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In Vitro Epithelial Differentiation of Human Induced Pluripotent Stem Cells for Vocal Fold Tissue Engineering

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Abstract

Objectives—We determined the feasibility and optimization of differentiating human induced pluripotent stem cells (hiPS) into nonkeratinized stratified squamous epithelial cells for vocal fold engineering.

Methods—hiPS were cultured and assessed for differentiation in 3 conditions: a 3-dimensional (3D) hyaluronic acid (HA) hydrogel scaffold, a 3D HA hydrogel scaffold with epidermal growth factor (EGF), and a 3D HA hydrogel scaffold cocultured with human vocal fold fibroblasts (hVFF). After 1, 2, and 4 weeks of cultivation, hiPS were selected for histology, immunohistochemistry, and/or transcript expression analysis.

Results—At 4 weeks, hiPS cultivated with hVFF or with EGF had significantly decreased levels of Oct 3/4, indicating loss of pluripotency. Immunofluorescence revealed the presence of pancytokeratin and of cytokeratin (CK) 13 and 14 epithelial-associated proteins at 4 weeks after cultivation in hiPS EGF and hiPS hVFF cultures. The transcript expression level of CK14 was significantly increased for hiPS hVFF cultures only and was measured concomitantly with cell morphology that was clearly cohesive and displayed a degree of nuclear polarity suggestive of epithelial differentiation.

Conclusions—We found that hiPS cultivated in 3D HA hydrogel with hVFF demonstrated the most robust conversion evidence to date of epithelial differentiation. Further work is necessary to focus on amplification of these progenitors for application in vocal fold regenerative biology.

Keywords

hiPS; hyaluronic acid hydrogel; induced pluripotent stem cell; tissue engineering; vocal fold

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INTRODUCTION

Vocal fold scarring is the foremost cause of voice disorders after vocal fold injury secondary to trauma, surgical treatment, and postinfection inflammation¹; however, the present treatment approaches are limited.² Tissue engineering research in general, and in vocal folds in particular, has flourished over the past decade. The majority of published work has addressed the lamina propria; fewer studies have focused on vocal fold epithelial regeneration. The epithelium of the vocal fold provides a physical and biochemical barrier that protects the vocal folds from external irritants and systemic stresses and maintains vocal fold hydration.^{3,4} When this epithelium is compromised, rapid and complete restoration of its integrity is crucial for normal function. Therapeutic options for regeneration and restoration of the epithelial barrier function after vocal fold injury are crucial to the comprehensive treatment of vocal fold scarring.

Two different approaches have been reported for the regeneration of vocal fold epithelium. Yamaguchi et al⁵ created an organotypic model of vocal fold mucosa with porcine fibroblasts, epithelial cells, and collagen I gel. Long et al⁶ constructed a vocal fold cover replacement utilizing adipose-derived mesenchymal stem cells, fibrin gel, and epidermal growth factor. There are drawbacks to both of these approaches that limit their translational potential, including the limited ability of the primary and mesenchymal cell lines that were used to proliferate and differentiate and the fact that this ability is further decreased after several passages.^{7,8} Alternatively, induced pluripotent stem cells (iPS) are capable of unlimited symmetric self-renewal, thus providing a limitless cell source for tissue engineering applications.^{9–12}

Induced pluripotent stem cells are generated from fibroblasts through the introduction of four transcription factors — Oct3/4, Sox2, c-Myc, and Klf4¹³ — and, because they are obtained from patient-derived somatic cells, they can prevent transplant rejection.¹³ There are precedents in the literature for the differentiation of iPS into adipocytes and osteoblasts,¹⁰ dendritic cells and macrophages,⁹ and chondrocytes^{11,12} for tissue engineering applications. Differentiation into any type of epithelium has not been well examined or established in iPS research. Only a few studies have addressed the possibility of epithelium differentiation of mouse iPS.^{14,15} Vocal fold epithelium is made up of nonkeratinized stratified squamous epithelial cells that are distinguished from other epithelia by the expression of cytokeratin (CK) 13 and CK14 in the suprabasal and basal layers, respectively. This pattern of expression can be found in other epithelia (ie, corneal, vaginal, and esophageal epithelia), but not in other respiratory epithelial cells. We are able to exploit these key morphological and phenotypic characteristics of vocal fold epithelium as a means of evaluating the differentiation potential of iPS.

