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Improvement of transfection with reprogramming factors in urine-derived cells

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Abstract: Human-induced pluripotent stem cells (iPSCs) are an accessible source of adult-derived, patient-specific pluripotent stem cells for use in basic research, drug discovery, disease modeling, and stem cell therapy. Improving the accessibility of methods to obtain iPSCs regardless of the cell source can enhance their clinical application. Therefore, our purpose is to report a simple protocol to obtain iPS-like cells from urine-derived renal epithelial cells (RECs) using different extracellular matrices and transfection reagents. In this study, we began by culturing urine-derived cells from healthy donors to establish a primary culture of renal epithelial cells, followed by their characterization. Subsequently, we generated iPS-like cells by transfecting renal epithelial cells (RECs) with vectors expressing Oct4, Sox2, L-Myc, Lin-28, and Klf4, and we compared the efficacy of different extracellular matrices and transfection reagents. The resultant iPS-like cells showed a human embryonic stem cell-like morphology and expressed the specific pluripotency markers Oct3/4, Nanog, Lin28, and Klf4. We concluded that Lipofectamine Stem Cell transfection reagent is more effective than FuGENE in obtaining iPS-like cells under the conditions tested. Moreover, the three matrices are similar in their efficiency of obtaining iPS-like cells. This report provides an experimental protocol for obtaining and generating iPS-like cells from urine samples for further cell therapy research on different human diseases.

Introduction

Since it has been shown that the human somatic cells can be reprogrammed to produce induced pluripotent stem cells (iPSCs) by expressing specific transcription factors, it has opened unprecedented opportunities in basic research, drug discovery, disease modeling, and stem cell therapy. The first report on iPSCs was published in 2006; iPSCs were generated by exposing murine fibroblasts to retroviruses expressing four reprogramming factors: Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). Then, the first generation of human iPSCs was constructed by the transduction of adult human fibroblasts with the same reprogramming factors (Takahashi *et al.*, 2007). Along with these reports, another research group generated human iPSCs by the transduction of human fetal fibroblasts with the Oct4, Sox2, Lin28, and Nanog reprogramming factors (Yu *et al.*, 2007). The hazard of retrovirus genome integration into the host genome raises the possibility of insertional mutagenesis and oncogene activation (Anson, 2004). Therefore, the production of virus-free iPSCs is a critical safety concern for their potential clinical application in cell and gene therapy. Okita and colleagues described an efficient method to generate virus-free iPSCs derived from mouse embryonic fibroblasts by transient plasmid expression (Okita *et al.*, 2008). Patient-specific iPSCs have been widely used to study the mechanisms of various diseases and test new pharmacological therapies in a variety of human diseases (Crandall and Lalande, 2013). These iPSCs can be generated from various cellular sources ranging from skin fibroblasts to blood cells; however, endometrial and urine-derived cells hold tremendous clinical interest because they can be obtained through noninvasive methods to minimize the collateral risks

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of biopsy in patients with diseases such as diabetes or hemophilia (Ding *et al.*, 2015). Due to variation between iPSC clones obtained from different cell sources, feeder-free systems, and culture conditions, it remains important to establish a simple protocol for obtaining iPSCs regardless of the cellular source or patient's background (Nagasaka *et al.*, 2017; Chin *et al.*, 2009).

This study aimed to report a simple protocol for obtaining iPS-like cells from urine-derived renal epithelial cells from healthy donors using nonviral vectors. Here, we present a comparison of commercial extracellular matrices and transfection reagents in urine-derived cells. Our results showed that the transfection reagent is a significant factor to consider in the efficacy of obtaining iPS-like cells; meanwhile, there was a similar efficacy among the three matrices tested. These results can be further used to support protocol selection for the generation of iPS-like cells from patients with different medical conditions.

Materials and Methods

Urine sample collection

Urine samples were obtained from three healthy male donors. This work was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee from the School of Medicine, Universidad Autónoma de Nuevo León (Monterrey, México) under Reg. HT17-00002. All volunteers who participated in this study signed written informed consent before donating urine samples.

Donors were asked to clean their urethral area with flushable, antibacterial, premoistened wipes, and discard the first urine stream into the toilet before collecting their urine in sterile containers. All samples (ranging between 100 and 200 mL each) were processed within 60 min post-collection.

Primary renal epithelial cell culture

Urine samples were transferred to 50 mL-tubes inside a culture hood and centrifuged at 300 × *g* for 10 min at room temperature. The supernatant was carefully discarded by pipetting inside a culture hood, leaving approximately 0.2 mL of urine in the tube, and the cell pellets were washed three times with 10 mL of 1X PBS containing 100 U/mL penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Lafayette, CO, USA, #15240062) at 300 × *g* for 10 min at room temperature. The cell pellet was resuspended in 1 mL of Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) (Gibco, Thermo Fisher Scientific, Lafayette, CO, USA, #11320033) supplemented with the Renal Epithelial Cell Growth kit (RECG) (ATCC, Manassas, VA, USA #PCS-400-040) and 100 U/mL penicillin/streptomycin. From this point onwards, this medium mixture is referred to as the primary medium. The cell suspension was seeded into 12-well plates coated with 0.1% L-gelatin (Sigma-Aldrich, St. Louis, MO, USA, #G1393) in 1X PBS. Every day during the first three days post-isolation, 1 mL of the primary medium was added. Beginning the next day, half of the medium was replaced with fresh renal epithelial cell basal medium (RECBM) (ATCC, Manassas, VA, USA, #PCS-400-030) supplemented with the RECG kit and 100 U/mL penicillin/streptomycin daily before visible cells/colonies appeared and after the establishment of a primary culture of renal epithelial cells (RECs). The first full change of medium was made after the first colonies were observed to maintain factor secretion from the urine-derived cells and avoid unnecessary stress.

