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Melanocytes derived from transgene-free human induced pluripotent stem cells.

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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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Melanocytes Derived from Transgene-Free Human Induced Pluripotent Stem Cells

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TO THE EDITOR

Defects in melanocytes have been implicated in the etiology of a variety of human skin diseases and disorders (Lin and Fisher, 2007; Fistarol and Itin, 2010; Rees, 2011). There is long-standing interest in studying the development and dysfunction of human melanocytes, but there has not been a reliable and accessible system to study early events in human melanocyte differentiation. An *in vitro* system that reliably and efficiently produces normal human melanocytes from embryonic stage cells would allow us to better dissect the physiological and pathological development of melanocytes. Recent advances in stem cell biology have led to the establishment of human induced pluripotent stem cell (hiPSC) techniques that enable researchers to reprogram somatic cells to the pluripotent state (Takahashi *et al.*, 2007). Differentiation of human and mouse pluripotent stem cells (PSCs) toward the melanocyte lineage has been reported (Yamane *et al.*, 1999; Pla *et al.*, 2005; Fang *et al.*, 2006; Nissan *et al.*, 2011; Ohta *et al.*, 2011; Yang *et al.*, 2011), but existing protocols have shortcomings that may limit their research and clinical applications. For example, the use of embryonic stem cells could lead to allogeneic immunoincompatibility of differentiated melanocytes and transplant recipients. In addition, the use of hiPSCs generated by integrative reprogramming strategies raises concerns about reactivation of retained transgenes, some of which are oncogenes. In addition, the current methods for melanocyte differentiation from hiPSCs require optimization in order to reproducibly generate high-purity melanocytes from multiple hiPSC lines.

We have established a strategy to produce human melanocytes *in vitro* for use as a platform for pigment cell research and the development of cell-based therapies. We first derived transgene-free hiPSCs from two distinct types of skin cells: human primary melanocytes (HMs) and human dermal fibroblasts

Abbreviations: hiPSC, human induced pluripotent stem cell; HM, human primary melanocyte; α-MSH, α-melanocyte-stimulating hormone; MITF, microphthalmia-associated transcription factor; PSC, pluripotent stem cell; SNP, single-nucleotide polymorphism

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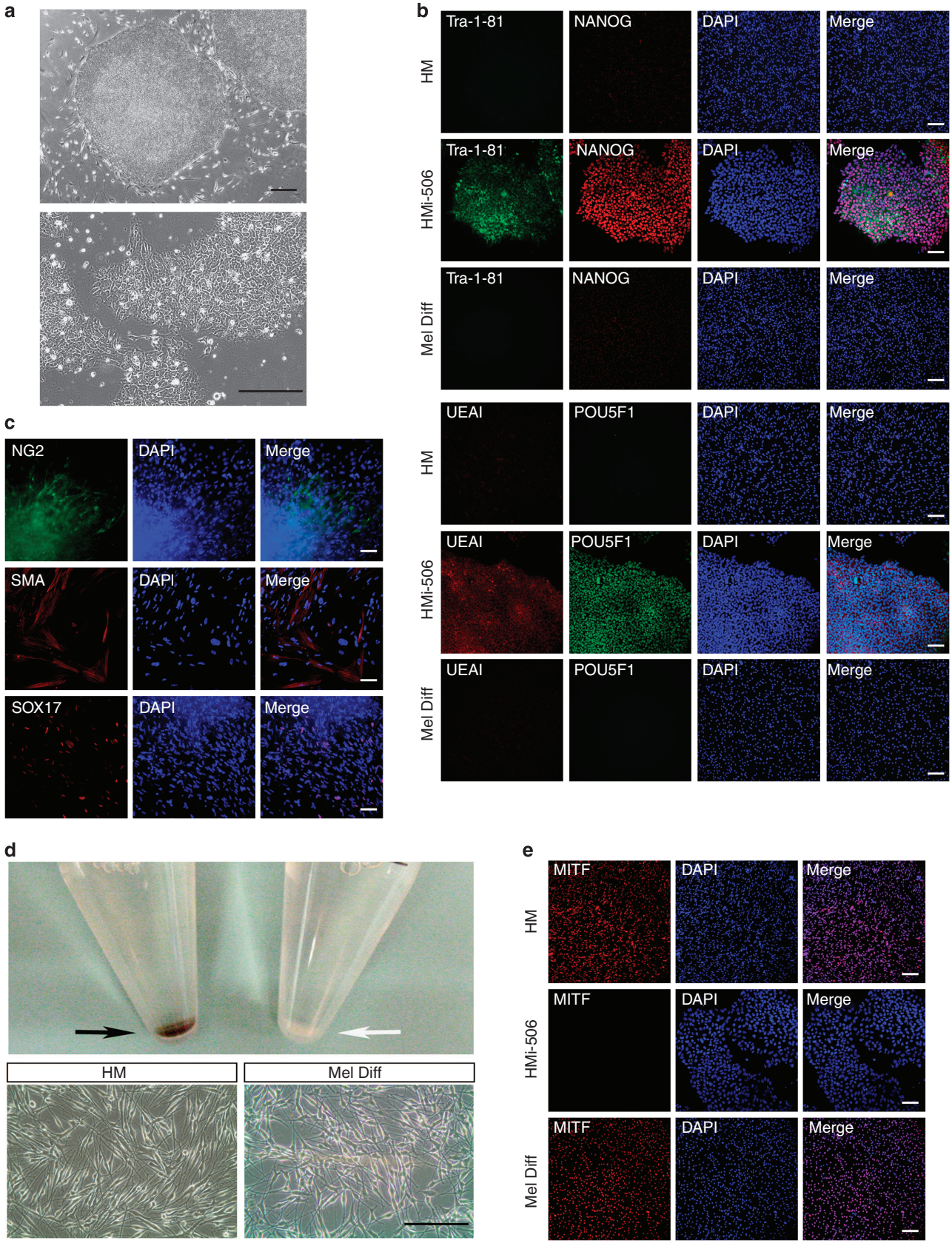

