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Generation of Induced Pluripotent Stem Cells from Urine

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ABSTRACT

Forced expression of selected transcription factors can transform somatic cells into embryonic stem cell (ESC)-like cells, termed induced pluripotent stem cells (iPSCs). There is no consensus regarding the preferred tissue from which to harvest donor cells for reprogramming into iPSCs, and some donor cell types may be more prone than others to accumulation of epigenetic imprints and somatic cell mutations. Here, we present a simple, reproducible, noninvasive method for generating human iPSCs from renal tubular cells present in urine. This procedure eliminates many problems associated with other protocols, and the resulting iPSCs display an excellent ability to differentiate. These data suggest that urine may be a preferred source for generating iPSCs.

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The transformation of somatic cells into induced pluripotent stem cells (iPSCs) using exogenous factors[1,2] also termed reprogramming, may be used for personalized regenerative medicine and can produce valuable *in vitro* models of human diseases or be used for toxicology screening.[3,4] Human iPSCs have been generated from multiple sources including skin (fibroblasts and keratinocytes), extraembryonic tissues or cord blood.[1,2,5–9] The reprogramming from these tissues has been achieved with varied frequencies, indicating that the cells of origin are an important determining factor. Researchers now also argue that iPSCs may retain cell-of-origin epigenetic memory[10] and accumulate other abnormalities as well.[11,12] Determining all of the cell types that iPSCs can be derived from, and defining their advantages or disadvantages, is therefore important. The ideal cell source should be easily accessible, susceptible, and universal (any age, sex, ethnic group, and body condition). The former consideration excludes many cell types used so far, whereas the latter eliminates neonatal tissues as in most countries they are not routinely stored. Dermal fibroblasts are possibly the most frequent cell type used for reprogramming. Yet, this requires biopsy, which sometimes encourages candidates to refuse donating tissue. Additionally, the procedure is contraindicated in life-threatening skin diseases (*e.g.*, severe epidermolysis bullosa) or burns. Recently, three groups reported the reprogramming of peripheral blood cells without $CD34^+$ cell mobilization.[13–15] The procedure is minimally invasive and requires small blood quantity. However, the efficiency was low (0.0008 to 0.1%) and the main target is mature T cells bearing specific T cell receptor rear-

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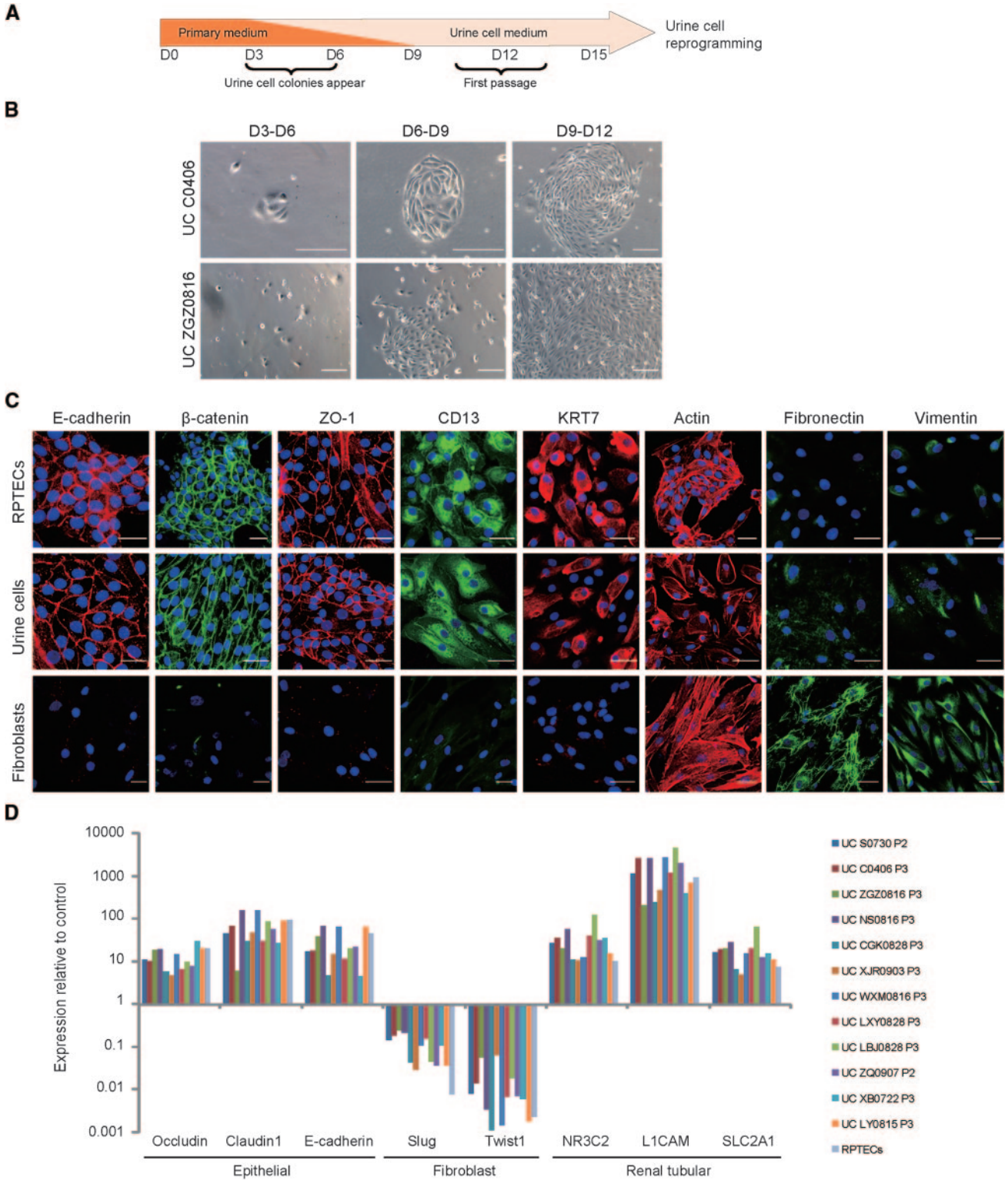

Figure 1. Collection and characterization of urine cells. (A) Scheme of urine sample collection. (B) Representative phase contrast photographs of urine cells (UC) at different points after collection. Top: type 1 cells. Bottom: type 2 cells. D, day (also hereafter). Scale bars, 100 μm. (C) Confocal immunofluorescence microscopy for the indicated markers in urine cells, renal proximal epithelial cells (RPTECs), and fibroblasts. E-cadherin and β-catenin are adherens junction markers; zonula occludens-protein 1 (ZO-1) is a tight junction marker; KRT7, keratin 7; scale bars, 40 μm. (D) Quantitative real-time PCR for the indicated markers in all donor cells used in this study; values are referred to skin fibroblasts. RPTECs were used as positive control. SLC2A1 is a renal proximal tubule solute carrier family 2-transporter.

Table 1. Summary of characterization for all urine cells reprogrammed to pluripotency in the course of this study and the resulting urinary induced pluripotent stem cell clones

