Generation of human induced pluripotent stem cells from urine samples

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Human induced pluripotent stem cells (iPSCs) have been generated with varied efficiencies from multiple tissues. Yet, acquiring donor cells is, in most instances, an invasive procedure that requires laborious isolation. Here we present a detailed protocol for generating human iPSCs from exfoliated renal epithelial cells present in urine. This method is advantageous in many circumstances, as the isolation of urinary cells is simple (30 ml of urine are sufficient), cost-effective and universal (can be applied to any age, gender and race). Moreover, the entire procedure is reasonably quick—around 2 weeks for the urinary cell culture and 3–4 weeks for the reprogramming—and the yield of iPSC colonies is generally high—up to 4% using retroviral delivery of exogenous factors. Urinary iPSCs (UiPSCs) also show excellent differentiation potential, and thus represent a good choice for producing pluripotent cells from normal individuals or patients with genetic diseases, including those affecting the kidney.

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INTRODUCTION

The conversion of somatic cells into embryonic stem cell (ESC)-like cells, also termed reprogramming, has attracted much attention since the milestone achievement by Takahashi and Yamanaka in 2006 (refs. 1–3). The implications of this technology are remarkable at all levels, from *in vitro* disease modeling to potential clinical transplantation and others[4–6], but a series of recently identified caveats (in particular mutations, copy number variations, epigenetic alterations and immunogenicity) have put the methodology into question[7–10]. To assess the generality of these issues, it is relevant to study whether different donor cell types produce iPSCs with different features. The reprogramming of cells with distinct embryonic origins (endoderm, mesoderm or ectoderm) may also be useful to understand better the molecular machinery that changes cell fate, and this knowledge can subsequently help improve the technique. To this end, multiple laboratories including ours have systematically reprogrammed multiple human cell types, including skin fibroblasts[1,3], keratinocytes[11], melanocytes[12], adipose stem cells[13–15], peripheral blood[16–18], periosteum membrane[13] and periodontal ligament[19], neural stem cells[20], hepatocytes[21], amniocytes[22,23] and cells from extraembryonic tissues (umbilical cord blood and matrix, and placental amniotic and chorionic mesenchymal cells (MCs))[13,24–26], among others.

For any method requiring a large number of cells for reprogramming, there are a number of issues to be addressed. The issue we came across most commonly was a reluctance to undergo an invasive procedure for donating samples. Blood is a relatively easy cell source to have access to, but the procedure is still invasive[27]. The use of hair follicles may not be strictly considered noninvasive either, but it is also easily accessible[28]. There are different tissues that can be obtained in a completely noninvasive way. Extraembryonic tissues[29] contain a variety of cells that can be efficiently used for producing iPSCs, for example, cord blood, but they are only available at the moment of birth unless they are properly stored. Moreover, although cells from cord blood are banked in many countries, the procedure is expensive and not universally applied. Notably, urine production is an essential part of human physiology at any age, and for any sex or ethnic origin, making it possible to collect samples under any circumstance except for renal failure. Thus, we envisaged that if it was regularly successful, the isolation of exfoliated cells present in urine could represent an excellent candidate for noninvasive reprogramming.

Urine as a cell source for generating pluripotent cells

The mammalian urinary apparatus is composed of two kidneys, two ureters, the bladder and the urethra, and it serves homeostatic functions through the controlled excretion of volume and electrolytes. Among other things, this is essential for maintaining the acid-base balance and an adequate blood pressure[30]. The basic structural and functional unit of the kidney is the nephron, a tubular-like structure that extends from the kidney cortex to the medulla[31]. The renal corpuscle is the initial part of the nephron and filters the blood supply from the renal circulation. Remarkably, it is estimated that each human kidney contains more than 1 million nephrons and processes ~100 liters of filtrate per day[30]. Yet, most of this volume is reabsorbed in the distal part of the nephron and only ~2 liters are normally excreted through the urethra as urine daily. Perhaps not surprisingly, given the massive tubular network, 2,000–7,000 cells are detached from this system daily and can be collected in urine[32]. Sutherland and Bain were the first to report the successful culture of exfoliated urinary cells[33], and multiple groups have reproduced this since[32,34–40]. These cells are epithelial-like, but their morphology can be rather regular or somehow elongated[32,37,40]. They mostly originate from the renal epithelium based on their gene expression profile, but urothelial cells can be detected as well[32]. Notably, Zhang and colleagues have