The goal of this study was to determine the feasibility of differentiating human iPS (hiPS) into stratified squamous epithelial cells for vocal fold regeneration. To mimic the in vivo environment, we cultured hiPS in a 3-dimensional hyaluronic acid (HA) hydrogel scaffold, which has been previously shown to have viscoelastic properties similar to those of human vocal fold mucosa in vitro^{16,17} and to be biocompatible and noninflammatory in vivo.¹⁸ To assess feasibility, we evaluated two common differentiation strategies: use of epidermal

growth factor (EGF) and co-culturing with human vocal fold fibroblasts (hVFF). We hypothesized that EGF signaling would influence gene and protein expression to produce CK13 and CK14, two vocal fold epithelium-associated proteins.⁶ We also hypothesized that cell-cell interactions in the microenvironment, created by co-culturing with hVFF, would be important in directing the site-specific differentiation of hiPS.

MATERIALS AND METHODS

CELL CULTURE

hiPS—We maintained hiPS (fibroblast-derived IMR90-4 line; WiCell, Madison, Wisconsin)¹⁹ on feeder-free surfaces using tissue culture dishes coated with Matrigel (BD Bioscience, San Jose, California) with mTeSR 1 (StemCell Technologies, Vancouver, Canada) medium, a defined, feeder-free maintenance medium for hiPS cells. Morphologically identifiable differentiated cells were mechanically removed, and cells were passaged every 6 to 7 days. Passage 53 cells were used throughout this investigation.

hVFF—Primary hVFF were established from normal human vocal folds, which were harvested from an autopsy of a 21-year-old donor within 24 hours of death.²⁰ Tissue was obtained with approval of the Institutional Review Board of the University of Wisconsin–Madison. Primary hVFF were isolated and cultured as described previously.²⁰ hVFF were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% non-essential amino acids (NEAA; Sigma-Aldrich, St Louis, Missouri) and 1% penicillin/streptomycin (Sigma-Aldrich). The hVFF cells were passaged every 6 to 7 days. Cells from passages 7 through 10 were used throughout this investigation.

Three-Dimensional Cell Culture—We prepared hiPS-hydrogel constructs as follows. An HA gelatin hydrogel, Glycosil (Glycosan BioSystems, Inc, Salt Lake City, Utah), was developed in conjunction with the Center for Therapeutic Biomaterials at the University of Utah.^{21–23} It is composed of polyethylene glycol diacrylate (PEGDA)–cross-linked thiolmodified HA (CMHA-S) and thiol-modified gelatin (Gtn-DTPH). Hydrogel was prepared by mixing 50 mg of CMHA-S in 3.5 mL deionized water (DG water; 1.4% wt/vol) and 0.262 mL of 10 mg of Gtn-DTPH in 1 mL DG water (1.0% wt/vol) with 0.405 mL of 50 mg of PEGDA in 0.61 mL DG water (8.2% wt/vol). The final concentrations of each solution were CMHA-S 1.2% (wt/vol), Gtn-DTPH 0.06% (wt/vol), and PEGDA 0.8% (wt/vol). hiPS at passage 53 were suspended in the reconstituted HA hydrogel solution at a density of 3.0×10^6 cells per milliliter. Two hundred microliters of solution containing hiPS was introduced into Millicell Culture Plate inserts (Millipore Co, Billerica, Massachusetts; 6.0×10^5 cells per insert) with a membrane filter (0.4- μ m pore size). hiPS-hydrogel constructs (Fig 1) were placed in 12-well polystyrene plates (BD Falcon, Franklin Lakes, New Jersey) with cell culture medium (mTeSR 1) added above and around the gel. Medium for the hiPS-hydrogel constructs placed in 12-well polystyrene plates was changed every day. For the growth factor condition, cell culture medium (DMEM–10% FBS) with EGF (R&D Systems, Minneapolis, Minnesota) at a final concentration of 100 ng/mL was added above and around the gel. For the hVFF HA hydrogel condition, inserts including hiPS hydrogel constructs were placed in 10-cm culture plates (BD Falcon) with hVFF treated

with mitomycin C (MMC) to halve their proliferation. Medium, DMEM–10% FBS, for the hiPS-hydrogel constructs placed in 10-cm culture plates was changed 3 times a week. At 1, 2, and 4 weeks after cultivation, hiPS from each condition were selected for histologic and immunohistochemical examination, RNA extraction, and quantitative polymerase chain reaction (PCR) analysis.