	Urine Cells												
	C0406	**S0730**	**LY0815**	**LXY0828**	**NS0816**	**XB0722**	**WXM0816**	**ZGZ0816**	**CGK0828**	**XJY0903**	**ZQ0907**	**LBJ0828**	**GM1020**
	Female, 24 years, Caucasian	Female, 24 years, Caucasian	Female, 25 years, Asian	Female, 27 years, Asian	Female, 28 years, Asian	Male, 27 years, Asian	Male, 25 years, Asian	Male, 27 years, Asian	Male, 24 years, Asian	Male, 28 years, Asian	Male, 26 years, Asian	Male, 27 years, Asian	Female, 65 years, Asian
Volume of urine	100 ml	100 ml	150 ml	140 ml	160 ml	50 ml	100 ml	60 ml	90 ml	200 ml	100 ml	100 ml	100 ml
Major cell type	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2	Type 2	Type 2	Type 1	Type 2	Type 2	Type 1	Type 1
qPCR	√	√	√	√	√	√	√	√	√	√	√	√	√
IF	√	√	√	ND	ND	√	√	√	√	ND	ND	ND	ND
Passage of UC for reprogramming	P2	P2	P3	P2	P2	P2	P3	P2	P3	P2	P3	P3	P2
Reprogramming efficiency	0.30%	4%	0.10%	0.30%	3%	0.10%	0.30%	3%	0.20%	2%	0.20%	0.20%	0.01%
Number of UiPSC colonies picked	4	11	2	2	5	2	2	3	2	2	2	2	2
UiPSC characterization													
qPCR	4/4	5/11	2/2	2/2	3/5	2/2	2/2	3/3	2/2	2/2	2/2	2/2	1/2
IF	2/4	2/11	ND	ND	ND	1/2	ND	ND	1/2	ND	ND	ND	ND
DNA methylation	2/4	2/11	1/2	1/2	1/5	2/2	2/2	1/3	2/2	1/2	1/2	1/2	1/2
Karyotype	1/4	1/11	1/2	1/2	1/5	1/2	1/2	1/3	1/2	1/2	1/2	1/2	1/2
DNA microarrays	1/4	1/11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
STR analysis	1/4	1/11	1/2	1/2	1/5	1/2	1/2	1/3	1/2	1/2	1/2	1/2	1/2
Differentiation													
EBs	2/4	2/11	ND	ND	ND	1/2	ND	ND	ND	ND	ND	ND	ND
neurons	2/4	1/11	1/2	1/2	1/5	1/2	1/2	1/3	ND	ND	ND	ND	ND
cardiomyocyes	2/4	2/11	1/2	1/2	2/5	1/2	1/2	1/3	1/2	1/2	ND	1/2	ND
hepatocytes	2/4	1/11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
teratomas	2/4	1/4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Quantitative real-time PCR (qPCR) and immunofluorescence (IF) analysis are routinely included all markers in Figures 1 and 2. Reprogramming efficiency refers to alkaline phosphatase-positive clones with human embryonic stem cell (ESC) morphology. Neuronal differentiation for urinary induced pluripotent stem cells (UiPSC) clones not mentioned in Figure 3 was only assessed morphologically; as for cardiomyocyte differentiation, we only verified the generation of beating clusters. √, done; UC, urine cells; STR, single tandem repeat; EBs, erythroid bodies.

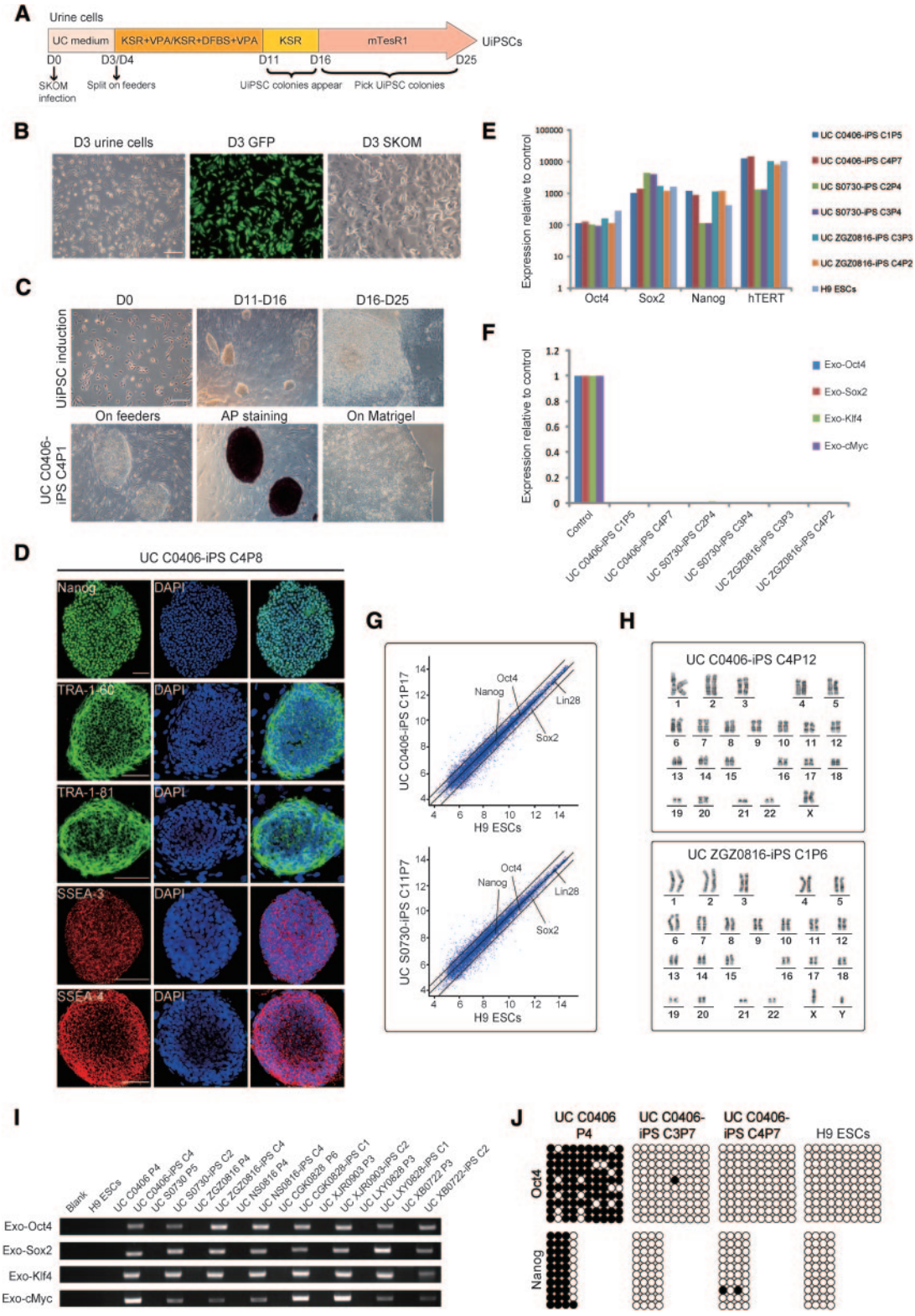

Figure 2. Generation of induced pluripotent stem cells (iPSCs) from urine cells. (A) Schematic representation of iPSC generation from urine cells (UC). SKOM refers to the four exogenous factors Sox2, Klf4, Oct4, and c-Myc. (B) Phase contrast and immunofluorescence photographs of urine cells at day 3 after infection with control green fluorescent protein (GFP) retrovirus. Cells infected with the exogenous factors are also included; note the early morphologic changes (clustering) indicative of reprogramming. Scale bar, 200 μm. (C) Top: representative phase contrast photographs of emerging urinary induced pluripotent stem cells (UiPSCs) colonies at different time points. Bottom: representative phase contrast photographs of passage (P) 1 UiPSCs grown on feeders (alkaline phosphatase [AP]

rangements, thus representing a caveat for some potential applications. Moreover, in rare cases giving/transfusing blood is not exempt of concerns (*e.g.*, because of religious beliefs), and the reprogramming may be problematic in patients with blood diseases (*e.g.*, hemophilia and leukemia) or immunodepression (*e.g.*, cancer and AIDS). In our search for optimal tissue sources, we have produced human iPSCs from periosteal membrane, adipose stem cells, and extraembryonic tissues.[8,9] Here we report the generation of iPSCs from exfoliated renal tubular cells present in urine.

The human kidney contains an extensive network of tubules whose total surface is bigger than the skin. As part of normal physiology approximately 2000 to 7000 cells from this tubular system and downstream parts of the urinary tract (ureters, bladder, and urethra) detach and are excreted in urine daily.[16] These cells—hereafter termed urine cells–-not only are not damaged but are fully functional and can be used for *in vitro* studies.[17] Besides, they can be collected anywhere without medical assistance and are easily expanded (Figure 1A). We hypothesized that if amenable to reprogramming, urine cells might be a valuable cell source that has some advantages compared with other cell types. We produced cell cultures from urine of several healthy individuals (Table 1). At first glance they consisted of squamous cells (likely from urethra) and a few blood cells (mostly erythrocytes), but after 3 to 6 days they were replaced by small colonies that grew quickly. These colonies corresponded to two main morphologies: type 1 or type 2 (Figure 1B), in agreement with previous reports on urine cell isolation.[18] Type 1 cells were more rounded and grew closely attached to neighbor cells, suggesting an epithelial phenotype. Type 2 cells were more elongated and grew more dispersed. In some sample collections all colonies corresponded to 1 of the 2 cell types, but in others they were mixed. The cell cultures were pooled upon reaching high density and split for further characterization and reprogramming. Those enriched in type 1 cells displayed well formed cell-cell junctions as assessed by immunofluorescence microscopy (Figure 1C). They were also positive for the intermediate filament keratin 7 (an epithelial marker) plus the renal proximal tubule marker CD13, and the distribution of actin was cortical (Figure 1C). Quantitative real-time PCR (qPCR) further supported a predominant epithelial origin (Figure 1D). Renal proximal epithelial cells and fibroblasts were used as controls for the immunofluorescence and qPCR. Type 2 cell-enriched cultures showed a rather similar immunofluorescence pattern, but the intensity was milder, and the distribution more patchy (data not shown); qPCR results were likewise comparable (Figure 1, C and D). In both cases we observed little staining for the fibroblastic-like markers fibronectin and vimentin (Figure 1C). Therefore, these results support that both cell types have epithelial origin and suggest that type 2 cells may arise from partial epithelial dedifferentiation.

