Human embryonic stem cell-derived keratinocytes exhibit an epidermal transcription program and undergo epithelial morphogenesis in engineered tissue constructs

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Human Embryonic Stem Cell-Derived Keratinocytes Exhibit an Epidermal Transcription Program and Undergo Epithelial Morphogenesis in Engineered Tissue Constructs

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Human embryonic stem (hES) cells are an attractive source of cellular material for scientific, diagnostic, and potential therapeutic applications. Protocols are now available to direct hES cell differentiation to specific lineages at high purity under relatively defined conditions; however, researchers must establish the functional similarity of hES cell derivatives and associated primary cell types to validate their utility. Using retinoic acid to initiate differentiation, we generated high-purity populations of keratin 14+ (K14) hES cell-derived keratinocyte (hEK) progenitors and performed microarray analysis to compare the global transcriptional program of hEKs and primary foreskin keratinocytes. Transcriptional patterns were largely similar, though gene ontology analysis identified that genes associated with signal transduction and extracellular matrix were upregulated in hEKs. In addition, we evaluated the ability of hEKs to detect and respond to environmental stimuli such as Ca^{2+}, serum, and culture at the air–liquid interface. When cultivated on dermal constructs formed with collagen gels and human dermal fibroblasts, hEKs survived and proliferated for 3 weeks in engineered tissue constructs. Maintenance at the air–liquid interface induced stratification of surface epithelium, and immunohistochemistry results indicated that markers of differentiation (e.g., keratin 10, involucrin, and filaggrin) were localized to suprabasal layers. Although the overall tissue morphology was significantly different compared with human skin samples, organotypic cultures generated with hEKs and primary foreskin keratinocytes were quite similar, suggesting these cell types respond to this microenvironment in a similar manner. These results represent an important step in characterizing the functional similarity of hEKs to primary epithelia.

Introduction

Pluripotent cells maintain the capacity to proliferate extensively *in vitro* and differentiate into lineages of the three embryonic germ layers. In the form of blastocyst-derived human embryonic stem (hES) cells,[1] these cell lines offer tremendous potential for use in scientific research, diagnostic, and clinical applications. Researchers have recently made significant advances in the directed differentiation of hES cells to lineages of interest, and in some cases these methods are capable of generating high-purity populations of specific cell types.[2–4] Incorporation of these derivatives into engineered tissues will permit better characterization of their functionality in a more *in vivo*-like microenvironment.[5] Systematic comparisons of differentiated cells to their primary, somatic counterparts in the context of these functional studies will be even more informative.[6] For example, will hES cell–derived progenitors respond appropriately to biochemical or biophysical signals in their environment? Will ES cell–derived precursors undergo terminal differentiation or display a more invasive phenotype given their embryonic origin? Researchers must address these issues to determine the similarity of and identify any disparities between hES cell derivatives and their somatic counterparts.

Epithelial cells of the ectoderm form the epidermis, mammary glands, cornea, and other tissues, performing various functions depending on their tissue of origin.[7] Although adult epithelia typically retain a high capacity for regeneration and have been effectively used in tissue engineering and cell biology studies, there is considerable interest in the generation of epithelial lineages from hES cells.[8–10] ES cell-derived epithelia may provide novel insights into the development of certain ectodermal tissues, as various disorders are attributed to genetic abnormalities in these cell types.[11,12] Further, epithelial

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cells often initiate aggressive tumors (i.e., carcinomas), and so the ability to generate epithelial derivatives from genetically modified clones would be particularly useful in studying the mechanisms of carcinogenesis.[13,14] Finally, the proliferative capacity of pluripotent human cells makes them an attractive source of material for clinical applications of tissue engineering.[3] In the end, each of these applications requires that hES cell-derived epithelia are functionally similar to somatic cells.

Various methods have been developed to generate keratinocytes and keratinocyte precursors from hES cells. Recently, we identified retinoic acid (RA) application as a potent means of directing hES cells to epithelial lineages when applied in the context of bone morphogenetic protein (BMP) signaling.[10] BMP-4 alone has been demonstrated to generate early ectodermal progenitors from hES cell lines grown on a secreted matrix.[8] As is the case for other lineages, marker expression alone is not sufficient to demonstrate cell-specific functionality; therefore, hES cell-derived keratinocytes (hEKs) must be characterized to determine their ability to terminally differentiate in complex microenvironments. Organotypic culture systems offer an effective means of gauging cellular differentiation *in vitro*, and various modifications of this technique are available.[15–18] By cultivating cells in more complex, tissue-specific microenvironments such as basement membrane analogs or at the air–liquid interface (ALI), researchers can more effectively assess cell function and tissue morphogenesis. Recently, Hewitt *et al.* cultivated a mixture of hES cell-derived epithelial precursors and accompanying mesenchymal cells at the ALI and successfully detected expression of keratins (K12) and basement membrane proteins, though no stratification was observed in this system.[19]

In our study we have used microarray analysis to quantitatively compare the transcriptional profile of hEKs with that of primary foreskin keratinocytes (PFKs) cultivated *in vitro*. Next, we evaluated the ability of hEKs to detect and respond to biochemical cues in submerged cultures, observing the induction of epithelial differentiation markers via immunofluorescence and immunoblotting. Finally, organotypic skin cultures were generated using hEKs to characterize the ability of these derivatives to undergo epithelial morphogenesis and terminally differentiate at the ALI.

