Human Induced Pluripotent Stem Cell–Derived Ectodermal Precursor Cells Contribute to Hair Follicle Morphogenesis *In Vivo*

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Well-orchestrated epithelial–mesenchymal interactions are crucial for hair follicle (HF) morphogenesis. In this study, ectodermal precursor cells (EPCs) with the capacity to cross talk with hair-inductive dermal cells were generated from human induced pluripotent stem cells (hiPSCs) and assessed for HF-forming ability *in vivo*. EPCs derived from three hiPSC lines generated with 4 or 3 factors (*POU5F1*, *SOX2*, *KLF4* +/− *MYC*) mostly expressed keratin 18, a marker of epithelial progenitors. When cocultured with human dermal papilla (DP) cells, a 4 factor 201B7 hiPSC-EPC line upregulated follicular keratinocyte (KC) markers more significantly than normal human adult KCs (NHKCs) and other hiPSC-EPC lines. DP cells preferentially increased DP biomarker expression in response to this line. Interestingly, 201B7 hiPSCs were shown to be ectodermal/epithelial prone, and the derived EPCs were putatively in a wingless-type MMTV integration site family (WNT)-activated state. Importantly, co-transplantation of 201B7 hiPSC-EPCs, but not NHKCs, with trichogenic mice dermal cells into immunodeficient mice resulted in HF formation. Human HF stem cell markers were detected in reconstituted HFs; however, a low frequency of human-derived cells implied that hiPSC-EPCs contributed to HF morphogenesis via direct repopulation and non–cell autonomous activities. The current study suggests a, to our knowledge, previously unrecognized advantage of using hiPSCs to enhance epithelial–mesenchymal interactions in HF bioengineering.

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INTRODUCTION

Hair follicle (HF) morphogenesis and regeneration depend on intensive and reciprocal interactions between epithelial and mesenchymal components (Hardy, 1992; Paus and Cotsarelis, 1999; Mikkola and Millar, 2006). Currently, attempts to regenerate HFs center on combining receptive epithelial and trichogenic dermal mesenchymal components and grafting them into an *in vivo* environment (for reviews, see Ohyama *et al.*, 2010; Yang and Cotsarelis, 2010).

In mice, bulge stem cells more efficiently reconstituted HFs than nonbulge cells in *in vivo* HF reconstitution assays (Blanpain *et al.*, 2004; Morris *et al.*, 2004), suggesting that preparation of highly receptive epithelial cells is pivotal to achieve efficacious HF regeneration (Stenn *et al.*, 2007). Similarly, human bulge stem cells can provide an optimal epithelial component. However, currently available isolation techniques (Ohyama *et al.*, 2006; Tiede *et al.*, 2009) are not efficient enough to collect the necessary number of cells for *in vivo* assays. Ehama *et al.* (2007) reported that neonatal human keratinocytes (KCs) more efficiently formed HF-like structures compared with adult human KCs at the same passage. HF neogenesis in adulthood could occur in higher vertebrates (Ito *et al.*, 2007); however, human HF morphogenesis proceeds mostly *in utero* (Stenn *et al.*, 2007). Conditioning KCs to an embryonic or neonatal state could improve the receptivity to hair-inductive signals.

Recently, successful generation of terminally differentiated KCs from human induced pluripotent stem cells (hiPSC) has been reported (Tolar *et al.*, 2011; Itoh *et al.*, 2011). By following the differentiation pathway of hiPSCs to mature KCs, hiPSC-derived precursor cells with high receptivity to hair-inductive dermal signals may be generated. The use of human embryonic stem cells requires significant ethical consideration. Thus, hiPSCs would be a more easily accessible source of epithelial component for HF bioengineering.

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Abbreviations: DP, dermal papilla; EB, embryoid body; EPC, ectodermal precursor cell; HF, hair follicle; hiPSC, human induced pluripotent stem cell; hiPSC-EPCs, human induced pluripotent stem cell–derived ectodermal precursor cells; KC, keratinocyte; MDC, mice neonatal dermal cells; NHKC, normal human adult keratinocyte; SHH, sonic hedgehog; WNT, wingless-type MMTV integration site family

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In this study, three lines of hiPSCs were, respectively, differentiated into ectodermal precursor cells (EPCs) expressing epithelial markers and were examined for their capacity to communicate with human dermal papilla (DP) cells. Subsequently, the ability of the most interactive precursor cells to contribute to HF morphogenesis *in vivo* was assessed.

RESULTS

Generation of hiPSC-EPCs from respective hiPSC lines

Established hiPSC lines generated with 4 (201B7; Takahashi *et al.* (2007) and WD39; Imaizumi *et al.* (2012)) or 3 (WDT2; Ohta *et al.* (2011)) reprogramming factors (POU class 5 homeobox 1 [*POU5F1*], SRY (sex determining region Y)-box2 [*SOX2*], kruppel-like factor 4 (gut) [*KLF4*] +/− v-myc myelocytomatosis viral oncogene homolog (avian) [*MYC*]) were converted into embryoid bodies (EBs; Figure 1a), exposed to retinoic acid to promote ectodermal lineages (Saitou *et al.*, 1995; Metallo *et al.*, 2008) and bone morphogenetic protein-4 (BMP4) to suppress neural lineages (Wilson and Hemmati-Brivanlou, 1995). Irrespective of derived hiPSC lines, 70–80% of EBs adopted a cystic morphology by day 5 (Figure 1b). Resultant EBs were plated onto collagen I–coated dishes and cultured in defined KC serum-free medium for an additional 6 days to form confluent epithelial cell–appearing colonies with mostly the same efficiency among starting hiPSC lines (Figure 1c). The protocol is summarized in Figure 1d (details provided in Supplementary materials and methods).

Inversely proportional to the shut down of the pluripotency marker *POU5F1,* marked upregulation of epithelial marker, keratin 18 (*KRT18*; Maurer *et al.*, 2008), as well as KC markers DeltaNp63 (*TP63*) and keratin 14 (*KRT14*; Itoh *et al.*, 2011), was observed (Figure 2a and b). Flow cytometric analyses demonstrated that the generated cells mostly expressed KRT18, and a small fraction of them expressed KC markers (Figure 2c). The status of generated cells was similar among three hiPSC lines: KRT18-positive cells, 96.2 ± 2.5–98.0 ± 1.8%; TP63-positive cells, 34.1 ± 7.8–37.2 ± 4.7%; and KRT14-positive cells, 8.6 ± 3.1–10.4 ± 4%.