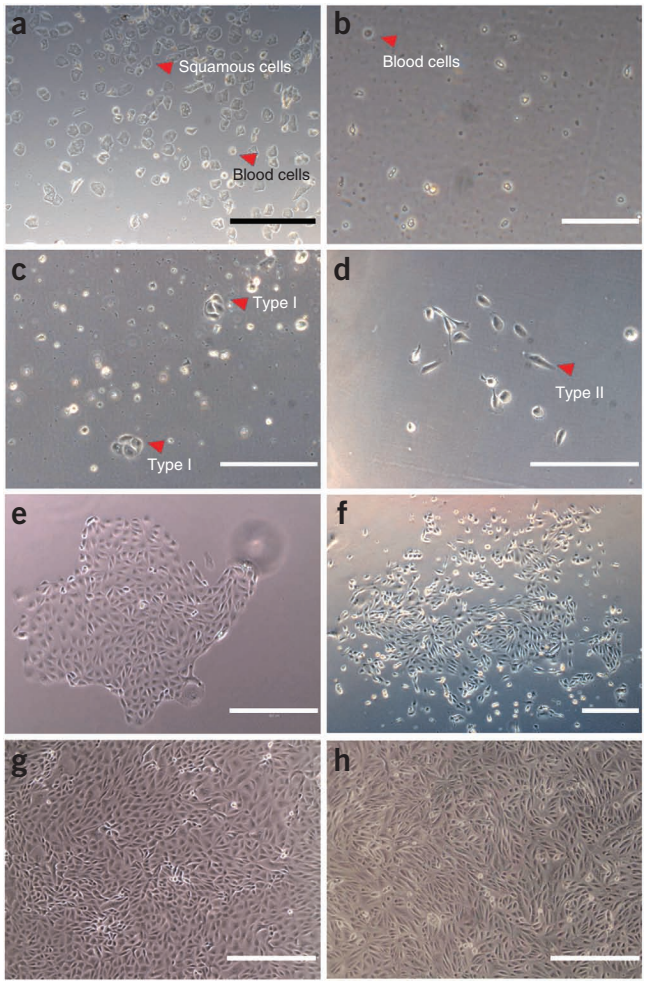

Figure 1 | Morphology of urinary cells at different time points after collection. (**a**) Fresh female urine samples mainly consist of squamous cells and a few blood cells (arrowheads). (**b**) Fresh male urine samples mainly contain a few blood cells (arrowhead). (**c**–**h**) Type I (**c**,**e**,**g**) and type II (**d**,**f**,**h**) urinary cell colonies (arrowheads) at days 4 (**c**,**d**), 9 (**e**,**f**) and 12 (**g**,**h**). Representative images are shown in **a**–**h**; scale bars, 400 µm.

also identified a subpopulation of urinary cells that express stem cell markers and have multidifferentiation potential[41,42].

Experimental design

We first adapted a method for urine sample collection and cell expansion that is simple, effective and affordable (the only cost is the culture medium). From 57 individuals, we were able to isolate a primary culture of cells from 42 individuals on the first attempt. Samples were contaminated in 1 out of 30 male samples and in 4 out of 27 female samples. A primary culture was isolated from five further individuals during second, third or fourth attempts at isolation. This means a total success rate of approximately 82%. Subsequently, we optimized our existing reprogramming procedures with retroviral vectors, devising a protocol that allowed the successful generation of UiPSCs from urinary cells on almost every occasion we have tried[43].

The primary medium for urinary cell isolation contains 10% (vol/vol) fetal bovine serum (FBS) to enhance the initial cell adherence and survival. As for the urinary cell expansion, we used RE (renal epithelial) proliferation medium in our recent report[43]. More recently, we have also successfully cultured urinary cells using RE/MC (renal epithelial/mesenchymal cell) proliferation medium (see MATERIALS, Reagent Setup and **Supplementary Table 1**). Urinary cells grow well with both media, but show higher proliferation rate in the latter. We normally observe three to ten small colonies per well (12-well plate) after several days of culture, irrespective of the sex of the donor. The total number of cells should reach ~100,000–200,000 cells within 2–3 weeks of culture and before the second passage.

In agreement with previous reports[32,37,40], the urinary cell colonies routinely show two types of morphology, namely type I and II (**Fig. 1**). Type I colonies have a more regular appearance with smooth-edged contours and cobblestone-like cell morphologies, whereas type II colonies are more randomly arranged. Type II cells tend to grow more quickly than type I cells. In our experience, both types of colonies can be present in the same sample collection, but either one or the other is usually more abundant. Cultures enriched in type I cells are slightly more frequent than type II cells. Cultures from the same individual can be enriched in type I or II colonies in different isolations. Cells in both types of colonies similarly express

Box 1 | Retrovirus production ● TIMING ~1 week

1. Thaw a vial of HEK 293T cells 5 d before infecting the urinary cells. Count the HEK 293T cells in the pellet and seed ~2 × 10^6 cells onto a 100-mm tissue culture dish in HEK 293T medium. Incubate the cells at 37 °C.
2. After 2 d, the cells should have reached more than 90% confluence. Next, aspirate the medium, gently wash the dish with 10 ml of washing buffer once, aspirate the buffer and add 1.5 ml of 0.05% (wt/vol) trypsin-EDTA. Incubate for 2 min at 37 °C and gently tap the tissue culture plate from the side to ensure that all cells are detached.

▲ **CRITICAL STEP** For efficient retrovirus production, it is essential that the HEK 293T cells are healthy.

3. Rinse the dish with 9 ml of HEK 293T medium and collect the volume into a 50-ml tube.
4. Centrifuge the mixture at 200*g* for 3 min at room temperature and discard the supernatant.
5. Resuspend in 10 ml of HEK 293T medium and count the cells using a hemocytometer or an automated cell counter.
6. Seed 4 × 10^6 cells in 10 ml of HEK 293T medium in 100-mm culture dishes and incubate them at 37 °C for 24 h.

▲ **CRITICAL STEP** Adjust the number of cells to seed according to the rate of proliferation (this may vary depending on the batch of FBS) and desired time of transfection. This is highly relevant, as HEK 293T cells have low adherence and otherwise will detach after transfection when they reach high confluence. Do not add antibiotics to the culture medium, as this may reduce the transfection efficiency.

(continued)

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Box 1 | (continued)

7. At 24 h after seeding the HEK 293T cells (they should have reached around 80% confluence), prepare the Lipofectamine 2000/DNA complexes for transfection according to the manufacturer's instructions. For a 100-mm culture dish, use 20 µg of the corresponding pMXs vector, 20 µg of pCL-Eco packaging vector and 45 µl of Lipofectamine 2000. In addition to *OCT4*, *SOX2*, *KLF4* and *c-MYC*, we also routinely include pMXs-GFP as a separate control for monitoring the efficiency. Therefore, five different dishes are needed.

! CAUTION Use a class II (or higher) biological safety cabinet and handle viral supernatants conscientiously and carefully.

8. Gently add the Lipofectamine/DNA complexes dropwise and uniformly onto the medium. Incubate at 37 °C overnight.

9. At 12 h after transfection, carefully remove the transfection medium from each 100-mm culture dish and add 10 ml of fresh HEK 293T medium. Incubate at 37 °C overnight.