Materials and Methods

Cell culture and differentiation

H9 hES cells[1] were cultured on irradiated mouse embryonic fibroblasts (MEFs) in unconditioned hES cell medium (UM) (Dulbecco's modified Eagle's medium (DMEM)/F12 containing 20% knockout serum replacer, 1× minimal essential medium (MEM) nonessential amino acids, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol) supplemented with 4 ng/mL basic fibroblast growth factor (bFGF) or in TeSR medium supplemented with 100 ng/mL bFGF and 20% knockout serum replacer. Conditioned medium (CM) was prepared by incubating irradiated MEFs overnight in UM without bFGF; before use, bFGF 4 ng/mL was added, and CM was sterile filtered. hES cells were transferred to Matrigel (BD Biosciences, San Jose, CA)-coated plates, cultured in CM for at least two passages before differentiation to remove MEFs from culture, and differentiated as previously described.[10] Briefly, cells on Matrigel were treated with 1 μM all-trans RA in UM for 7 days, changing medium daily. RA-induced cells were passaged using Dispase (2 mg/mL) onto gelatin-coated plates in defined keratinocyte serum-free medium (DSFM). After 2–4 weeks, differentiated cells were subcultured onto gelatin-coated plates using trypsin–ethylenediaminetetraacetic acid (EDTA) (0.5 g/L trypsin + 0.2 g/L EDTA; Invitrogen, Carlsbad, CA) to obtain high-purity epithelial cell populations. Cells at this stage were initially seeded at 20,000 cells/cm^2; in subsequent passages hEKs were plated at 5000 cells/cm^2. PFKs were obtained from Invitrogen and cultured in parallel with hEKs in DSFM on porcine gelatin or human collagen IV (Sigma, St. Louis, MO)-coated plates. Similar morphology and growth kinetics were observed when using either matrix. In some cases the cultured epithelial cells were cultured in DSFM with 1 mM Ca^{2+} or flavinoid adenine dinucleotide (FAD) medium, which consisted of 3:1 F12/DMEM basal medium, 2.5% fetal bovine serum, 0.4 μg/mL hydrocortisone (Sigma), 8.4 ng/mL cholera toxin (Sigma), 5 μg/mL insulin (Sigma), 24 μg/mL adenine (Sigma), 10 ng/mL epidermal growth factor, and 1× antibiotic/antimycotic with or without 0.66 mM Ca^{2+}, to induce differentiation. All cell culture reagents were obtained from Invitrogen, unless otherwise noted.

Flow cytometry

Cells were detached from culture plates using trypsin–EDTA, fixed in 1% paraformaldehyde for 10 min at 37°C, and permeabilized in flow cytometry buffer (phosphate-buffered saline [PBS] with 2% fetal calf serum, 0.1% NaN_3, and 0.1% Triton X-100). Primary antibodies were incubated overnight in flow cytometry buffer; control samples were included using no primary antibody. After a 1 h secondary staining the cells were analyzed on a FACScalibur flow cytometer using CellQuest software.

Microarray sample preparation

Total RNA was extracted from subcultured hEKs (three independent differentiation experiments) or PFKs using the RNeasy Mini kit (Qiagen, Valencia, CA). Ten micrograms of total RNA was used to synthesize cDNA with a Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen). One microgram each of hEK and PFK cDNA was labeled with Cy3- and Cy5-labeled wobble nonamers, respectively, and 6 μg of each labeled cDNA from parallel cultures was competitively hybridized to Nimblegen arrays according to the manufacturer's instructions (Roche NimbleGen, Madison, WI). The full human genome array design was 385 K 2006-08-03_HG18_60mer_expr, which targets over 47,000 genes with eight probes/target.

Microarray data analysis

After robust multiarray analysis normalization, array data were processed using Arraystar software (DNASTAR, Madison, WI). A Student's *t*-test and false discovery rate (FDR) (Benjamini Hochberg) method were used to identify differentially expressed genes.[20] For most discussion and analysis, we used a gene set obtained with a *p*-value cutoff of 0.05 and at least a fourfold difference in expression levels. For gene ontology (GO) analysis we performed functional annotation of our gene set using the DAVID tool (http://david.abcc.ncifcrf.gov/).[21] Annotated GO terms were limited to those with an expression analysis systematic explorer score less than 0.001. Redundant annotations were eliminated for brevity. Array

data were deposited in the Gene Expression Omnibus at www.ncbi.nlm.nih.gov/geo/ (series GSE17265).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was conducted as previously described.[10] Briefly, 1 μg of RNA was used to generate cDNA using oligo-dT primers and Omniscript RT (Qiagen). qPCR was performed using Quantitect SYBR Green PCR kit with 1 μL cDNA and gene-specific primers on an iCycler (Bio-Rad, Hercules, CA) with an annealing temperature of 54°C. Relative expression levels were calculated as $2^{\Delta\Delta CT}$ after normalizing to the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Gene-specific primers are listed in Supplemental Table S1 (available online at www.liebertonline.com/ten).

Antibodies and immunostaining

Postconfluent cells in monolayer culture were fixed in 4% paraformaldehyde for 20 min, rinsed in PBS, quenched with 100 mM glycine, and incubated in blocking buffer (5% chick serum with 0.4% [v/v] Triton X-100 in PBS) for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C in blocking buffer, and after subsequent washes in PBS, fluorophore-conjugated secondary antibodies were applied for 1 h at room temperature. Nuclear staining was accomplished using Hoechst dyes. Primary antibodies used for immunostaining and immunohistochemistry (IHC) comprised rabbit anti-keratin 14+ (K14) polyclonal (Lab Vision, Fremont, CA), mouse anti-Mucin 1 (MUC1) monoclonal (clone GP1.4; Lab Vision), mouse anti-K19 monoclonal (clone A53-B/A2.26; Lab Vision), mouse anti-p63 monoclonal (clone 4A4; Lab Vision), mouse anti-K10 monoclonal (clone DE-K10; Lab Vision), mouse anti-Ki67 monoclonal (clone MM1; Vector Laboratories), mouse anti-filaggrin monoclonal (clone FLG01; Lab Vision), mouse anti-involucrin monoclonal (clone SY5; Lab Vision), and goat anti-involucrin polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA). Species-specific secondary antibodies were obtained from Invitrogen and were conjugated to Alexa 488, 594, and 647 dyes. Immunofluorescence images were acquired on an Olympus IX70 microscope using MetaVue imaging software.