These findings indicated the successful generation of cell populations committed to ectodermal lineage, resembling epithelial progenitors. Thus, we designated the cells obtained with our protocol as ''hiPSC-derived ectodermal precursor cells (hiPSC-EPCs).''

Interestingly, when each hiPSC-EPC population was exposed to high-calcium conditions, upregulation of KC terminal differentiation markers (Yuspa *et al.*, 1989; Bilousova *et al.*, 2011; Itoh *et al.*, 2011) was observed, especially in 201B7 EPC lines (Supplementary Figure S1 online). These findings implied that hiPSC-EPCs contained the cells with KC differentiation potential.

hiPSC-EPCs bidirectionally cross talk with human DP cells mimicking the interactions in the hair bulb

In the bulb of anagen HFs, the DP sends inductive signals to hair matrix cells to produce the hair shaft (Ohyama *et al.*, 2010). To assess whether each hiPSC-EPC line could interact with human DP cells and, if yes, which was the most potent responder line, a coculture system was established (Figure 3a). In this system, normal human adult KCs (NHKCs) without coculture were used as the baseline. The stimuli from coexisting DP cells increased the expression of hair differentiation

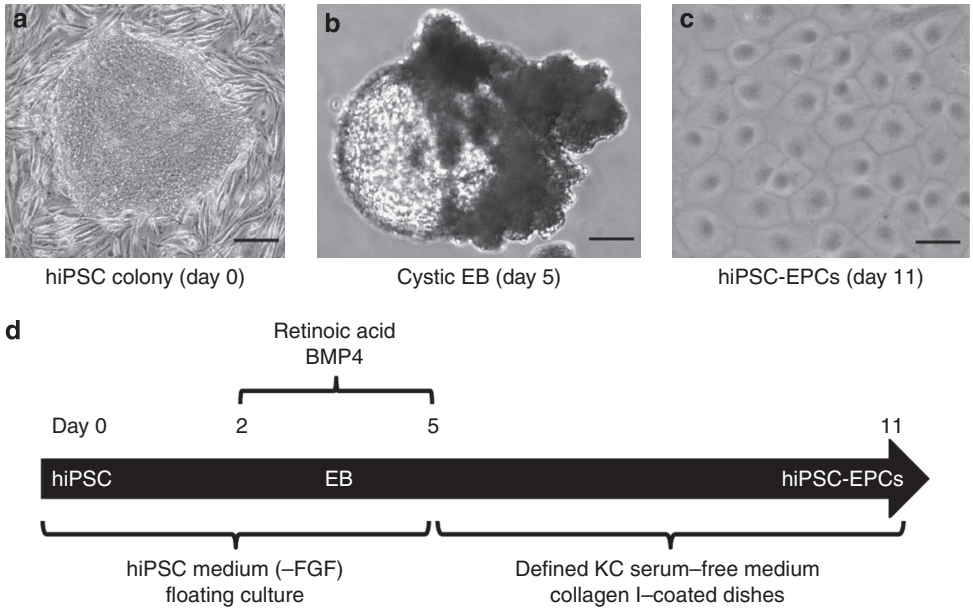

Figure 1. Protocol established for human induced pluripotent stem cell–derived ectodermal precursor cell (hiPSC-EPC) generation. (**a**–**c**) Morphological characteristics of hiPSCs during protocol, and their descendant cells. The differentiation protocol consists of (**a**) detachment of hiPSC colonies from feeder layer, floating culture of resultant embryoid body (EB) with retinoic acid and bone morphogenesis protein-4 (BMP4) to form (**b**) cystic EBs, and culture of EBs on collagen I–coated plates in defined keratinocyte (KC) serum-free medium to form (**c**) hiPSC-EPCs. Bar = 30 μm (**a**, **b**), 10 μm (**c**). (**d**) Summary of the established protocol. FGF, basic fibroblast growth factor.