We infected urine cells at passage 2 to 3 with retroviruses producing Sox2, Klf4, Oct4, and c-Myc (Figure 2A). Infection efficiency was high as shown by parallel transduction with retroviruses producing green fluore2scent protein (GFP), and in cells expressing the factors, we observed early morphology changes indicative of reprogramming[19] (Figure 2B). In total, samples from 12 young adults of either Chinese or Caucasian origin were reprogrammed to iPSCs, 7 corresponding to males and 5 to females. Characterization of the primary culture and the resulting iPSCs (hereafter named UiPSCs) is summarized in Table 1. Small colonies normally appeared at day 11 to 16 post-transduction (Figure 2, A and C), sometimes later. Many of these colonies progressively adopted human embryonic stem cell (ESC)-like morphology and were picked between days 16 and 25 (Figure 2, A and C). The reprogramming efficiency varied among donors, but in general was high, between 0.1 and 4% (Table 1). We produced UiPSCs from a 65-year-old woman as well, but the efficiency was approximately 0.01% (Table 1). Moreover, we could freeze and thaw urine cells from several donors before transduction without impairing reprogramming efficiency significantly. Urine cells could also be infected at later passages albeit with a drop in efficiency (*e.g.*, 0.3% for passage 5 compared with 3% for passage 2 using donor ZGZ0816 or 0.05% for passage 4 compared with 0.3% for passage 2 using donor UCC0406). After colony expansion (Figure 2C, bottom), UiPSCs were characterized by standard procedures including alkaline phosphatase staining (AP) (Figure 2C), immunofluorescence for human ESC markers (Figure 2D), and qPCR for endogenous ESC genes plus negligible expression of the transgenes (Figure 2, E and F). DNA microarrays demonstrated global gene expression close to H9 ESCs (Figure 2G) and we detected normal karyotype (Figure 2H), transgene integration in the genomic DNA (Figure 2I), and demethylation of the proximal Oct4 and Nanog promoters (Figure 2J). Single tandem repeat analysis of donor urine cells and UiPSCs showed matched origin

staining is also included) or Matrigel. Scale bar, 200 μm. (D) Confocal immunofluorescence microscopy for the indicated human embryonic stem cell (ESC) markers of a representative UiPSC clone; scale bars, 100 μm. (E) Quantitative real-time PCR (qPCR) for endogenous ESC transcription factors in the indicated UiPSCs. hTERT indicates human telomerase reverse transcriptase. Values are referred to donor urine cells; H9 ESCs were used as control. (F) qPCR showing silencing of the exogenous transgenes in the indicated UiPSC clones; values are referred to transduced cells extracted at day 6. (G) Scatter plot of DNA microarrays data for 2 representative UiPSC clones and H9 ESCs. (H) Semiquantitative PCR showing integration of the exogenous transgenes in the genome of the indicated UiPSC clones, urine donor cells, water, and H9 ESCs were included as controls. (I) Normal karyotype of representative male and female UiPSC clones. (J) Bisulfite sequencing analysis for the Oct4 and Nanog proximal promoters in 2 representative UiPSC clones from the same donor.

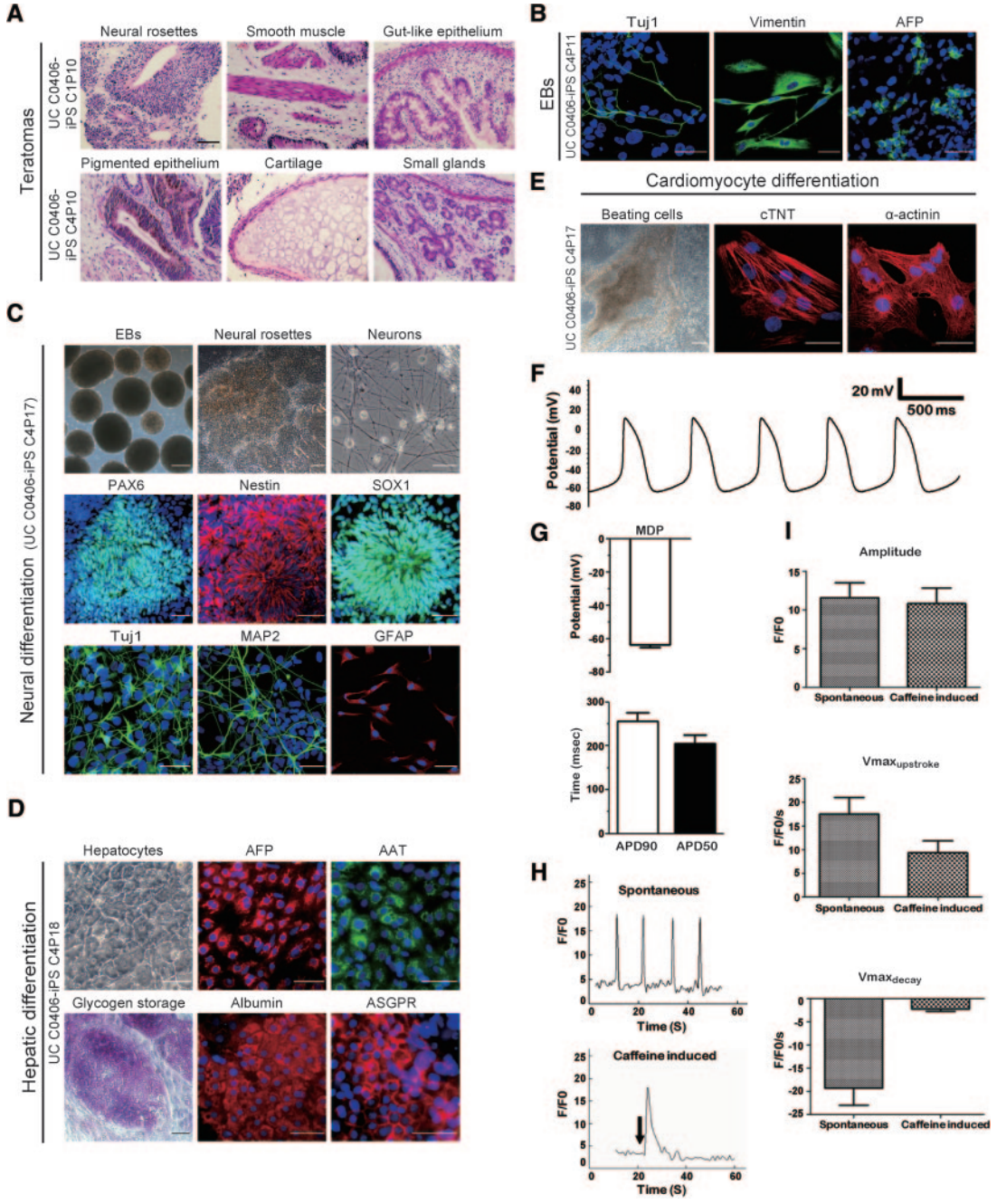

Figure 3. Multidifferentiation potential of urinary induced pluripotent stem cells (UiPSCs). (A) Teratomas comprising derivatives of the 3 germ layers. Scale bar, 100 μm. (B) Confocal immunofluorescence microscopy for markers of the 3 germ layers in differentiating embryoid bodies (EBs) of a representative UiPSC clone. AFP stands for α-fetoprotein; scale bars, 50 μm. (C) Top, from left to right: phase contrast photographs of EBs growing in suspension, neural rosettes, and neurons produced from a representative UiPSC clone; scale bars, 50 μm. Middle: confocal immunofluorescence microscopy for the indicated markers of neural rosettes produced from the same UiPSC clone; scale bars, 50 μm. Bottom: confocal immunofluorescence of neurons and astrocytes (glial fibrillary acidic protein [GFAP]) produced from the same UiPSC clone; scale bars, 50 μm. (D and E) Phase contrast and immunofluorescence photographs of hepatocytes and cardiomyocytes produced from representative UiPSC clones; scale bars, 50 μm (phase contrast of cardiomyocytes), 200 μm (phase contrast of hepatocytes) and 50 μm (all immunofluorescence photographs). AAT stands for α-1 antitrypsin. Glycogen accumulation was detected with periodic acid-Schiff staining. ASGPR stands for asialoglycoprotein receptor. (F) Representative spontaneous action potential tracing recorded by whole-cell patch clamp technique. Data correspond to clone UC C0406-iPS-C4P17 (also in G through I); similar results were observed with UC C0406-iPS-C1P17. (G) Top: maximal diastolic potential (MDP). Bottom: action potential duration at 90 and 50% repolarization (APD90 and APD50). (H) Representative tracings of rhythmic spontaneous and caffeine-induced calcium transients. (I) Amplitude, maximal upstroke velocity (Vmax upstroke), and maximal decay velocity (Vmax decay) of calcium transients.