Immunoblotting

Cellular protein was harvested from postconfluent cells incubated in the specified media and quantified as previously described.[10] Equal amounts of protein were resolved on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% milk in tris-buffered saline (TBS), membranes were probed with primary goat anti-involucrin (Santa Cruz Biotechnology) and rabbit anti-K14 (Lab Vision) polyclonal antibodies overnight and stained with horseradish peroxidase-conjugated secondary antibodies for 1 h. The proteins levels were detected by chemiluminescence (Pierce, Rockford, IL), and protein loading was verified by probing against β-actin.

Organotypic skin culture

Dermal constructs were prepared by combining high-concentration rat tail collagen I (4 mg/mL; 80% final volume; BD Biosciences) and 10×DMEM (10% final volume; Sigma) on ice and adjusting the pH with 1 N NaOH. Normal human dermal fibroblasts were resuspended in ice-cold fetal bovine serum (10% final volume; 500,000 cells/mL final volume) and added to the collagen mixture. Two milliliters of the collagen/fibroblast mixture was added to 1 μm pore size inserts in a six-well plate and incubated at 37°C to gel for 1 h. After gelation, the wells were flooded with prewarmed FAD medium. The dermal constructs were gently detached from the sides of wells using a Pasteur pipette to facilitate contraction over 1–2 days. About $2–3\times10^6$ epithelial cells was seeded on contracted gels in 100 μL of FAD medium and allowed to attach for 2 h before flooding the wells. After 2 days in submerged culture, organotypic cultures were rinsed with stratification medium (FAD medium with 0.66 mM Ca^{2+}) and transferred to deep well plates so that epithelial cells were at the ALI. The constructs were maintained at the ALI for 2–3 weeks before analysis.

Histology and IHC

Organotypic skin cultures were removed from well inserts and embedded in 2% agar with 1% formalin. The agar-encased tissues were fixed overnight in 10% formalin at 4°C and stored in 70% ethanol before embedding in paraffin. Sections were cut at 5 μm and mounted on charged slides. The slides were deparaffinized in three changes of xylene and dehydrated in graded alcohols to water. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Antigen retrieval was completed according to the manufacturer's instructions for each antibody. After antigen retrieval, the slides were blocked in 5% goat serum and incubated for 1 h with titered primary antibodies. After washing, slides were incubated with secondary antibodies for 1 h, stained with hematoxylin, eosin, and/or 3,3′-diaminobenzidine, and mounted with antifade reagent.

Statistical analysis

Data analysis of microarray experiments was conducted as described earlier. All results were reproduced in at least three independent experiments.

Results

Global transcriptional analysis of hEKs and PFKs

To characterize the similarity between hEKs and neonatal keratinocytes, we performed microarray analysis of *in vitro*-cultivated hEKs and PFKs. Three independent hES cell cultures were differentiated as described earlier and analyzed along with parallel PFK cultures. The purity of all cultures was verified via cell morphology and K14 staining by flow cytometry (Fig. 1A, B). Although unsorted populations were used, extensive characterization of these derivative populations has demonstrated the absence of undifferentiated hES cells and high purity of epithelial markers.[10] Initially, we performed a stringent analysis to identify differentially expressed genes, limiting the output to those which had FDR-adjusted *p*-values less than 0.01. Under these restrictions less than 10 genes were differentially expressed (*IGF2, CAPN6, SFRP2, HAND2, FKBP10, TCF7L2, ZP3, HOXC11*, and *RPS4Y1*). In subsequent analysis using a *p*-value cutoff of 0.05 and FDR test we obtained ~700 transcripts (some

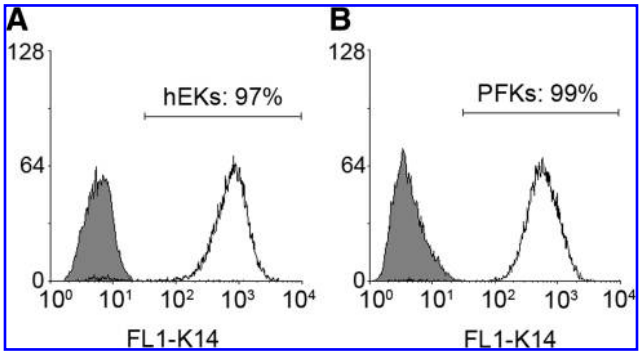

FIG. 1. Retinoic acid-induced differentiation of hES cells yields K14+ populations of similar purity to primary keratinocytes. Flow cytometry analysis of K14 expression in hEK (**A**) and PFK (**B**) cultures demonstrated that both achieved >95% K14+ purity. hES, human embryonic stem; hEKs, hES cell-derived keratinocytes; K14, keratin 14+; PFKs, primary foreskin keratinocytes.

referring to the same gene) whose expression differed at least fourfold between the cell types.