Primary renal epithelial cell characterization

To characterize the cell morphology by immunofluorescence, 6×10^4 RECs were seeded into a 24-well plate. After 12 h, the cells were fixed with methanol/acetone, washed with 1X PBS and blocked with 3% normal goat serum (Invitrogen, Thermo Fisher Scientific, Lafayette, CO, USA, #31872) for 30 min at 4°C. Cells were washed and incubated with the following primary antibodies overnight at 4°C: anti-cytokeratin (1:100; Dako, Agilent, Santa Clara, CA, USA #Z0622), anti-E-cadherin (1:100; BD Biosciences, San Jose, CA, USA, #610181), β-catenin (1:100; BD Biosciences, San Jose, CA, USA, #610181), anti-ZO-1 (1:100; Invitrogen, Thermo Fisher Scientific, Lafayette, CO, USA, #33-9100), anti-CD10 (1:100; Biocare medical, Pacheco, CA, USA, # CM129C), and anti-CD13 (1:100; R&D Systems, Minneapolis, MN, USA, #MAB3815). The next day, the cells were washed with 1X PBS and incubated with the following secondary antibody for 3 h at 4°C in the dark: goat anti-mouse CF594A (1:200; Biothium, Fremont, CA, USA, #20111). Cellular nuclei were stained with DAPI (286 nM in 1X PBS, Thermo Fisher Scientific, Lafayette, CO, USA, #D1306) for 10 min and washed with 1X PBS. The slides were mounted with VectaShield® Vector Laboratories Inc., Burlingame, CA, USA) and analyzed by fluorescence microscopy (Leica) before photographic documentation with the QCapture Pro 7 program (QImaging).

Reprogramming of renal epithelial cells

Renal epithelial cells with less than 4 passages were subjected to dedifferentiation into pluripotent stem cells by transfection. RECs were seeded at a density of 6×10^4 into a 24-well plate coated with three different extracellular matrices: Matrigel® (1:100; Corning, Santa Barbara, CA, USA, #354277), Geltrex® (1:100; Gibco, Thermo Fisher Scientific, Lafayette, CO, USA, #A14133-01), and vitronectin (1:100; Gibco, Thermo Fisher Scientific, Lafayette, CO, USA, #A14700). After 48 h, RECs were transfected with the Epi5 Episomal iPSCs Reprogramming kit® (Invitrogen, Thermo Fisher Scientific, Lafayette, CO, USA, #A15960) using Lipofectamine stem cell (Invitrogen, Thermo Fisher Scientific, Lafayette, CO, USA, #STEM00001) or FuGENE HD® (Promega, Madison, WI, USA, #E2311) transfection reagent, following the manufacturer's instructions. At 24 h post-transfection, the medium was changed to mTeSRI® basal medium (STEMCELL Technologies, Cambridge, MA, USA, #85851) supplemented with mTeSRTM1 5X (STEMCELL Technologies, Cambridge, MA, USA, #85852), and 100 U/mL penicillin/streptomycin. The cells were monitored daily to detect the appearance of the first iPS-like colonies. Each colony showing the appropriate morphology reported in the literature was selected, picked up with a needle, and seeded in a 96-well plate coated with Matrigel, Geltrex, or vitronectin and maintained with mTeSRTM1® basal medium supplemented with mTeSRTM1 5X for human pluripotent stem cells to continue the clonal expansion. When the colonies reached the appropriate confluence, the cells were dissociated, resuspended in ReLeSR®

stem cell reagent (STEMCELL Technologies, Cambridge, MA, USA, #05872) and seeded into a 48-well plate to continue clonal expansion. The iPS-like colonies were expanded for two months prior to further assays.

iPS-like cells characterization

iPS-like expanded colonies were seeded into a 24-well plate coated with Matrigel, Geltrex or vitronectin and incubated until 80% confluence was reached. Then, the cells were fixed with methanol/acetone, washed with 1X PBS, and blocked with 3% normal goat serum for 30 min at 4°C. Cells were washed and incubated with the following primary antibodies overnight at 4°C: anti-Nanog (1:50; R & D Systems, Minneapolis, MN, USA, #AF1997), anti-Lin28 (1:50; R&D Systems, Minneapolis, MN, USA, #AF3757), anti-Oct4 (1:100; R&D Systems, Minneapolis, MN, USA, #MAB17591), and anti-Klf4 (1:50; R & D Systems, Minneapolis, MN, USA, #AF3640). The next day, the cells were washed with 1X PBS and incubated with the corresponding secondary antibody for 3 h at 4°C in the dark as follows: anti-mouse CF594A (1:200; Biothium, Fremont, CA, USA #20111) or anti-goat NL557 (1:200; R&D Systems, Minneapolis, MN, USA, #NL999). Cellular nuclei were stained with DAPI for 10 min and washed with 1X PBS. The slides were mounted with VectaShield® and analyzed by fluorescence microscopy (Leica, DM1000), followed by photographic documentation with the Qcapture Pro 7 program (QImaging). Representative microscope fields are shown.

Analysis of gene pluripotency expression in iPS-like cells

For the analysis of gene expression, RNA isolation from clones obtained of iPS-like cells was performed with the GeneJet RNA Purification kit® (Thermo Fisher Scientific, Lafayette, CO, USA, #K0731) according to the manufacturer's instructions. One microgram of the RNA transcripts was used for cDNA synthesis using the Maxima First Strand cDNA Synthesis kit® for RT-PCR (Thermo Fisher Scientific, Lafayette, CO, USA, #K1641). Once the cDNA was obtained, the RT-qPCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix® (Thermo Fisher Scientific, Lafayette, CO, USA, #K0221) with the following oligonucleotides: Oct3/4, Sox2, Nanog, Lin28, Nodal, Rex1, and GAPDH. GAPDH was used to normalize the gene expression (Tab. 1).

qPCR reactions were performed using a StepOne Real-Time PCR System (Applied Biosystems). Calculations were made using the $2^{-\Delta\Delta Ct}$ threshold cycle method and normalized to the expression of the endogenous gene GAPDH.

Statistical analysis

The results were analyzed by one-way ANOVA and Tukey's multiple comparisons test and plotted using Prism software v.6 (GraphPad, Inc., San Diego, CA, USA). $p < 0.05$ indicates statistical significance.

Results

Establishment of primary renal epithelial cell culture from the urine samples of healthy donors

The isolation and culture of renal epithelial cells generate colonies with different morphologies named types 1 and 2. Type 1 cell colonies have irregular shapes and consist of spindle-like cells. In contrast, type 2 cell colonies are smooth (Dörrenhaus *et al.*, 2000). To obtain renal epithelial cells, we isolated urine-derived cells from the samples of healthy donors. Within the first day after isolation, the cell morphology was similar to that of squamous cells and predominated by small, round cells. After 6 to 9 days, small colonies with irregular and smooth edges were observed, which was consistent with renal epithelial cell morphology. At 12 to 15 days after their isolation, cells were harvested after no more than four passages to continue the expansion of the renal cells to further characterize and reprogram the cells (Figs. 1a and 1b).

