SUPPLEMENTAL INFORMATION

SUPPLEMENTAL MATERIALS AND METHODS

Collection and primary culture of UCs

UCs were collected and cultured as described by Zhou. In brief, immediately after collecting 100 ml of urine from the healthy donors in sterile urine cups (Gongdong Medical equipment Co., Zhejiang, China), the exfoliated renal epithelial cells present in the urine were spun down, re-suspended, and cultured in a primary medium containing DMEM/high glucose (Corning Life Sciences) and Ham’s F12 nutrient (Gibco by Life Technology™) mix (1:1), which was supplemented with 10% (vol/vol) FBS (Gibco by Life Technology™), 100 U ml⁻¹ penicillin (Hyclone), 100 ug ml⁻¹ streptomycin (Hyclone), REGM SingleQuot kit (Lonza) supplements, and 2.5 μg ml⁻¹ amphotericin B (Sigma-Aldrich) during the first four days. From Day 5 (D5), instead of the primary medium, the RE proliferation medium (REGM BulletKit) (Lonza) was used for urinary cell expansion until the cell density reached 80–90%. The expanded UCs were either immediately processed for cell reprogramming or cryopreserved in a freezing medium (CellBanker®), followed by storage at -196 °C in liquid nitrogen for later examination or reprogramming. UCs at less than passage three were used for UiPSC generation.

Generation of UiPSCs

UiPSCs were generated by infecting the UCs with a combination of retroviruses
encoding the human transcription factors SOX2, OCT-4, KLF4, and c-MYC, as described by Takahashi. In brief, 6×10^4 UCs at less than passage three were seeded in a six-well dish (Costar, Corning life sciences) and infected with a combination of retroviruses that were produced by 293T cells (Cell Bank, Shanghai, China) transfected with pMXs-Sox2, pMXs-Oct4, pMXs-Klf4, pMXs-c-Myc, and pCL-Eco obtained from Addgene. After culturing in RE proliferation medium (REGM BulletKit) (Lonza) for six days, the infected cells were seeded on mitomycin C (MMC; Sigma-Aldrich)-treated mouse embryonic fibroblasts (MEF) (Cell Bank, Shanghai, China) and cultured in ESC medium/dFBS until UiPSCs colonies appeared. The ESC medium/dFBS, which contained DMEM nutrient mix F12 (Gibco by Life Technology TM) supplemented with 20% KSR (Gibco by Life Technology TM), 1% GlutaMAX (Invitrogen), 1% NEAA (Invitrogen), 100 μM β-Mercaptoethanol (Invitrogen), and 10 ng/ml bFGF (Peprotech), was then replaced with mTesR medium (Stemcell). After 2-3 weeks, the UiPSCs colonies were mechanically picked and cultured in an mTesR medium (Stemcell) on a matrigel (BD Biosciences)-coated plate and passaged with 0.5mM EDTA in PBS. The UiPSCs were either immediately processed for the examination of their stem cell characteristics or cryopreserved in a mTesR medium (Stemcell) that was supplemented with 10% dimethylsulfoxide (DMSO, Sigma-Aldrich), followed by storage in liquid nitrogen at -196 °C for use in later experiments.

**Stem cell culture**
The UiPSCs, iPSCs derived from fibroblasts (from Junfeng Ji’s lab), and H9 human ESCs (Cell Bank, Shanghai, China) were cultured in feeder-free conditions in mTesR medium (Stemcell) on a matrigel-coated plate. The culture medium was changed every day, and the cells were passaged every four days with 0.5 mM EDTA in PBS.4

**Immunofluorescence examination of UiPSCs**

For immunofluorescence examination, the UiPSCs seeded on the matrigel (BD Biosciences)-coated plate were fixed with paraformaldehyde (PFA; Sigma-Aldrich) (4% in PBS), permeabilized with 0.4% Triton X-100 (Sigma-Aldrich), and incubated overnight with the following primary antibodies: mouse anti-SSEA (1/75) (Abcam), rabbit anti-Sox2 (1/250) (Millipore), mouse anti-human Tra-1-81 (1/100) (Millipore), and goat anti-human Nanog (1/75) (R&D). Subsequently, the cells were incubated for 1.5 h with AlexaFluor-555-labeled secondary antibody (1/1000) (Invitrogen), and the nuclei were labeled with 4’, 6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml; Sigma-Aldrich). Finally, images were captured using an Olympus IX71 microscope (Olympus) that was equipped with DP2-BSW software (Olympus) and prepared using Image J software and Microsoft PowerPoint (2007).