▲ CRITICAL STEP Split the urinary cells for infection 24 h after initiating the transfection of HEK 293T cells. They will receive the first round of retroviruses after another 24 h.

▲ CRITICAL STEP Check the signal produced by pMXs-GFP with a fluorescence microscope, and ensure that nearly 100% of HEK 293T cells have been transfected.

10. At 36 h after transfection, carefully collect the viral supernatant for each transcription factor (~10 ml) using an automatic pipette and transfer them to a 15-ml tube. Add 10 ml of fresh HEK 293T medium to each dish gently.

! CAUTION Drop the medium onto the sides of the dishes, being careful so as to prevent the cells from detaching.

11. Filter the viral supernatant using a 0.45-µm syringe filter.

12. Add polybrene (final concentration of 8 µg ml^{-1}) to the viral supernatants before infection. This viral supernatant is now ready for infecting the urinary cells (first round of infection; Step 19 of main PROCEDURE).

13. After another 24 h, collect again the viral supernatant as described in steps 10–12 (of this box), and then dispose of the HEK 293T cells. This viral supernatant is now ready to infect cells as the second round of infection (Step 21 of the main PROCEDURE).

■ PAUSE POINT Viral supernatants can be kept at 4 °C for 24 h. This is useful when the preparation of the urinary cells for infection and the retrovirus production were not properly coordinated, but involves partial loss of efficiency.

epithelial markers based on mRNA analysis and immunofluorescence staining, although the intensity of the latter tends to be milder in type II colonies (**Supplementary Table 2**). In accordance with previous reports[32,40], we found that type I and II cells also express RE markers (**Supplementary Table 2**), whereas we did not detect expression of the urothelial marker uroplakin III.

In our experience, there is enough number of urinary cells for retroviral infection at passage 1 or 2 (the latter more frequently), and infection at this point ensures higher iPSC generation efficiency. According to our own procedure, transfection of the 293T human embryonic kidney (HEK 293T) cells with retroviral vectors producing the exogenous factors should be started 24 h before splitting the urinary cells for infection (**Box 1**). The production of supernatants containing high retrovirus titer is crucial to the success of reprogramming. We recommend using a GFP control retroviral vector for monitoring transfection and infection efficiency.

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MATERIALS

REAGENTS

- Urine (see Reagent Setup) **! CAUTION** Properly inform the donors and obtain a signed consent form.
- Washing buffer (see Reagent Setup)
- Dulbecco's phosphate-buffered saline (DPBS) buffer (Gibco, cat. no. C14190500BT)
- Amphotericin B solution (Sigma, cat. no. A2942)
- Penicillin/streptomycin solution (Hyclone, cat. no. SV30010)
- Primocin (InvivoGen, cat. no. ant-pm-1)
- Gelatin, 0.1% (wt/vol) solution (Millipore, cat. no. ES-006-B)
- Primary medium (see Reagent Setup)
- RE proliferation medium (see Reagent Setup)
- MC proliferation medium (see Reagent Setup)
- RE/MC proliferation medium (see Reagent Setup)
- DMEM/high glucose (Hyclone, cat. no. SH30022.01B)
- Ham's F-12 nutrient mix (Gibco, cat. no. 11765-054)
- Renal epithelial cell growth medium (REGM) SingleQuot kit supplement and growth factors (Lonza, cat. no. CC-4127)
- FBS (PAA, cat. no. A15-101)
- REGM BulletKit (Lonza, cat. no. CC-3190)
- GlutaMAX (Invitrogen, cat. no. 35050)
- Non-essential amino acid solution (NEAA; Invitrogen, cat. no. 11140)
- Recombinant human fibroblast growth factor-basic (bFGF; Peprotech, cat. no. 100-18B)
- Recombinant human epidermal growth factor (EGF; Peprotech, cat. no. AF-100-15)
- Recombinant human platelet-derived growth factor-AB (PDGF-AB; Peprotech, cat. no. 100-00AB)
- BSA (PAA, cat. no. K31-011)
- Trypsin-EDTA (0.25%; Gibco, cat. no. 25200114)
- Defined trypsin inhibitor (Invitrogen, cat. no. R-007-100)
- TrypLE Select (1×; Gibco, cat. no. 12563-011)
- Antibodies for urinary cell identification (**Supplementary Table 3a**)
- Quantitative RT-PCR primers for urinary cell identification (**Supplementary Table 3b**)
- Freezing medium for urinary cells (CELLBANKER, cat. no. CELLBANKER 2)
- HEK 293T cells (ATCC, cat. no. CRL-11268)
- HEK 293T medium (see Reagent Setup)
- Trypsin-EDTA (0.05%; Gibco, cat. no. 25300120)
- pMXs retroviral vectors producing human *OCT4* (official name: *POU5F1*), *SOX2*, *KLF4* and *c-Myc* (offical name: *MYC*) (Addgene, cat. nos. 17217, 17218, 17219 and 17220)

- pMXs plasmid producing GFP was prepared by us
- Lipofectamine 2000 (Invitrogen, cat. no. 11668019)
- Polybrene (Sigma, cat. no 107689)
- Mitomycin C (MMC; Sigma, cat. no. M4287)
- MMC-treated mouse embryonic fibroblasts (MEFs; see Reagent Setup)
- CF-1 mice (Charles River Laboratories International, strain code 023)
- MEF medium (see Reagent Setup)
- Defined FBS (dFBS; Hyclone, cat. no. SH30070.03)
- Human ESC medium (see Reagent Setup)
- Human dFBS medium (see Reagent Setup)
- Human ESC/dFBS medium (see Reagent Setup)
- DMEM nutrient mix F12 (Gibco, cat. no. C11330500BT)
- KnockOut serum replacement (KSR; Gibco, cat. no. 10828028)
- β-Mercaptoethanol (Invitrogen, cat. no. 21985023) **! CAUTION** It is toxic by inhalation and skin contact. Handle with care.
- mTeSR1 basal medium (Stemcell, cat. no. 05850)
- mTeSR1 5× supplement (Stemcell, cat. no. 05850)
- mTeSR1 medium (see Reagent Setup)
- Valproic acid (VPA; Sigma, cat. no. 05194)
- Dispase (Invitrogen, cat. no. 17105041)
- Matrigel human ESC-qualified matrix (BD Biosciences, cat. no. 354277)
- Cryostor cell cryopreservation medium CS10 (Sigma, cat. no. C2874)
- Liquid nitrogen