Reports have indicated that renal cells express epithelial markers as cadherin, cytokeratin, zonula occludens-protein 1 (ZO-I), and others (Zhou *et al.*, 2012). Immunofluorescence confirmed that primary cultures of RECs express the epithelial-specific markers: cytokeratin, E-cadherin, β-catenin, ZO-I, and proximal tubular epithelial markers: neutral endopeptidase (CD10) and aminopeptidase N (CD13) (Fig. 2). The expression of epithelial-specific marker proteins indicated the successful establishment of a primary renal epithelial culture from the urine samples of healthy donors. We observed an isolation rate of 75% among all the donor samples containing cells that proliferated to colonies.

Renal epithelial cell reprogramming using nonviral vectors and different extracellular matrices

Somatic cells can be reprogrammed to iPSCs by overexpressing certain defined transcription factors. iPSCs can be generated by using nonintegrative vectors, such as adenovirus or plasmids, with the latter preferable for clinical applications (Stadtfeld *et al.*, 2008; Okita *et al.*, 2013). We transfected RECs at passage 3 with nonviral vectors expressing the reprogramming factors Oct4, Sox2, Lin28, L-Myc, and Klf4 to induce cell dedifferentiation into pluripotent stem cells. Morphological changes are indicative of cell reprogramming. After 12 days of transfection, we observed small colonies made up of polyhedral cells with large nuclei and scarce cytoplasm, which are similar to the morphology criteria of ESC (embryonic stem cell)-like colonies (Nagasaka *et al.*, 2017). By day 20, compact colonies were observed, selected, picked up under the microscope, and expanded for further characterization (Figs. 3a and 3b).

Three different extracellular matrices and two transfection reagents were tested. Cells seeded on Matrigel, Geltrex or vitronectin generated more iPS-like colonies when transfected with Lipofectamine stem cell reagent than with FuGENE reagent. Statistical analysis revealed no significant difference between the extracellular matrices; moreover, upon transfection reagent analysis, we detected that Lipofectamine stem cell reagent is significantly more effective than FuGENE in obtaining iPS-like colonies from urine-derived renal epithelial cells in these conditions (Fig. 3c).

iPS-like cells derived from RECs express pluripotency markers

Next, we aimed to determine whether the initial iPS-like colonies could maintain their pluripotent state after clonal expansion. Immunofluorescence confirmed that the iPS-like cells with no more than three passages expressed the

TABLE 1

Oligonucleotides used for qPCR

Oligonucleotides	Forward	Reverse
Oct3/4	F-GACAGGGGGAGGGGAGGAGCTAGG	R-CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2	F- GGGAAATGGGAGGGGTGCAAAAGAGG	R-TTGCGTGAGTGTGGATGGGATTGGTG
Lin28	F-GGAGGCCAAGAAAGGGAATATGA	R-AACAATCTTGTGGCCACTTTGACA
Nanog	F-CAGCCCCGATTCTTCCACCAGTCCC	R-CGGAAGATTCCCAGTCGGGTTCAC
Nodal	F-GGGCAAGAGGCACCGTCGACATCA;	R-GGGACTCGGTGGGGCTGGTAACGTTTC
Rex1	F-CAGATCCTAAACAGCTCGCAGAAT	R-CAGATCCTAAACAGCTCGCAGAAT
GAPDH	F-GTGGACCTGACCTGCCGTCT	R-GGAGGAGTGGGTGTCGCTGT

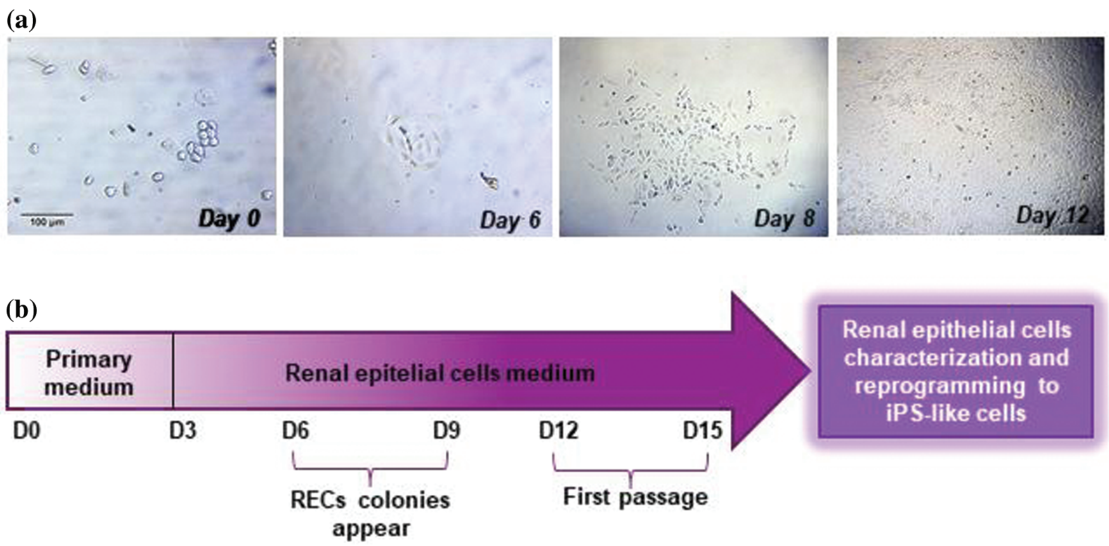

FIGURE 1. Primary culture of renal epithelial cells. (a) Microphotographs show a timeline culture of a colony of RECs obtained from the urine of healthy donors. Scale bar = 100 µm. (b) Diagram showing the timeline of the methodology used to establish primary cultures of RECs.

following pluripotency-specific proteins after their expansion: Oct4, Klf4, Lin28, and Nanog (Fig. 4). Moreover, to confirm the pluripotency of the iPS-like cells obtained, we conducted a qPCR assay to assess the expression of ESC markers. The results confirmed that the iPS-like cells expressed pluripotency-specific genes: *oct3/4*, *sox2*, *lin28*, *nanog*, *nodal*, and *rex1* (Fig. 5). Of the genes analyzed, *nanog*, *nodal*, and *rex1* were not used to reprogram the RECs. Due to the expression of pluripotency-associated transcription markers, we can conclude that iPS-like cells were successfully generated from urine-derived renal epithelial cells.

Discussion

The human kidney contains an extensive network of tubules; those cells from the renal tubular system and urinary tract are detach and excreted daily in the urine (Rahmoune *et al.*, 2005).