**Alkaline phosphatase (AP) staining**

In order to analyze the alkaline phosphatase (AP) activity, the UiPSCs were fixed with paraformaldehyde (PFA; Sigma-Aldrich) (4% in PBS) and incubated in an AP staining solution (mix Fast Red Violet [FRV]) with Naphthol AS-BI phosphate solution
and water in a 2:1:1 ratio (FRV:Naphthol:water) by using the Alkaline Phosphatase Detection Kit (Millipore, Billerica, USA) according to the manufacturer’s protocol. Finally, images were captured using an Olympus CKX41 microscope (Olympus) that was equipped with Toup View software.

**Teratoma assay for UiPSCs**

NOD/SCID mice (6-8 weeks old, male) were purchased from Silaike Experimental Animal Co. (Shanghai, China) and kept in the Animal Housing Facility at Zhejiang University. All animal experiments were approved by the Institutional Animal Care and Use Committee at Zhejiang University.

For the intramuscular injections, $1 \times 10^6$ UiPSCs were injected into the muscle center in the hind-leg quadriceps along the long axis of the mice. The tumors were isolated, fixed in formalin, and embedded in paraffin for 6-8 weeks after the injection. The samples were then sectioned into 5 μm slices using a microtome (Microtome RM2245; Lecia, Germany). Hematoxylin-eosin staining was performed on the sections. The images were then captured using a Leica DM 4000B microscope.

**Western blot analysis**

A human lens was obtained from a 20-year-old male who died from an accident and donated his organs. The mature LB samples at D25 and human lens were lysed with extraction buffer. The protein concentration was then determined by using a spectrophotometer (Bio-Rad iMark Microplate Reader). Thirty μg extracts from each
sample were loaded in designated lanes. All proteins were separated and transferred onto a PVDF blotting membrane. The membranes with targeted proteins were then incubated overnight with α-A crystalline (Enzo), α-B crystalline (Enzo), β-crystalline (Santa Cruz), LC3B (Sigma-Aldrich) and GAPDH (Abcam) primary antibody at a 1:1000 dilution. After incubation with horseradish peroxidase (HRP)-conjugated second antibody (Cell Signaling) (1:5000), the images were developed using the ECL detection system (Millipore) and the ChemiDoc™ MP imaging system (Bio-Rad). They were prepared using Microsoft PowerPoint (2007).

**Quantitative real-time polymerase-chain-reaction (qRT-PCR) assay**

**UiPSCs and ESCs.** The total RNA from the undifferentiated UiPSCs and ESCs was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s guidelines and then dissolved in RNase-free H2O. RNA quality and concentration were determined using spectrophotometers (NanoDrop 2000c, Thermo Scientific). RNA samples were examined via RT-quantitative (q)PCR to validate the expression of *SOX2, OCT4,* and *NANOG* using SYBR Premix Ex TagTM (RR420A) on an ABI Fast 7500 RT-PCR system according to the manufacturer’s protocols (TaKaRa).

**Human lens epithelial cells (HLECs).** The human epithelial cell line SRA 01-04, which was obtained from the Riken cell bank (Tsukuba, Japan), was cultured in Dulbecco’s modified essential medium (DMEM) (Corning Life Sciences) supplemented with 10% fetal bovine serum (FBS) (Gibco) in a humidified 37°C incubator with a 5% CO2 atmosphere. The total RNA from the HLECs was extracted,
qualified, and quantified as described above.

**LB.** At various time points during LB differentiation, all RNA from the cell samples was extracted, qualified, and quantified as previously described. The expression of a selection of genes (EYA1, DLX3, SIX1, SOX1, PROX1, α-A crystallin, α-B crystallin, β-crystallin, γ-crystallin, MIP, FOXE3, and PAX6) was also examined as previously described.

Normalization of data was as following. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The quantification cycle (Ct) was obtained, and the ΔCt value was calculated with Ct (target gene) − Ct (GAPDH). ΔΔCt (log2 Ratio) was calculated with ΔCt (control group) − ΔCt (target group).

All the primers used in the present study were described previously,\(^1\), the others were designed in Primer Bank, or taken from Primer Bank (SOX1 and FOXE3). They were listed in Table.S1.

Real-time PCR samples were also used for running gel.

**Methylene blue staining**

The LBs were prepared as described above for TEM examination. The LBs were sectioned in LKB 11800 PYRAMITOME. The sections were then stained with 1% methylene blue in PBS and observed under Leica DM 2500 microscopy.