in all cases (Table S1A). If the circumstances were such, the latter excludes the unlikely contamination and reprogramming of cells from a sexual partner. To prove that these UiPSCs are pluripotent we performed nonspecific differentiation through teratomas (Figure 3A) and embryoid bodies (EBs) (Figure 3B and Supplemental Figure S1A). Next we did directed-UiPSC differentiation into neural lineages (neural stem cell-like cells, neuron-like cells, and astrocyte-like cells) (Figure 3C); hepatocyte-like cells (Figure 3D and Supplemental Figure S1B); and cardiomyocyte-like cells (Figure 3E and Supplemental Figure S1C), which was verified by immunofluorescence microscopy for the appropriate markers and qPCR (for the hepatocytes and cardiomyocytes). Neural differentiation was produced for 12 UiPSCs corresponding to 11 donors, hepatocytes for four clones of three donors, and cardiomyocytes for 14 clones of 11 donors (Table 1). During cardiomyocyte differentiation the proportion of spontaneously beating EBs was high (between 30 and 75%); a representative recording is included in Supplemental Video S1. Likewise, electrophysiology measurement of action potentials and calcium transients (Figure 3, F through I) showed behavior similar to that of cardiomyocytes produced from human ESCs or fibroblast-derived iPSCs (data not shown).

In conclusion, we have generated iPSCs from an easily accessible source in a totally noninvasive manner, and the quality of our cell lines seems excellent according to standard criteria. It may be arguable to say that urine collection is easier than a skin biopsy or a blood draw in healthy individuals. However, in at least some circumstances (except when there is renal insufficiency) the procedure seems advantageous operationally. It will be relevant to see whether—due to less direct exposure to radiation—urine cells produce iPSCs bearing less somatic cell mutations and copy number variations than iPSCs from skin.[11,12] Moreover, if it is true that iPSCs have memory of the donor tissue,[10] then one could say that UiPSCs should be the best source for producing renal cells[20] that can be used for transplantation or disease modeling. Collecting urine for creating UiPSCs may be as well an interesting option for certain genetic diseases (*e.g.*, von Hippel-Lindau syndrome)[21] in which the remaining wild-type allele is mainly mutated in the kidney. Clearly, nonintegration and mouse feeder-free approaches would be required to ultimately produce clinical grade iPSCs,[22,23] but it is likely that such approaches can be applied to urine cells. Caveats such as lack of the strictest sterility are a concern not only during urine collection but for any *ex vivo* cell source and do not represent a technical obstacle for UiPSC generation provided that some basic norms of hygiene are taken and antibiotics included in the early stages of culture. In fact urine is sterile before it reaches the urethra, and we only collect the middle stream of the micturition. Therefore, in at least some situations urine samples may be considered a preferred source for iPSC derivation.

CONCISE METHODS

The collection and culture of urine cells is described in detail in the *Supplemental Methods*. The ethics committee of the Guangzhou Institutes of Biomedicine and Health (in Guangzhou, China) or the University of Natural Resources and Life Sciences (in Vienna, Austria) approved this and other procedures, and signed consent forms are available upon request. Skin fibroblasts were purchased from Coriell cell repository (AG06299) and maintained in DMEM (Invitrogen) + 10% (vol/vol) FBS (Hyclone). Retroviral plasmids producing human Oct4, Sox2, Klf4, and c-Myc transcription factors were purchased from Addgene. Viral supernatants were harvested on 2 consecutive days starting 48 hours after transfection. Urine cells at passages 2 to 4 were trypsinized and seeded on six-well dishes, and 60,000 cells were added per well. Cells were infected with viral supernatants generated by transfection of HEK293T cells (using Lipofectamine 2000, Invitrogen) with retroviral pMXs vectors (Addgene) containing the cDNAs of human Oct4, Sox2, Klf4, and c-Myc. Two rounds of infection were performed successively (of 12 hours each) as described before.[7] Polybrene (Sigma) was added to increase infection efficiency. After the second round of infection the tissue culture medium of the transduced cells was changed to urine cell medium. Infection efficiency was monitored separately and was close to 100% as demonstrated by transduction with GFP-expressing vectors. On day 3 or 4, cells transduced with the reprogramming factors were trypsinized, and their number was counted. Routinely, 50,000 cells were seeded onto a layer of feeders in a 10-cm culture dish, using human ESC medium (F12 + 20% Knock-out Serum Replacement + 10 ng/ml basic fibroblast growth factor + nonessential amino acids [all from Invitrogen], L-glutamine, and β-mercaptoethanol, which was renewed daily. On day 5, the medium was changed to human ESC medium + 1 mM valproic acid (VPA; Sigma) or half ESC medium+ half dFBS medium (consisting of DMEM high glucose [Invitrogen] + 20% human defined fetal bovine serum [dFBS, Hyclone] + VPA. In both cases VPA was added only from day 5 to 12. From day 12 to day 16 we used human ESC medium and then mTesR1 medium (StemCell) until the last day of the experiment. After infection the medium was renewed daily in all stages. From day 16, those colonies that were big enough and identifiable as human ESC-like (*i.e.*, flat morphology with defined borders and big nuclei containing prominent nucleoli), could be picked mechanically and expanded in human ESC medium on feeders or on mTesR1 medium on Matrigel. Details of UiPSC characterization procedures are also provided in *Supplemental Methods*.

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DISCLOSURES

None.

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See related editorial, "Induced Pluripotent Stem Cells from Human Kidney," on pages 1179–1180.

Supplemental Information for this article is available online at http://www.jasn.org/.

Supplementary Methods:

Urine collection and cell expansion

Appropriate containers (up to 500 ml) were sterilized before urine collection. Donors were asked to wash extensively their urethral area, including the labiae in women, with flushable pre-moistened wipes, and to direct the first portion of the urine stream into the toilet and not the container. The first micturition of the day was routinely avoided. Besides, only the mid stream of urine was later on collected into sterile containers. The usual volume of specimens was 150-200 ml. Urine samples were then transferred into 50 ml tubes inside a tissue culture hood and these tubes were centrifuged at 400 g for 10 minutes at room temperature. The supernatant was carefully discarded inside the tissue culture hood, leaving approximately 1 ml or less of urine in the tube. Pellets were individually resuspended and all the 50 ml tubes derived from one sample collection pooled into one single 50 ml tube. Around 10 ml of PBS containing ampothericin B and penicillin/streptomycin were added to prevent growth of contaminant funghi or facultative bacteria residing in the urethra (before that urine is sterile). The samples were centrifuged at 400 g for 10 minutes. The supernatant was discarded, leaving only around 0.2 ml of sample. Around 1 ml of primary medium was added to resuspend the cell pellet. The recipe for the primary medium contained DMEM/Ham´s F12 1:1 (Hyclone), 10 % of fetal bovine serum (FBS; PAA), SingleQuot Kit CC-4127 REGM (Lonza), ampothericin B and penicillin/streptomycin. The cells were then transferred onto 12 well plates coated with L-gelatine in 1 ml of primary medium. The first 2 days a few hundred µl of primary medium were added to retain the antibiotics concentration and keep the nutrition level up. The following days the medium was carefully changed to REBM (Renal Epithelial Basal

Medium, Lonza) medium containing SingleQuot Kit CC-4127 REGM (Lonza) (the combination of the 2 is referred to as urine cell medium), the procedure was never carried out completely to maintain factors secreted by the urine cells and avoid unnecessary stress. Visible cells/colonies appeared routinely after 3-6 days, typically 3-5 per sample on average. The first full media change was made after the first cells/colonies were seen. Cells were then split onto a bigger surface aided by 0.25% trypsin containing 1 mM EDTA when the culture grew confluent. RPTECs were obtained from a biopsy and maintained in urine cell medium.

