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Induction of Human Embryonic and Induced Pluripotent Stem Cells Into Urothelium

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ABSTRACT

In vitro generation of human urothelium from stem cells would be a major advancement in the regenerative medicine field, providing alternate nonurologic and/or nonurological tissue sources for bladder grafts. Such a model would also help decipher the mechanisms of urothelial differentiation and would facilitate investigation of deviated differentiation of normal progenitors into urothelial cancer stem cells, perhaps elucidating areas of intervention for improved treatments. Thus far, in vitro derivation of urothelium from human embryonic stem cells (hESCs) or human induced pluripotent stem (hiPS) cells has not been reported. The goal of this work was to develop an efficient in vitro protocol for the induction of hESCs into urothelium through an intermediary definitive endoderm step and free of matrice and cell contact. During directed differentiation in a urothelial-specific medium ("Uromedium"), hESCs produced up to 60% urothelium, as determined by uroplakin expression; subsequent propagation selected for 90% urothelium. Alteration of the epithelial and mesenchymal cell signaling contribution through noncell contact coculture or conditioned media did not enhance the production of urothelium. Temporospatial evaluation of transcription factors known to be involved in urothelial specification showed association of IRF1, GET1, and GATA4 with uroplakin expression. Additional hESC and hiPS cell lines could also be induced into urothelium using this in vitro system. These results demonstrate that derivation and propagation of urothelium from hESCs and hiPS cells can be efficiently accomplished in vitro in the absence of matrices, cell contact, or adult cell signaling and that the induction process appears to mimic normal differentiation. Stem Cells Translational Medicine 2014;3:610–619

INTRODUCTION

Augmentation or creation of a new bladder is often necessary for children and adults with bladder disorders or cancer. Although human and animal studies have demonstrated the feasibility of using bioengineered autologous tissue (1), fabrication of bladder grafts for patients with cancer would ideally come from a nonurologic and/or nonurological source. The ability to induce human embryonic stem cells (hESCs) or human induced pluripotent stem (hiPS) cells into urothelium would provide a major clinical advancement in the regenerative medicine field. Scientifically, a model with which to more precisely study urothelial differentiation would be beneficial for understanding deviations from normal processes that lead to the development of bladder cancer, such as the transformation of normal urothelial stem cells into cancer stem cells (2).

The luminal surface of the urinary tract is lined by a unique epithelium, urothelium, that provides a barrier against urine toxins (3). The urothelium is composed of three types of urothelial cells—basal, intermediate, and superficial/umbrella—that are positioned from the basement membrane to the lumen. From basal stem cell to terminally differentiated umbrella cell, these cell layers model an outward progression of increasing cell differentiation and decreasing cell proliferation. The near impermeability of urothelium is due to specialized proteins called uroplakins (UPs). Four UP subtypes form specific heterodimers (UP1a/UP2 and UP1b/UP3) that assemble in the superficial cells to form crystalline plaques on the luminal surface of the bladder (4). Thus, UP expression and plaque formation are markers for urothelial cell differentiation.

Although the exact process of embryonic stem cell (ESC) differentiation into mature urothelium remains murky, at least two milestones of differentiation are passed: definitive endoderm (DE) and adult urothelial stem cell (5–7). Passage through DE involves expression of the early endoderm transcription factor SOX17 and its downstream targets, FOXA1 and FOXA2 (8, 9). Terminal differentiation of urothelium, as well as adipocytes, is mediated by the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) (10, 11). Specifically, PPARγ activity induces uroplakin expression through the modulation

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of intermediary transcription factors FOXA1 and IRF1, which have cognate binding sites in UP promoters [12]. In the murine bladder, Grainyhead factor GET1/GRHL3 is highly expressed in urothelium. Evaluation of Get1-null mice shows that GET1, which directly targets the Up2 gene, is necessary for urothelial terminal differentiation [13].