We divided these genes according to the cell type in which they were highly expressed and then subjected each set to GO analysis. These data are summarized in Table 1. Both cell types were found to express transcripts associated with development (e.g., organ, tissue, and anatomical structure), which is not surprising given the embryonic and neonatal origin of each cell type. GO terms that were uniquely upregulated in hEKs were related to cell signaling, adhesion, and extracellular matrix; none of which are surprising given the extensive amount of time hES cell precursors have been maintained *in vitro*. Blood vessel and muscle development were also identified, suggesting potential contamination of particular cell types or possibly abnormal differentiation. The GO terms that correlated with genes upregulated in PFKs included ectoderm/epidermal development, chemotaxis, response to external stimulus, and several biosynthetic pathways. Many of these processes are associated with the phenotype of activated keratinocytes.[22,23] Example genes for

Table 1. Gene Ontology Analysis of Transcripts That Differ Significantly Between Human Embryonic Stem Cell-Derived Keratinocytes and Primary Foreskin Keratinocytes

GO term	*Description*	*Count*[a]	%[b]	p-*Value*[c]
Upregulated in hEKs				
GO:0032502	Developmental process	81	36.65	3.69 E −15
GO:0048513	Organ development	39	17.65	5.14 E −09
GO:0009653	Anatomical structure morphogenesis	34	15.38	9.91 E −08
GO:0031012	Extracellular matrix	18	8.14	2.07 E −07
GO:0005576	Extracellular region	37	16.74	2.11 E −07
GO:0007165	Signal transduction	67	30.32	5.36 E −06
GO:0007155	Cell adhesion	24	10.86	8.12 E −06
GO:0009887	Organ morphogenesis	17	7.69	9.62 E −06
GO:0007166	Cell surface receptor-linked signal transduction	39	17.65	5.79 E −05
GO:0016337	Cell–cell adhesion	13	5.88	6.45 E −05
GO:0005509	Calcium ion binding	26	11.76	2.57 E −05
GO:0005515	Protein binding	100	45.25	8.59 E −05
GO:0042692	Muscle cell differentiation	6	2.71	2.06 E −04
GO:0001944	Vasculature development	10	4.52	2.13 E −04
GO:0007167	Enzyme-linked receptor protein signaling pathway	12	5.43	2.47 E −04
GO:0016055	Wnt receptor signaling pathway	8	3.62	4.06 E −04
GO:0005604	Basement membrane	7	3.17	1.26 E −04
Upregulated in PFKs				
GO:0044271	Nitrogen compound biosynthetic process	9	4.00	2.27 E −05
GO:0006935	Chemotaxis	10	4.44	3.29 E −05
GO:0032502	Developmental process	58	25.78	5.04 E −05
GO:0007398	Ectoderm development	10	4.44	5.49 E −05
GO:0009605	Response to external stimulus	20	8.89	6.36 E −05
GO:0006725	Aromatic compound metabolic process	9	4.00	8.91 E −05
GO:0005615	Extracellular space	17	7.56	1.67 E −04
GO:0008544	Epidermis development	9	4.00	1.97 E −04
GO:0048856	Anatomical structure development	41	18.22	2.94 E −04
GO:0009888	Tissue development	13	5.78	3.03 E −04
GO:0008652	Amino acid biosynthetic process	6	2.67	3.04 E −04
GO:0016477	Cell migration	11	4.89	7.03 E −04
GO:0032501	Multicellular organismal process	60	26.67	7.40 E −04
GO:0001525	Angiogenesis	8	3.56	8.95 E −04
GO:0004252	Serine-type endopeptidase activity	9	4.00	9.37 E −04

[a]Number of genes in the set included within the GO term.
[b]% of genes in the set included within the GO term.
[c]*p*-Values refer to expression analysis systematic explorer score of significance for GO analysis.
GO, gene ontology; hEKs, human embryonic stem cell-derived keratinocytes; PFKs, primary foreskin keratinocytes.

Table 2. Differentially Expressed Genes Between Human Embryonic Stem Cell-Derived Keratinocytes and Primary Foreskin Keratinocytes

NCBI gene ID	*Description*	*qPCR relative expression*[a]	*Array relative expression*[b]
Signal transduction-associated genes			
IGF2	Insulin-like growth factor 2	(330, 2408)	(55, 260)
KIT	c-Kit (receptor tyrosine kinase)	NE	70
SFRP2	Secreted frizzled-related protein 2	NE	(9, 63)
IGFBP3	IGF-binding protein 3	(10, 380)	30
FGFR2	Fibroblast growth factor receptor 2	(6, 22)	6
WNT5A	Wnt 5A protein	NE	6
Basement membrane-associated genes			
LAMA5	Laminin 5, alpha chain	(34, 262)	6
FN1	Fibronectin 1	NE	36
Embryonic-associated genes			
CAPN6	Calpain 6 (expressed in placenta)	NE	61
HAND2	Heart and neural crest expressed 2	NE	(5, 50)
Epithelial-associated genes			
KRT19	Keratin 19	(7, 57)	10
TGM2	Transglutaminase 2	NE	(49, 260)
KRT16	Keratin 16	(0.069, 0.046)	0.20
KRT6	Keratin 6	NE	0.11

[a]Relative expression levels were expressed as $2^{\Delta\Delta CT}$. When significantly different values were observed across the three replicates, low and high values are presented within parentheses.

[b]Relative expression of transcripts from microarrays is listed after robust multiarray analysis normalization. In some cases, different probes against the same gene yielded significantly different values; low and high values are presented within parentheses.

NE, not evaluated; qPCR, quantitative polymerase chain reaction.

each GO term are listed in Supplemental Table S2 (available online at www.liebertonline.com/ten).