UiPSC characterization

AP staining, transgene integration, karyotyping, and bisulfate sequencing were done as described[1,2]. For DNA methylation experiments filled circles indicate methylation and open circles demethylation; the percentage of C (cytosine)-T (thymidine) conversion out of the CpG context was bigger than 95%. STR analysis was performed using an Applied Biosystems Genetic Analyzer (ABI3130, ABI). Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen) and total RNA was extracted using Trizol (Invitrogen). qPCR was performed using a Thermal Cycler DiceTM Real Time System (ABI7300, ABI) and SYBR Green Premix EX TaqTM (Takara); beta actin was used for normalization and all items were measured in triplicate. All primers used in this study are included in ***Table S1B***. DNA microarrays were performed using Illumina's Human HT-12 V4.0 Expression Beadchip according to the manufacturer's instructions. Chips were scanned using Illumina BeadChip Reader and data analyzed using illumina BeadStudio Application. Data have been deposited in the GEO database (accession number is in process). For teratomas, 2×10^6 UiPSCs were injected subcutaneously or intramuscularly

into the right hind leg of immuno-compromised NOD-SCID mice. Tumors were excised 8-10 weeks later, fixed, and embedded in paraffin, sectioned and stained with hematoxylin/eosin. For EB differentation, iPSCs on feeder were treated with dispase (Invitrogen) and collected by scraping. After centrifugation, cell pellets were re-suspended in human ESC medium without bFGF and grown for 8 days in non-adherent dishes. Forming EBs were then transferred to gelatin-coated dishes to allow differentiation for another 8 days before processing for immunofluorescence analysis.

Immunofluorescence microscopy

Cells were fixed in 4% paraformaldehyde for 30 minutes, washed, blocked and permeabilized in blocking solution (PBS containing 3% normal goat serum and 0.2% Triton X-100) for 30 minutes to 2 hours. Then they were incubated with primary antibodies in blocking solution at 4°C overnight, washed 3 times and incubated with the corresponding secondary antibodies for 1 hour at room temperature. Cells were washed twice and stained with DAPI (Sigma) for 5 minutes in order to visualize the nuclei (in blue). A Leica TCS SP2 Spectral confocal microscope (Leica Microsystems GmbH) was used for observation and photographing. Representative fields are shown in the corresponding Figures. Before immunofluorescence, beating areas were cut out with scissors, collected into a 1.5 ml tube with low calcium PBS, and left for 30 minutes at room temperature. These cell clumps were transferred into PBS containing 0.5-1 mg/ml collagenase 2 and incubated at 37°C for 30-40 minutes. The digestion was terminated with cardiomyocyte medium (Knockout DMEM [GIBCO] + 20% FBS [PAA], non-essential amino acids, and beta mercaptoethanol). The samples were centrifuged and the pellet resuspended in cardiomyocyte medium. Cell suspensions were plated on gelatine-

coated coverslips and cultured at 37°C for at least 2 days before fixation and immunofluorescence. Primary antibodies (and phalloidin for staining the actin cytoskeleton) used in this paper are listed in ***Table S1C***.

Tissue-specific differentiation and electrophysiological measurements

Neuronal, hepatocyte and cardiomyocyte differentiation were performed as described[3-5]. N2, B27, RPMI 1640 and hepatoZYME-SPF were purchased from Invitrogen, Heparin from Sigma, EGF, Activin A and oncostatin M from R&D Systems, BMP2, FGF4, HGF and KGF from PeproTech, and dexamethasone from Enzo Life Sciences. Periodic acid Schiff's staining was performed using a kit purchased from Polysciences. Electrophysiological characterization of iPSC-derived cardiomyocytes (at day 23) was done using standard whole-cell patch-clamp to record the action potential phenotypes (HEKA Instruments Inc.)[6]. Patch pipettes were prepared from 1.5-mm thin-walled borosilicate glass tubes using a Sutter micropipette puller P-97 and had typical resistances of 3–5 MΩ when filled with an internal solution containing (mM): 110 K^+ aspartate, 20 KCl, 1 $MgCl_2$, 0.1 Na-GTP, 5 Mg-ATP, 5 Na_2-phosphocreatine, 5 EGTA, 10 HEPES, and pH adjusted to 7.3 with KOH. The external Tyrode's bath solution consisted of (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 0.4 KH_2PO_4, 1.8 $CaCl_2$, 10 glucose, 5 HEPES, and pH adjusted to 7.4 with NaOH. Spontaneous electrical activity was measured whereas the iPSC-derived cardiomyocytes were left passive without current input. Twenty consecutive action potentials from spontaneously firing iPSC-derived cardiomyocytes were recorded to ensure stable waveforms for analysis. Data were corrected for the liquid junction potentials of +15.9 mV. Calcium transients were detected with confocal calcium imaging using a protocol described previously[7]. Briefly, isolated iPSC-derived

cardiomyocytes were loaded with 5 μM Fluo-3 AM (Invitrogen) for 25 minutes at 37°C in Tyrode solution containing (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 0.4 KH_2PO_4, 1.8 $CaCl_2$, 10 glucose, 5 HEPES at pH 7.4. Calcium transients were recorded with a confocal imaging system (Olympus Fluoview System version 4.2 FV300 TIEMPO) mounted on an upright Olympus microscope (IX71) and then quantified as background subtracted fluorescence intensity changes normalized to the background subtracted baseline fluorescence. Data were fed into the Felix 32 (Photon Technology International) software for analysis. Video recording of beating cardiomyocytes was done using a Canon Digital IXUS 70 camera (Canon).

Supplementary references:

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Supplementary Figure legends:

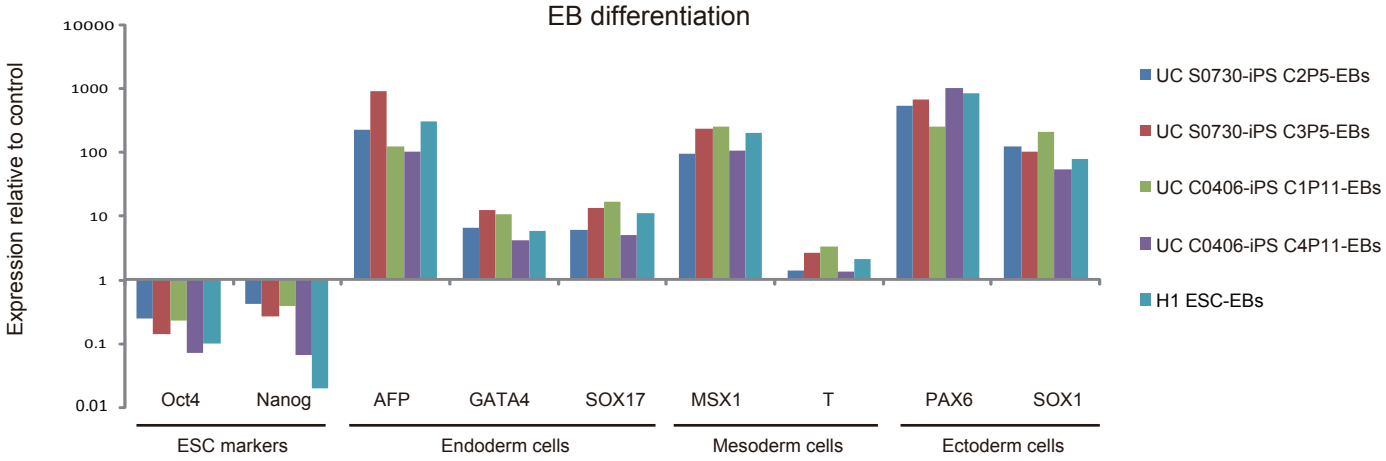
Figure S1-**A**, qPCR for the indicated markers in differentiating EBs produced from the indicated UiPSC clones; H1 ESCs were added as a control. Values are referred to the respective undifferentiated UiPSC clones. **B**, qPCR for the indicated markers at different points of directed hepatocyte differentiation of the indicated UiPSC clones. Values are referred to the respective undifferentiated UiPSCs. **C**, qPCR for the indicated markers after directed cardiomyocyte differentiation of the indicated UiPSC clones; H1 ESCs were added as a control. Values are referred to the respective undifferentiated UiPSC clones.