Various studies have derived mouse urothelium from progenitor cell sources. Through tissue recombination experiments, Oottamasthien et al. demonstrated the ability of embryonic mouse bladder mesenchyme to induce murine ESC differentiation into urothelium [14]. A temporospatial analysis showed early expression of FOXA1 and FOXA2, markers of DE, then loss of FOXA2 and persistence of FoxA1 with UP expression [15]. Murine embryoid bodies were also induced into epithelium with keratinocyte serum-free medium and 3T3-conditioned medium on collagen membranes. Upon subsequent transplantation into nude mice, the tissue constructs expressed UPs and therefore contained urothelium [16]. In vitro, Mauney et al. demonstrated that all-trans retinoic acid (RA) mediates murine ESC specification to urothelium via a GATA4/6 signaling axis [17].

Human urothelium has been transdifferentiated from bone marrow mesenchymal and adipose-derived stem cells in vitro using urothelial cell conditioned medium and urothelial cell co-culture [18, 19]. In addition, human urothelial cells were differentiated in vivo from a human embryonic germ cell-derived cell line that was grown on a transplanted rat bladder graft [20]. However, in cell derivation of human urothelium from hESCs or hiPS cells has not yet been investigated.

The previous studies of induction of mouse urothelium were performed using various collagen matrices or mesenchymal substrates; however, we and others have demonstrated the ability to affect urothelial cell proliferation, differentiation, and UP expression in vitro without matrix support or cell contact by adjusting culture conditions with regard to PPARγ, epidermal growth factor (EGF), calcium, and bovine pituitary extract [10, 12, 21–24]. Using these matrix- and cell-contact-free conditions, we developed an in vitro protocol for induction of hESCs and hiPS cells into DE and then urothelium, which mimics known urothelial differentiation steps and is not reliant on signals from adult epithelial or mesenchymal cells.

**Materials and Methods**

This research was conducted after receiving approval from the University of California Davis stem cell research oversight committee.

**hESC Maintenance and DE Induction**

The H9 hESC line was grown on irradiated mouse embryonic fibroblasts (GlobalStem, Inc., Rockville, MD, http://www.globalstem.com) in H9 medium consisting of 80% knockout Dulbecco’s modified Eagle’s medium: Nutrient Mixture F12, 20% knockout serum replacement (Invitrogen, Carlsbad, CA, http://www.invitrogen.com); 4 ng/ml basic fibroblast growth factor (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com), 1 mM Glutamax, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acid solution. Passages 39–60 were used for experiments. At intermittent times, H9 ESCs were evaluated for Oct4, Nanog, and Tra160 expression to confirm pluripotency. H9 colonies were also occasionally karyotyped and used in a standard teratoma assay to test for chromosomal abnormality. H9-derived DE and urothelium (p0) were also tested for biosafety using an orthotopic teratoma assay, whereby cells were transplanted into the bladder wall of NOD/SCID/interleukin-2 receptor γ knock-out mice via subepithelial injection.

Induction of hESCs into DE was performed as described by D’Amour et al. [5] in RPMI medium (Mediatech, Inc., Manassas, VA, http://www.corning.com) supplemented with GlutaMAX (Invitrogen), penicillin/streptomycin, and different concentrations of fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA, http://www.gembio.com). On the initiation of differentiation, hESCs were briefly washed phosphate-buffered saline (Gibco, Grand Island, NY, http://www.invitrogen.com) and treated with 0% FBS for 24 hours, then 0.2% FBS for the second 24 hours, and finally 2% FBS for all subsequent days of differentiation. Between days 3 and 12, cells were treated with 100 ng/ml of recombinant human Activin A (R&D Systems).

**Differentiation of DE Into Urothelium**

The medium of DE colony plates was changed to RPMI-based “Uromedium” [21] supplemented with Clonetics Single Quot (hCC-4131) [60 μg/ml of bovine pituitary extract, 0.1 ng/ml of human EGF, 5 μg/ml of insulin, 0.5 μg/ml of hydrocortisone, 30 μg/ml of genaminicin, 15 ng/ml of amphotericin; Lonza, Walkersville, MD, http://www.lonza.com], plus 30 ng/ml cholera toxin A (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) and 2% FBS (Gemini Bio-Products). Plates were incubated in a 37°C humidified atmosphere with 5% carbon dioxide, and the medium was changed every 2–3 days. Transfer to Uromedium was designated day zero. To induce terminal differentiation on day 18, troglitazone (1 μM; a PPARγ activator) was added and human EGF was removed. On day 21, plated cells were fixed for ICC or cells were harvested for flow cytometry or polymerase chain reaction (PCR).