EQUIPMENT

- Forma Class II, A2 biological safety cabinet (Thermo Fisher Scientific, cat. no. 1287)
- Forma Series II, water-jacketed CO_2 incubator, set at 37 °C, 5% CO_2 (Thermo Fisher Scientific, cat. no. 3111)
- Inverted tissue culture microscope with phase-contrast, epifluorescence, ×5, ×10 and ×20 objectives (Zeiss, model Axiovert 40 CFL)
- Tissue culture centrifuge with swinging rotor and adaptors for 15- and 50-ml tubes (Zonkia, cat. no. SC-2544)
- Aspirator tube assembly (Thermo Scientific, cat. no. 92249)
- Automatic pipette
- Tissue culture dishes, 100 mm
- Tissue culture plates, 24, 12 and 6 wells
- Cell counter or hemocytometer
- Millex syringe-driven filter unit, 0.22 and 0.45 μm (Millipore, cat. nos. SLGP033RB and SLHV033RB)
- Syringes, 10 and 50 ml (Double-Dove, cat. no. 101223)
- Conical tubes, 15 and 50 ml (Corning, cat. nos. 430790 and 430828)
- Cryovials (Nalgene, cat. no. 5000)

REAGENT SETUP

Urine Urinary cell collection is a very quick procedure compared with other cell sources. If possible, ask the donor to drink abundant water ~1 h before collection. It is also recommended that he/she cleans the urethral area with premoistened wipes immediately before collection. The first stream of urine should be discarded and the mid-stream collected into the container. **▲ CRITICAL** To improve the survival rate, urine should not be collected during the first micturition[40]. A volume of 30 ml of urine is enough for efficient urinary cell isolation from most people, but increasing the volume produces a higher yield.

Washing buffer Washing buffer is DPBS supplemented with 100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin and 500 ng ml^{-1} of amphotericin B. To prepare 500 ml of buffer, mix 2.5 ml of penicillin/streptomycin and 1 ml of amphotericin B, and fill it up to 500 ml with DPBS. Primocin can be added instead of amphotericin B and penicillin/streptomycin. Store the buffer at 4 °C and use it within several weeks.

Primary medium Primary medium contains DMEM/high glucose and Ham's F12 nutrient mix (1:1), supplemented with 10% (vol/vol) FBS, 100 of U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, the REGM SingleQuot kit supplements and 2.5 μg ml^{-1} amphotericin B. To prepare 500 ml of medium, mix 50 ml of FBS, 2.5 ml of penicillin/streptomycin, 1 ml of amphotericin B and each supplement vial of the REGM SingleQuot kit, and fill it up to 500 ml with half DMEM/high glucose and half Ham's F12 nutrient mix. **! CAUTION** Rinse each supplement vial carefully with primary medium to avoid any loss. **▲ CRITICAL** Store the medium in the dark at 4 °C and use it within 2 weeks.

RE proliferation medium To prepare 500 ml of medium, add the entire amount of each supplement vial of the REGM BulletKit to the RE cell basal medium contained in the same kit. **! CAUTION** Rinse each supplement vial carefully with RE proliferation medium to avoid any loss. **▲ CRITICAL** Store the medium in the dark at 4 °C and use it within 2 weeks.

MC proliferation medium MC proliferation medium contains DMEM/high glucose supplemented with 10% (vol/vol) FBS, 1% (vol/vol) GlutaMAX, 1% (vol/vol) NEAA, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 5 ng ml^{-1} bFGF, 5 ng ml^{-1} PDGF-AB and 5 ng ml^{-1} EGF. To prepare 500 ml of medium, mix 50 ml of FBS with 5 ml of GlutaMAX, 5 ml of NEAA, 2.5 ml of penicillin/streptomycin, 100 μl of bFGF, 250 μl of PDGF-AB and 50 μl of EGF. Filter-sterilize with a 0.22-μm syringe filter, and fill it up to 500 ml with DMEM/high glucose. **▲ CRITICAL** Store the medium in the dark at 4 °C and use it within 2 weeks.

RE/MC proliferation medium RE and MC medium are mixed in a 1:1 ratio. To prepare 500 ml of medium, mix 250 ml of RE proliferation medium with 250 ml of MC proliferation medium. **▲ CRITICAL** Store the medium in the dark at 4 °C and use it within 2 weeks.

HEK 293T medium DMEM/high glucose containing 10% (vol/vol) FBS and 1% (vol/vol) GlutaMAX. To prepare 500 ml of medium, mix 50 ml of FBS with 5 ml of GlutaMAX, and fill it up to 500 ml with DMEM/high glucose. Store the medium at 4 °C and use it within several weeks.

MEF medium MEF medium is DMEM/high glucose containing 10% (vol/vol) FBS, 1% (vol/vol) GlutaMAX, 1% (vol/vol) NEAA, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. To prepare 500 ml of medium, mix 50 ml of FBS with 5 ml of GlutaMAX, 5 ml of NEAA and 2.5 ml of penicillin/streptomycin, and fill it up to 500 ml with DMEM/high glucose. Store the medium at 4 °C and use it within several weeks.