Table S1-Results of STR analysis for the indicated urine donor cells and UiPSCs (**A**), plus list of primers (**B**) and antibodies used in this study (**C**).

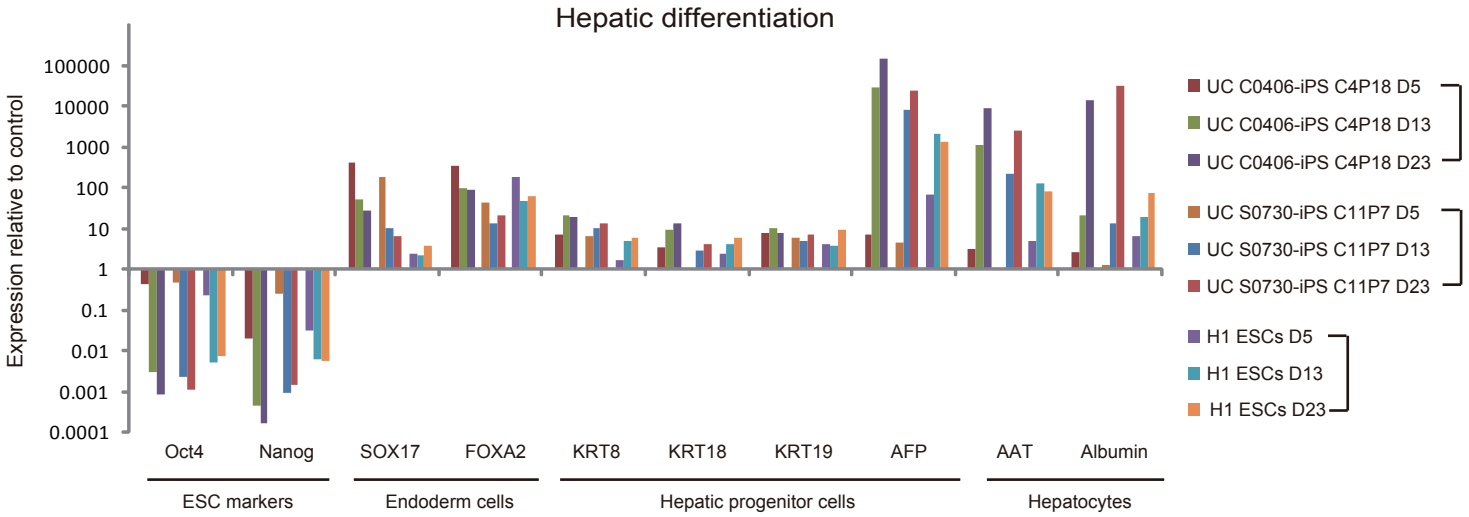
Video S1-Spontaneous beating of cardiomyocytes generated from a representative UiPSC clone (UC C0406- iPS C4P17).

Zhou *et al.* Fig S1

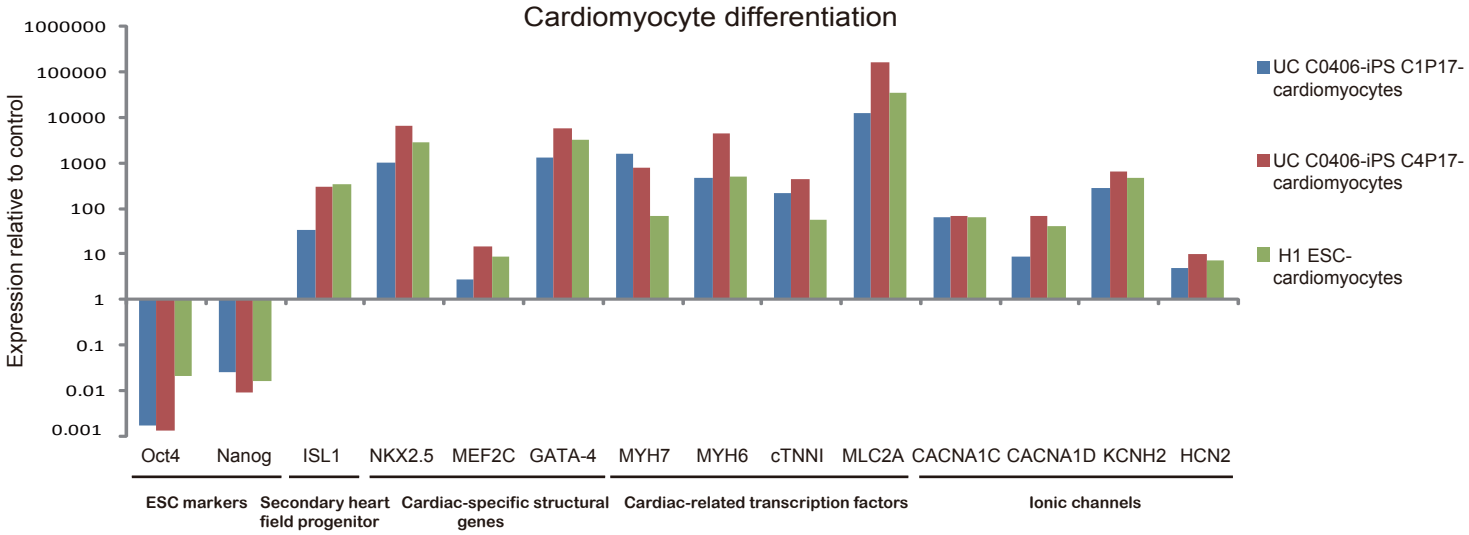
A



B



C



Zhou *et al*. Table S1A

STR for UiPSCs characterization

Sample Name	Marker	Dye	Allele 1	Allele 2	Sample Name	Marker	Dye	Allele 1	Allele 2
UC S0730 P4	D3S1358	B	15	18	UC S0730-iPS C11	D3S1358	B	15	18
	TH01	B	9	9.3		TH01	B	9	9.3
	D21S11	B	28	30		D21S11	B	28	30
	D18S51	B	13	16		D18S51	B	13	16
	Penta E	B	7	10		Penta E	B	7	10
	D5S818	G	12	13		D5S818	G	12	13
	D13S317	G	8	12		D13S317	G	8	12
	D7S820	G	8	11		D7S820	G	8	11
	D16S539	G	11	12		D16S539	G	11	12
	CSF1PO	G	10	12		CSF1PO	G	10	12
	Penta D	G	8	11		Penta D	G	8	11
	AMEL	Y	X			AMEL	Y	X	
	vWA	Y	16	16		vWA	Y	16	16
	D8S1179	Y	10	14		D8S1179	Y	10	14
	TPOX	Y	8	9		TPOX	Y	8	9
	FGA	Y	22	25		FGA	Y	22	25
UC ZGZ0816 P4	D3S1358	B	14	16	UC ZGZ0816-iPS C1	D3S1358	B	14	16
	TH01	B	9.3	9.3		TH01	B	9.3	9.3
	D21S11	B	30	32.2		D21S11	B	30	32.2
	D18S51	B	13	14		D18S51	B	13	14
	Penta E	B	18	20		Penta E	B	18	20
	D5S818	G	9	11		D5S818	G	9	11
	D13S317	G	10	11		D13S317	G	10	11
	D7S820	G	8	12		D7S820	G	8	12
	D16S539	G	9	11		D16S539	G	9	11
	CSF1PO	G	11	11		CSF1PO	G	11	11
	Penta D	G	13	13		Penta D	G	13	13
	AMEL	Y	X	Y		AMEL	Y	X	Y
	vWA	Y	15	16		vWA	Y	15	16
	D8S1179	Y	13	13		D8S1179	Y	13	13
	TPOX	Y	8	8		TPOX	Y	8	8
	FGA	Y	22	22		FGA	Y	22	22
UC LY0815 P4	D3S1358	B	15	17	UC LY0815-iPS C1	D3S1358	B	15	17
	TH01	B	9	9.3		TH01	B	9	9.3
	D21S11	B	30	32.2		D21S11	B	30	32.2
	D18S51	B	14	17		D18S51	B	14	17
	Penta E	B	11	15		Penta E	B	11	15
	D5S818	G	11	13		D5S818	G	11	13
	D13S317	G	8	8		D13S317	G	8	8
	D7S820	G	11	12		D7S820	G	11	12
	D16S539	G	9	9		D16S539	G	9	9
	CSF1PO	G	10	12		CSF1PO	G	10	12
	Penta D	G	10	11		Penta D	G	10	11
	AMEL	Y	X			AMEL	Y	X	
	vWA	Y	17	20		vWA	Y	17	20
	D8S1179	Y	10	13		D8S1179	Y	10	13
	TPOX	Y	8	9		TPOX	Y	8	9
	FGA	Y	23	24		FGA	Y	23	24
	D3S1358	B	16	17		D3S1358	B	16	17