Standard urothelial culture protocols utilize keratinocyte basal medium (KBM). We sought to avoid transfer of the DE cells from an RPMI- to a KBM-based medium. Experiments were carried out to compare urothelial growth on RPMI- and KBM-based media by tetrazolium dye colorimetric method (MTT assay) and analysis of cellular proliferation. No differences were detected.

**Propagation of H9-Induced Urothelium**

For passage and expansion of H9-derived urothelial cells, cultures were switched to a KBM-based Uromedium (Lonza) (rather than RPMI-based) to enrich for and favor long-term urothelial cell growth in vitro. At day 21 after induction (p0), nonterminally differentiated cultures were released from adherence using 0.5% Dispase II (Life Technologies, Rockville, MD, http://www.lifetech.com) and replated for expansion in KBM-based Uromedium. Upon reaching approximately 80% confluence, H9-derived urothelial cells were serial passaged out to 4 passages (p4) before being cryopreserved. At each passage, H9-derived urothelial cells were fixed for intracellular cytokine flow cytometry (ICC) or intracellular flow cytometry analysis of UP expression.

**Culture With All-Trans Retinoic Acid**

DE colonies were grown as described above in the presence of RA (Sigma-Aldrich) added to a final concentration of 10 μM with media changes every 2 days.
Noncontact, Coculture With Human Urothelium

Adult human urothelial cells (ScienCell, Inc., Carlsbad, CA, http://www.sciencellonline.com) were seeded on poly-L-lysine 75-cm² tissue culture dishes with universal collection medium (ScienCell, Inc.) supplemented with penicillin/streptomycin and growth factors. After the colonies reached 70% confluence, they were washed, harvested, centrifuged, resuspended, and seeded on Transwell permeable membranes (0.4-µm pore size) in six-well plates and incubated overnight. The next day, Transwell inserts were placed in six-well chambers of DE colonies and grown with the DE (noncontact) in RPMI-based Uromedium for 21 days, as described above. A negative control was run with DE cells grown in the bottom well without human urothelial cells.

Culture With Urothelial Cell-Conditioned Medium

Adult human urothelial cells (ScienCell Inc.) were grown in RPMI-based Uromedium. After colonies reached 70% confluence, medium was collected every 3 days, pooled and filtered through a 0.24-µm filtration unit (Corning Enterprises, Corning, NY, http://www.corning.com). DE colonies were grown for 21 days in conditioned medium, as described above.

Culture With Bladder Smooth Muscle Cell-Conditioned Medium

Smooth muscle cells were isolated from human cadaveric bladders and cultured in 75-cm² tissue culture flasks in smooth muscle cell medium (Lonza). Cells were passaged and expanded; conditioned medium from smooth muscle cells was collected every 2-3 days and filtered through a 0.24-µm filter. DE colonies were grown for 21 days in conditioned medium, as described above.

Other hESC and iPSC Cell Lines

The above experiments were performed a minimum of three times for the H9 hESC line. After analysis of results, induction of the following lines into urothelium using Uromedium alone was evaluated (n = 1-3): H1 (hESC; WiCell Research Institute, Madison, WI, http://www.wicell.org), HuES8 (hESC; Harvard University), IMR90-4 hiPSCs (source: human foreskin; WiCell), and hiPSCs derived from CD34⁺ hematopoietic stem cells (hESC; source: human cord blood, lentiviral transduction of OCT4/SOX2/KLF4/MYC) [25].

Immunocytochemistry

Cultured cells were fixed for 15 minutes at room temperature in 4% wt/vol paraformaldehyde. Cells were then washed several times in Tris-buffered saline and blocked in Tris-buffered saline/0.25% wt/vol Triton X-100 (Sigma-Aldrich) containing 3% normal donkey serum (Jackson Laboratory, Bar Harbor, ME, http://www.jax.org). Primary antibodies and concentrations used are listed in supplemental online Table 1 under “Supporting Information.” Appropriate secondary antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) were used for detection. DAPI (4',6-diamidino-2-phenylindole) was used as a nuclear counterstain.