**Immunofluorescence examination during LB differentiation**

During LB differentiation, the cell samples were fixed with paraformaldehyde (PFA; Sigma-Aldrich) (4% in PBS), permeabilized with 0.4% Triton X-100 (Sigma-Aldrich),
and incubated overnight with the following primary antibodies: rabbit anti-SIX1 (1:200) (Cell Signaling), rabbit anti-PROX1 (1:750) (Abcam), rabbit anti-FOXE3 (1:100) (Sigma-Aldrich), rabbit anti-Collagen IV (1:100) (Abcam), rabbit anti-E-cadherin (1:100) (Cell Signaling), rabbit anti-α-A crystalline (1:100) (Enzo), rabbit anti-α-B crystalline (1:100) (Enzo), rabbit anti-β-crystallin (1:50) (Santa Cruz), rabbit anti-γ-crystallin (1:50) (Santa Cruz), rabbit anti-AQP0 (MIP) (1:100) (Santa Cruz), and mouse anti-chicken PAX6 (1:200) (DSHB). Subsequently, the cells were incubated for 1.5 h with AlexaFluor-555-labeled secondary antibody (1/1000; Invitrogen). Finally, 4′, 6-diamidino-2-phenylindole (DAPI) (0.5 µg/ml; Sigma-Aldrich) was added to label the nuclei, and the samples were mounted in Vectashield (Vector Laboratories), captured using an Olympus IX71 microscope (Olympus) equipped with DP2-BSW software (Olympus) or a Leica TCS SP8 confocal microscope (Leica), and prepared with Image J software and Microsoft PowerPoint (2007).

The fixed LBs (D25) were stained with a membrane stain DiOC6 (3,3′-dihexyloxacarbocyanine iodide) (Sigma-Aldrich) for the cellular arrangement analysis.

LBs on D25 were fixed with paraformaldehyde (PFA; Sigma-Aldrich) (4% in PBS), embedded in paraffin and sectioned to 3 µm slices using a microtome (Leica RM2245). After permeabilization with 0.4% Triton X-100, sections were incubated overnight with rabbit anti-α-A crystalline (1:100) (Enzo), rabbit anti-α-B crystalline
(1:100) (Enzo), rabbit anti-β-crystallin (1:50) (Santa Cruz), rabbit anti-γ-crystallin (1:50) (Santa Cruz), rabbit anti-AQP0 (MIP) (1:100) (Santa Cruz), followed by AlexaFluor-555-labeled secondary antibody (1/1000; Invitrogen) for 1.5 h and DAPI for 10 mins. Images were captured with a Leica TCS SP8 confocal microscope (Leica), and prepared with Image J software and Microsoft PowerPoint (2007).

All positive and negative specific immunolabelings were repeated in more than three independent experiments.

**Transmission electron microscopy (TEM)**

The LBs from D25 were first fixed with 2.5% glutaraldehyde in phosphate buffer (0.1M, pH7.0) for 4 h, followed by postfixing with 1% OsO4 in phosphate buffer (0.1 M, pH7.0) for 2 h. The samples were then dehydrated, infiltrated, and embedded in Spurr’s resin. Finally, the LBs were sectioned in Leica EM UC7 ultratome. The sections were stained with uranyl acetate and alkaline lead citrate for 5 and 10 min, respectively. The images of the sectioned whole LBs were taken with a Hitachi Model H-7650 TEM and prepared using Microsoft PowerPoint (2007).

**Analysis of LBs’ magnifying ability**

The magnifying ability of the LBs was analyzed as previously described. In brief, a 35-mm culture dish with the LBs (or the rat lens) was placed on a paper on which an “X” had been printed. The dish was then placed and fixed on a dissecting microscope (Olympus) to ensure a constant distance between the “X” and the LBs (or the rat lens).
Using a Pro-MicroScan digital camera (Olympus) that was equipped with Toup View software, photographs of the “X” underneath the culture medium alone, the rat lens, and each LB were then taken. To quantify the magnification factor of each LB (or the rat lens), the central width of the “X” in the photos was measured underneath the medium alone, the rat lens, and the LBs. Finally, the average magnification factor of the LBs (or the rat lens) was determined by calculating the ratio between the average central width of the “X” under the LBs (or the rat lens) and the central width of the “X” under the culture medium alone \( \text{magnification factor} = \frac{\text{average central width of the “X” under the LBs (or the rat lens)}}{\text{central width of the “X” under the culture medium alone}} \). Here, the rat lens was used as a positive control.