GENE EXPRESSION ANALYSIS

RNA Extraction and Reverse Transcription—Total RNA was isolated with an RNeasy Micro-Kit (Qiagen, Hilden, Germany). Samples were treated with RNase-Free DNase Set (Qiagen) to remove traces of genomic DNA contamination. Samples of complementary DNA were synthesized with Omniscript reverse transcription (RT) kit (Qiagen) by use of Oligo-dT primer and Random 6 mer primer according to the manufacturer's instructions. RT reaction components were 1xBuffer RT, 0.5 mmol/L deoxyribonucleotide (dNTP) mix, 1 μ mol/L Oligo-dT, 10 μ mol/L Random 6 mer, 10 units of RNase inhibitor, and 4 units of Omni script Reverse Transcriptase.

Conventional PCR—To confirm that hiPS were in an undifferentiated state before the experiments, we performed conventional PCR analysis with a PCR reaction mix consisting of GoTaq DNA Polymerase (Promega, Madison, Wisconsin) with 0.5 μ mol/L of each primer (see Table). The cycling protocol for conventional PCR was 95°C for 2 minutes (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds) $\times$ 30 cycles in 20 μ L reaction mixture. PCR products underwent electrophoresis on a 2% agarose gel followed by ethidium bromide staining. Primer sets for glyceraldehyde 3-phosphate dehydrogenase and β -actin were used as positive controls. Primer sets for Oct3/4 and Nanog, markers of undifferentiated cells, were used to verify the hiPS undifferentiated state. All conventional PCR was performed in triplicate.

Quantitative RT-PCR Analysis—Quantitative real-time PCR for CK14 and Oct3/4 genes was performed with specific primer pairs (see Table) and LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Corp, Indianapolis, Indiana) by use of a LightCycler 1.5 (Roche Diagnostics), with amplification of β -actin as the housekeeping gene. Amplification was carried out for 95°C for 10 minutes (95°C for 10 seconds, 55°C for 7 seconds, 72°C for 7 seconds) for 45 cycles in a 20 μ L reaction mixture containing 1xMaster Mix, 5 μ L cDNA, and 0.5 μ mol/L of each primer. All RT-PCR was performed in triplicate.

HISTOLOGY AND IMMUNOFLUORESCENCE

hiPS-hydrogel constructs were either fixed with 4% paraformaldehyde, processed, and embedded in paraffin for routine hematoxylin and eosin (H&E) staining, or fresh-frozen in optimal cutting temperature (OCT) compound (Tissue Tek; Sakura Finetek Inc, Torrance, California) for immunofluorescence. Paraffin-embedded samples were cut at a thickness of 5 μ m per section and prepared for H&E staining by a standard protocol. We cut OCT compound-embedded samples at a thickness of 20 μ m per section and processed them for immunofluorescence using standard techniques. Briefly, sections were permeabilized with 0.1% Triton X-100, blocked with 5% normal goat serum, and incubated in the appropriate