Image J Quantification of Uroplakin Staining

ICC-stained slides were visualized under a Nikon Eclipse E400 fluorescence microscope (Nikon, Tokyo, Japan, http://www.nikon.com) and photographed with an RTKE Diagnostic Instrument Spot camera (Spot Imaging Solutions, Sterling Heights, MI, http://www.spotimaging.com). Using a ×10 objective lens, images were taken of six to eight fields per well of a six-well plate for each uroplakin stain and culture condition. Fields where chosen based on preselected points on a grid. In the case of overlapping colonies, that particular preselected point was skipped and an image was taken of the next dictated point. Fluorescence was quantified in ImageJ [26] using the Color Threshold and Analyze Particles tools (National Institutes of Health, Bethesda, MD, http://rsbweb.nih.gov/ij/). Negative controls for each fluorescent stain were used to set the color thresholds, and the values were applied to test samples. The area of staining was generated as a number of pixels, and for each condition, the ratio of U to DAPI pixels was calculated and averaged (n = 6-8). Using Prism 6 software (GraphPad Software, Inc., San Diego, CA, http://www.graphpad.com), the average ratio of U to DAPI was graphed as a percentage and plotted with SEM values (n = 3 experiments for each condition). Prism 6 was also used to run statistical analyses of the data. Differences between conditions were compared using unpaired t tests and an analysis of variance (ANOVA) test; a p value of <.05 was considered significant.

Real-Time Quantitative PCR

RNA was isolated using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany, http://www.qiagen.com), following the manufacturer’s instructions. RNA was reverse transcribed using random hexamer primers and oligo(dT) with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, http://www.bio-rad.com), following the recommended protocol from the manufacturer. Specific cDNA targets were amplified using iQ SYBR Green Supermix (Bio-Rad) on an i-Cycler instrument (Bio-Rad). β-Actin and GAPDH were used as reference genes. The relative levels of mRNA were quantified using the 2−ΔΔCT method [27]. Forward primers are listed in supplemental online Table 2 under “Supporting Information.”

Intracellular Flow Cytometry

Cell cultures were harvested via 0.5% Dibase II (Roche, Indianapolis, IN, http://www.roche.com) and stained with LIVE/DEAD Fixable Near-IR dye (Invitrogen), as per the manufacturer’s instructions. Cells were fixed with flow cytometry fixation buffer (R&D Systems), per manufacturer’s instructions, then permeabilized, blocked and stained in flow cytometry permeabilization/wash buffer I (R&D Systems) with 10% human and 10% donkey sera. The following primary antibodies were used in a multicolor flow cytometry analysis panel: UP2a and UP1b (rabbit; Abcam, Cambridge, U.K., http://www.abcam.com), UP2 and UP3 (goat; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), IF1 (mouse; Abcam), GATA4 AlexaFluor 488 (mouse; BD Biosciences, San Diego, CA, http://www.bdbsciences.com) and Sox17AlexaFluor 488 (mouse; R&D Systems), FoxA2 (mouse; BD Biosciences), and GRL13/GET1 (goat; Santa Cruz). Negative controls were prepared using matched isotype controls or secondary antibody-only stains, as appropriate. Data were collected on an LSRII Fortessa cell analyzer (BD Biosciences), and analysis was performed with FlowJo software (TreeStar, Ashland, OR, http://www.treestar.com).
Figure 1. Illustration of the in vitro culture system used for the differentiation of hESCs into urothelium. hESCs were treated with increasing gradients of FBS for 3 days and then Activin A for 9 days to form DE. DE was directed toward urothelium via culture in Uromedium, a urothelial-specific medium, or other tested conditions. Various markers for pluripotency, multipotency, and differentiation were assessed at various time points during the entirety of the culture. Abbreviations: DE, definitive endoderm; EGF, epidermal growth factor; FBS, fetal bovine serum; hESCs, human embryonic stem cells; PPARγ, peroxisome proliferator-activated receptor γ; RA, retinoic acid.

