Materials and methods

Reprogramming fibroblasts into iPSCs

Fibroblasts were infected by Sendai virus (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)). Transfected fibroblasts (approximately 1×10^5 cells per nucleofection) were directly plated onto 10 cm feeder-seeded dishes in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific), which contained 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). The culture medium was changed every other day. On day 12 post-transfection, the medium was replaced with hPSM (Ai et al., 2020). Colonies that morphologically resembled iPS colonies became gradually visible 15 days after infection. iPS cell lines were cultured on X-ray-inactivated CF-1 mouse embryonic fibroblasts (MEFs) in hPSM and denoted as P in this work.

hPSC medium was comprised of DMEM/F12 (Thermo Fisher Scientific) supplemented with15% KSR (Gibco, Life Technologies, Grand Island, NY, USA), 10 ng/mL bFGF (Peprotech, USA), 0.1 mM β-Me (Sigma Aldrich, St. Louis, MO, USA), non-essential amino acids (NEAA, Gibco), 20% PS-Gro Medium (Stem RD).

Derivation of ESCs/ntESCs/EPSCs from cynomolgus monkey blastocyst

Normal cynomolgus monkey blastocysts were obtained as previously described (Niu et al., 2014), and nuclear transferred blastocysts were obtained according to the literature (Liu et al., 2018). These monkey blastocysts (5–6 days post-fertilization) were transiently treated by 0.5% protease (Sigma) to remove the zona pellucida. Whole blastocyst was seeded on feeders in hPSM, 5iLA (Theunissen et al., 2014) or EPS medium (Yang et al., 2017) supplemented with 10 μmol/L Y27632 (StemCell). Medium was pre-warmed in a humidified incubator under 20% O_2 and 5% CO_2 at 37 ℃ for at least one hour before the embryos were seeded. 72 hours later, the embryo attached to the feeder layer and the medium was completely refreshed with 5 μmol/L Y27632. Henceforth, the medium was refreshed every 2 days until ESC-like outgrowth was visible. Generally, ESC-like outgrowth became evident on Day 7–14. After treating the embryo for about 10 min with 1 mg/mL Collagenase type IV (Gibco), the outgrowth was mechanically picked, dissociated into small clumps, and seeded onto fresh prepared feeders in corresponding medium with 5 μmol/L Y27632. After 3–5 days, the outgrowth displayed typical conventional ESC colony morphology and could be passaged and expanded with Collagenase type IV. ESC line derived from normal blastocysts in hPSM was denoted as E, ESC line derived from normal blastocysts in 5iLA was denoted as NE, EPSC line derived from normal blastocysts was denoted as EP, ntESC line derived from nuclear transferred blastocyst was denoted as NTE in this work.

Derivation of TSCs from cynomolgus EPSCs

Cynomolgus EPSCs were single-cell dissociated by TrypLE Express, and $0.5–1.0\times10^6$ cells were seeded onto a pre-coated 6-well plate and cultured in TSCs medium (Dong et al.). Cells were cultured in 5% CO_2 and 20% O_2, and passaged upon 80%–100% confluency at a ratio of 1:2 to 1:4 (volume ratio). Medium was changed every 2 days. Between passage 5 and 10, highly proliferative TSCs emerged. TSC line was denoted as EPT.

Derivation of cynomolgus naïve-like PSCs from iPSCs

Cynomolgus iPSCs were trypsinized and plated at 5×10^4 cells per well on MEF-coated 6-well plate in hPSM supplemented by 10 μmol/L Y27632. 24h later, medium was replaced with 5iLA supplemented with 8 ng/mL bFGF. After 10 days resetting, PSC colonies with dome-shaped morphology were readily isolated and expanded in 5iLA medium. The established line was denoted as PN in this work.

Derivation of cynomolgus EPSCs from iPSCs

Derivation of Cynomolgus EPSCs from iPSCs was performed as described in Yang, Y. et al (2017). Briefly, cynomolgus iPSCs were trypsinized and plated at 5×10^4 cells per well on MEF-coated 6-well plate in hPSM supplemented by 10 μmol/L Y27632. 12h later, medium was replaced with EPS medium. Medium was changed daily until dome-shaped colonies emerged. After 3–5 passages, cells could be readily expanded. The established line was denoted as PEP in this work.