antibody containing 1% bovine serum albumin (BSA) for 60 minutes at room temperature, followed by incubation with secondary antibodies for another 60 minutes at room temperature in the dark. Nuclei were labeled with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, California). Four antibodies were used: anti-PanCK primary mouse monoclonal antibody (1:300 dilution, ab6401; Abcam, Inc, Cambridge, Massachusetts), anti-CK13 primary rabbit polyclonal antibody (1:100 dilution, ab97327; Abcam, Inc), anti-CK14 primary rabbit polyclonal antibody (1:400 dilution, RB-9020; Thermo Scientific, Fremont, California), and anti-stage-specific embryonic antigen 1 (SSEA-1) primary mouse monoclonal antibody (1:50 dilution, sc-217020; Santa Cruz Biotechnology, Inc, Santa Cruz, California). PanCK, CK13, and CK14 staining allowed us to demonstrate epithelial differentiation. SSEA-1 staining was used to confirm cell differentiation from an undifferentiated cell. Slides were observed with a fluorescence microscope (E600; Nikon, Melville, New York). Two species-specific secondary antibodies were used: Alexa Fluor 488-labeled goat anti-rabbit (1:1,000 dilution; A-11008, Invitrogen, Grand Island, New York) and Alexa Fluor 488-labeled goat anti-mouse (1:1,000 dilution; A-11001, Invitrogen). We took all fluorescent images with a digital camera (DP71; Olympus, Tokyo, Japan), maintaining equal exposure time and post-image processing. Histologic and immunologic analysis was performed by a blinded independent pathologist.

STATISTICAL ANALYSIS

Statistical analysis was completed for the quantitative PCR results. We normalized the sample results separately by taking the average of the hiPS-only condition, the hiPS EGF condition, and the hiPS hVFF condition for the 2- and 4-week cultivations over messenger RNA (mRNA; CK14 and Oct3/4) expression levels compared to hiPS in 2-dimensional culture as a control. This accommodated differences in mRNA expression rates between the conditions. Ratios were transformed by use of 2 to the negative CT before analysis. First, 2-way analysis of variance was conducted to compare the expression levels between groups across different time points. Then, post hoc protected Fisher's least significant difference testing was performed. All analyses were carried out with SAS 9.2 (SAS Institute, Cary, North Carolina) software. A p value of less than 0.05 was considered statistically significant in the 2-tailed statistical tests.

RESULTS

CONFIRMATION OF hiPS UNDIFFERENTIATED STATE

To confirm that hiPS were in an undifferentiated state after dozens of generations, we performed conventional PCR analysis before our in vitro experiments. As shown in Fig 2, Nanog and Oct3/4, markers of undifferentiated cells, were detected in our hiPS cultures. In contrast, CK13 and CK14, two vocal fold epithelium-associated proteins, were not detected in these cultures (Fig 2).

EPITHELIAL DIFFERENTIATION OF hiPS

Gene Expression by Quantitative RT-PCR—For CK14 transcript levels, there were significant differences between various conditions of culture and between various times ($p < 0.0001$), as well as a significant interaction effect between condition and time ($p = 0.0015$;

Fig 3A). At the 2-week time-point, CK14 levels were significantly greater for the hiPS hVFF condition than for the hiPS EGF ($p < 0.0001$) and hiPS-only ($p < 0.0001$) conditions. There were no significant differences in CK14 levels between the hiPS EGF and hiPS-only conditions ($p = 0.1053$). At 4 weeks, CK14 expression in the hiPS hVFF condition continued to be significantly greater than that in the hiPS-only condition ($p < 0.0375$), but there was no significant difference between the hiPS hVFF and hiPS EGF conditions ($p = 0.1395$). There were no significant differences in CK14 levels between the hiPS EGF and hiPS-only conditions ($p = 0.4645$).

For Oct3/4 transcript levels, there were significant differences for condition ($p < 0.001$) and time ($p < 0.0029$). There was not a significant interaction effect ($p = 0.3646$; Fig 3B). At 2 weeks, there were significantly greater Oct3/4 gene levels for the hiPS hVFF condition than for the hiPS EGF condition ($p < 0.008$), but no difference between the hiPS hVFF and hiPS-only conditions ($p = 0.06$). Levels for the hiPS EGF condition were significantly less than those for the hiPS-only condition ($p = 0.0002$). At 4 weeks, Oct3/4 transcript levels were significantly less for the hiPS hVFF condition ($p = 0.0078$) and the hiPS EGF condition ($p = 0.0011$) than for the hiPS-only condition. There were no significant differences between the hiPS hVFF and hiPS EGF conditions ($p = 0.3012$).