Human ESC medium Human ESC medium contains DMEM nutrient mix F12 supplemented with 20% (vol/vol) KSR, 1% (vol/vol) GlutaMAX, 1% (vol/vol) NEAA, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 100 μM β-mercaptoethanol and 10 ng ml^{-1} bFGF. To prepare 500 ml of medium, mix 100 ml of KSR with 5 ml of GlutaMAX, 5 ml of NEAA, 2.5 ml of penicillin/streptomycin, 500 μl of β-mercaptoethanol and 200 μl of bFGF. Filter-sterilize with a 0.22-μm syringe filter, and fill it up to 500 ml with DMEM nutrient mix F12. **▲ CRITICAL** Store the medium at 4 °C and use it within 2 weeks.

Human dFBS medium dFBS medium contains DMEM/high glucose containing 20% (vol/vol) dFBS, 1% (vol/vol) GlutaMAX, 1% (vol/vol) NEAA, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 100 μM β-mercaptoethanol and 10 ng ml^{-1} bFGF. To prepare 500 ml of medium, mix 100 ml of dFBS with 5 ml of GlutaMAX, 5 ml of NEAA, 2.5 ml of penicillin/streptomycin, 500 μl of β-mercaptoethanol and 200 μl of bFGF. Filter-sterilize with a 0.22-μm syringe filter and fill it up to 500 ml with DMEM/high glucose. **▲ CRITICAL** Store the medium at 4 °C and use it within 2 weeks.

Human ESC/dFBS medium Human ESC and human dFBS medium are mixed in a 1:1 ratio. To prepare 500 ml, mix 250 ml of human ESC medium with 250 ml of human dFBS medium. **▲ CRITICAL** Store the medium at 4 °C and use it within 2 weeks.

mTeSR1 medium mTeSR1 basal medium and mTeSR1 5× supplement. To prepare 500 ml, add the entire contents of the supplemental bottle to the mTeSR1 basal medium. **▲ CRITICAL** Store the medium at 4 °C and use it within 2 weeks.

Dispase Resuspend the dispase in DMEM nutrient mix F12 to a final concentration of 1 mg ml^{-1}. Filter-sterilize with a 0.22-μm syringe filter. Next, make 10-ml aliquots and store dispase at −20 °C for up to 6 months.

VPA Resuspend VPA in DMEM nutrient mix F12 to a final concentration of 200 mM. Filter-sterilize with a 0.22-μm syringe filter. Next, make 500–1,000-μl aliquots and store VPA at −20 °C for up to 6 months.

Gelatin-coated dishes Add an appropriate volume of 0.1% (wt/vol) gelatin solution to the tissue culture plates. Ensure that the entire bottom surface is covered with liquid and incubate the plates for ~30 min at 37 °C. Aspirate the gelatin solution and wash once using washing buffer before use.

Matrigel preparation and coating Thaw the Matrigel slowly overnight at 4 °C. Use prechilled tips and tubes to prepare 0.25-ml aliquots and store them at −80 °C for up to 6 months. To prepare Matrigel-coated plates, thaw a 0.25-μl aliquot on ice for 1–2 h and resuspend it in 25 ml of cold DMEM nutrient mix F12 medium using prechilled pipettes; next, add the Matrigel to the tissue culture plates (1 ml is enough for one well of a six-well plate) and incubate at 37 °C for 1 h. Aspirate the Matrigel and wash it once with DMEM

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nutrient mix F12 before use. ▲ CRITICAL The Matrigel should be kept cold throughout the whole process or it will solidify.

MMC inactivation of MEFs To prepare MMC, resuspend 2 mg of MMC in 200 ml of MEF medium and filter-sterilize with a 0.22-μm syringe filter. MMC can be stored at 4 °C for 2 weeks or at −20 °C for 2 months. To inactivate MEFs, grow cells (isolated from day 13.5 of pregnant CF-1 mice) up to passage 3 in MEF medium, add 10 ml of MMC per 10-mm tissue culture dish and incubate for 2–3 h. After the inactivation, wash the cells three times with washing buffer, trypsinize using 0.25% (wt/vol) trypsin-EDTA and count the cell number. Centrifuge the mixture at 200*g* for 5 min at room temperature (20–22 °C) and plate the cells onto gelatin-coated tissue culture plates in MEF medium and incubate them at 37 °C. The cells can also be frozen and stored at −80 °C or in liquid nitrogen for months.

Growth factor reconstitution Reconstitute all growth factors in DPBS with 0.1% (wt/vol) BSA to the following stock concentrations: EGF 50 μg ml^{-1}, bFGF 25 μg ml^{-1} and PDGF-AB 10 μg ml^{-1}. ! CAUTION Store the mixture at 4 °C and use it within 1 week. For long-term storage (up to 6 months), make aliquots and store them at −80 °C.

PROCEDURE

Urine sample collection ● TIMING ~10 min

1| Collect urine with a container of proper volume (**Supplementary Fig. 1**) as described in Reagent Setup.

▲ CRITICAL STEP The container must be sterile; we recommend using disposable plastic-sealed receptacles (**Supplementary Fig. 1**).

■ PAUSE POINT Urine is a hostile environment for cells owing to its abnormal osmotic pressure, low pH and toxic metabolites (e.g., in the case of regular smokers), and there is risk of contamination if it is not handled adequately. Ideally, cells should be isolated immediately after sample collection. If the delay is unavoidable, refrigeration of the sample at 4 °C (e.g., on ice) will help preserve cell viability for some time. In this regard, we have successfully isolated cells from urine kept refrigerated for up to 4 h.

Isolation of urinary cells ● TIMING ~30 min plus incubation time

2| Transfer the urine samples to sterile 50-ml tubes (**Supplementary Fig. 1**).

! CAUTION This step and all subsequent steps involving cell handling should be performed inside a tissue culture hood to avoid contamination.

3| Centrifuge the tubes at 400*g* for 10 min at room temperature.

4| Carefully aspirate the supernatant, leaving only 1 ml in the tube.

5| Resuspend the pellets gently and individually in the remaining 1 ml of urine, and then collect them (if there are multiple tubes of urine) into a single 50-ml tube.