Immunostaining

Cells were fixed in 4% paraformaldehyde (Dingguo, Beijing, China) at room temperature for 15 min, then blocked with phosphate buffered saline (PBS, Corning, Massachusetts, USA) containing 0.2% Triton X-100 (Sigma) and 3% BSA (Sigma) at room temperature for 45 min. The cells were then incubated with primary antibodies at 4 °C overnight, then with secondary antibodies at room temperature for 1 h. The nuclei were stained with DAPI (Roche, Basel, Switzerland). The following antibodies were used: anti-OCT4 (1:500, volume ratio, Abcam, Cambridge, UK), anti-Nanog (1:400, volume ratio, Abcam), anti-SOX2 (1:500, volume ratio, Abcam), anti-CK7 (1:300, volume ratio, Abcam), anti-CDX2 (1:500, volume ratio, Abcam) and anti-GATA3 (1:500, volume ratio, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Flow cytometry analysis

2×10^6 Cynomolgus iPSCs, H293T and MEFs were respectively singularized and resuspended. 5 μL PE anti-human CD298 antibody and PE anti-human β2-microglobulin antibody were added to each suspension, followed by 15–20 min incubation under room temperature in the dark. Subsequently, 2 mL cell staining buffer was added to each sample, and rinsing was conducted by centrifuging the samples at 350 *g* for 5 min. Repeat the rinsing step and resuspended the cells in 200 μL buffer for flow cytometry analysis.

Hashtag multiplexing sample preparation

Labeling buffer was constituted by adding 1% bovine serum albumin (BSA) to PBS buffer. Resuspension buffer was constituted by adding 0.04% (volume ratio) BSA to PBS buffer. Both labeling buffer and resuspension buffer were placed on ice or at 4 °C before further steps.

For each sample, antibody mix was constituted by adding the corresponding Hashtag antibody (Biolegend, TotalSeqTM-B Reagents) to 50 μL labeling buffer for a final concentration of 0.4 μg/μL. It was recommended to use 0.1–1 μg antibody per $2–10\times10^6$ cells. The antibody mix was centrifuged at 14000*g*, 4 °C for 10 min before use.

Cells were dissociated and singularized using accutase at 4 °C. For each sample, 5×10^6 cells were resuspended in 50 μL labeling buffer and incubated with 5 μL Human TruStain FcX (Biolegend, California,

USA) at 4 °C for 10 min. The suspension was then gently mixed with the corresponding antibody mix supernatant for 10 times and incubated at 4 °C for 30 min. After incubation, 1.4 mL labeling buffer was added before the suspension was centrifuged at 400g, 4 °C for 5 min. Further, the supernatant was removed, and the cells were rinsed with 1.5 mL labeling buffer twice. Finally, the samples were resuspend with resuspension buffer to an ultimate density of 1–2×10^6 cells/mL.

10x library establishment and sequencing

We obtained the single-cell suspension with qualified cell viability (>85%) evaluated by trypan blue staining and Countess II. All samples were mixed together at 5 times of loading amount and left on ice before use. The one-in-five volume of pooled single-cell suspension was loaded onto the 10x Genomics single-cell-B chip. The cDNA library was prepared according to the standard manufacturer's protocol from 10x Genomics Single Cell 3' v3 Reagent Kits with Feature Barcoding technology for Cell Surface Protein, and then sequenced on an Illumina Novaseq PE150 System.

Sequencing data pre-processing

Data obtained from Illumina Novaseq PE150 was divided into 4 groups based on different indexes in DNA library, denoted as Read1, Read2, GEL and CSP. GEL was the 3'-gene expression library, and CSP was the membrane surface marker library. The 28bp front end of Read1 contained information of cell barcode and UMI, based on which Read2 was demultiplexed. Quality control of raw data was evaluated by FastQC. Clean data was filtered using Trimmomatic by the following criteria: the beginning and end bases that scored under 3 in a read were remove; every 4 bases were grouped, and the groups that had an average score under 15 were removed. The clean data was again evaluated by FastQC.

10x Genomics data processing

10x Genomics data were normalized and analyzed by Cell Ranger. We mapped the collected reads to cynomolgus reference genome (*Macaca fascicularis*) and corrected the transcripts using GTF annotation file. Cell counts, read counts and gene counts of the clean data were collected and demultiplexed according to barcode by Cell Ranger.

After normalization, the top 2000 genes with greatest variable coefficient were identified by FindVariableFeatures function, from which top 20 DEGs were selected for clustering analysis by FindClusters function. Results were plotted using UMAP method.

FindMarkers function of the R Seurat package was used to screen for the upregulated DEGs ($P<0.05$) and Gene Ontology (GO) analysis was performed by the clusterProfiler package.

In vivo monkey embryo RNA-seq data

The RNA-seq data from 29 embryo samples was downloaded from Liu, D. et al. (Genome Res. 28, 1481–1493, 2018). DEGs from 4 different blastocyst stages (EB, MB, LB, and HB) were calculated using FindAllMarkers function,and intersectioned with the DEGs from our samples. Similarities between our ESC component of samples and EB, MB, LB, and HB were ranked using AddModuleScore.