Urine-derived cells are a source of somatic cells obtained without invasive intervention, which is the principal advantage of urine-derived cells over other somatic cells sources, such as mesenchymal stem cells, fibroblasts, or blood cells. Therefore, urine-derived cells have a prospective clinical future to generate iPSCs-specific of patients (Manaph *et al.*, 2018).

In this study, we performed the isolation of urine-derived cells from healthy adult donors, the establishment of primary cultures of renal epithelial cells, and their further reprogramming into iPS-like cells using nonviral vectors expressing Oct4, Sox2, L-Myc, Lin-28, and Klf4. In addition, we compared the abilities of different commercial extracellular matrices and transfection reagents to support the efficient acquisition of iPS-like colonies.

There have been several reports of urine-derived cells isolated from patients with different disease conditions (Chen *et al.*, 2013; Park *et al.*, 2015; Afzal and Strande, 2015). Our study is based on a previously reported culture method (Zhou *et al.*, 2011). We used DMEM/F-12 supplemented with RECG factors as a primary medium and renal epithelial cell basal medium (ATCC) containing RECG factors (ATCC) as urine-derived cell culture medium; meanwhile, Zhou and coauthors used DMEM/F-12 and renal epithelial cell basal medium (Lonza) supplemented with SingleQuot factors® (Lonza). We observed colonies with irregular and smooth edges that appeared after 3–6 days of culture, which is consistent with the observations of Zhou *et al.* (2011). RECs cultures were established from over 75% of the isolates from healthy donor samples that contained viable cells; this percentage is higher than the isolation rates of 37% and 52% reported from healthy donor samples (Dörrenhaus *et al.*, 2000; Belik *et al.*, 2008).

Next, we seeded renal epithelial cells in Matrigel, Geltrex or vitronectin and dedifferentiated the RECs into iPS-like cells using nonviral reprogramming factors. iPS-like cells obtained

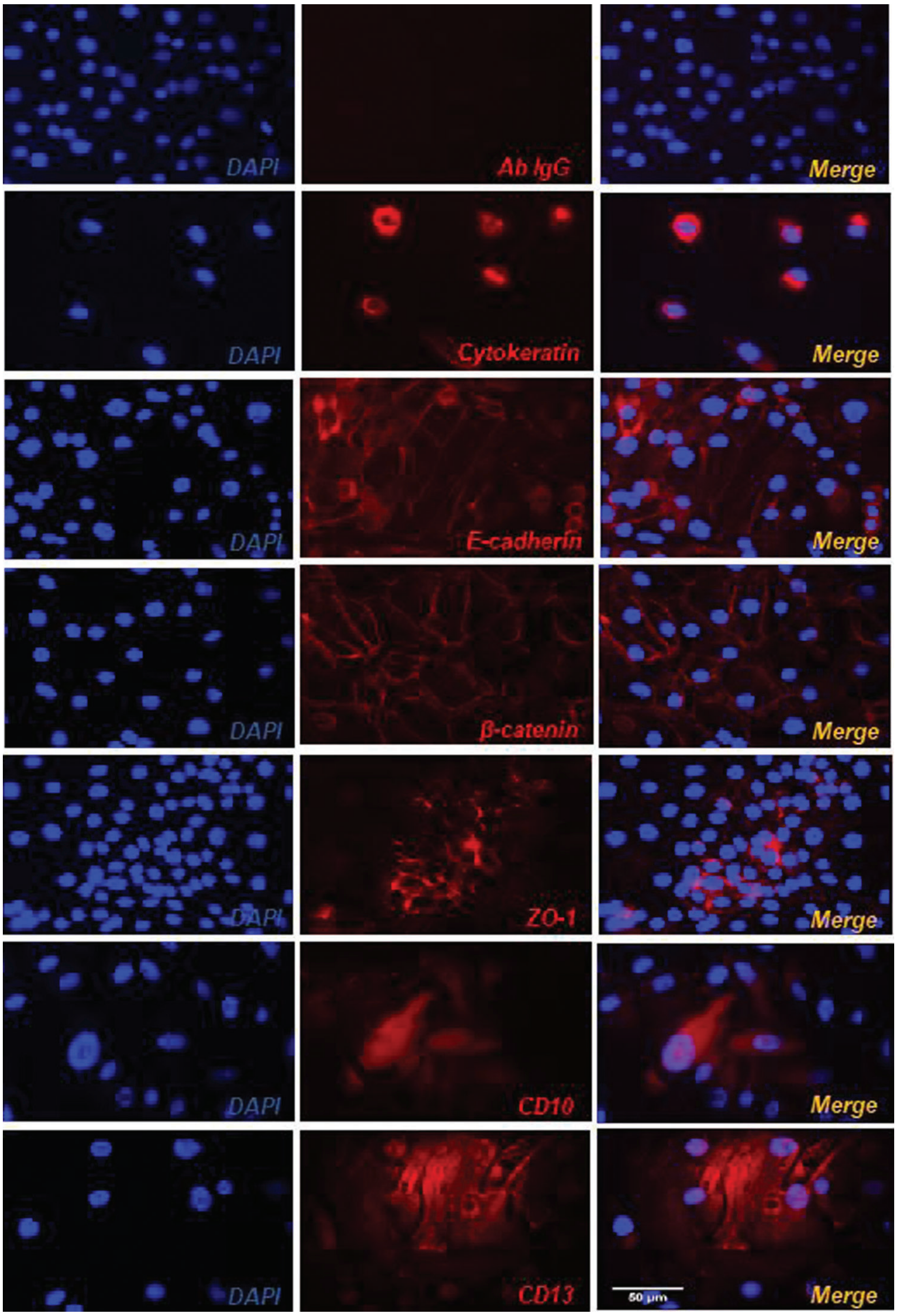

FIGURE 2. Expression of renal epithelial cell-specific markers.
RECs were obtained from urine samples from healthy donors. Microphotographs show positive signals (red channel) for cytokeratin, E-cadherin, β-catenin, ZO-1, which are specific markers for epithelial cells; CD10 and CD13, which are specific markers for renal epithelial cells, detected by immunofluorescence. Nuclei were stained with DAPI. Ab IgG refers to a non-specific IgG antibody as a control. Scale bar = 50 µm.

from the culture with Matrigel, Geltrex, or vitronectin extracellular matrices had an hESC (human embryonic stem cell)-like morphology with central and prominent nuclei, scarce cytoplasm, and small and polyhedral colonies. Importantly, the iPS-cell colonies expressed the pluripotency markers Oct4, Klf4, Nanog, and Lin28. Additionally, we confirmed by qPCR the expression of pluripotency genes *oct3/4*, *sox2*, *nanog*, *lin28*, *nodal*, and *rex1*. Of the genes analyzed, *nanog*, *nodal*, and *rex1* were not used to reprogram the RECs. Nevertheless, further assays are required for the exhaustive characterization of the iPS-like cells generated with each experimental condition.