Figure 1. For caption see page 2106.

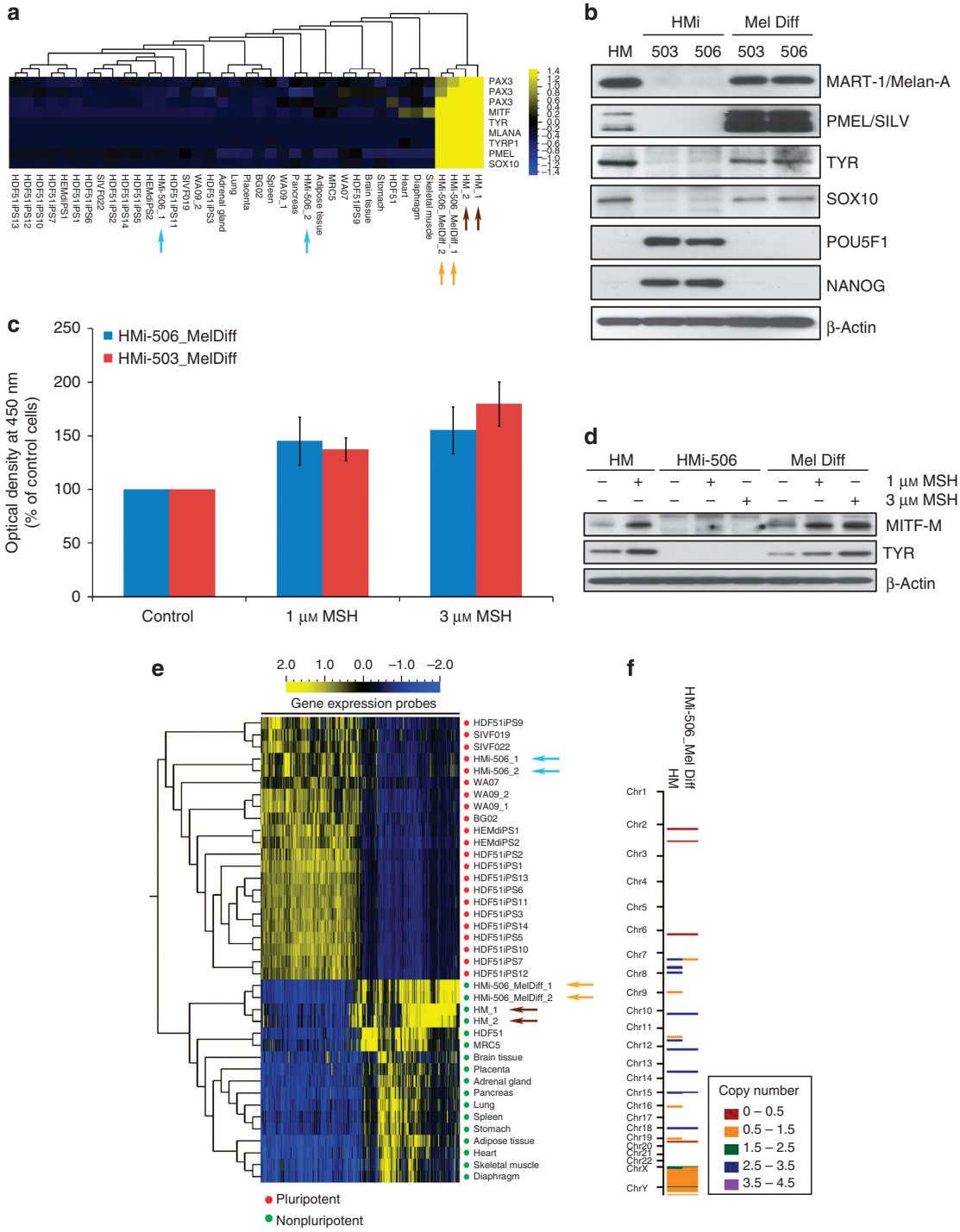

Figure 2. Continued on following page.

Figure 1. Generation and differentiation of transgene-free human induced pluripotent stem cell (hiPSCs). (**a**) HMi-506 cells generated from human primary melanocyte (HM) cells using a Sendai virus–based reprogramming system were cocultured with mouse embryonic fibroblast feeder cells (upper panel) and in feeder-free conditions (lower panel). (**b**) Immunofluorescence staining showed that biomarkers of pluripotency, Tra-1-81, NANOG, OCT4/POU5F1, and UEA-I lectin, were strongly positive in HMi-506 cells but absent in their differentiated derivatives (Mel Diff) and HM cells. (**c**) Embryoid bodies from HMi-506 cells contained cells from all three germ layers. NG2-positive cells: ectoderm; smooth muscle actin (SMA)–positive cells: mesoderm; SOX17-positive cells: endoderm. (**d**) The HMi-506_Mel Diff cells displayed pigmentation and morphology typical of HM cells. The black arrow indicates a pigmented cell pellet of HMi-506_Mel Diff cells, whereas the white arrow indicates undifferentiated HMi-506 cells. (**e**) Immunofluorescence staining showed that microphthalmia-associated transcription factor (MITF) was expressed in HM cells and HMi-506_Mel Diff cells and absent in HMi-506 cells. DAPI, 4′,6-diamidino-2-phenylindole. Scale bars = 100 μm.

Figure 2. Molecular and functional characterization of the melanocyte-like differentiated cells. (**a**) Heat map and dendrogram of melanocytic biomarkers showing that these transcripts were preferentially expressed in human primary melanocyte (HM) cells and HMi-506_Mel Diff cells. Brown arrows, HM samples; turquoise arrows, undifferentiated HMi-506 samples; orange