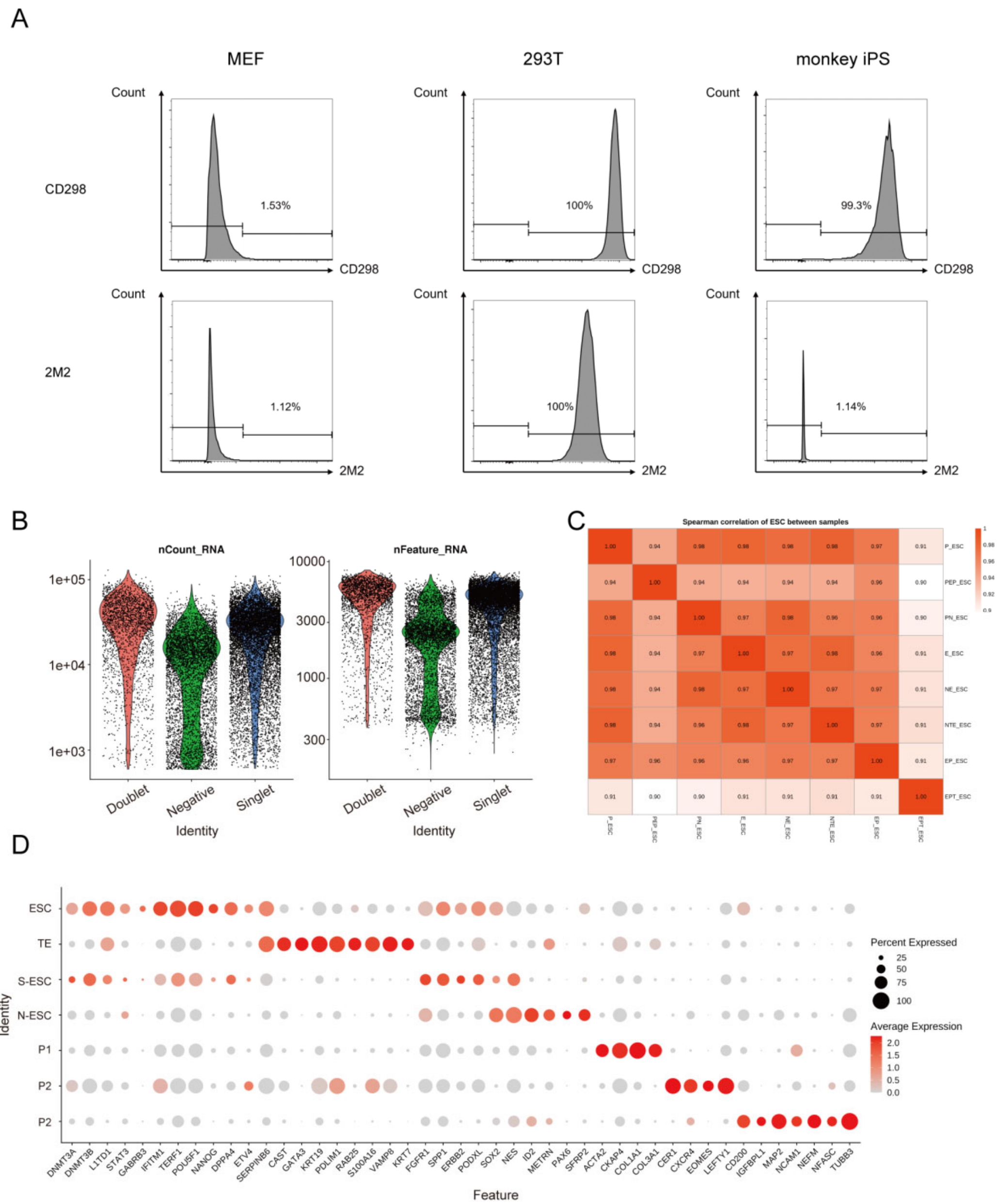

Fig. S1 Sample multiplexing of various monkey pluripotent stem cell lines based on 10× genomics. (a) Antibody targeting CD298 exclusively recognize primate cells but not mouse embryonic fibroblasts (MEF). Antibody targeting 2M2 specifically binds to human cells and not monkey cells. Collectively, data indicated potential application of Hashtag for monkey stem cells; (b) Violin plot of RNA counts and gene counts of the barcoded cells, of which nCount RNA indicates the number of RNA counts and nFeature RNA indicates the number of gene counts; (c) Spearman correlation of ESCs between samples; (d) Dot plot displaying differentially expressed genes to annotate subpopulations in each sample.