Morphological Analyses—Cells subjected to all conditions were successfully cultured and stained with H&E at 1, 2, and 4 weeks. For the hiPS-only and hiPS EGF conditions, cells demonstrated little histologic evidence of epithelial differentiation at 1, 2, or 4 weeks and little evidence of intercellular adhesion (Fig 4). For cells cultured in hiPS with hVFF, there was, again, little histologic evidence of epithelial differentiation at 1 or 2 weeks. However, at 4 weeks, the cells were more clearly cohesive and displayed a degree of nuclear polarity suggestive of epithelial differentiation.

Immunohistochemical Analyses—For the hiPS-only condition, there was no expression of PanCK, CK13, or CK14 throughout the experimental period (Figs 5–7). For the hiPS EGF condition, there was no expression of PanCK, CK13, or CK14 at 1 or 2 weeks after induction. Whereas expression of PanCK and CK13 was detected at 4 weeks after induction, expression of CK14 was not. For the hiPS hVFF condition, no expression of PanCK, CK13, or CK14 was detected at 1 or 2 weeks after induction. Expression of PanCK, CK13, and CK14 was detected at 4 weeks after induction. Expression of SSEA-1, a marker for monitoring the transition of undifferentiated stem cells to a differentiated state, was not detected throughout the experimental period for the hiPS-only condition (Fig 8). For the EGF HA hydrogel and hVFF HA hydrogel conditions, no expression of SSEA-1 was detected at 1 or 2 weeks after induction. In contrast, the expression of SSEA-1 was detected at 4 weeks of cultivation for both the EGF HA hydrogel and hVFF HA hydrogel conditions.

DISCUSSION

This is the first feasibility report of the differentiation of hiPS into epithelial cells for vocal fold applications. Our results provide substantiation that hiPS have the potential to differentiate into vocal fold epithelium, as demonstrated by H&E and gene and protein expression of vocal fold epithelium markers. Further evidence of cell differentiation was

shown by the expression of SSEA-1, a marker for monitoring transition of undifferentiated stem cells to a differentiated state.²⁴ Our results indicate that hiPS are compatible with HA hydrogel, and grafting was tracked for 4 weeks. Furthermore, we report enhanced differentiation with hVFF co-culture.

Progress in tissue engineering is dependent upon cell sourcing and cell tissue characterization. Specifically, success is influenced by the availability of distinct cell sources for tissue regeneration, cell characterization, and development of universal donor cell lines. Autologous somatic cells are limited. Numerous studies have focused on using stem cells such as mesenchymal stromal cell (MSC) lines and embryonic stem (ES) cell lines as potential cell sources.^{25–27} These studies have shown that MSCs and ES cells have the potential for vocal fold lamina propria regeneration. Although MSCs are relatively safe, these cells have limited ability to proliferate and differentiate.⁷ On the other hand, ES cells are capable of unlimited proliferation and differentiation into any tissue or cell; however, transplant rejection and ethical issues have posed problems for tissue engineering applications using ES cells. Use of iPS generated from skin fibroblasts by the introduction of 4 transcription factors resolves these problems.¹³ As these cells are capable of unlimited symmetric self-renewal, there are limited ethical concerns, and the use of iPS obtained from patient-derived somatic cells can prevent transplant rejection.

Differentiation into stratified squamous epithelium has not been well examined or established with hiPS. In this investigation, at 4 weeks, hiPS co-cultured with hVFF displayed enhanced epithelial differentiation, as marked by morphology (cell adhesion). Adhesion is a differentiating feature of epithelial cells.²⁸ Adhesion was noted concomitantly with increases in transcription levels and the immunohistologic presence of stratified squamous epithelium cytokeratins. Co-culturing of hiPS with organ-matched mesenchyme has been shown to permit proliferation and self-renewal of progenitors.²⁹ In co-culture, hVFF may be secreting signaling and paracrine factors that alter and promote hiPS differentiation. It has been well documented that MSCs possess the ability to participate in the tissue repair and regeneration process and to stimulate proliferation and differentiation of resident progenitor cells through a variety of paracrine mechanisms.³⁰ As found in our previous study, hVFF have the same cell surface markers, immunophenotypic characteristics, and differentiation potential as MSCs.³¹ hVFF also provide a rich source of glycosaminoglycans, proteoglycans, elastin, and collagen molecules that influence the migration, growth, differentiation, and activity of neighboring cells.^{32,33} Consequently, we propose that the cell-cell interactions in the microenvironment, mimicking the in vivo environment, created by co-culturing with hVFF in 3-dimensional HA hydrogel may be important in directing the site-specific differentiation of hiPS. Specific mechanisms responsible for mesenchymal-mediated differentiation of hiPS into stratified squamous epithelial cells require further investigation.