arrows, HMi-506_Mel Diff samples. (**b**) Expression of melanocytic and pluripotency biomarkers was detected by western blotting. (**c**) Melanin production was increased in a dose-dependent manner in the differentiated derivatives treated with α-melanocyte-stimulating hormone (α-MSH) for 48 hours. Columns indicate mean of three independent experiments and bars indicate SD. (**d**) Protein expression of TYR and MITF-M in cells subjected to α-MSH treatment for 24 hours detected by western blotting. (**e**) Unsupervised hierarchical clustering of gene expression profiles from 22 human pluripotent stem cell (hPSC) samples and 17 samples of human nonpluripotent cells. Arrows were colored as in **a**. (**f**) Copy number variation (CNV) analysis was performed using single-nucleotide polymorphism (SNP) genotyping data, and indicated that no significant additional CNVs arose during reprogramming or differentiation.

(HDF51) (Figure 1a and Supplementary Figure S1a online). We used a nonintegrative reprogramming approach mediated by Sendai virus–based vectors independently encoding *POU5F1*, *SOX2*, *KLF4*, and *MYC* (Fusaki *et al.*, 2009; Macarthur *et al.*, 2012). As shown in Figure 1b and Supplementary Figure S1b online, biomarkers of cellular pluripotency, including endogenous OCT4/POU5F1, NANOG, Tra-1-81, and UEA-I (Wang *et al.*, 2011), were positive in HMi-506, HMi-503, and HDF51i-509 hiPSCs. Cells were also shown to be pluripotent using a gene expression diagnostic test (PluriTest; Muller *et al.*, 2011), by differentiation into cells that express biomarkers relevant to all three germ layers *in vitro* (Figure 1c and Supplementary Figure S1c, S1d and S1e online) and by generation of teratomas (Supplementary Figure S1d online).