6| Add 10 ml of washing buffer (**Supplementary Fig. 1**).

7| Centrifuge the samples at 200*g* for 10 min at room temperature.

8| Carefully discard the supernatant, leaving only ~0.2 ml plus the pellet.

9| Add 1 ml of primary medium to resuspend the cell pellet, and then transfer the volume into a single well of a 12-well plate (coated beforehand with 0.1% (wt/vol) gelatin; **Supplementary Fig. 1**). The cells should have an appearance similar to that shown in **Figure 1a**,**b**.

▲ CRITICAL STEP Coating the culture plates with gelatin increases the initial adherence of urinary cells, which is also relevant for subsequent passages.

▲ CRITICAL STEP Female urine samples routinely contain more urine sediment than male samples. This mainly consists of squamous cells (likely from the urethra) that do not attach to the culture plates (**Fig. 1a**). A few blood cells (mostly erythrocytes) can sometimes be observed in male and female samples, especially when the age of the donor is advanced (**Fig. 1a**,**b**). These cell types gradually disappear after aspirating the medium in Step 12.

10| Add 1 ml of primary medium to the cells and incubate the cells at 37 °C for 24 h.

Urinary cell expansion ● TIMING ~2 weeks

11| Add 1 ml of primary medium to the culture on the next 3 d (i.e., 24, 48 and 72 h after plating). This and other volumes below are adjusted to a 12-well plate. Do not remove any medium.

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12| Approximately 96 h after plating, aspirate most of the medium (now should be ~4 ml), leaving ~1 ml, and then add 1 ml of proliferation medium.

13| Change half of the proliferation medium daily, leaving the other half intact.
! CAUTION If the tissue of origin is of particular interest for the reprogramming experiment (for example, for comparative studies of epigenetic memory of the donor tissue[44]), we recommend performing cell sorting when the total number of cells reaches approximately 100,000–200,000 to obtain a homogenous kidney epithelial cell population, as described by others[45,46].
▲ **CRITICAL STEP** Small colonies (of approximately two to four cells) should appear from 72 to 120 h (3–5 d) after plating and grow steadily (**Fig. 1c**,**d**). We normally observe three to ten small colonies per well, irrespective of the sex of the donor. Colonies with either regular (type I) or elongated (type II) morphology are routinely observed (**Fig. 1c**–**h**). Both types of colonies mainly consist of RE cells on the basis of immunofluorescence and gene expression analysis (see discussion in the INTRODUCTION and also **Supplementary Table 2**).

14| Check the plates under the microscope daily and continue to incubate until cells reach 80–90% density.
? TROUBLESHOOTING

15| When the urinary cell culture becomes dense enough for passaging (80–90% confluency; around 9–12 d after plating), split all the cells onto a new well of a 12-well plate for further expansion. This is considered passage 1. The cells tolerate 0.25% (wt/vol) trypsin-EDTA well, but to stop the reaction we do not use medium containing FBS and instead add trypsin inhibitor. TrypLE Select can also be used for the same purpose. Continue to incubate the cells and change the medium every other day. If at this point there are enough cells (~100,000 cells as assessed by counting), then they can instead be split onto a well of a six-well plate and used for infection directly (Step 17).
▲ **CRITICAL STEP** Do not allow the culture to become too dense, because this will reduce cell viability after passaging.

16| When the culture reaches 80–90% confluency, split all the cells 1:4 into the preferred size of plates for further expansion or onto a six-well plate for infection (Step 17). This is passage 2. Continue to passage cells in this way if required.
▲ **CRITICAL STEP** The highest efficiency of iPSC generation is achieved with urinary cells at passage 1–3, especially for aged or diseased individuals. Type II cells can be reprogrammed at later passages with higher efficiency than type I.
▲ **CRITICAL STEP** The life span of urinary cells varies between the two types of urinary cell colonies. Whereas type I cell colonies normally stop proliferating around passage 5, type II colonies can be successfully cultured up to passage 10.
■ **PAUSE POINT** Alternatively, the urinary cells can be frozen at −80 °C or in liquid nitrogen for later use. To do this, resuspend a cell pellet in 1 ml of CELLBANKER, which is devoid of FBS.

Retrovirus infection and UiPSC generation ● TIMING 3–4 weeks
17| Split 60,000 urinary cells (passages 1–4) per well in six-well dishes, or defrost a vial (in this case it is recommended to add more cells, as there may be some death) and incubate at 37 °C for 24 h.

18| Check that the urinary cells are ~30% confluent 24 h after seeding (**Fig. 2a**). Aspirate the medium and add 2 ml of fresh RE proliferation medium in each well.

19| Add 2 ml of viral supernatant for each transcription factor in each well. Accordingly, when using the four Yamanaka factors, the total volume will be 8 ml. Incubate the cells at 37 °C. This is considered day 0 of infection (**Fig. 2a**).

20| After 12–16 h, aspirate the culture medium and add 2 ml of fresh RE proliferation medium in each well and let the cells recover for another 8–12 h.

21| Repeat Step 19 to perform a second round of infection and incubate at 37 °C for 12 h.