Figure 2. The expression patterns of pluripotency, multipotency, and differentiation markers during the induction of H9 human embryonic stem cells to urothelium. Real-time polymerase chain reaction was used to analyze the expression of (A) pluripotency markers, OCT4 and NANOG (A); definitive endoderm markers SOX17 and FOXA2, mesoderm marker Brachyury, and ectoderm marker ZIC1 (B); and uroplakins (subtypes UP1a, UP1b, UP2, UP3a, UP3b) (C). (D) Flow cytometry analysis of SOX17 and FOXA2 expression was used to quantify the amount of definitive endoderm derived from Activin A-treated H9 human embryonic stem cells. SOX17 and FOXA2 expressions (solid lines) are shown relative to matched isotype controls (shaded histograms).

RESULTS

Differentiation of Urothelium From H9 Human Embryonic Stem Cells Through a Definitive Endoderm Intermediary Step

Undifferentiated H9 hESCs were induced to become urothelium through a definitive endoderm intermediary step, as illustrated in Figure 1. At strategic time points during the differentiation process, real-time PCR was used to investigate the expression profiles of various markers for pluripotency, multipotency, and differentiation. As expected, undifferentiated H9 expressed high levels of the pluripotency genes OCT4 and NANOG, and expression decreased on Activin A treatment and subsequent differentiation. H9 cells also lacked expression of differentiation markers SOX17, FOXA2, uroplakins, and ZIC1, further confirming their undifferentiated state. On differentiation into DE, a concurrent increase of DE-specific genes, SOX17 and FOXA2, accompanied downregulation of the pluripotency genes (Fig. 2B). Subsequently, the expressions of
SOX17 and FOXA2 were downregulated as DE was induced into urothelium. The mesodermal marker Brachyury was expressed at very low levels in H9 and was further downregulated during differentiation to DE and then urothelium. In contrast, ZIC1, an ectodermal marker, was upregulated during the urothelial induction phase. Although flow cytometry analysis of SOX17 and FOXA2 consistently showed that approximately 80% of cells become DE on Activin A treatment of H9 (Fig. 2D), this culture system also supported some ectodermal cell lineage differentiation, as demonstrated by ZIC1 mRNA expression. However, ZIC1 expression was minimal and limited to a few nonurothelial colonies after initial induction (p0), as determined by ICC analysis (supplemental online Fig. 1). Because real-time PCR data are not quantitative but rather are merely comparative, the 40-fold increase in ZIC1 expression over the near-zero expression in DE does not accurately reflect the actual production of ectodermal cells in this culture system.

The production of urothelium from DE was determined by analyzing the expression of uroplakins, urothelial cell-specific differentiation markers, at various time points over the 21-day UroMedium-driven induction period (Fig. 1). Induced cultures were analyzed by ICC for all uroplakin subtypes (UP1a, UP1b, UP2, UP3) at days 6, 12, 18, and 21; DE was designated day 0. Figure 3A shows the emergence of UP-expressing colonies as early as 6 days after DE. Uroplakin expression in individual colonies appeared to increase out to day 21. In addition, a brightness image of urothelial colonies at day 18 displayed the typical cobblestone morphology of cultured urothelium (Fig. 3C). Real-time PCR analysis further confirmed the UP expression pattern observed by ICC. All subtypes of human UPs were found to be upregulated during the urothelial induction phase. Uroplakin expression emerged by day 6 and increased out to day 21 (Fig. 2C); however, as described below, temporal analysis of intracellular UPS by flow cytometry suggested even earlier induction of urothelium, by day 1 after DE.

Flow cytometry was also used to assess the production of urothelium at day 21, the end of culture. Intracellular staining for each individual UP subtype showed that a proportion of the cell...
population expressed UP1a, UP1b, UP2, and UP3, as determined by the unimodal shift of UP expression over the shaded negative control (Fig. 3C). Although expression of all UPs could be detected by day 21, UP1a and UP1b were more highly expressed than were UP2 and UP3. The histograms in supplemental online Figure 2 show the specificity of the UP intracellular staining.