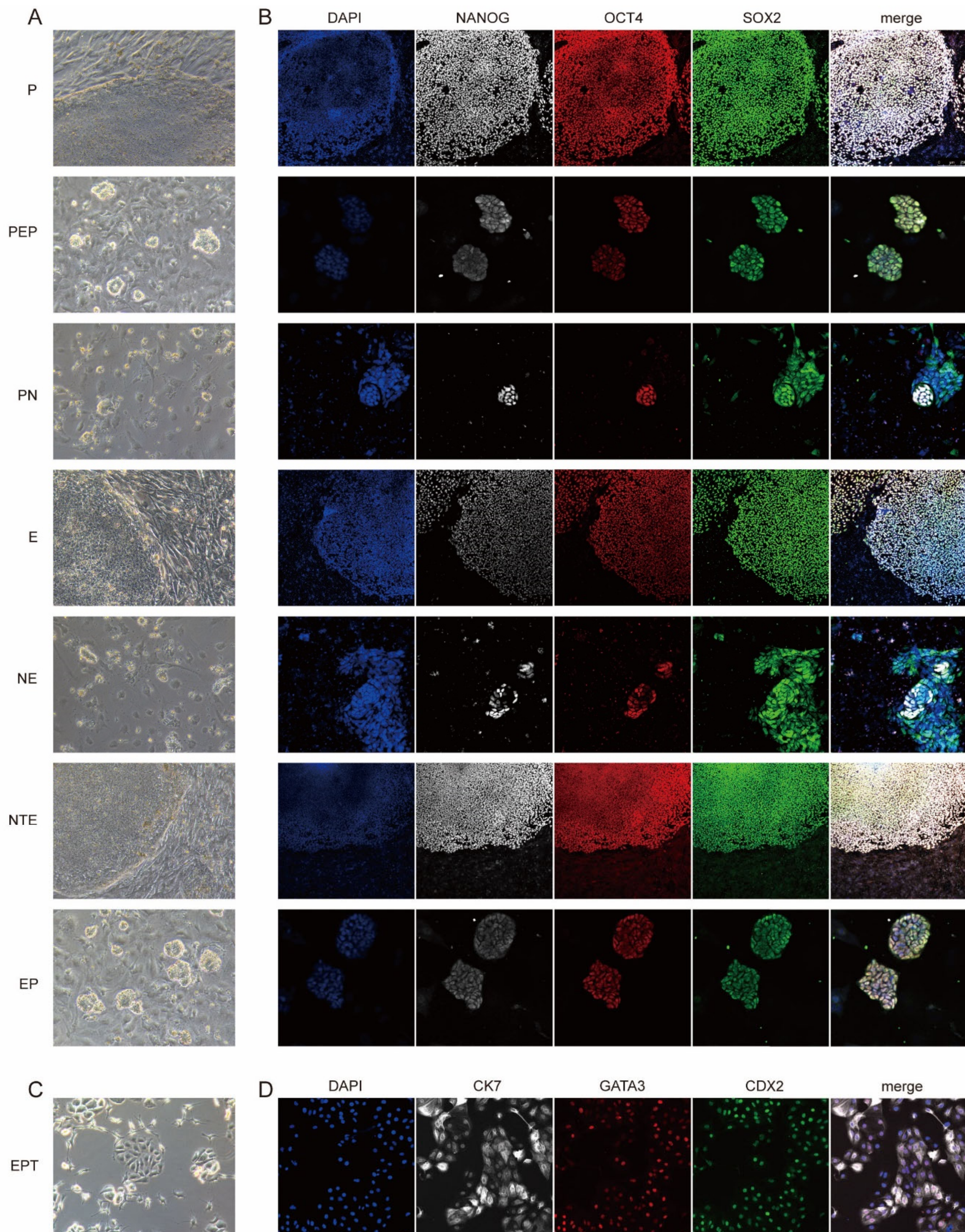

Fig. S2 Representative images of eight monkey pluripotent stem cell lines. Bright field images of monkey pluripotent stem cells maintained in different stem cell (a) or trophoblast (c) culture conditions, and immunostaining of gene markers indicating cell identity (b and d). P denotes monkey iPSCs cultured in conventional human stem cell medium; PEP denotes monkey iPSCs cultured in

extended pluripotent stem medium; PN denotes monkey iPSCs cultured in human naive medium; E denotes monkey ESCs derived from blastocyst in conventional human stem cell medium; NE denotes monkey ESCs derived from blastocyst in human naïve medium; NTE denotes nucleus transferred monkey PSCs cultured in conventional human stem cell medium; EP denotes monkey ESCs derived from blastocyst in extended pluripotent stem medium; EPT denotes monkey cells derived from blastocyst in trophoblast stem cell medium.

Reference

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