22| At 12 h after the second infection, aspirate off medium and add fresh RE proliferation medium. Continue to incubate the cells and replace the medium with fresh RE proliferation medium daily. Examine the infection efficiency by checking the expression of the pMXs-GFP virus with a fluorescence microscope. Ensure that nearly 100% of the cells are GFP positive at 72 h after infection. Both type I and II urine cells can be infected efficiently (**Fig. 2b**).
? TROUBLESHOOTING

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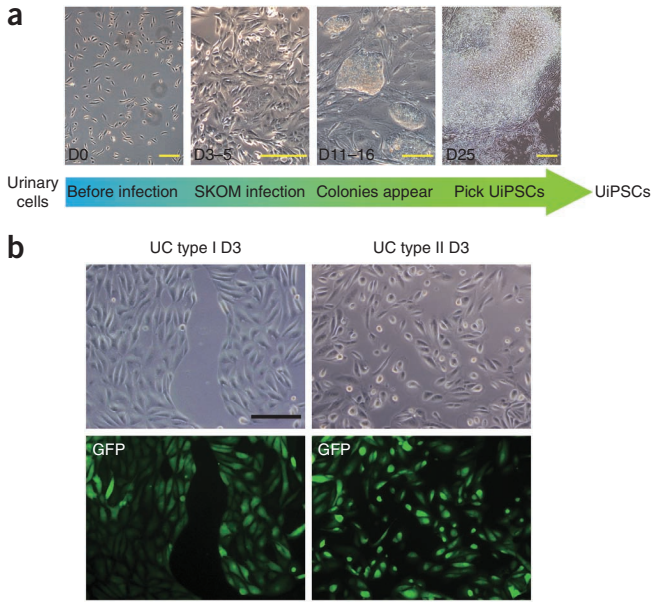
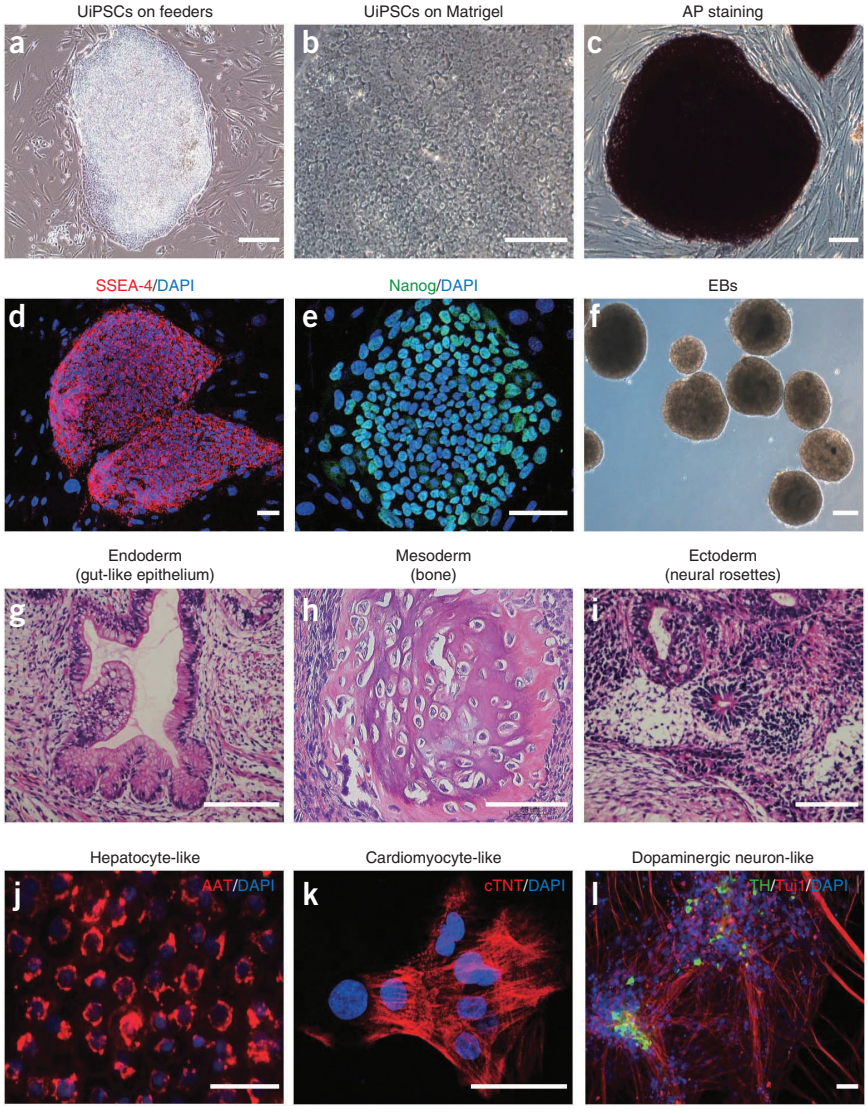


Figure 2 | Retrovirus infection and UiPSC generation. (**a**) Schematic representation of the kinetics of reprogramming using urinary cells (UCs) and four exogenous factors. Phase-contrast photographs for representative stages are shown. SKOM indicates *SOX2*, *KLF4*, *OCT4* and *c-MYC*. (**b**) Representative phase-contrast and immunofluorescence photographs of UCs at day 3 (D3) after infection with GFP retroviruses. Both type I and II UCs can be infected with high efficiency. Scale bars, 200 μm.

23| Check the infected urinary cells under the microscope daily. Urinary cells infected with the four exogenous factors should show a strong morphology change (the size becomes smaller and the cells more tightly packed) and increased proliferation 2–3 d after the second infection (**Fig. 2a**).
▲ **CRITICAL STEP** Urinary cells may become senescent after the infection, in which case the morphology change will not be obvious. In these cases, the experiment should be discarded. Early passages of urinary cells increase the chance of overcoming the senescence barrier. In addition, type II cells proliferate quicker and in general tolerate better the infection procedure.
? TROUBLESHOOTING



24| If the morphology change and proliferation of the infected urinary cells is substantial, then plate out 4×10^6 irradiated MEFs using MEF medium on gelatin-treated 100-mm plates as feeder cells for UiPSC induction.
▲ **CRITICAL STEP** The feeders should only be plated 1–2 d before splitting the infected urinary cells (Steps 25–28).