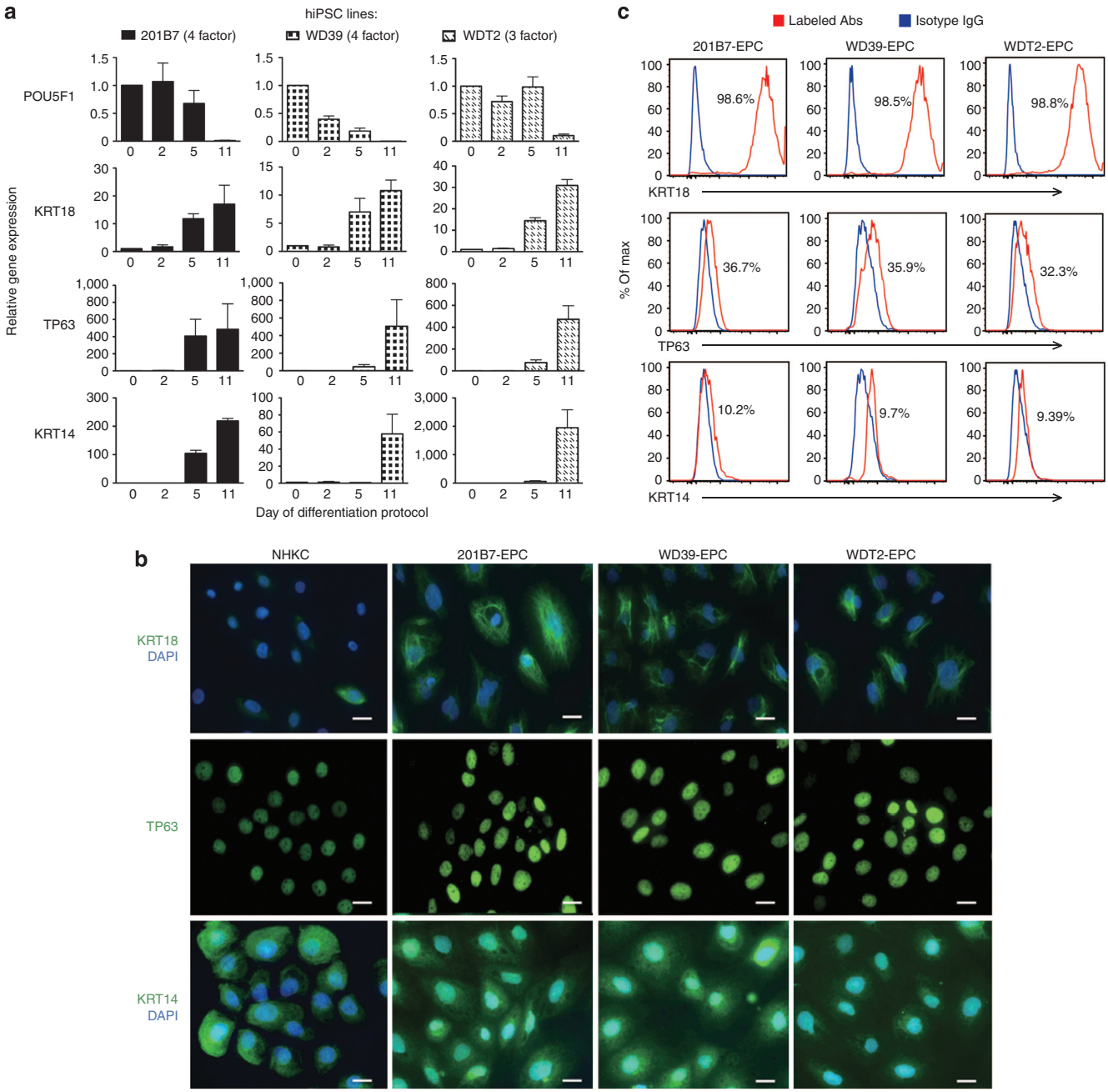

Figure 2. Characterization of three lines of human induced pluripotent stem cell–derived ectodermal precursor cells (hiPSC-EPCs). (**a**) Downregulation of multipotency marker, *POU5F1*, and increased expression of epithelial/keratinocyte lineage genes *KRT18, TP63*, and *KRT14* in each hiPSC line during the differentiation protocol. (**b**) Normal human keratinocytes (NHKCs) and day 11 hiPSC-EPC colonies demonstrated positive immunoreactivities for KRT18. NHKCs and the cell at the periphery of hiPSC-EPC colonies showed TP63- and KRT14-positive staining. (**c**) Flow cytometric analyses of day 11 hiPSC-EPCs derived from three respective hiPSC lines. Bar = 10 μm. Mean ± SEM ($n = 3$). DAPI, 4′,6-diamidino-2-phenylindole.

markers representing respective follicular epithelial components, including keratin 75 (*KRT75*), msh homeobox 2 (*MSX2*), lymphoid enhancer-binding factor 1 (*LEF1*), and trichorhinophalangeal syndrome type I (*TRPS1*; Rendl *et al.*, 2005; Kobielak and Fuchs, 2006; Porter, 2006; Gu and Coulombe, 2007; Fantauzzo *et al.*, 2008) in NHKCs ($P<0.05$; Figure 3b). When three lines of hiPSC-EPCs were cocultured, each line differentially responded to DP signals (Figure 3c, d). Compared to relative upregulation in NHKCs: 201B7 hiPSC-EPCs increased all markers tested, WD39 hiPSC-EPCs upregulated only *KRT75* and WDT2 hiPSC-EPCs downregulated all markers (Figure 3c). Importantly, 201B7 hiPSC-EPCs upregulated *KRT75* and *TRPS1* compared with NHKCs, suggesting their higher receptiveness to DP signals.

Epithelial–mesenchymal interactions are bidirectional in nature. Thus, we next evaluated whether hiPSC-EPCs could