UC NS0816 P4	TH01	B	9	9	UC NS0816-iPS C3	TH01	B	9	9
	D21S11	B	30.2	32		D21S11	B	30.2	32
	D18S51	B	13.2	20		D18S51	B	13.2	20
	Penta E	B	15	20		Penta E	B	15	20
	D5S818	G	12	12		D5S818	G	12	12
	D13S317	G	8	11		D13S317	G	8	11
	D7S820	G	8	10		D7S820	G	8	10
	D16S539	G	12	12		D16S539	G	12	12
	CSF1PO	G	12	12		CSF1PO	G	12	12
	Penta D	G	9	9		Penta D	G	9	9
	AMEL	Y	X			AMEL	Y	X	
	vWA	Y	14	17		vWA	Y	14	17
	D8S1179	Y	11	11		D8S1179	Y	11	11
	TPOX	Y	8	8		TPOX	Y	8	8
	FGA	Y	21	23		FGA	Y	21	23
UC XB0722 P5	D3S1358	B	15	16	UC XB0722-iPS C2	D3S1358	B	15	16
	TH01	B	8	9		TH01	B	8	9
	D21S11	B	32.2	33.2		D21S11	B	32.2	33.2
	D18S51	B	15	15		D18S51	B	15	15
	Penta E	B	23	23		Penta E	B	23	23
	D5S818	G	13	13		D5S818	G	13	13
	D13S317	G	10	11		D13S317	G	10	11
	D7S820	G	11	13		D7S820	G	11	13
	D16S539	G	11	11		D16S539	G	11	11
	CSF1PO	G	9	9		CSF1PO	G	9	9
	Penta D	G	7	7		Penta D	G	7	7
	AMEL	Y	X	Y		AMEL	Y	X	Y
	vWA	Y	14	15		vWA	Y	14	15
	D8S1179	Y	13	13		D8S1179	Y	13	13
	TPOX	Y	8	8		TPOX	Y	8	8
	FGA	Y	21	23		FGA	Y	21	23
UC WXM0816 P4	D3S1358	B	15	17	UC WXM0816-iPS C2	D3S1358	B	15	17
	TH01	B	9	9		TH01	B	9	9
	D21S11	B	29	29.2		D21S11	B	29	29.2
	D18S51	B	13	20		D18S51	B	13	20
	Penta E	B	5	10		Penta E	B	5	10
	D5S818	G	11	13		D5S818	G	11	13
	D13S317	G	8	10		D13S317	G	8	10
	D7S820	G	11	11		D7S820	G	11	11
	D16S539	G	9	12		D16S539	G	9	12
	CSF1PO	G	9	10		CSF1PO	G	9	10
	Penta D	G	9	14		Penta D	G	9	14
	AMEL	Y	X	Y		AMEL	Y	X	Y
	vWA	Y	17	18		vWA	Y	17	18
	D8S1179	Y	13	15		D8S1179	Y	13	15
	TPOX	Y	11	11		TPOX	Y	11	11
	FGA	Y	19	20		FGA	Y	19	20
	D3S1358	B	14	14		D3S1358	B	14	14
	TH01	B	6	7		TH01	B	6	7
	D21S11	B	30.2	30.2		D21S11	B	30.2	30.2
	D18S51	B	20	20		D18S51	B	20	20
	Penta E	B	17	17		Penta E	B	17	17
	D5S818	G	11	16		D5S818	G	11	16
	D13S317	G	8	8		D13S317	G	8	8

UC CGK0828 P5	D7S820	G	11	12	UC CGK0828-iPS C2	D7S820	G	11	12
	D16S539	G	10	10		D16S539	G	10	10
	CSF1PO	G	11	11		CSF1PO	G	11	11
	Penta D	G	9	9		Penta D	G	9	9
	AMEL	Y	X	Y		AMEL	Y	X	Y
	vWA	Y	14	18		vWA	Y	14	18
	D8S1179	Y	10	12		D8S1179	Y	10	12
	TPOX	Y	8	8		TPOX	Y	8	
	FGA	Y	21	24		FGA	Y	21	24
UC LBJ0828 P4	D3S1358	B	15	16	UC LBJ0828-iPS C1	D3S1358	B	15	16
	TH01	B	8	9		TH01	B	8	9
	D21S11	B	28.2	29.2		D21S11	B	28.2	29.2
	D18S51	B	15	22		D18S51	B	15	22
	Penta E	B	10	12		Penta E	B	10	12
	D5S818	G	9	11		D5S818	G	9	11
	D13S317	G	8	9		D13S317	G	8	9
	D7S820	G	12	12		D7S820	G	12	12
	D16S539	G	10	11		D16S539	G	10	11
	CSF1PO	G	10	12		CSF1PO	G	10	12
	Penta D	G	10	10		Penta D	G	10	10
	AMEL	Y	X	Y		AMEL	Y	X	Y
	vWA	Y	14	17		vWA	Y	14	17
	D8S1179	Y	11	14		D8S1179	Y	11	14
	TPOX	Y	8	9		TPOX	Y	8	9
	FGA	Y	19	23		FGA	Y	19	23
UC ZQ0907 P4	D3S1358	B	15	17	UC ZQ0907-iPS C1	D3S1358	B	15	17
	TH01	B	7	9		TH01	B	7	9
	D21S11	B	28.2	30.2		D21S11	B	28.2	30.2
	D18S51	B	14	15		D18S51	B	14	15
	Penta E	B	12	17		Penta E	B	12	17
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	D13S317	G	11	13		D13S317	G	11	13
	D7S820	G	11	12		D7S820	G	11	12
	D16S539	G	10	12		D16S539	G	10	12
	CSF1PO	G	10	15		CSF1PO	G	10	15
	Penta D	G	9	11		Penta D	G	9	11
	AMEL	Y	X	Y		AMEL	Y	X	Y
	vWA	Y	15	16		vWA	Y	15	16
	D8S1179	Y	12	13		D8S1179	Y	12	13
	TPOX	Y	8	8		TPOX	Y	8	8
	FGA	Y	20	24		FGA	Y	20	24
UC LXY0828 P5	D3S1358	B	14	16	UC LXY0828-iPS C1	D3S1358	B	14	16
	TH01	B	9	10		TH01	B	9	10
	D21S11	B	29.2	30.2		D21S11	B	29.2	30.2
	D18S51	B	13	14		D18S51	B	13	14
	Penta E	B	11	15		Penta E	B	11	15
	D5S818	G	13	14		D5S818	G	13	14
	D13S317	G	10	13		D13S317	G	10	13
	D7S820	G	8	10		D7S820	G	8	10
	D16S539	G	9	12		D16S539	G	9	12
	CSF1PO	G	10	10		CSF1PO	G	10	10
	Penta D	G	9	10		Penta D	G	9	10
	AMEL	Y	X			AMEL	Y	X	
	vWA	Y	16	19		vWA	Y	16	19

