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.


The transformation of somatic cells into induced pluripotent stem cells (iPSCs) using exogenous factors1,2 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 memory10 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. Derma 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-
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. 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

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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])
In our search for optimal tissue sources, we have produced human iPSCs from peristomal membrane, adipose stem cells, and extraembryonic tissues.\(^e\)\(^g\) 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.\(^\text{16}\) These cells—hereafter termed urine cells—not only are not damaged but are fully functional and can be used for in vitro studies.\(^\text{17}\) Besides, they can be collected fully functional and can be used for cells—not only are not damaged but are These cells—hereafter termed urine cells—which are not damaged and can be isolated from urine daily.\(^\text{16}\)

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.\(^\text{18}\) 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 fluorescent protein (GFP), and in cells expressing the factors, we observed early morphology changes indicative of reprogramming\(^\text{19}\) (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 posttransduction (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.
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 non Specific 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. Moreover, if it is true that iPSCs have memory of the donor tissue, then one could say that UiPSCs should be the best source for producing renal cells 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) 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 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. 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 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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REFERENCES


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 amphotericin B and penicillin/streptomycin were added to prevent growth of contaminant fungi 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), amphotericin 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, \(2 \times 10^6\) 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 differentiation, 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 described³⁻⁵. 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 MgCl₂, 0.1 Na-GTP, 5 Mg-ATP, 5 Na₂-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 MgCl₂, 0.4 KH₂PO₄, 1.8 CaCl₂, 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 previously⁷. 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 MgCl₂, 0.4 KH₂PO₄, 1.8 CaCl₂, 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:**


**Supplementary Figure legends:**

*Figure SI-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.  

*Figure SI-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.  

*Figure SI-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 SI*-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 SI*-Spontaneous beating of cardiomyocytes generated from a representative UiPSC clone (UC C0406- iPS C4P17).
### Zhou et al. Table S1A

**STR for UiPSCs characterization**

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Zhou et al. Table S1C

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