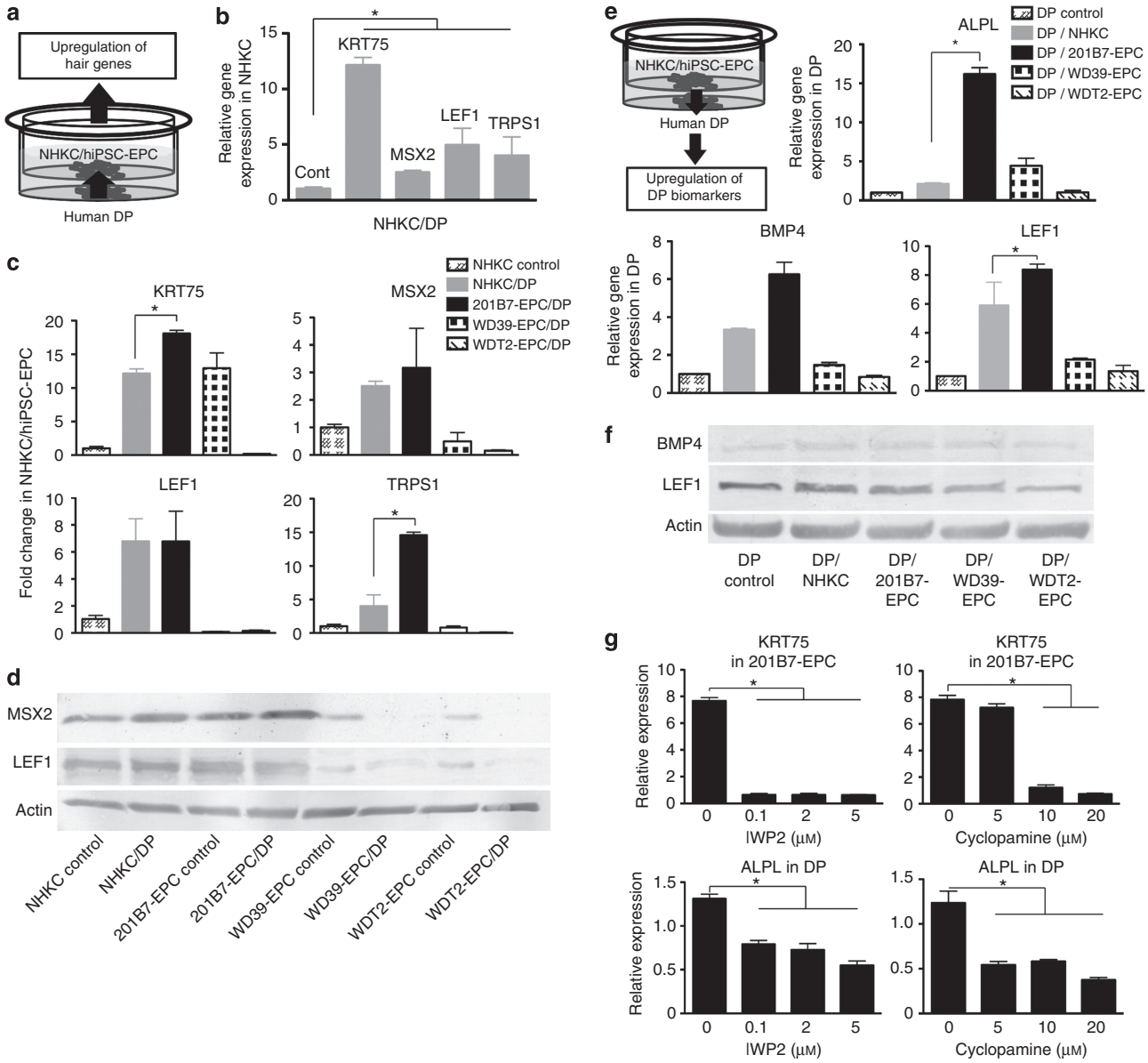

Figure 3. Human induced pluripotent stem cell–derived ectodermal precursor cells (hiPSC-EPCs) bidirectionally cross talk with human dermal papilla (DP) cells. (**a**) Illustration of the coculture system. (**b**) Normal human keratinocytes (NHKCs) upregulated hair-related genes in this system and served as positive controls. (**c**) Each hiPSC-EPC line differentially upregulated hair-related gene expression in coculture. 201B7 hiPSC-EPCs more intensely increased *KRT75, MSX2,* and *TRPS1* expression than NHKCs. (**d**) Confirmation by western blot analysis. (**e**) DP biomarkers were upregulated in DP cells when cocultured with NHKC/hiPSC-EPCs. 201B7 hiPSC-EPCs more strongly enhanced DP biomarker expression than NHKCs. (**f**) Confirmation by western blot analysis. (**g**) WNT and sonic hedgehog pathway inhibition downregulated *KRT75* in 201B7 hiPSC-EPCs and *ALPL* in DP cells, respectively, in coculture. *$P<0.05$ for (**b**, **c**, **e**, **g**). BMP4, bone morphogenetic protein-4; Cont, control; LEF1, lymphoid enhancer-binding factor 1; KRT75, keratin 75; MSX2, msh homeobox 2; TRPS1, trichorhinophalangeal syndrome type I.

ameliorate DP cell properties. Alkaline phosphatase, liver/bone/kidney (*ALPL*; Handjiski *et al.*, 1994; McElwee *et al.*, 2003; Iida *et al.*, 2007), *LEF1* (Kishimoto *et al.*, 2000), and *BMP4* (Rendl *et al.*, 2008) were selected as DP biomarkers, and their expression levels were monitored. After the coculture with NHKCs, DP cells upregulated *BMP4* and *LEF1* and slightly increased *ALPL* expression (Figure 3e). Strikingly, 201B7 hiPSC-EPCs markedly upregulated all three DP biomarkers compared with NHKCs, whereas such an increase was not observed in WD39 or WDT2 hiPSC-EPCs (Figure 3e and f).

Wingless-type MMTV integration site family (WNT) and sonic hedgehog (SHH) signaling pathways were known to have crucial roles in the interaction between hair matrix KCs and DP cells (Ohyama *et al.*, 2010). Interestingly, a WNT inhibitor, IWP 2 (Chen *et al.*, 2009), and a SHH inhibitor, cyclopamine (Nanba *et al.*, 2003), dose dependently downregulated *KRT75* in 201B7 hiPSC-EPCs and *ALPL* in

DP cells in coculture (Figure 3g), whereas these inhibitors did not exhibit any inhibitory effect on intrinsic expression of these genes in 201B7 hiPSC-EPCs or DP cells (Supplementary Figure S2 online). The expression of WNT or SHH downstream genes, including *AXIN2*/*PTCH1*/*GLI1*, were not clearly suppressed in a dose-dependent manner (data not shown); however, the above findings suggested that epithelial–mesenchymal interactions in this coculture system were mediated, at least in part, by those signaling pathways.

These findings implied that hiPSC-EPCs, in particular 201B7 hiPSC-EPCs, were capable of replicating the epithelial–mesenchymal cross talk in HFs. Such an interaction was not observed when non-induced hiPSCs were cocultured with human DP cells (Supplementary Figure S3 online) and when DP cells were replaced by human dermal fibroblasts (data not shown).

201B7 hiPSCs exhibited innate ectodermal/epithelial-prone differentiation and the traits of WNT-activated state when converted to EPCs

In vitro studies elucidated functional differences between 201B7 and WD39/WDT2 hiPSC-EPCs. To probe their natural fate, hiPSC lines were converted into EBs and allowed to differentiate without any induction. All lines downregulated pluripotency markers *SOX2* and *POU5F1* following EB conversion and differentiation (Figure 4a). Interestingly, 201B7 hiPSCs more readily expressed microtubule-associated protein 2 (*MAP2*), an ectoderm marker, and epithelial markers *KRT8* and *KRT18* than other hiPSC lines (Figure 4a). On the other hand, WD39 and WDT2 hiPSCs were prone to express endoderm (SRY-box 17 (*SOX17*), forkhead box A2 (*FOXA2*), α-fetoprotein (*AFP*)) and mesoderm (msh homeobox 1 (*MSX1*), T, brachyury homolog (mouse) [*BRACHURY (T)*]) markers (Figure 4a; Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). Thus, 201B7 hiPSCs differentiated more along the ectoderm/epithelial lineages compared with WD39 and WDT2 hiPSCs.