In the hiPS EGF condition, expression of CK13 and PanCK was detected at 4 weeks after cultivation. Cells stained with H&E demonstrated no histologic evidence of epithelial differentiation throughout the experimental period. Taken together, these results indicate a diminished effect of EGF on epithelial differentiation. Concomitantly, we measured a significant decrease in messenger RNA expression of Oct3/4 at 2 and 4 weeks, indicating

differentiation. Long et al⁶ have reported the effectiveness of 10 ng/mL EGF for epithelial differentiation utilizing adipose-derived MSCs. However, it has been suggested that 10 ng/mL EGF alone is inadequate to produce organized epithelial differentiation.⁶ Taketani and Oka³⁴ reported the effectiveness of EGF for proliferation of epithelial cells, and the effect of EGF was maximal at 50 to 100 ng/mL. Heo et al³⁵ reported that EGF increased [³H]thymidine incorporation, a method of estimating cell proliferation rate, in a time- and dose-dependent manner in ES cells. The maximum increase in the [³H]thymidine incorporation was observed with 100 ng/mL EGF; 10 ng/mL EGF increased [³H]thymidine incorporation only slightly. Interestingly, there have been differing dosages of EGF utilized for cell differentiation.^{36,37} EGF at concentrations ranging from 100 to 200 ng/mL induced differentiation of type A spermatogonia.³⁶ EGF at 50 ng/mL showed optimal α -smooth muscle actin expression related to keratocyte differentiation.³⁷ It has been reported that the effects of mitogenic EGF signaling exhibit a non-monotonic or biphasic dose response curve: EGF at a low concentration such as 10 ng/mL elicits a mitogenic signaling pathway to stimulate cell proliferation, whereas at a high concentration such as 500 ng/mL, EGF inhibits cell growth.³⁸ These findings suggest that the most effective dose of EGF could be different in different tissues or cells. Differences in the effectiveness of various EGF concentrations may be due to unknown EGF quality or to differences in cell types, marker indexes, or experimental conditions. To determine the most effective concentration of EGF for hiPS vocal fold epithelial differentiation, dose response studies need to be undertaken.

A limitation of our present study is the lack of quantitative real-time PCR for the CK13 gene that would provide further support to the proteins measured with immunofluorescence. CK13, together with CK14, distinguishes vocal fold epithelium from other respiratory tract epithelia. In our quantitative PCR, we observed two peaks for the dissociation curve analysis for CK13, but only one band on agarose gel (data not shown). The reasons for this discrepancy may include the primer design, the real-time PCR conditions, or a small quantity of CK13 expression. More investigation is necessary to optimize the validity of real-time PCR measurements for the CK13 gene.

CONCLUSIONS

hiPS hydrogel constructs co-cultured with hVFF demonstrated the strongest evidence of nonkeratinized stratified squamous epithelium differentiation after 4 weeks of cultivation in vitro. Our findings substantiate the need for further investigation into mesenchymal-mediated differentiation of hiPS as a possible cell source for vocal fold regeneration therapy.

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Fig 1.

Fabrication of human induced pluripotent stem cell(s) (hiPS)–hydrogel constructs to induce epithelial differentiation in vitro. hiPS were cultured in hyaluronic acid (HA) hydrogel as 3-dimensional scaffold. hiPS hydrogel constructs were **A**) cultivated in isolation, **B**) treated with epidermal growth factor (EGF), or **C**) co-cultured with human vocal fold fibroblasts (hVFF).

Fig 2.

For confirmation of undifferentiated hiPS after dozens of generations, conventional polymerase chain reaction (PCR) analysis was performed before in vitro experiments. Nanog and Oct3/4, markers of undifferentiated cells, were detected from hiPS cultures. In contrast, cytokeratin (CK) 13 and CK14, two vocal fold epithelium– associated proteins, were not detected. GAPDH — glyceraldehyde 3-phosphate dehydrogenase.