Over the years, progress has been made in defining the molecular arrangement of components in the basement membrane due to variation between iPSCs lines obtained with different feeder-free systems and culture conditions (Amit *et al.*, 2004; Sun *et al.*, 2009; Nagasaka *et al.*, 2017). Interactions between cells and ECMs have important roles in the regulation of cell functions, and their composition change with different cell types and phenotypes (Frantz *et al.*, 2010). Cai *et al.* (2015) show that the surfaces coated with ECMs from bone marrow mesenchymal stem cells (MSCs), dermal fibroblasts, and osteoblasts promoted cell adhesion more strongly than surfaces coated with ECMs from osteosarcoma cells. Additionally, the ECMs promoted the proliferation of MSCs while they inhibited the proliferation of osteosarcoma cells. Therefore, the coating cell source is important for investigating the effect of

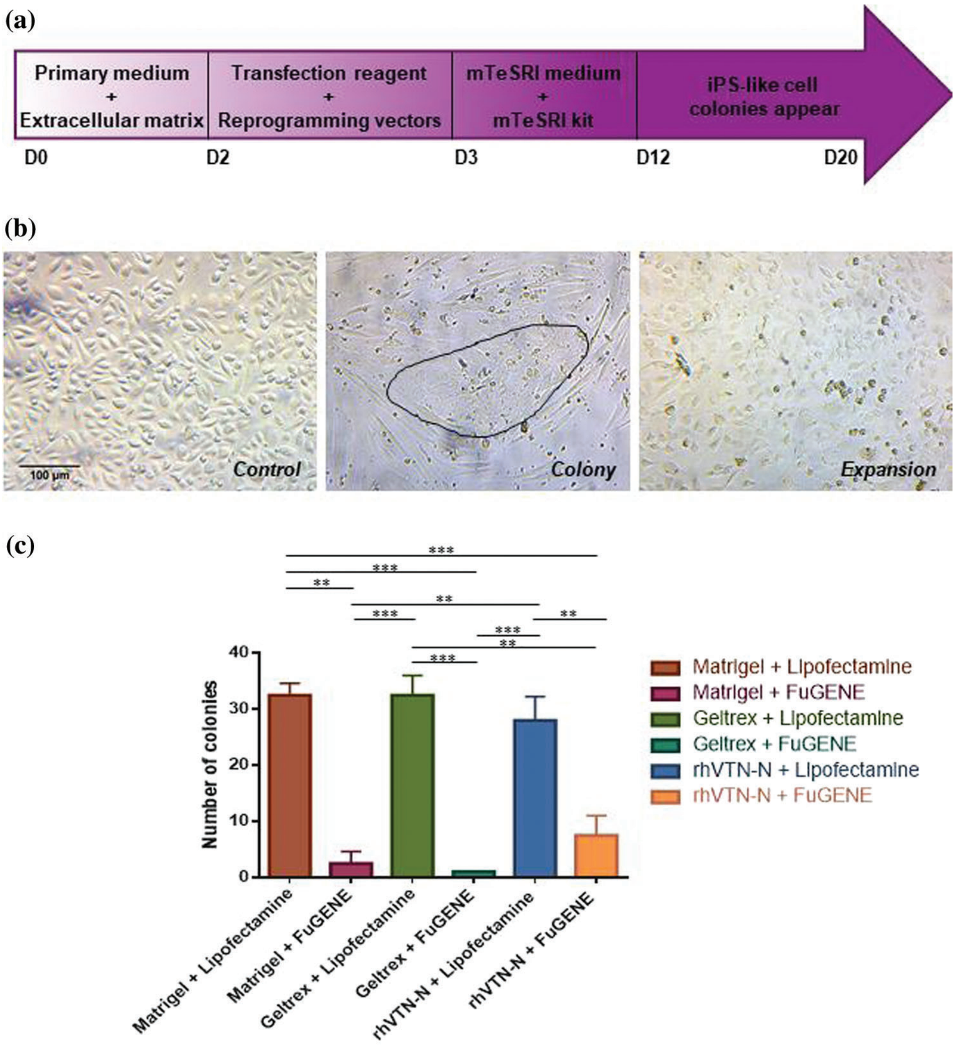

FIGURE 3. Comparison of extracellular matrices and transfection reagents to improve iPS-like colony generation.
(a) Diagram showing the timeline of the methodology used to establish iPS-like colonies. (b) A representative iPS-like colony. Reference control: renal epithelial cells. Colony: an iPS-like colony in RECs transfected with pCE-hOCT3/4 (Oct4), pCE-hSK (Sox2, Klf4), and pCE-hUL (L-Myc, Lin28). Expansion: expansion of the iPS-like colonies. Scale bar = 100 µm. (c) The number of iPS-like colonies obtained with the extracellular matrices Matrigel, Geltrex, and vitronectin combined with Lipofectamine or FuGENE transfection reagent. Significant differences among the groups: ***Matrigel + Lipofectamine *vs.* Matrigel + FuGENE; **Matrigel + Lipofectamine *vs.* rhVTN-N + FuGENE; *** Matrigel + Lipofectamine *vs.* Geltrex + FuGENE; **Matrigel + FuGENE *vs.* rhVTN-N + Lipofectamine; ***Matrigel + FuGENE *vs.* Geltrex + Lipofectamine; **rhVTN-N + Lipofectamine *vs.* rhVTN-N + FuGENE; ***rhVTN-N + Lipofectamine *vs.* Geltrex + FuGENE; **rhVTN-N + FuGENE *vs.* Geltrex + Lipofectamine; ***Geltrex + Lipofectamine *vs.* Geltrex + FuGENE. ANOVA one-way and Tukey's multiple comparisons test were performed; $^{**}p < 0.01$; $^{***}p < 0.001$. Mean ± SD are shown.

specific or a combination of a few proteins from ECMs. In another study, Rojas and coauthors found that a fibrinogen matrix improved cardiac iPSC retention in an experimental model of ischemic heart failure (Rojas *et al.*, 2015).