To evaluate whether such a characteristic is unique to 201B7 hiPSCs, three additional possible ectodermal-prone hiPSC lines (Nakagawa *et al.*, 2008; Okita *et al.*, 2011) were differentiated by the same protocol. Interestingly, 414C2 hiPSCs expressed differentiation markers in a manner similar to 201B7 hiPSCs (Supplementary Figure S4a online). However, in coculture, 414C2 hiPSC-EPCs responded to DP signals to upregulate *KRT75*, *MSX2*, and *TRPS1*, but not

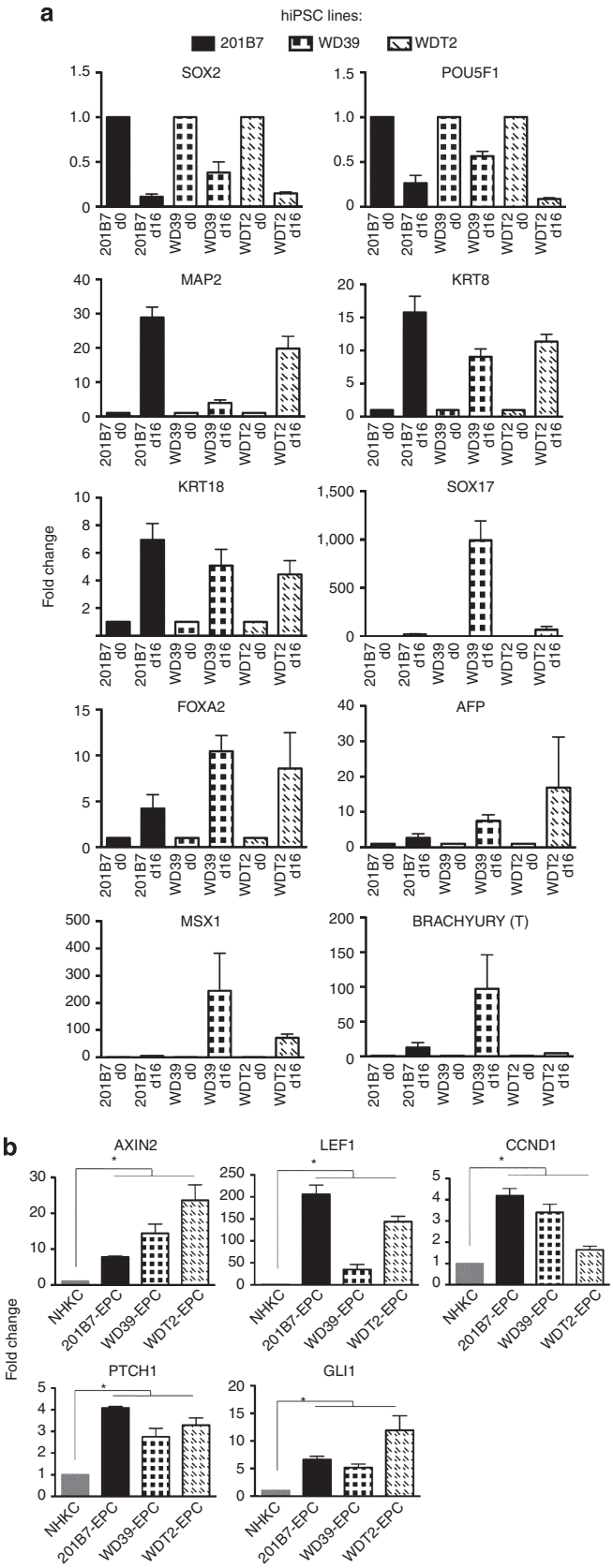

Figure 4. Characterization of the intrinsic properties of human induced pluripotent stem cell (hiPSC) lines and derived hiPSC-derived ectodermal precursor cell (EPC) lines. (**a**) Embryoid body formation downregulated pluripotency markers *SOX2* and *POU5F1* in all hiPSC lines, indicating successful induction of differentiation. 201B7 hiPSCs more highly upregulated ectoderm (*MAP2*) and epithelial (*KRT8* and *KRT18*) markers than other lines. WD39 and WDT2 hiPSCs were more prone to express endoderm (*SOX17*, *FOXA2*, and *AFP*) and mesoderm (*MSX1* and BRACHURY (*T*)) markers compared with 201B7 hiPSCs. (**b**) hiPSC-EPCs more intensely upregulated WNT and sonic hedgehog (SHH) signaling genes than NHKCs. The differential expression of *AXIN2*, *LEF1*, and *CCND1* implied that 201B7 hiPSC-EPCs were in a higher activated WNT state than other hiPSC-EPCs, whereas such a trend was not observed in SHH signaling gene expression (*$P<0.05$).

LEF1 (Supplementary Figure S4b online). In addition, 414C2 hiPSC-EPCs failed to upregulate DP biomarkers (Supplementary Figure S4c online).

Previous studies demonstrated that the activation of WNT signaling dispose KCs to HF fate (Zhang *et al.*, 2008). Interestingly, all hiPSC-EPCs expressed higher levels of WNT signaling genes than NHKCs (Figure 4b). 201B7 EPCs expressed a lower level of WNT-negative regulator axin 2 (*AXIN2*; Jho *et al.*, 2002) but higher levels of *LEF1* and WNT downstream target cyclin D1 (*CCND1*; von Gise *et al.*, 2011) compared with WD39 and WDT2 EPCs, implying that 201B7 EPCs might be in a higher WNT-activated state than other lines (Figure 4b). SHH signaling genes also have crucial roles in HF morphogenesis (Nanba *et al.*, 2003). Patched 1 (*PTCH1*) and GLI family zinc finger 1 (*GLI1*) were higher in all hiPSC-EPC lines compared with NHKCs, although upregulation was not distinctively intense in 201B7 EPCs (Figure 4b).

These findings implied that intrinsic functional differences among individual hiPSC lines exist, which may explain why 201B7 hiPSC-EPCs better communicated with DP cells *in vitro*.