To further understand transcriptional differences between the hEK and PFK cultures we selected individual genes that were overexpressed in a given cell type and validated the results using qPCR. These data are presented in Table 2 along with other genes with significant fold changes from our 95% confidence/FDR test. Transcripts associated with signal transduction that were highly expressed in hEKs included insulin-like growth factor 2 (*IGF2*), insulin-like growth factor-binding protein 3 (*IGFBP3*), fibroblast growth factor receptor 2 (*FGFR2*), Wnt-related genes (*WNT5A* and *SFRP2*), and *KIT*, a receptor tyrosine kinase that binds stem cell factor. hEKs also upregulated the genes encoding basement membrane proteins such as laminin 5 and fibronectin. Some epithelial genes were also differentially expressed. For example, *KRT19* and *TGM2* transcriptions were elevated in hEKs, whereas *KRT6* and *KRT16* were highly expressed in PFK cultures. The complete list of differentially expressed genes ($p < 0.05$) is available in Supplemental Table S3 (available online at www.liebertonline.com/ten).

Finally, we evaluated the genes specific to both hES cells and keratinocytes that were not differentially expressed based on our FDR confidence value. *NANOG* and *POU5F1*, which encode the Oct4 transcription factor, were minimally expressed in both cell types and detected at similar levels. Various cytokeratin markers of the epithelial lineage were transcribed at comparable levels, including *KRT5, KRT14*, and *KRT18*. Overall, these data provide supporting evidence that RA-mediated differentiation of hES cells can elicit a keratinocyte-like phenotype. To more definitively evaluate the function of these progenitors we attempted to induce terminal differentiation of hEKs by manipulating the microenvironment.

Induction of terminal differentiation by soluble factors

hES cell-derived epithelia in monolayer culture routinely began to stratify and undergo terminal differentiation after reaching confluence.[10] Cells in suprabasal layers expressed MUC1 (Fig. 2A, B), a marker detected in more terminally differentiated populations of epithelial tissues.[24] Although the surrounding basal cells uniformly expressed K14 (Fig. 2C), the presence of this protein was markedly decreased in suprabasal growths and replaced by K19 (Fig. 2D). Though not typically expressed in the epidermis, K19 expression is more commonly observed in embryonic epidermis[25] and is regulated by RA in keratinocytes *in vitro*.[26] However, primary keratinocytes have been shown to express K19 suprabasally in culture.[27] To gauge the response of hEKs to soluble factors, we added Ca^{2+} (1 mM) to hEKs in DSFM or changed the medium to serum-containing FAD medium with (0.66 mM) or without Ca^{2+}. Both Ca^{2+} and FAD medium significantly increased the levels of involucrin which was detected in cellular lysates (Fig. 2E). Involucrin is expressed suprabasally in stratified squamous epithelia and is involved in the crosslinking of cell membrane proteins in keratinocytes during terminal differentiation.[28]

Incorporation of hEKs into organotypic skin cultures

Although soluble factors were capable of inducing limited differentiation in submerged cell cultures, organotypic skin cultures offer a much more effective means of characterizing keratinocyte function.[15,16] Adhesion of epithelia on a dermal compartment containing fibroblasts helps to maintain progenitor function, and placement of keratinocytes at the ALI enhances stratification and terminal differentiation.[15] To this end we formed dermal constructs by incorporating normal human dermal fibroblasts within gelled collagen and added

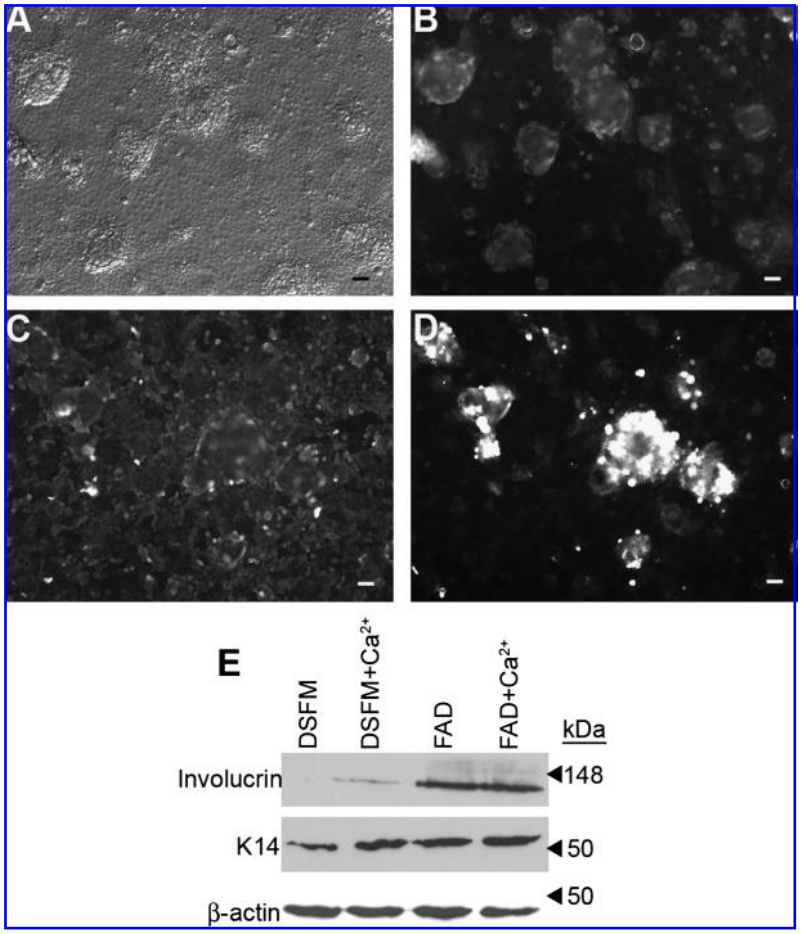

FIG. 2. Terminal differentiation of hEK cells in submerged cultures. (**A**) Phase contrast and (**B**) immunofluorescent images of confluent hES cell-derived epithelial cells stained against Mucin 1. (**C, D**) Confluent culture of hES cell-derived epithelia stained for K14 (**C**) and K19 (**D**). Images were taken at different focal planes. Note suprabasal cells stained positive for K19. Scale bars = 50 μm. (**E**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis/western blot analyses of hES cell-derived epithelia treated with the specified media for 24 h.