Matrigel/Geltrex, one of the most widely used extracellular matrices for the feeder-free growth of undifferentiated hESCs, is extracted from Engelbreth-Holm-Swarm mouse tumor and consists of a mixture of laminin, collagen IV, heparan sulfate proteoglycan, and nidogen-1 (Kleinman *et al.*, 1982; Stojkovic *et al.*, 2005). Individual basement membrane components have been examined in other reports. Cells seeded on surfaces coated with laminin, collagen IV, and fibronectin results on compact colonies of hESCs, although cultures maintained on fibronectin or collagen IV did not contain as many colonies as those maintained on Matrigel or laminin (Xu *et al.*, 2001), which suggest the importance of all basement membrane components for the establishment of undifferentiated hESC colonies.

Additionally, the expression and function of iPSCs integrin extracellular matrix receptors have been investigated on iPSCs cultures with feeder layers, Matrigel, or vitronectin to understand the interaction between iPSCs and extracellular matrix components. Cultures maintained on Matrigel require β1 integrins for adhesion, while cultures maintained on vitronectin require αvβ5 for adhesion. In contrast, blockade of β1 integrins did not affect adhesion to vitronectin, and the inhibition of αvβ5 did not affect adherence to Matrigel. However, integrins β1 and αvβ5 were shown to mediate iPSCs proliferation on vitronectin, whereas only β1 was required for iPSCs proliferation on Matrigel (Rowland *et al.*, 2010). Furthermore, we have shown that more iPSC colonies were obtained with Matrigel than with vitronectin. This observation could be due to the high content of laminin and collagen IV in Matrigel, which interacts with iPSCs through β1 integrins such as α2β1, α3β1, α7β1, and α11β1 to promote cell adherence, while only αvβ5 integrins are responsible for adherence to vitronectin (Braam *et al.*, 2008; Miyazaki *et al.*, 2008).

The selection of the optimal transfection method is essential for the efficient establishment of iPSC colonies. The transfection capability of Lipofectamine 2000 and FuGENE was reported in mouse embryonic stem cells (Tamm *et al.*, 2016). Additionally, Lipofectamine 3000 was used to report a simple protocol for transfection of MSCs, a cell type known to be difficult to transfect (de Carvalho *et al.*, 2018). In this study, we compared the capability of Lipofectamine stem cell reagent with FuGENE reagent to establish a simple method to obtain iPS-like cells from RECs, which are difficult to transfect. We observed more iPS-like colonies using Lipofectamine Stem Cell reagent than FuGENE reagent regardless of whether Matrigel,

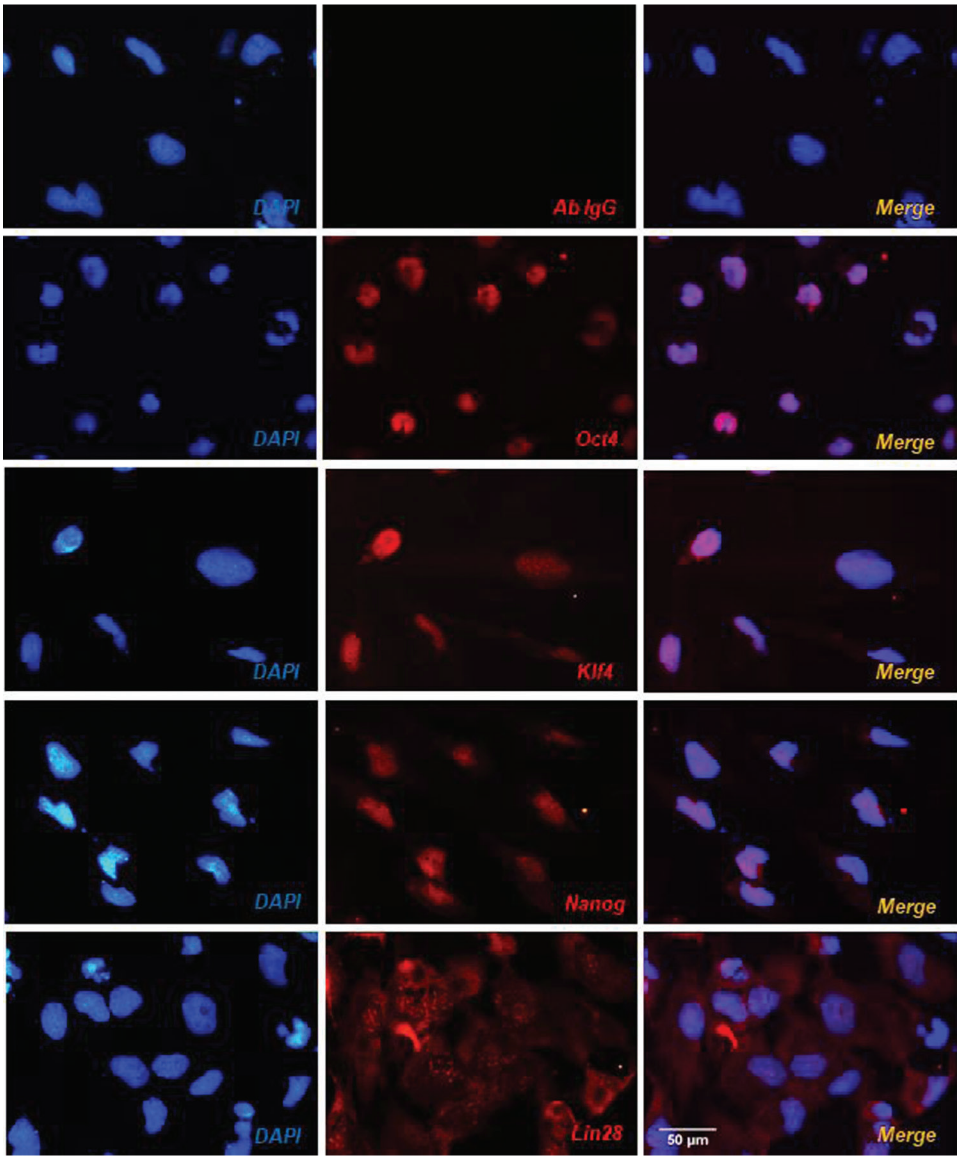

FIGURE 4. iPS-like cells characterization.
Characterization of iPS-like cells by immunofluorescence. Microphotographs show positive signals (red channel) for the pluripotency markers Oct4, Klf4, Nanog, and Lin28. Nuclei were stained with DAPI. Ab IgG refers to a non-specific IgG antibody as a control. Scale bar = 50 µm.