At day 21 after induction, nonterminally differentiated cultures were passaged and derived urothelial cells were maintained and expanded out to four passages. Because KBM-based medium is the typical growth medium for urothelium, we switched from the induction RPMI-based Urometer to KBM-based Urometer at the point of initial passage. KBM-based Urometer further selected for urothelial cell growth on passaging, as demonstrated by the increase in UP1a expression by intracellular flow cytometry analysis of passaged cultures (passages 1–3), as compared with the initial induction (p0) (Fig. 3D).

The Influence of Signal Modulation on the Production of Urothelium In Vitro

To determine whether altering cell signaling could increase the production of urothelium in this in vitro system, various culture conditions were investigated. To activate GATA4/6 signaling, all-trans retinoic acid was added to Urometer. In addition, adult urothelial cell conditioned medium, adult smooth muscle cell conditioned medium, and noncontact coculture with adult urothelial cells were evaluated. Using an ImageJ-based method to quantify ICC images of day 21 H9-derived cultures, the relative amount of UP expression to colony surface area was determined. As shown in Figure 4, all subtypes of UPs were expressed under all conditions tested. UP1a and UP1b expression ranged from approximately 10% to 60%, with UP2 and UP3 being expressed relatively less. Statistical analyses showed there to be no significant differences among Urometer; RA-treated, urothelial cell conditioned medium; and urothelial cell coculture conditions (unpaired t tests p > .05 and ANOVA). Although an ANOVA test suggested smooth muscle cell conditioned medium to be significantly less productive than the other four conditions, individual t tests showed no difference between smooth muscle cell conditioned medium and any other individual condition (p > .05). Thus, no tested condition was better able to induce urothelial differentiation beyond that induced with Urometer in vitro.

In Vitro Derivation of Urothelium From hESCs Recapitulates Normal Urothelial Cell Differentiation

The transcription factors IRF1, GATA4, GE1, and FOXA2 have been previously identified as being involved in urothelial cell specification. To determine whether urothelial cell differentiation in this culture system mimics that of other systems or in vivo cell specification, these transcription factors were analyzed for their coexpression with UPs during the induction of urothelium from H9 hESCs. Specifically, cells were analyzed for the expression of UP, IRF1, GATA4, GE1, and FOXA2 within the first 4 days of urothelial differentiation by multicolor flow cytometry analysis. IRF1 and GE1 were expressed at days 1 and 4 after DE, respectively, and coexpressed with UP1b in approximately 20–30% of cells (Fig. 5A, 5C). GATA4 expression was maximally detected on day 4 but to a much lesser extent than either IRF1 or GE1; however, it did appear that GATA4 and UP1b were coexpressed in a small percentage of cells (Fig. 5B). FOXA2 was found to be coexpressed with UP out to day 4 (Fig. 5D). Because FOXA2 is expressed in DE and during urothelial cell differentiation but downregulated in mature urothelium, the expression of FOXA2 in urothelial cells was also analyzed at day 21 (Fig. 5D, far right). Interestingly, FOXA2 was still expressed in approximately 20% of UP-expressing cells at day 21. ICC analysis further confirmed the coexpression of IRF1 with UP1b and GATA4 with UP2 in some cells induced at day 1 (Fig. 5F).