Co-grafting of 201B7 hiPSC-EPCs and trichogenic mice dermal cells in *in vivo* environment generated HF structures

Finally, ''patch'' hair reconstitution assay was performed by co-grafting 201B7 hiPSC-EPCs and trichogenic mice neonatal dermal cells (MDCs) into subcutaneous spaces of nude mice ($n=4$). NHKCs were similarly co-transplanted for comparison ($n=4$). Impressively, when 201B7 hiPSC-EPCs were co-grafted with trichogenic MDCs, cysts containing multiple HFs were formed, whereas NHKCs failed to form such structures (Figure 5a, Table 1). A small fibrotic cyst with several HF structures were formed by MDCs alone in one out of four experiments, possibly because of minor mice epithelial cell contamination (Figure 5b, Table 1). Both hiPSC-EPCs and normal mice KCs co-grafted with MDCs reconstituted morphologically normal HFs with sebaceous glands (Figure 5c–e). Interestingly, the HFs formed in the 201B7 hiPSC-EPC patch were of a significantly larger diameter ($60.4 \pm 14.8\,\mu m$) compared with those formed from the positive controls ($23.4 \pm 5.5\,\mu m$; $P<0.05$).

Importantly, positive reactivity for human-specific nuclear antigen, which is globally expressed in normal human HFs (Figure 5f), was preferentially detected in hair shafts and matrix of HFs in 201B7 hiPSC-EPC patches (Figure 5g), whereas such staining was not detected in HFs reconstituted only with murine cells (Figure 5h). Around 15–20% of cells were found to be of human origin in a human signal positive HF. These observations were further confirmed by the positive staining of human-specific cytoplasmic marker

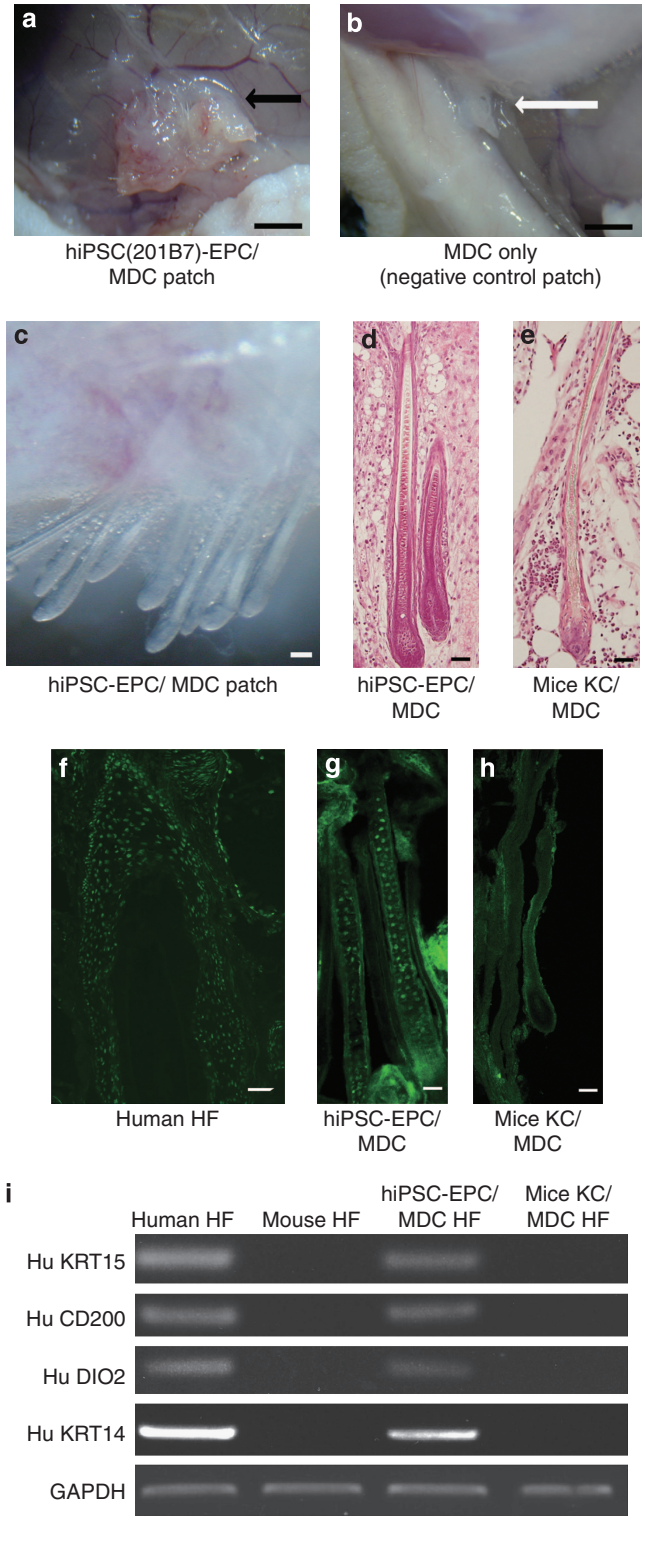

Figure 5. Co-grafting of 201B7 human induced pluripotent stem cell–derived ectodermal precursor cells (hiPSC-EPCs) and mice dermal cells generated hair follicle structures. (**a**, **b**) 201B7 hiPSC-EPCs formed a follicular cyst (arrow) when co-transplanted with mice dermal cells (MDCs). (**b**) Grafting only MDCs resulted in fibrotic cyst (arrow) formation. (**c**) Morphology of hair follicles (HFs) formed in 201B7 hiPSC-EPC patches. (**d**, **e**) Normal pilosebaceous unit structure in 201B7 hiPSC-EPC (**d**) and positive control (**e**) patches. (**f**, **g**, **h**) Positive human nuclear antigen staining was detected in normal human HFs (**f**) and in the 201B7 hiPSC-EPC patch (**g**) but not in the control patch (h). (**i**) Human (Hu)-specific hair stem cell and basal keratinocyte (KC) gene expression in HFs of 201B7 hiPSC-EPC patch. Bars = 0.25 cm (**a**, **b**), 50 μm (**c**–**h**). (**d**, **e**) Hematoxylin and eosin staining. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1. Summary of *in vivo* hair reconstitution assays

Epithelial component	Mice dermal cells	Number of experiments	Efficiency of HF formation[1] (%)	Gross phenotype of structures formed	HF density (HFs per cyst)	Human HF stem cell marker expression
Mouse neonatal KCs	Combined	4	4/4 (100%)	Cyst filled with HFs	>50	(−)
Human adult KCs	Combined	4	0/4 (0%)	No structures formed	NA	NA
201B7 hiPSC-EPCs	**Combined**	**4**	**4/4 (100%)**	**Cyst filled with HFs**	**>50**	**(+)**
201B7 hiPSCs without differentiation	Combined	2	2/2 (100%)	Cyst with HFs and marked fat tissue	>50	(−)
201B7 hiPSC-embryoid body (day 5 of KC differentiation)	Combined	2	2/2 (100%)	Cyst with HFs and marked fat tissue	>50	(−)
None	As is	4	1/4 (25%[2])	Fibrotic cyst	<10	(−)

Abbreviations: HF, hair follicle; hiPSC-EPCs, human induced pluripotent stem cell–derived ectodermal precursor cells; KC, keratinocytes; NA, not applicable.
[1]The number of sites with HF formation observed/the number of grafts performed.
[2]In one sample, a few HF structures were observed in a fibrotic cyst possibly because of minor mice epithelial cell contamination.
Bold indicates the positive and main finding of this study.