hEKs to the contracted rafts at confluence in FAD medium. Minimal differentiation occurred when culturing tissue constructs in DSFM (with or without Ca^{2+}). In this medium the keratinocytes migrated to form a web-like pattern and failed to maintain coverage of the dermal compartment (unpublished observations). After 3 weeks of culture at the ALI the organotypic skin cultures were processed for analysis. PFKs were cultured in the organotypic model in parallel, and human skin served as a positive control for IHC.

Histological analysis was conducted to observe tissue structure and marker localization of cells in organotypic culture. Hematoxylin and eosin staining highlighted the epithelial and dermal compartments as well as areas of stratification and cornification in the constructs generated using hEKs or PFKs (Fig. 3A, B, respectively). In addition, structures reminiscent of intracellular bridges were observed in high-power frames of the epithelium (Fig. 3C). The cells continued to proliferate after 3 weeks at the ALI, as determined by Ki67+ cells present throughout the epithelium (Supplemental Fig. S1A, available online at www.liebertonline.com/ten). Keratinocytes were numerous in the basal epithelial layers and also stained positively for p63 (Fig. 3D, E), a transcription factor known to be downregulated during terminal differentiation.[29] However, epithelial cells in human skin were smaller and densely packed and had a significantly greater nuclear:cytoplasmic ratio (particularly in the basal layers) compared with those in our engineered constructs (Fig. 3D–F).

Squame-like cells were present in the suprabasal layers of hEK and PFK-containing tissues, suggesting that hES cell-derived epithelia are capable of terminal differentiation (Fig. 3A, B). Although this cornification was reproducibly observed in multiple cultures, coverage over the collagen raft was not as uniform when compared with organ cultures of primary keratinocytes. In addition, differences were observed in the wetness of hEK- versus PFK-containing organotypic cultures, with hEKs displaying significant decreases in water retention. In contrast, increases in terminal differentiation markers of the skin were detected throughout the epithelium of hES cell-derived organotypic cultures. Stratified cell layers in hES cell-derived and, to a lesser extent, PFK-derived tissue expressed K10 and involucrin; however, K10 was more present in the suprabasal layers of hEK-containing tissue rather than immediately above the basal layer (Fig. 3G–L). Notably, basal keratinocytes did not stain positive in any tissues. Punctate filaggrin staining was present in the uppermost cell layers of hEK-derived tissues (Fig. 3M), indicative of the formation of keratohyalin granules.[30] Filaggrin-positive cells were also observed in engineered tissues made from PFKs (Fig. 3N), though filaggrin staining was strongest in human skin (Fig. 3O). The differential expression of this marker between hES cell-derived and *in vivo* tissues may indicate differences in cell phenotype or deficiencies in the tissue engineering process. Although differences between hES cell-derived and primary tissues were noted, the fact that cells from different differentiation experiments reproducibly executed their