In Vitro Differentiation of Urothelium From hiPSC Cells

The vast majority of patients requiring bladder grafts would benefit most from tissue derived from nonautologous or nonurologic sources. To extend the proof-of-concept of deriving urothelium from H9 hESCs in vitro, two alternative hESC lines (H1, HuES8) and two hiPSC cell lines (human CD34+ HSC-derived iPSCs, IMR90-4) were used in this culture system. On day 21 of induction in Urometer, cultures derived from these hESCs and hiPSC cells were analyzed for the expression of uroplakins by flow cytometry (Fig. 6). UP1a and UP1b were distinctly expressed in each of the four cell types tested and to similar levels as seen in H9 hESC-derived cultures. The expression of uroplakins was also confirmed by ICC for H1, HSC-derived iPSC, and IMR90-4 lines (supplemental online Fig 3). Thus, urothelium can be derived from multiple sources using this in vitro culture system.
Figure 5. Uroplakins coexpress with transcription factors known to be involved in urothelial cell fate specification. (A–D): H9 human embryonic stem cell-induced cultures were analyzed during the first 4 days of urothelial induction for the expression of UP1b and transcription factors IRF1, GATA4, GET1, and FOXA2 by intracellular flow cytometry analysis. From left to right, expression of each protein (solid blue line histogram) is depicted relative to its negative control (shaded gray histogram). Bivariate dot plots display the relationship between UP1b and each transcription factor (gray dots represent the negative control; blue dots represent antibody stain). Quadrant gates are set based on the negative controls. (D): At far right, the second bivariate plot for FOXA2 depicts the relationship with UP1b at day 21. All flow data are representative of n = 3 experiments. (E): HepG2 cells were used as a positive control for expression of the noted transcription factors. (F): Induced H9 human embryonic stem cell cultures were analyzed at day 1 after DE by intracellular cytokine flow cytometry for the coexpression of UP1b (green) with IRF1 (red) and UP2 (green) with GATA4 (red). DAPI (4',6-diamidino-2-phenylindole; blue) was used as a nuclear counterstain. Magnification ×20.
**DISCUSSION**

Currently, when a patient needs replacement or enlargement of the bladder, the surgeon's only choice is intestinal tissue. Because the functions of the gastrointestinal epithelium and the urothelium are diametric in nature, ensuing complications from such grafting practices make intestinal tissue a less-than-optimal choice. Storage of urine in a reservoir lined by intestinal epithelium is associated with urinary stone formation, electrolyte abnormalities, and neoplasia. In the next few decades, there is a strong possibility that patients with benign urologic disease will have bioengineered tissue available for reconstruction. In dogs, acellular bladder matrices that have been recellularized with urothelial and smooth muscle cells exhibit better regeneration on implantation than their acellular counterparts [28], and bladder tissue constructs used in human clinical trials have thus far been derived from autologous, adult cells [1].

The majority of patients in need of replacement bladder tissue have urothelial cancer. Because bladder cancer is multifocal and is classified as a “field” defect with premalignant urothelium scattered throughout the bladder, autologous urothelium from a cancer patient for bioengineering purposes would not be safe. Consequently, the need for nonautologous urothelium for optimal bladder tissue construction is evident. With future application of umbilical cord stem cell banking, hESC banking, and hiPS cell availability becoming mainstream, we envision multiple potential sources of induced urothelium that may carry less risk of malignancy and thus allow more patients to benefit from advances in tissue engineering [29].

For translational purposes, an in vitro system devoid of matrices, cell contact, and animal products or cells is necessary. We have established an in vitro protocol that produces urothelium from hESCs, through an intermediary DE step, in culture conditions free of matrices, cell contact, and adult cell signaling. By generating urothelium in vitro, we avoid the direct implantation of hESCs in vivo and the associated potential for malignancy. As an initial safety measure, H9-induced urothelium (p0) was transplanted for an orthotopic teratoma assay, of which the results were negative. The next foreseeable step is to adapt this culture system to a truly xenogenic free in vitro system. Although a serum-free system for induction of hESCs into DE has been described previously [30], establishment and maintenance of human urothelium in vitro can also be achieved without serum [31]. Thus, the potential for serum-free induction of urothelium from hESCs in vitro is promising.

The data presented in this paper suggest that this in vitro culture system does indeed support a directed process of differentiation from hESCs to DE to urothelium. The downregulation of pluripotency and the upregulation of appropriate multipotency (DE) and differentiation gene expression, as elucidated by real-time PCR, demonstrate the appropriate transitions of undifferentiated hESCs to DE to urothelium.