(Supplementary Figure S5 online). In addition, HFs harvested from 201B7 hiPSC-EPC patches ($n=4$) expressed human-specific HF stem cell marker genes *KRT15, CD200,* and *DIO2* (Ohyama *et al.*, 2006), and a basal KC marker *KRT14*, whereas such messages were not detectable in controls ($n=4$; Figure 5i). Thus, 201B7 hiPSC-EPCs repopulated in HF structures, but with low efficiency. When the same assay was performed using undifferentiated 201B7 hiPSC and day 5 EBs, similar HF formation was observed (Table 1). However, human-derived cells were scattered only in connective and fat tissue (data not shown), and the reconstituted HFs did not express human-specific markers (Supplementary Figure S6 online), suggesting that 201B7 hiPSCs and derived cells supported HF formation via non–cell autonomous activities.

DISCUSSION

Recent studies have reported successful induction of fully differentiated KCs from hiPSCs (Itoh *et al.*, 2011); however, hiPSC-EPCs might be more preferable than hiPSC-KCs for testing our hypothesis that less committed cells better interact with the hair-inductive mesenchyme. At the same time, the use of hiPSC-EPCs represents the major drawback of the current study because of the limitation in hiPSC-EPC characterization. On the basis of global KRT18 expression, hiPSC-EPCs resembled epithelial progenitors (Maurer *et al.*, 2008). The presence of putative KC lineage cells in hiPSC-EPCs was also suggested. Yet, these findings could only conclude that hiPSC-EPCs represent a heterogeneous cell population comprising putatively multipotent ectodermal derivatives. Considering that noninduced hiPSCs failed to communicate with DP cells in coculture, commitment to ectodermal lineage seems to be necessary to interact with trichogenic mesenchyme. To clarify which hiPSC-EPC subset contributed to HF formation, hiPSC-EPCs need to be sorted into functionally distinct subsets before being tested. Identification of cell surface markers that enable hiPSC-ECP sorting is in progress. Accordingly, NHKCs may not be an appropriate control for hiPSC-EPCs in some experiments. Ideally, functional comparisons should be performed among isolated KC-prone hiPSC-EPC subpopulation, hiPSC-KCs (Itoh *et al.*, 2011), and NHKCs. Unfortunately, at least in our hands, sufficient number of hiPSC-KCs (Itoh *et al.*, 2011) for patch assay have not been successfully harvested. Whether the HF-prone nature is intrinsic to hiPSC-EPCs or shared with hiPSC-derived KCs represents the next important questions to be addressed.

Unlike the increase in HF marker expression in hiPSC-EPCs evoked by DP cells, the differential upregulation of DP genes induced by hiPSC-EPCs was not fully reflected at the protein level, leaving the possibility that the functional differences among EPCs may not be as evident as those predicted at the gene expression levels. However, the findings supported the importance of establishing an assay to pre-assess intrinsic properties of individual hiPSC lines. We are aware that the current system capitulated limited aspects of the complex cross talk in HFs (Ohyama *et al.*, 2010; Yang and Cotsarelis, 2010). Considering inconsistent upregulation or down-regulation of WNT and SHH downstream genes in the inhibition experiments, we could not exclude the possibility that the increase in hair follicle KC/DP-related gene expression resulted from intracompartmental cross talk. Reconstruction of the *in vivo* anatomical relationship between KC and DP cells by folliculoid spheres (Havlickova *et al.*, 2009) or three-dimensional skin equivalents (Havlickova *et al.*, 2004) may be necessary to accurately assess the interaction between hiPSC-EPCs and DP cells.

An obvious question is which underlying mechanism enabled 201B7 hiPSC-EPCs to better communicate with DP cells. hiPSC lines were generated either from facial (201B7) or scalp (WD39 and WDT2) dermal fibroblasts, which might have influenced their properties. The 201B7 hiPSC line was shown to be ectodermal/epithelial prone, but another hiPSC line with similar properties failed to achieve an equivalent level of bidirectional interaction with DP cells *in vitro,* implying that the ectodermal/epithelial-prone nature alone is not a definitive factor. Other innate characteristics, including the possible WNT-activation status, might have contributed to the

capacity to regenerate HFs, which was demonstrated in, but may not be limited to, the 201B7 EPC line.

Previous studies in which MDCs or rat DP cells were combined with KCs of other species reported chimeric HF formation (Ferraris *et al.*, 1997; Ehama *et al.*, 2007; Kobayashi *et al.*, 2010). Different HF reconstitution assays were adopted in these studies (Ferraris *et al.*, 1997; Ehama *et al.*, 2007), and the repopulation efficiency of human cells in HFs cannot be directly compared with that in this study. Nevertheless, the direct contribution of hiPSC-EPCs to HF structures seems to be very low. In fact, despite successful detection of human-specific HF stem cell gene expression, intensive immunohistological examination failed to clearly locate human-derived cells in the bulge region of regenerated HFs. As human-derived cells were detected mainly in the shaft and the matrix, it is possible that the epithelial compartment of HFs was originally composed of hiPSC-EPCs, but was gradually replaced by mice epithelial cells contaminated in MDCs or newly recruited bone marrow cells (Ehama *et al.*, 2007; Tamai *et al.*, 2011). We harvested patch structures at earlier time points; however, we have not obtained any finding to support this speculation so far. Failure of HF reconstitution by 201B7 hiPSC-EPCs and human DP cells in a pilot study and the limited supply of human DP cells forced us to use MDCs. As DP cells lose intrinsic properties in culture, the use of freshly isolated human DPs or functionally restored DP cells (Ohyama *et al.*, 2012) may solve the complexity of the hybrid HF reconstitution assay.