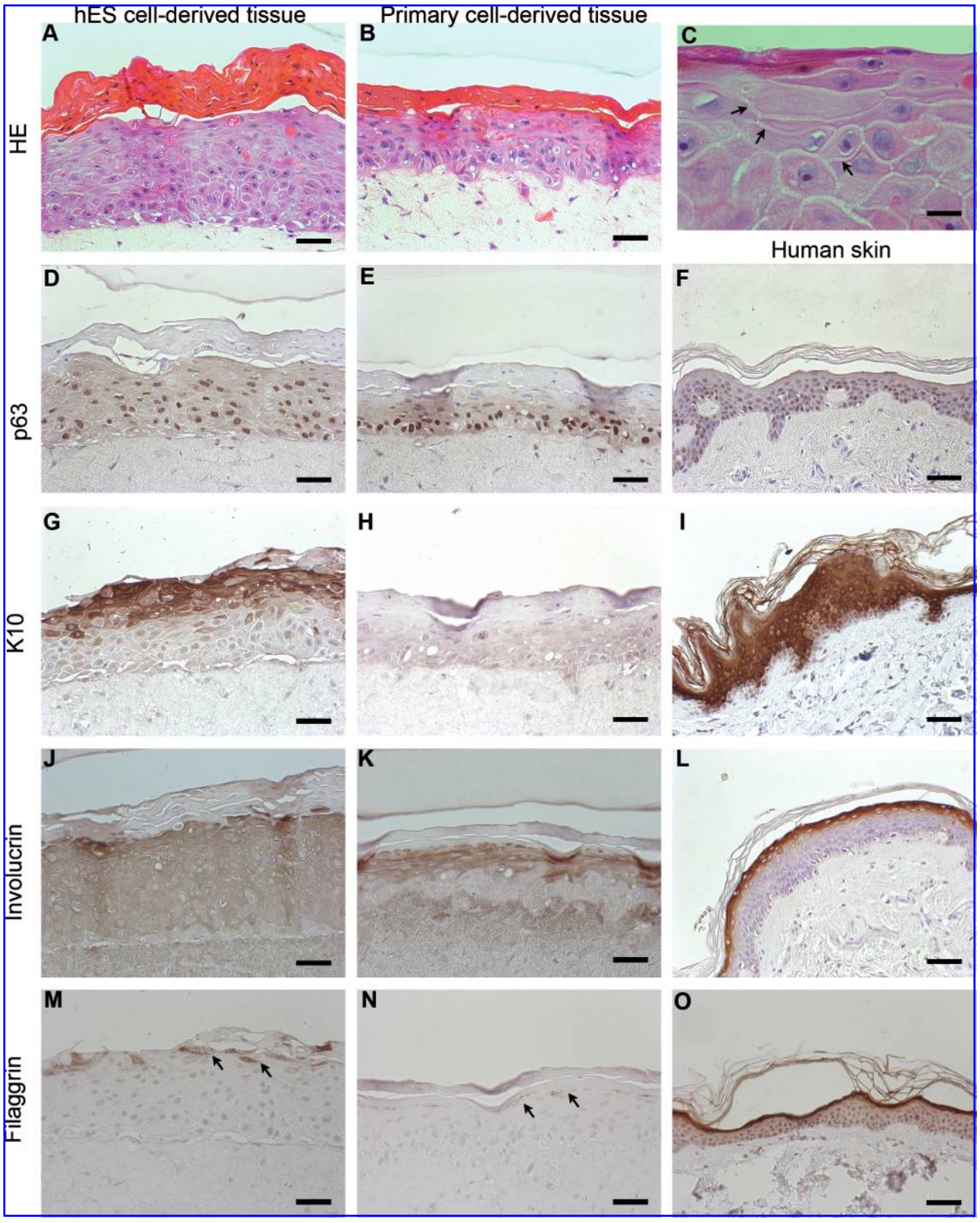

FIG. 3. HE staining and immunohistochemistry analyses of organotypic skin cultures cultured at the air–liquid interface for 2–3 weeks. (**A, C, D, G, J, M**) Organotypic cultures engineered with hES derivatives. (**B, E, H, K, N**) Organotypic tissues engineered with foreskin keratinocytes. (**F, I, L, O**) Human skin. (**A–C**) HE staining depicts stratified and cornified epithelial morphology in hES cell-derived epithelium (**A, C**) and foreskin keratinocyte engineered tissue (**B**). Intracellular bridges are evident in (**C**) (see arrows). (**D–O**) Immunohistochemistry analysis against the listed antigen reveals localization of markers within the epithelium of engineered hES cell-derived epithelium (**D, G, J, M**), foreskin keratinocyte-derived epithelium (**E, H, K, N**), and primary human skin (**F, I, L, O**). Images were taken from at least three independent experiments. Negative controls containing no primary antibody were included for all analysis. Arrows indicate specific areas of filaggrin staining (**M, N**). Scale bars = 50 μm for all images except (**C**). Scale bar = 16 μm for (**C**). HE, hematoxylin and eosin.

terminal differentiation program provides evidence for their ability to respond to complex microenvironments, including dermal stroma and the ALI. The high-efficiency methods used to generate hEKs therefore makes them an attractive tool for studying human epidermal differentiation and epithelial morphogenesis.

Discussion

Treatment of undifferentiated hES cells with RA in the presence of endogenous BMP signaling is an effective means of producing high-purity populations of epithelial cells.[10] We have previously demonstrated the utility of this method in generating epithelial cells of ectodermal origin from hES cells.[10] In our work we performed a quantitative comparison of hEKs and an analogous primary cell type, foreskin keratinocytes, to identify any gross differences in their respective transcriptional networks. We then evaluated the ability of hEKs to respond to microenvironmental cues such as culture medium, Ca^{2+}, or the ALI. We further characterized these hES cell-derived and primary epithelia in organotypic skin cultures to observe and compare the histology and expression of terminal differentiation markers in engineered tissues.

Overall, PFKs and hEKs exhibited a relatively similar transcriptional phenotype. Although hEKs were exposed to a relatively high concentration of RA early during differentiation, the lack of RA-associated genes or the ontologies present in the upregulated hEKs gene set provides evidence that this dosage does not directly interfere with cellular function. Despite the male and female origin of PFKs and hEKs, respectively, we detected less than 20 differentially expressed genes on the Y chromosome. Further, our most stringent analysis ($p < 0.01$ with FDR test) yielded only 10 differentially expressed genes. Although researchers have conducted global transcriptional analyses of hES cells before or during differentiation,[31–33] few studies have compared high-purity differentiated populations with their associated primary cell types.[34] Interestingly, comparable statistical analyses of different hES cell lines (using similar levels of significance) yielded larger sets of differentially expressed genes than we observed here.[33] Although these results provide strong evidence for the similarity of H9-derived hEKs and primary keratinocytes, some key differences between the two cell types are noteworthy and offer insight into our findings using organotypic cultures.