We newly developed two differentiation protocols based on previously reported methods. One protocol involves an aggregation-in-suspension step, whereas the other does not (Supplementary Figure S2 online). Both protocols generated cells displaying typical melanocyte morphology and pigmentation (Figure 1d) from hiPSCs after 30 days of directed differentiation, suggesting that the aggregation-in-suspension step is dispensable. The melanin granules that accumulated at the dendritic tips of differentiated cells were intensely stained by Fontana–Masson staining, indicating that the pigmentation of these cells was due to melanogenesis (Supplementary Figure S3 online). In addition, MITF (microphthalmia-associated transcription factor), a marker for melanocyte progenitors, was expressed in more than 90% of the differentiated derivatives after 30 days (Figure 1e and Supplementary Figure S4 online), which appears to be a higher differentiation efficiency than other reported protocols (Nissan *et al.*, 2011; Ohta *et al.*, 2011). As expected, MITF was not detected in the undifferentiated hiPSCs, and was present in the primary melanocytes (Figure 1e). Notably, our protocols resulted in similarly high levels of melanocyte differentiation for all four independent hiPSC lines examined, highlighting their reproducibility.

Other melanocytic biomarkers including *TYR* (*tyrosinase*), *MLANA* (melan-A), *TYRP1* (*tyrosinase-related protein 1*), *PMEL* (*premelanosome protein*), *PAX3* (*paired box 3*), and *SOX10* (*SRY-box 10*) were highly expressed in the differentiated derivatives (similar to primary melanocytes, Figure 2a and b). The melanin content and cell signaling involved in melanin production in the differentiated derivatives was increased by treatment with α-melanocyte-stimulating hormone (α-MSH) in a dose-dependent manner (Figure 2c and d and Supplementary Figure S5 online). These findings indicate that the differentiated derivatives possess molecular features of *bona fide* melanocytes and accurately mimic their ability to respond to α-MSH, which is the factor that activates melanogenesis and enhances skin pigmentation during the tanning response (Thody, 1999).

Genome-wide gene expression profiling and unsupervised hierarchical clustering revealed that the melanocytes (HMi-506_Mel Diff_1 and HMi-506_Mel Diff_2) differentiated from the HMi-506 cells were closely clustered with HMs and were distinct from all undifferentiated hiPSC samples (Figure 2e). As genetic abnormalities may occur in hiPSC genomes during the reprogramming and differentiation processes, we tested the genomic stability of the cells by comparing the differentiated derivatives with the parental primary melanocytes using high-resolution single-nucleotide polymorphism (SNP) genotyping and copy number variation analysis. As shown in Figure 2f, the HMi-506_Mel Diff derivatives and parental cells showed highly similar genotyping profiles, showing that the cellular genome remained stable during reprogramming and differentiation.

Similar to human melanocytes *in vivo*, the differentiated derivatives in semiautologous skin reconstructs were located at the dermis–epidermis interface and interspersed with keratinocytes (Supplementary Figure S6a, S6b, S6c and S6d online), indicating their ability to integrate with the skin tissue of transplant recipients. Similar to the autologous dermal fibroblasts used for generating transgene-free hiPSCs, the differentiated derivatives stimulated limited proliferation of peripheral blood mononuclear cells that were isolated from the blood of the same individual in a mixed lymphocyte reaction assay (Supplementary Figure S6e online). These results attest to the clinical advantages of melanocytes differentiated from hiPSCs using the reprogramming and differentiation approaches described here.

In this study, we have demonstrated that genetically stable melanocytes can be efficiently differentiated from transgene-free hiPSCs generated from two different types of cutaneous cells. This differentiation protocol takes less time than previously reported melanocytic differentiation protocols, and we showed that it is equally effective for multiple independent hiPSC lines. We performed a thorough investigation of the differentiated cells, including genome-wide gene expression analysis and SNP genotyping in addition to functional assays. Our approach can serve as an unlimited source of custom human

melanocytes that can be used for novel approaches for modeling human skin disease (e.g., melanoma and vitiligo) and to provide material for transplantation.

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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Three-Dimensional Morphology of Touch Domes in Human Hairy Skin by Correlative Light and Scanning Electron Microscopy

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TO THE EDITOR

The presence of tactile structures in human hairy skin was first reported by Pinkus in 1902. He discovered distinct epidermal disc-like structures with nerves and ''Tastzellen'' (i.e., Merkel cells) at the base of the epidermis, and named these structures ''Haarscheiben'' because of their close association with hair follicles. These structures were revisited by later investigators (Kamide, 1955; Kawamura *et al.*, 1964). Recent researchers have also reported the histology of the human ''Haarscheiben'' (Moll *et al.*, 2005; Reinisch and Tschachler, 2005), whereas some other investigators showed that Merkel cells

Abbreviations: K20, keratin 20; LM, light microscopy; SEM, scanning electron microscopy; 3D, three-dimensional

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