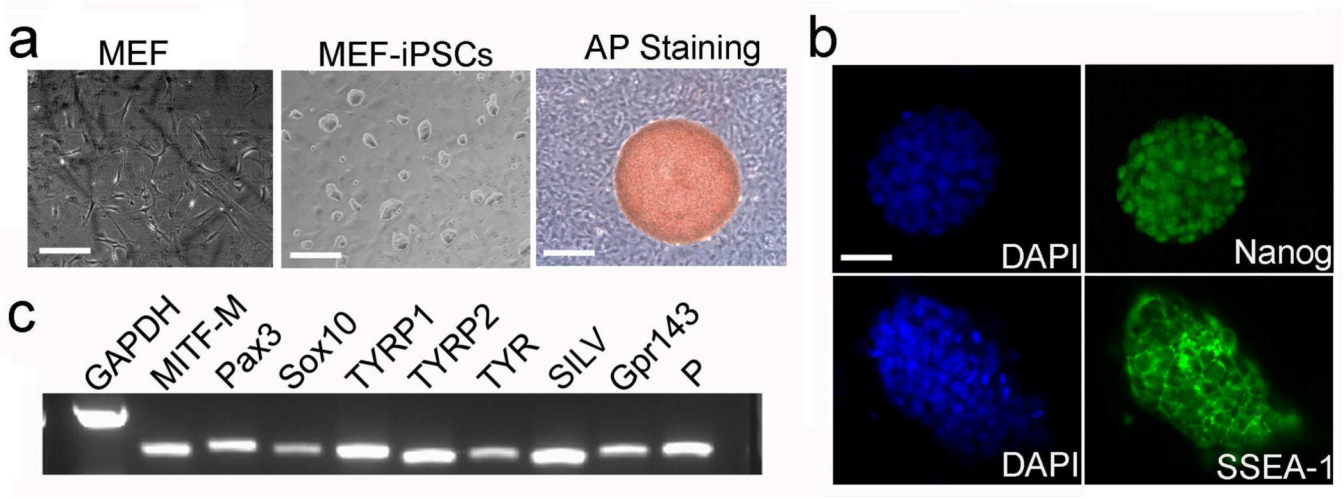

Figure 6. Generation of iPSCs with virus-free system and characterization of melanocyte derived from virus-free iPSCs

(a). Morphology of MEFs and iPSCs. Left panel represents the morphology of MEFs; middle panel represents the morphology of iPSCs; right panel represents AP staining of a representative iPSC colony. Bar in MEF and MEF-iPSC indicates 150 μm; bar in AP staining 500 μm. (b). Immunostaining of iPSCs with antibodies against Nanog and SSEA-1. Bar indicates 200 μm. (c). 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).*