The cellular microenvironment plays a key role in determining cellular fate,[5,35] and by manipulating *in vitro* culture conditions we were able to characterize the ability of hEKs to detect and respond to different stimuli. We previously detected definitive epidermal proteins, including K10 and filaggrin,[10] in submerged culture. The cells that proliferated and lost contact with the underlying matrix expressed additional markers of terminal epithelial differentiation, including MUC1 and K19, although these proteins are not typically expressed in the adult epidermis.[24,25] Further, culture of hEKs in the presence of Ca^{2+}, serum, or both caused a significant increase in involucrin expression. These factors, in contrast to RA,[36,37] are well-known inducers of keratinocyte differentiation and provide additional evidence that hEKs exhibit an epidermal phenotype.[28,38,39]

The most effective *in vitro* methodologies for analysis of epidermal differentiation are organotypic cultures. Recently, Hewitt *et al.* cultured a mixture of H9 hES cell-derived ectodermal (K18+) and mesenchymal precursors at the ALI, detecting basement membrane proteins and K12 in tissue constructs.[19] However, neither K14 nor p63 were observed in their engineered tissues, indicating that these precursors had not yet obtained a basal epithelial phenotype. In our organotypic cultures, histological observation demonstrated the ability of hEKs' propensity to terminally differentiate, as both stratification and areas of cornification were evident. The cells continued to proliferate and maintained p63 expression in the basal layers after 3 weeks at the ALI; however, the basal compartment was less distinct in hEK constructs compared with human skin or PFK-containing tissues. Although terminal differentiation markers such as involucrin, K10, and filaggrin were localized to the suprabasal layers, the delineation of differentiation compartments and strength of signal were lacking compared with *in vivo* samples.

Some results from our microarray analysis are consistent with these findings, including elevated transcript levels associated with signal transduction. In particular, these genes included *IGF2, FGFR2*, and genes involved in Wnt signaling. Several other reports have identified instability in the imprinting of the paternally inherited IGF2 allele in hES cells.[34,40] Although elevated *IGF2* transcripts may have come from contaminating embryonic or placental cell types, the importance of IGF signaling in maintaining hES cells in the undifferentiated state could result in selection of cells that overexpress this gene during extended *in vitro* culture.[41] Similar adaptations may have also contributed to the overexpression of FGFR2- and Wnt-related proteins such as SFRP2, Wnt3, Wnt5A, and Wnt6, as both pathways are active in undifferentiated hES cell cultures.[42–44] Any adaptations that might have occurred in the undifferentiated state of hES cell growth might still be present after differentiation to the epithelial lineage. The IGF, FGF, and Wnt signaling pathways are typically associated with the growth state. For example, TCF/LEF-mediated transcription (downstream of the canonical Wnt pathway) is a key driver of epithelial cell proliferation in the skin.[45] Elevated signaling along these pathways might ultimately interfere with the terminal differentiation program of keratinocytes, resulting in decreased expression of some markers relative to primary tissues and an inability to abruptly initiate differentiation programs.

We also observed elevated expression of extracellular matrix genes in our arrays. Our PFKs were recently derived from *in vivo* tissue containing stroma that presumably secrete extracellular matrix and help to maintain the basement membrane. In contrast, hEKs have been maintained *in vitro* for an extensive period of time,[1] and repeated passaging and cryopreservation may have selected for cells that can secrete their own matrix. Extended cultivation *in vitro* may have resulted in the observed upregulation of basement membrane transcripts, and the presence of matrix proteins in suprabasal layers of tissue constructs could interfere with their stratification program. In other words, ES cells and their derivatives may be more fixed in a progenitor-like state as a result of *in vitro* selection over time. Another potential indicator of *in vitro* adaptation in hEKs is the relatively higher

expression of biosynthetic genes in PFKs. Upon activation *in vivo*, keratinocytes express K6/K16 and proliferate extensively.[46] hEKs, which have been maintained in rich media for many passages,[1] may have an enhanced ability to utilize cellular precursors (e.g., amino acids) present in tissue culture media.

Finally, elevated expression of embryonic genes such as *CPN6* and *HAND2* could indicate the presence of contaminating placental or embryonic cell types that pervaded in culture. Indeed, the profile of keratin transcription and translation is quite different between embryonic epithelium and the epidermis.[47] Human fetal skin does not begin keratinization of the interfollicular epidermis until ~24 weeks of gestation, and filaggrin is not detected at significant levels until that time.[25] In addition, K19 is expressed only in some basal cells of the adult epidermis but is detected at high levels during embryonic development in the basal compartment and the periderm, a single-layered epithelium that covers the epidermis early during development.[25] K19 was significantly upregulated in hEKs and, unlike in PFK constructs, was detected throughout the epithelium of our engineered tissues (Supplemental Fig. S1B, available online at www.liebertonline.com/ten). Given the embryonic origin of our differentiated cells, these populations may be somewhat heterogeneous, containing progenitor cells which take on phenotypes of the periderm or more mature epidermal cells. Finally, moisture retention (based on visual observation) was significantly diminished in hEK constructs compared with PFK constructs. Interestingly, organotypic cultures made up of mixed epithelial populations containing as much as 75% hEKs looked relatively dry and well differentiated (unpublished observation). Although the lack of constitutive markers prevented us from identifying each cell type in histological samples, this result provides evidence that hEKs do not interfere with somatic cellular processes or display an invasive phenotype.

Our comparative analysis of hEKs and PFKs demonstrated that hEKs display key hallmarks of epithelial morphogenesis and exhibit a transcriptional program similar to that of PFKs. Although these data provide evidence for the resemblance of hEKs to primary keratinocytes, several questions remain to be answered. In the context of grafted tissue, are the surrounding somatic cells capable of inducing more adult phenotypes from hES cell-derived cells? Also, the pluripotent origin of these cells may endow them with greater plasticity than somatic epithelial cells. As such, tissue-specific *in vivo* stroma/matrices may allow the cells to cross-differentiate, forming other stratified epithelia (e.g., corneal and oral). In summary, our results further demonstrate the ability of hES cells to generate functional epithelial cells and highlight the importance of characterizing hES cell derivatives in the appropriate microenvironments to better establish cellular phenotypes.

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Disclosure Statement

No competing financial interests exist.

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