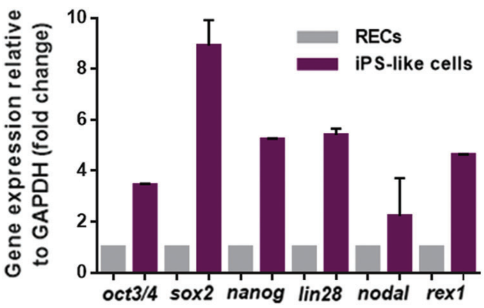

FIGURE 5. Gene expression of pluripotency markers.
iPS-like cells were analyzed by real-time PCR. The graph shows the expression of pluripotency genes *oct3/4*, *sox2*, *nanog*, *lin28*, *nodal*, and *rex1*. Reference control was RECs. GADPH was used for normalization. Mean ± SD are shown.

Geltrex, or vitronectin was used under the experimental conditions tested. However, there are still more challenges to be addressed, such as increasing transfection efficacy on cells that are difficult to transfect.

There are ethical controversies and risks reported in the currently used viral methods to induce pluripotency in somatic cells for clinical applications, in addition to the risk of obtaining somatic cells from vulnerable patients, such as patients with abnormal hemorrhagic diseases or kidney diseases (Zhou and Zeng, 2013; Ji *et al.*, 2017; Molinari and Sayer, 2019). In this report, we tested a non-viral method for generating integration-free iPS-like cells under different feeder-free culture conditions and transfection reagents using urine-derived cells, which are obtained without invasive intervention.

Compared to currently used non-viral methods that use sophisticated transfection methods to obtain iPSCs from urine-derived cells and other somatic cells obtained through invasive procedures on different diseases (Lee *et al.*, 2017; Li *et al.*, 2016), the conditions tested here represent an improvement in iPSCs technology for experimental research due to the accessibility to obtain iPS-like cells from urine-derived cells using different transfection reagents and extracellular matrices. We report results using different conditions to obtain iPS-like cells, which can be very useful when deciding which method to use in experimental research.

Conclusions

A human urine-derived renal epithelial cell primary culture from healthy donors was performed. Additionally, iPS-like cells were obtained from a primary culture of RECs by a virus-free and feeder-free system. We concluded that Lipofectamine Stem Cell transfection reagent is more effective than FuGENE in obtaining iPS-like colonies from urine-derived cells under the conditions reported. Moreover, the three matrices are comparable in their efficiency to obtain iPS-like cells. This report provides an experimental protocol to obtain and generate iPS-like cells from urine samples for further cell therapy research on different human diseases.

Author Contributions

All authors made substantial contributions to this study. ORR, MLA, JPT, and EPG participated in data collection and manuscript preparation, ORR, MLA, JPT, AVO, ASD, OSC, and RMOL, participated in the analysis and interpretation of data, LVC, HRR, AGG, OSC, participated in manuscript preparation and revision, MLA and RMOL designed the study and participated in manuscript preparation. All authors read and approved the final version of the manuscript.

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References

Afzal MZ, Strande JL (2015). Generation of induced pluripotent stem cells from muscular dystrophy patients: efficient integration-free reprogramming of urine-derived cells. *Journal of Visualized Experiments* **95**: 52032.

Amit M, Shariki C, Margulets V, Itskovitz-Eldor J (2004). Feeder layer- and serum-free culture of human embryonic stem cells. *Biology of Reproduction* **70**: 837–845. DOI 10.1095/biolreprod.103.021147.

Anson DS (2004). The use of retroviral vectors for gene therapy-what are the risks? A review of retroviral pathogenesis and its relevance to retroviral vector-mediated gene delivery. *Genetic Vaccines Therapy* **2**: 9. DOI 10.1186/1479-0556-2-9.

Belik R, Follmann W, Degen GH, Roos PH, Blaszkewicz M, Knopf HJ, Golka K (2008). Improvements in culturing exfoliated urothelial cells *in vitro* from human urine. *Journal of Toxicology and Environmental Health Part A* **71**: 923–929. DOI 10.1080/15287390801988871.

Braam SR, Zeinstra L, Litjens S, Ward-van Oostwaard D, van den Brink S, van Laake L, Lebrin F, Kats P, Hochstenbach R, Passier R, Sonnenberg A, Mummery CL (2008). Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. *Stem Cells* **26**: 2257–2265. DOI 10.1634/stemcells.2008-0291.

Cai R, Kawazoe N, Chen G (2015). Influence of surfaces modified with biomimetic extracellular matrices on adhesion and proliferation of mesenchymal stem cells and osteosarcoma cells. *Colloids and Surfaces B: Biointerfaces* **126**: 381–386. DOI 10.1016/j.colsurfb.2014.11.050.

de Carvalho TG, Pellenz FM, Laureano A, da Rocha Silla LM, Giugliani R, Baldo G, Matte U (2018). A simple protocol for transfecting human mesenchymal stem cells. *Biotechnology Letters* **40**: 617–622. DOI 10.1007/s10529-018-2505-8.

Chen Y, Luo R, Xu Y, Cai X, Li W, Tan K, Huang J, Dai Y (2013). Generation of systemic lupus erythematosus-specific induced pluripotent stem cells from urine. *Rheumatology International* **33**: 2127–2134. DOI 10.1007/s00296-013-2704-5.

Chin MH, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, Ambartsumyan G, Aimiuwu O, Richter L, Zhang J, Khvorostov I, Ott V, Grunstein M, Lavon N, Benvenisty N, Croce CM, Clark AT, Baxter T, Pyle AD, Teitell MA, Pelegrini M, Plath K, Lowry WE (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell* **5**: 111–123. DOI 10.1016/j.stem.2009.06.008.

Crandall L, Lalande M (2013). Is urine the next source of stem cells? *Regenerative Medicine* **8**: 235–236. DOI 10.2217/rme.13.24.

Ding Z, Sui L, Ren R, Liu Y, Xu X, Fu L, Bai R, Yuan T, Hao Y, Zhang W, Pan H, Liu W, Yu H, Esteban CR, Yu X, Yang Z, Li J, Wang X, Belmonte JCI, Liu GH, Yi F, Qu J (2015). A widely adaptable approach to generate integration-free iPSCs from non-invasively acquired human somatic cells. *Protein & Cell* **6**: 386–389. DOI 10.1007/s13238-014-0117-1.

Dörrenhaus A, Müller JI, Golka K, Jedrusik P, Schulze H, Föllmann W (2000). Cultures of exfoliated epithelial cells from different locations of the human urinary tract and the renal tubular system. *Archives Toxicology* **74**: 618–626. DOI 10.1007/s002040000173.