	D8S1179	Y	14	15		D8S1179	Y	14	15
	TPOX	Y	8	9		TPOX	Y	8	9
	FGA	Y	21	24		FGA	Y	21	24
UC XJY0903 P4	D3S1358	B	16	17	UC XJY0903-iPS C1	D3S1358	B	16	17
	TH01	B	6	9		TH01	B	6	9
	D21S11	B	30	31.2		D21S11	B	30	31.2
	D18S51	B	13	14		D18S51	B	13	14
	Penta E	B	15	15		Penta E	B	15	15
	D5S818	G	11	11		D5S818	G	11	11
	D13S317	G	8	10		D13S317	G	8	10
	D7S820	G	11	13		D7S820	G	11	13
	D16S539	G	9	11		D16S539	G	9	11
	CSF1PO	G	10	12		CSF1PO	G	10	12
	Penta D	G	9	9		Penta D	G	9	9
	AMEL	Y	X	Y		AMEL	Y	X	Y
	vWA	Y	17	18		vWA	Y	17	18
	D8S1179	Y	15	17		D8S1179	Y	15	17
	TPOX	Y	9	11		TPOX	Y	9	11
	FGA	Y	21	24.2		FGA	Y	21	24.2
UC C0406 P2	D3S1358	B	16	17	UC C0406-iPS C4	D3S1358	B	16	17
	TH01	B	8	9.3		TH01	B	8	9.3
	D21S11	B	33.2	35.2		D21S11	B	33.2	35.2
	D18S51	B	19	20		D18S51	B	19	20
	Penta E	B	17	23		Penta E	B	17	23
	D5S818	G	12	15		D5S818	G	12	15
	D13S317	G		13		D13S317	G		13
	D7S820	G	10	13		D7S820	G	10	13
	D16S539	G	12	13		D16S539	G	12	13
	CSF1PO	G	11	12		CSF1PO	G	11	12
	Penta D	G	10	13		Penta D	G	10	13
	AMEL	Y	X			AMEL	Y	X	
	vWA	Y	20			vWA	Y	20	
	D8S1179	Y	13	14		D8S1179	Y	13	14
	TPOX	Y	8	11		TPOX	Y	8	11
	FGA	Y	21	22		FGA	Y	21	22
UC GM1020 P2	D3S1358	B	16	17	UC GM1020-iPS C1	D3S1358	B	16	17
	TH01	B	9	9		TH01	B	9	9
	D21S11	B	29	30		D21S11	B	29	30
	D18S51	B	12	21		D18S51	B	12	21
	Penta E	B	11	19		Penta E	B	11	19
	D5S818	G	11	12		D5S818	G	11	12
	D13S317	G	9	12		D13S317	G	9	12
	D7S820	G	9	11		D7S820	G	9	11
	D16S539	G	10	12		D16S539	G	10	12
	CSF1PO	G	11	11		CSF1PO	G	11	11
	Penta D	G	12	13		Penta D	G	12	13
	AMEL	Y	X	X		AMEL	Y	X	X
	vWA	Y	14	17		vWA	Y	14	17
	D8S1179	Y	12	13		D8S1179	Y	12	13
	TPOX	Y	8	8		TPOX	Y	8	8
	FGA	Y	23	24		FGA	Y	23	24

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Primer list

Name	Sense	Antisense
Oct4	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT
Sox2	CCCAGCAGACTTCACATGT	CCTCCCATTTCCCTCGTTTT
Nanog	TGAACCTCAGCTACAAACAG	TGGTGGTAGGAAGAGTAAAG
hTERT	TGTGCACCAACATCTACA AG	GCGTTCTTGGCTTTCAGGAT
exo-Oct4	GGGTGGACCATCCTCTAGAC	CCAGGTCCGAGGATCAAC
exo-Sox2	GGGTGGACCATCCTCTAGAC	GGGCTGTTTTTCTGGTTG
exo-Klf4	GGGTGGACCATCCTCTAGAC	GGAAGTCGCTTCATGTGG
exo-cMyc	GGGTGGACCATCCTCTAGAC	CCTCGTCGCAGTAGAAATAC
AFP	ATTGGCAAAGCGAAGCTG	GCTGTGGCTGCCATTTTT
GATA4	CAGAAAACGGAAGCCCAA	TTGCTGGAGTTGCTGGAAG
SOX17	ACGGAATTTGAACAGTAT	CAGGATAGTTGCAGTAAT
MSX1	TGCCTCGCTCTACGGTGCCT	GGCTGGAGGAATCGGCTGGC
T	GTGGGCCTGGAGGAGAGCGA	TTGTCCGCCGCCACGAAGTC
PAX6	TTGCTTGGGAAATCCGAG	TGCCCGTTCAACATCCTT
SOX1	TTTCCCCTCGCTTTCTCA	TGCAGGCTGAATTCGGTT
Occludin	TGCCGCGTTGGTGATCTTT	GCCCAGGATAGCACTCACTATT
Claudin1	AGCTGCAAAATGTACGACTCG	CAGGAGCAAGCTACCAAGGT
E-cadherin	CCCACCACGTACAAGGGTC	ATGCCATCGTTGTTCACTGGA
Slug	ATATTCGGACCCACACATTACCT	GCAAATGCTCTGTTGCAGTGA
Twist1	GTCCGCAGTCTTACGAGGAG	GCTTGAGGGTCTGAATCTTGCT
KRT7	AGACGGAGTTGACAGAGCTG	GGATGGCCCGGTTCATCTC
SCNN1A	TCGAGTTCCACCGCTCCTA	GCCAGTACATCATGCCAAAGG
NR3C2	CACAGCACTGGTTCCTCAG	TTTGCCTGCTAAGCGGTTGA
L1CAM	CAAGCCCGAAGTGCAGTTC	CTGGCAAAGCAGCGGTAGAT
CD13	GCACAATCATCGCACTGTCAG	CGCTTTACTTTGGTCCAAGGT
SLC2A1	CTTTTCTGTTGGGGGCATGAT	CCGCAGTACACACCGATGAT
SLC2A2	CTGCTCAACTAATCACCATGCT	GGTCCCAATTTTGAAAACCCCA
FOXA2	GCGACCCCAAGACCTACAG	GGTTCTGCCGGTAGAAGGG
HEX	CGCTAAATGGAGGAGACT	CTGTTCACTGGGCAAATC
ALBUMIN	GTGGAAGAGCCTCAGAAT	TTGGTGTAACGAACTAATAGC
AAT	AGACCCTTTGAAGTCAAGCGACC	CCATTGCTGAAGACCTTAGTGATGC
KRT8	GATCTCTGAGATGAACCGGAACA	GCTCGGCATCTGCAATGG
KRT18	GGCATCCAGAACGAGAAGGAG	GCGGGCATTGTCCACAGTAT
KRT19	AACGGCGAGCTAGAGGTGA	TTCCGTCTCAAACTTGGTTCG
ISL1	GAGGGTTTCTCCGGATTTGG	TCCCATCCCTAACAAAGCATGT
MLC 2A	CAGGCCCAACGTGGTTCTT	CCATCACGATTCTGGTCGATAC
NKX 2.5	ACCCTGAGTCCCCTGGATTT	TCACTCATTGCACGCTGCAT
MLC-2V	CCTTGGGCGAGTGAACGT	GGGTCCGCTCCCTTAAGTTT
MYH7	GGCAAGACAGTGACCGTGAAG	CGTAGCGATCCTTGAGGTTGTA
MYH6	TCTCCGACAACGCCTATCAGTAC	GTCACCTATGGCTGCAATGCT
CTNNI	CCAACTACCGCGCTTATGC	CTCGCTCCAGCTCTTGCTTT
MEF-2C	TAACTTCTTTTCACTGTTGTGCTCCTT	GCCGCTTTTGGCAAATGTT
KCNQ1	CGCGGAAGCCTTACGATGT	GAACAGTGAGGGCTTCCCAAT
KCNH2	CGTGCTGCCTGAGTACAAGCT	TGTGAAGACAGCCGTGTAGATGA
HCN2	CACCTGCTACGCCATGTTCA	CTGGCAGCTTGTGGAAGGA
CACNA1C	AAGGCTACCTGGATTGGATCAC	GCCACGTTTTCGGTGTTGAC
CACNA1D	GGGCAATGGGACCTCATAAATAA	TTACCTGGTTGCGAGTGCATTA

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Antibody list

Antibody	Company	Catalog Number
E-cadherin	BD Bioscience	610181
β-catenin	BD Bioscience	610153
ZO-1	Invitrogen	339100
CD13	GeneTex	GTX62507
KRT7	GeneTex	GTX40206
Rhodamine phalloidin	Invitrogen	R415
Fibronectin	Sigma	F6140
Vimentin	Sigma	V5255
Nanog	R&D systems	AF1997
Tra-1-60	Millipore	MAB4360
Tra-1-81	Millipore	MAB4381
SSEA-3	Abcam	MC631
SSEA-4	Abcam	MC813
Tuj1	Sigma	T8860
PAX6	DSHB	NA
Nestin	Millipore	AB5922
SOX1	R&D systems	AF3369
Map2	Millipore	AB5622
GFAP	Sigma	G9269
cTnT	Thermo scientific	MS-295-P0
a-Actinin	Sigma	A7811
AAT	Invitrogen	18-0002
Albumin	R&D systems	MAB1455
ASGPR	Santa Cruz	Sc-52623
AFP	Sigma	A8452