**Statistical analysis**

A one-way ANOVA was performed using the SPSS software (Version 17.0, SPSS). The ANOVA was followed by Least Significant Difference (LSD) Post-Hoc Multiple Comparison when more than two groups had to be compared. Statistical significance was defined at \(*P < 0.05\) and \(**P < 0.01\).
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL FIGURE LEGENDS

**Figure S1. Generation of iPSCs from UCs.** (A) Time schedule of iPSC generation. (B) Morphologies of two types of UCs. Representative images show type I and type II UC colonies on D5 (arrows) and on D9 (arrows). (C) Representative images of UCs before retrovirus infection and iPSC colonies before mechanical selection (arrow). (D) UiPSCs passaged on matrigel for more than 30 passages were able to form embryonic bodies (EBs) and were positive for alkaline phophatase (AP) staining. Scale bars: (B) 100 μm; (C) for UCs, 100 μm; (C) for iPSCs, 500 μm; (D) for UiPSCs on Matrigel, 100 μm; (D) for EB, 50 μm; (D) for AP staining, 40 μm.

**Figure S2. Characterization of UiPSCs.** (A) Immunofluorescence examination showed that the UiPSCs were positive for embryonic stem-cell antigens, including Nanog, SSEA4, Sox2, and TRA1-81. (B) Quantitative real-time PCR (qRT-PCR) analysis showed that similar to ESCs (grey bar), the UiPSCs strongly expressed Sox2, Oct4, and Nanog (black bar), in contrast to the human epithelial cells (HLECs) (white bar). Date are presented as mean ± SEM (n = 3 independent experiments). ***, P<0.01 vs gene expression in HLECs; ND means not detected. (C-D) Teratoma assay conducted to analyze the differentiation ability of UiPSCs. Teratomas indicated by the red dotted frames were observed six to eight weeks after the UiPSCs were injected into the hind legs of NUD mice (C). H&E analysis revealed that UiPSCs were able to differentiate into three germ layers (D). Scale bars: (A) (D)100 μm; (C) 1 cm.
**Figure S3. Mature LBs generated only from the ‘fried egg’ cell clusters.** (A) Comparison of UiPSC differentiation process in the ‘non-fried egg’ condition and in the ‘multiple fried egg’ condition (single ‘fried egg’ condition in Figure 1C) on D11, D14, and D25. (B) Mature LBs could only be generated from the ‘multiple fried egg’ condition (and single ‘fried egg’ condition in Figure 1B). Scale bar: (A) 100 µm; (B) 3 mm.

**Figure S4. ESCs differentiating into LBs.** (A) Representative images of mature LBs derived from ESCs after 25 days of differentiation. (B) Representative images at various time points showing ESCs (D0); differentiating ESCs before selection, when the epithelial-like cells appeared (arrows) and with the selected parts of cells being indicated by the red dotted frames (D6); differentiating ESCs with a ‘fried egg’-like morphology (D11), showing the existence of ‘differentiating cells’ (D-Cells) and ‘supporting cells’ (S-Cells); and the first appearance of immature LBs (D14) (arrow) and mature LBs (D25) during LB formation. The red square frame indicates a lens-like and transparent structure. Magnified pictures (100× and 200×) provided increased detail at every stage. (C) After 25 days of differentiation, only the LBs in the ‘selected cells’ condition showed a transparent morphology, while those in ‘non-treated cells’ and ‘remaining cells’ conditions were simply small cell clusters. Scale bar: (A) 3 mm; (B) 100 µm (40×), 50 µm (100×), 30 µm (200×); (C) 100 µm.
Figure S5. **Generation efficiency of LB formation is influenced by UiPSC cell densities.** (A) Representative images of LBs when various numbers of differentiating UiPSCs colonies were seeded on the six-well plates. Scale bars: 1 cm; (B) Quantification data regarding LB generation efficiency, as analyzed on D25, when the number of seeded differentiating UiPSC colonies was 0~15 (white bar), 15~50 (grey bar), and 50~150 (black bar), respectively. Bars represent mean ± SEM (n=4). *, P<0.05.

Figure S6. **Analysis of the expression patterns of αA, αB, and β-crystallins in mature LBs on D25 by Western blot.** The expression patterns of αA, αB, and β-crystallins in mature LBs were similar to their expression patterns in human lens (HLs), which served as the positive control.

Figure S7. **Analysis of the expression of lens specific markers.** Each lens specific genes and a reference gene (GAPDH) at earlier vs late points was analyzed by real-time PCR and running gel.

Figure S8. **Analysis of the expression patterns of LC3B in mature LBs on D25 by Western blot.** The expression patterns of autophagy marker LC3B in mature LBs were analyzed. Human lens (HLs) served as a control.