The process of producing human urothelium with this in vitro system recapitulates certain known standards of urothelial differentiation. FOXA2 is a marker of multipotent DE, which is subsequently decreased during urothelial cell maturation [15]. As anticipated, FOXA2 and UP were coexpressed in cells within days of Urothelial medium induction. Interestingly, a proportion of UP-expressing cells still expressed FOXA2 after PPAR γ activation and 21 days in culture. This, in addition to the observation that UP1a and UP1b were consistently expressed at higher levels than UP2 and UP3, suggests a relatively immature phenotype of the induced urothelium. Because cultured urothelium typically takes on an immature phenotype, this rightfully and likely reflects the incomplete maturation of the hESC-derived urothelium [32]. Consistent with this observation and the fact that cultured urothelium does not express large quantities of UPs at the cell surface, we were not able to efficiently detect or use fluorescence-activated cell sorting hESC-derived urothelium via extracellular UPs.

Because we were unable to purify the induced urothelium using extracellular UP-based fluorescence-activated cell sorting, we used serial passaging and medium-based selection techniques to enhance purification. In doing so, we successfully increased the purity of our cultures to approximately 90% urothelium. These hESC-derived urothelial cells have been passaged out to passage 4 and cryopreserved and will be used as a source of nonautologous urothelial cells in our current and future bladder tissue engineering endeavors.

In the urothelium produced in this system, the expression of UP was found to be temporospatially associated with IRF1 and GET1, two transcription factors previously identified as mediators of urothelial differentiation. Whereas GATA4 signaling has been shown to strongly drive urothelial specification from mouse ESCs [17], the association of UP and GATA4 in hESC-derived urothelium was nominal. GATA4 was not significantly expressed in the culture and its coexpression in UP-expressing cells was limited to a small percentage of the population. Whether this disparity is due to species divergence, culture system differences, or timing is not yet clear.
Although a mechanistic evaluation of urothelial differentiation was not our intent, future investigations using overexpression and/or RNA inhibition to define the roles of the specified transcription factors in differentiation could be of interest. Much like UP proteins, transcription factors such as IRF1 and Gct1 could serve as indicators of targeted differentiation and warrant further study. More comprehensively, this in vitro system will allow for more detailed and controlled investigations into the biological processes of urothelial differentiation.

From a translational perspective, the ability to ultimately adapt this induction protocol from hESCs to iPSCs, and likely to other progenitor cell types, will allow for the derivation of urothelium from sources with features that are best able to meet the needs of an individual's medical condition (e.g., autologous/nonautologous, urologic/nonurologic, patient derived). Consequently, the ability to efficiently make human urothelium from hESCs and hiPS cells holds great promise in producing a source of bioengineered urothelium for those requiring urologic reconstruction.

CONCLUSION

The current study demonstrates that derivation of urothelium from hESCs and iPSCs can be sufficiently accomplished in vitro in the absence of matrices, cell contact, or adult cell signaling and that the induction process appears to mimic normal differentiation. Although the translational application of using hESCs and particularly iPSCs in tissue engineering is still being validated, the ability to produce urothelium in vitro from these sources is an accomplishment that holds important implications for the future bioengineering of bladder tissue for patients with bladder cancer.

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AUTHOR CONTRIBUTIONS

S.L.O. and R.T.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; A.L. and J.N.: collection and/or assembly of data, data analysis and interpretation; J.H.L.: collection and/or assembly of data; E.A.K.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

E.A.K. is a compensated consultant with Allergan, consultant for clinical trials, and has received compensated research funding.

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From “Highly Efficient Differentiation of Functional Hepatocytes From Human Induced Pluripotent Stem Cells,” by Xiaocui Ma, Yuyou Duan, Benjamin Tschudy-Seney, Garrett Roll, Iman Saramipoor Behbahan, Tijess P. Ahuja, Vladimir Tolstikov, Charles Wang, Jeannine McGee, Shiva Khoobyari, Jan A. Nolta, Holger Willenbring, and Mark A. Zern, published in Stem Cells Translational Medicine 2012;1:409-419.

Definitive endoderm cells were stained with the primary antibodies goat anti-SOX17 (green) (top left inset) and FOXA2 (pink) (bottom left inset) at day 8 after the induction of human induced pluripotent stem cells to definitive endoderm. The differentiated cells were then stained with the primary antibodies monoclonal antibody against α-fetoprotein (red) (middle inset) at day 9 and goat anti-albumin (green) (right inset) at day 25 after starting the differentiation of definitive endoderm toward hepatocytes.