Fig 3.

Messenger RNA transcript expression for **A)** CK14 and **B)** Oct3/4. Expression levels were normalized and expressed as fold change in hiPS expression. **A)** Expression of CK14 at 2 weeks was significantly greater for hiPS hVFF condition than for hiPS-only and hiPS EGF conditions. At 4 weeks, CK14 was significantly greater for hiPS hVFF condition than for hiPS-only condition. **B)** Expression of Oct3/4 at 2 weeks was significantly greater for hiPS-only and hiPS hVFF conditions than for hiPS EGF condition. At 4 weeks, Oct3/4 was significantly greater for hiPS-only condition than for hiPS EGF and hiPS HVFF conditions. Asterisk — $p < 0.005$.

Fig 4.

Hematoxylin-eosin morphology of constructs. For hiPS-only condition, cells demonstrated no histologic evidence of epithelial differentiation throughout experimental period. For hiPS EGF condition, cells demonstrated no histologic evidence of epithelial differentiation throughout experimental period. For hiPS hVFF condition, at 1 and 2 weeks after induction, cells demonstrated no histologic evidence of epithelial differentiation, but at 4 weeks, cells were more clearly cohesive and displayed certain degree of nuclear polarity, suggestive of epithelial differentiation. Scale bars — 100 μ m.

Fig 5. Immunofluorescent analysis of pan-cytokeratin (PanCK)-expressing cells in vitro. No PanCK was detected in hiPS-only condition throughout experimental period. For hiPS EGF condition, no expression of PanCK was detected at 1 or 2 weeks after cultivation, but it was detected at 4 weeks. For hiPS hVFF condition, no expression of PanCK was detected at 1 or 2 weeks after cultivation, but it was detected at 4 weeks. Scale bars — 100 μ m.

Fig 6.

Immunofluorescent analysis of CK13-expressing cells in vitro. CK13 was not detected in hiPS-only condition throughout experimental period. In hiPS EGF condition, no expression of CK13 was detected at 1 or 2 weeks after cultivation, but it was detected at 4 weeks. In hiPS hVFF condition, no expression of CK13 was detected at 1 or 2 weeks after cultivation, but it was detected at 4 weeks. Scale bars — 100 μ m.

Fig 7.

Immunofluorescent analysis of CK14-expressing cells in vitro. CK14 was not detected in hiPS-only condition throughout experimental period. For hiPS EGF condition, there was no expression of CK14 detected throughout experimental period. For hiPS hVFF condition, no expression of CK14 was detected at 1 or 2 weeks after cultivation, but it was detected at 4 weeks. Scale bars — 100 μ m.

Fig 8.

Immunofluorescent analysis of stage-specific embryonic antigen 1 (SSEA-1)–expressing cells in vitro. SSEA-1 was not detected in hiPS-only condition throughout experimental period. For hiPS EGF condition, SSEA-1 was not detected at 1 or 2 weeks after cultivation, but it was detected at 4 weeks. For hiPS hVFF condition, SSEA-1 was not detected at 1 or 2 weeks after cultivation, but it was detected at 4 weeks. Scale bars — 100 μ m.

Table**PRIMER SEQUENCES AND PRODUCTS OF REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION**

Target	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Product
GAPDH	TGA AGG TCG GAG TCA ACG GAT T	TTG ACG GTG CCA TGG AAT TTG	172
β-actin	TGG CAC CCA GCA CAA TGA AGA TCA	TCA TAC TCC TGC TTG CTG ATC CAC	130
Oct3/4	GTA TTC AGC CAA ACG ACC ATC T	TCT GCT TTG CAT ATC TCC TGA A	140
Nanog	GAA CTC TCC AAC ATC CTG AAC C	TCC CTG GTG GTA GGA AGA GTA A	174
CK13	CTG GAT GAG CTC ACT CTG TCT AAG	CTT CAT CTC CTC TTC ATG GTT CTT	102
CK14	ATT GAG GAC CTG AGG AAC AAG A	GTC TCA TAC TTG GTG CGG AAG T	113

GAPDH — glyceraldehyde 3-phosphate dehydrogenase; CK — cytokeratin.