Frantz C, Stewart KM, Weaver VM (2010). The extracellular matrix at a glance. *Journal of Cell Science* **123**: 4195–4200. DOI 10.1242/jcs.023820.

Ji X, Wang M, Chen F, Zhou J (2017). Urine-derived stem cells: the present and the future. *Stem Cells International* **2017**: 1–8. DOI 10.1155/2017/4378947.

Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR (1982). Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry* **21**: 6188–6193. DOI 10.1021/bi00267a025.

Lee YM, Zampieri BL, Scott-McKean JJ, Johnson MW, Costa ACS (2017). Generation of integration-free induced pluripotent stem cells from urine-derived cells isolated from individuals with down syndrome. *Stem Cells Translational Medicine* **6**: 1465–1476. DOI 10.1002/sctm.16-0128.

Li D, Wang L, Hou J, Shen Q, Chen Q, Wang X, Du J, Cai X, Shan Y, Zhang T, Zhou T, Shi X, Li Y, Zhang H, Pan G (2016). Optimized approaches for generation of integration-free iPSCs from human urine-derived cells with small molecules and autologous feeder. *Stem Cell Reports* **6**: 717–728. DOI 10.1016/j.stemcr.2016.04.001.

Manaph NPA, Al-Hawaas M, Bobrovskaya L, Coates PT, Zhou XF (2018). Urine-derived cells for human cell therapy. *Stem Cell Research & Therapy* **9**: 222. DOI 10.1186/s13287-018-0974-2.

Miyazaki T, Futaki S, Hasegawa K, Kawasaki M, Sanzen N, Hayashi M, Kawase E, Sekiguchi K, Nakatsuji N, Suemori H (2008). Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells. *Biochemical and Biophysical Research Communications* **375**: 27–32. DOI 10.1016/j.bbrc.2008.07.111.

Molinari E, Sayer JA (2019). Using human urine-derived renal epithelial cells to model kidney disease in inherited ciliopathies. *Translational Science of Rare Diseases* **4**: 87–95. DOI 10.3233/TRD-190034.

Nagasaka R, Matsumoto M, Okada M, Sasaki H, Kanie K, Kii H, Uozumi T, Kiyota Y, Honda H, Kato R (2017). Visualization of morphological categories of colonies for monitoring of effect on induced pluripotent stem cell culture status. *Regenerative Therapy* **6**: 41–51. DOI 10.1016/j.reth.2016.12.003.

Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S (2008). Generation of mouse induced pluripotent stem cells without viral vectors. *Science* **322**: 949–953. DOI 10.1126/science.1164270.

Okita K, Yamakawa T, Matsumura Y, Sato Y, Amano N, Watanabe A, Goshima N, Yamanaka S (2013). An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. *Stem Cells* **31**: 458–466. DOI 10.1002/stem.1293.

Park CY, Kim DH, Son JS, Sung JJ, Lee J, Bae S, Kim JH, Kim DW, Kim JS (2015). Functional correction of large factor VIII gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR-Cas9. *Cell Stem Cell* **17**: 213–220. DOI 10.1016/j.stem.2015.07.001.

Rahmoune H, Thompson PW, Ward JM, Smith CD, Hong G, Brown J (2005). Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes. *Diabetes* **54**: 3427–3434. DOI 10.2337/diabetes.54.12.3427.

Rojas SV, Martens A, Zweigerdt R, Baraki H, Rathert C, Schecker N, Rojas-Hernandez S, Schwanke K, Martin U, Haverich A, Kutschka I (2015). Transplantation effectiveness of induced pluripotent stem cells is improved by a fibrinogen biomatrix in an experimental model of ischemic heart failure. *Tissue Engineering Part A* **21**: 1991–2000. DOI 10.1089/ten.tea.2014.0537.

Rowland TJ, Miller LM, Blaschke AJ, Doss EL, Bonham AJ, Hikita ST, Johnson LV, Clegg DO (2010). Roles of integrins in human induced pluripotent stem cell growth on Matrigel and vitronectin. *Stem Cells and Development* **19**: 1231–1240. DOI 10.1089/scd.2009.0328.

Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008). Induced pluripotent stem cells generated without viral integration. *Science* **322**: 945–949. DOI 10.1126/science.1162494.

Stojkovic P, Lako M, Przyborski S, Stewart R, Armstrong L, Evans J, Zhang X, Stojkovic M (2005). Human-serum matrix supports undifferentiated growth of human embryonic stem cells. *Stem Cells* **23**: 895–902. DOI 10.1634/stemcells.2004-0326.

Sun N, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F, Hu S, Cherry AM, Robbins RC, Longaker MT, Wu JC (2009). Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 15720–15725. DOI 10.1073/pnas.0908450106.

Takahashi K, Yamanaka S (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**: 663–676. DOI 10.1016/j.cell.2006.07.024.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**: 861–872. DOI 10.1016/j.cell.2007.11.019.

Tamm C, Kadekar S, Pijuan-Galitó S, Annerén C (2016). Fast and efficient transfection of mouse embryonic stem cells using non-viral reagents. *Stem Cell Reviews and Reports* **12**: 584–591. DOI 10.1007/s12015-016-9673-5.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnology* **19**: 971–974. DOI 10.1038/nbt1001-971.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**: 1917–1920. DOI 10.1126/science.1151526.

Zhou T, Benda C, Duzinger S, Huang Y, Li X, Li Y, Guo X, Cao G, Chen S, Hao L, Chan YC, Ng KM, Cy Ho J, Wieser M, Wu J, Redl H, Tse HF, Grillari J, Grillari-Voglauer R, Pei D, Esteban MA (2011). Generation of induced pluripotent stem cells from urine. *Journal of the American Society of Nephrology* **22**: 1221–1228. DOI 10.1681/ASN.2011010106.

Zhou T, Benda C, Dunzinger S, Huang Y, Ho JC, Yang J, Wang Y, Zhang Y, Zhuang Q, Li Y, Bao X, Tse HF, Grillari J, Grillari-Voglauer R, Pei D, Esteban MA (2012). Generation of human induced pluripotent stem cells from urine samples. *Nature Protocols* **7**: 2080–2089. DOI 10.1038/nprot.2012.115.

Zhou Y, Zeng F (2013). Integration-free methods for generating induced pluripotent stem cells. *Genomics, Proteomics & Bioinformatics* **11**: 284–287. DOI 10.1016/j.gpb.2013.09.008.