Although direct participation in HF structures was not observed, co-grafting of 201B7 hiPCSs and EBs with MDCs also gave rise to HFs. The observations that HF formation was achieved only in the presence of hiPSCs and derived cells suggested that these cells promoted HF regeneration via non–cell autonomous activities emanating from the cells. Mesenchymal and adipose-derived stem cells secrete mediators and support tissue regeneration (Meirelles Lda *et al.*, 2009). Similarly, hiPSCs and derived cells might have released factors promoting HF formation, for instance, BMP6, WNT10B, or high-mobility group box 1 (Rendl *et al.*, 2008; Tamai *et al.*, 2011; Li *et al.*, 2012). Identification of responsible factors may be possible by secretome analysis of hiPSC-EPC-conditioned culture medium (Skalnikova *et al.*, 2011).

In aggregate, the current study suggested a, to our knowledge, previously unrecognized advantage of using hiPSCs for the enhancement of epithelial–mesenchymal interactions to achieve HF bioengineering. These data also highlight the necessity of the development of organ-specific *in vitro* screening methods, such as the coculture system in this study, which enables efficient assessment of functional aspects of hiPSC lines before full-scale, demanding investigations.

MATERIALS AND METHODS

Generation of hiPSCs

Lines of hiPSCs retrovirally induced with 4 (201B7 and WD39) or 3 (WDT2) factors (*POU5F1*, *SOX2*, *KLF4* +/− *MYC*) were generated from dermal fibroblasts derived from the facial or scalp dermis and characterized as previously described (Takahashi *et al.*, 2007; Ohta *et al.*, 2011; Imaizumi *et al.*, 2012). Information of additional hiPSC lines and the protocol for the maintenance of hiPSCs was provided in Supplementary materials and methods. Passage 20–35 hiPSC lines were selected for differentiation studies (Hussein *et al.*, 2011). All procedures were approved by the Institutional Review Board of Keio University (Protocol No. 17-75(3)) and carried out in accordance with the university's ethical guidelines. All human donors provided written informed consent in accordance with the Declaration of Helsinki Priniciples.

Generation of hiPSC-EPCs

EBs were formed (Metallo *et al.*, 2008; Bilousova *et al.*, 2011) and were kept for 2 days in floating culture; they were then exposed to 1 μM all-trans retinoic acid (Sigma, St Louis, MO) and 25 ng ml^{-1} BMP4 (R&D Systems, Minneapolis, MN) for an additional 3 days and transferred onto collagen I–coated plates (Asahi Glass, Tokyo, Japan) in defined KC serum-free medium (Life technologies, Carlsbad, CA) at 5% CO_2 and 37 °C for a further 6 days. The detailed protocol was provided in Supplementary materials and methods. For terminal KC differentiation assays, day 11 hiPSC-EPCs were either passaged into low (<0.1 mM)- or high (1.2 mM)-calcium conditions.

Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCRs were performed as previously described (Kobayashi *et al.*, 2010). Details were provided in Supplementary materials and methods. All primers were listed in Supplementary Table S1 online.

Immunocytochemistry

Immunocytochemistry was performed using primary mouse monoclonal antibodies: anti-human keratin 18 (1:100, ab668, Abcam, Cambridge, UK), anti-human tumor protein p63 (1:100, ab735, Abcam), or anti-human keratin 14 (1:100, ab7800, Abcam). Details were provided in Supplementary materials and methods.

Flowcytometric analysis

Flowcytometric analyses were performed as previously described (Ohyama *et al.*, 2006) using BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ). See Supplementary materials and methods for details.

Coculture of KCs/hiPSC-EPCs and DP cells

KC/hiPSC-EPC and DP coculture was established as previously described (Inui *et al.*, 2002) with modifications. Briefly, adult human KCs derived from the epidermis or hiPSC-EPCs (2.5×10^5 cells) were seeded onto collagen-coated permeable transwell inserts (Corning, Corning, NY) and cocultured with 80% confluent passage 2 human DP cells in DMEM:F12 (3:1). On day 2 or 4 (WNT and SHH inhibition experiment), total RNA and protein was extracted. See Supplementary materials and methods for detail.

Western blot

Western blot analysis was performed as described in Supplementary materials and methods using goat anti-β actin (1:1,000, ab8229, Abcam); rabbit anti-human LEF1 (1:200, ab22884, Abcam); rabbit anti-mouse/human MSX2 (1:100, sc-15396, Santa Cruz biotechnology, Santa Cruz, CA); and goat anti-human BMP2/4 (1:100, AF355, R&D systems) primary antibodies.

Assessment of intrinsic differentiation pathways in individual hiPSC lines

EBs were formed from individual hiPSC lines and cultured on gelatin. On day 0 and day 16, total RNA was isolated to assess differentiation marker expression by real-time PCR (Takahashi *et al.*, 2007). Details were described in Supplementary materials and methods.

Patch assay

Patch grafting assays were performed as described previously (Zheng *et al.*, 2005; Kobayashi *et al.*, 2010). Mouse KCs generated from epidermal sheets (Kobayashi *et al.*, 2010) and adult human KCs were used as controls. Each cell mixture composed of 2.5×10^6 epithelial and 1×10^7 dermal cells was subcutaneously injected into BALB/$c^{nu/nu}$ nude mice. Host mice were killed 2–3 weeks after grafting in order to harvest regenerated structures. See Supplementary materials and methods for details.

Human-specific antigen detection

Specimens were incubated with Alexa Fluor 488–conjugated mouse monoclonal anti-human nuclei (1:100, MAB1281, EMD Millipore, Billerica, MA) or anti-human cytoplasm antibodies (1:00, STEM121, StemCells, Newark, CA) and observed under a confocal microscope. Details were described in Supplementary materials and methods.

Statistical analysis

Statistical differences were identified by two-sided Student's *t*-test. A *P*-value of <0.05 was considered significant.

CONFLICT OF INTEREST

Shinya Yamanaka is a member without salary of the scientific advisory boards of iPierian, iPS Academia Japan, Megakaryon Corporation, and Retina Institute Japan. The remaining 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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