Figure 3 | UiPSCs show robust differentiation potential. (**a**–**c**) Representative phase-contrast photographs of UiPSCs grown on feeders or Matrigel, and alkaline phosphatase (AP) staining of UiPSCs grown on feeders. (**d**,**e**) Representative immunofluorescence staining patterns of UiPSCs using antibodies against SSEA-4 and Nanog. Cell nuclei were counterstained with DAPI. (**f**) Representative phase contrast photograph of embryoid bodies (EBs) produced with UiPSCs. (**g**–**i**) Representative photographs of teratomas produced by injecting UiPSCs into immunocompromised mice. Sections were stained with hematoxylin/eosin. (**j**–**l**) Representative immunofluorescences of hepatocyte-like cells, cardiomyocyte-like cells and dopaminergic neuron–like cells derived from UiPSCs. The antibodies used are anti-AAT (alpha1-antitrypsin) for hepatocyte-like cells, anti-cTNT (troponin T type 2) for cardiomyocyte-like cells, anti-TH (tyrosine hydroxylase) and anti-TUJ1 (βIII-tubulin) for dopaminergic neuron–like cells. Cell nuclei were counterstained with DAPI. Further information on how these characterizations were performed can be found in refs. 43,54 (for panels **a**–**c**) and 43,55 (for panels **d**–**l**). Scale bars, 100 μm.

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25| When the urinary cells reach 80–90% confluence (normally on days 3–5 after infection), and if the morphology change is substantial, trypsinize them using 0.25% (wt/vol) trypsin-EDTA and resuspend them in 10 ml of human ESC/dFBS medium.

26| Centrifuge the suspension at 200*g* for 5 min at room temperature.

27| Resuspend in 4 ml of human ESC/dFBS medium and count the cells.

28| Aspirate the medium from the feeder layers prepared in Step 24 and add ~8 ml of human ESC/dFBS medium. Next, add 5×10^4 to 1×10^5 infected urinary cells per 100-mm culture dish and incubate at 37 °C.

29| After 2 d, aspirate the medium and add fresh human ESC/dFBS medium + VPA (final concentration 1 mM). Continue to incubate the cells for 7 d, replacing the medium daily with fresh human ESC/dFBS medium + VPA (final concentration 1 mM).
▲ **CRITICAL STEP** Add VPA added to the induction medium for only 7 d; otherwise, this will result in toxicity to the reprogramming cells and feeder cells.

30| From day 16 after infection, replace the medium daily with fresh mTesR1 medium.

31| Seven days after human ESC-like colonies first appear (normally this is around days 16–25 after infection), pick the human ESC-like colonies mechanically onto feeder cells or Matrigel-coated plates (**Fig. 3a**,**b**). In some cases, the colonies may arise more slowly and therefore should be picked later as well.
▲ **CRITICAL STEP** Do not pick the human ESC-like colonies when they appear, as they will probably differentiate. Allow them to grow for at least 1 week after they appear (**Fig. 2a**).
? TROUBLESHOOTING

32| Culture and characterize the UiPSC colonies according to laboratory preference or as reported previously in *Nature Protocols*[47–49].

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
14	No urinary cell colonies are observed	Insufficient urine volume	Collect more volume Perform several collections and culture them in the same well
22,23	Weak GFP fluorescence or little morphology change is observed in the infected cells at days 2–3	Low viral titer Senescent cells	Repeat Concentrate the viral supernatants Use urinary cells at an earlier passage and/or culture in RE/MC proliferation medium
31	No human ESC-like colonies appear	Problem with medium or feeder preparation Low efficiency of reprogramming	Prepare fresh medium and new feeders Use urinary cells at an earlier passage and/or culture in RE/MC proliferation medium Add more cells onto the feeders at days 3–5 Continue reprogramming beyond day 25

● **TIMING**
Step 1, urine sample collection: 10 min
Steps 2–10, isolation of urinary cells: 30 min, plus 24 h of incubation
Steps 11–16 urinary cell expansion: 2 weeks (or until enough number of urine cells are obtained for UiPSCs generation)
Steps 17–32, retrovirus infection and UiPSCs generation: 3–4 weeks (until mechanical picking of iPSCs)
Box 1, retrovirus production: ~1 week

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ANTICIPATED RESULTS

A single collection of urine from an individual normally produces enough urinary cells for inducing reprogramming after ~2 weeks of culture. This time can be shortened by pooling multiple fresh urinary cell collections from the same donor. The protocol for reprogramming is highly reproducible and yields UiPSC clones on most of the occasions. Notably, the resulting UiPSCs are ESC-like and show excellent differentiation potential into lineages derived from the three germ layers[43] both *in vitro* and *in vivo* (**Fig. 3**). It has been recognized that iPSCs produced from different tissues have different propensities to differentiate into given tissues owing to the persistence of epigenetic memory of the donor cells[44]. It remains to be studied whether UiPSCs have a special propensity to differentiate back into RE cells, but this might be advantageous because methods for kidney differentiation are currently inefficient[50]. Song *et al.*[51] and Montserrat *et al.*[52] have reported the generation of iPSCs from kidney mesangial cells and renal proximal tubular epithelial cells (in both cases, obtained by means of a biopsy), respectively, and it would be interesting to compare their characteristics with UiPSCs. It will also be relevant to study whether, as a result of potential differences in genomic stability, UiPSCs are more or less prone to accumulate genomic alterations than iPSCs derived from other tissues. Of note, the protocol presented here is based on retroviral vectors, but the adaptation to nonintegrating approaches[53] is currently in progress in our laboratories.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS M.A.E., J.G. and R.G.-V. conceived the original idea. M.A.E., T.Z., C.B. and S.D. devised the protocol. J.G. and R.G.-V. provided advice for urinary cell isolation. T.Z., C.B. and S.D. performed most of the experiments. Y.H. and Y.L. contributed to these experiments. J.C.H., J.Y., Y.W., Y.Z., Q.Z. and X.B. contributed to the more recent adaptations of the protocol and participated in patient-specific UiPSC generation. M.A.E. supervised the experiments. M.A.E., H.-F.T., J.G. and R.G.-V. provided financial support. D.P. provided infrastructure support. M.A.E., T.Z. and C.B. wrote the manuscript and approved the final version.

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