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Supplemental Information

Generating functional cells through enhanced interspecies chimerism with human pluripotent stem cells

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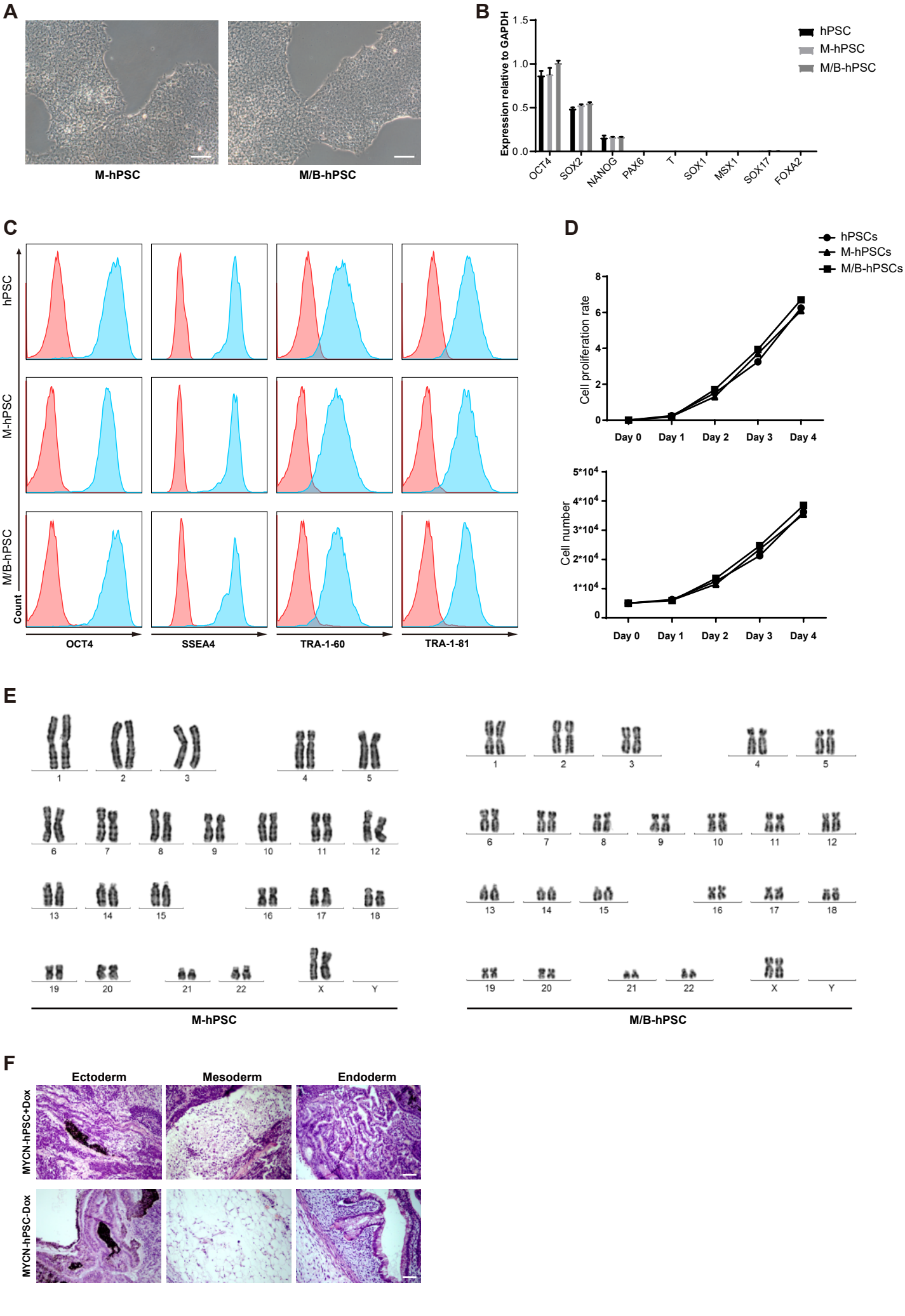
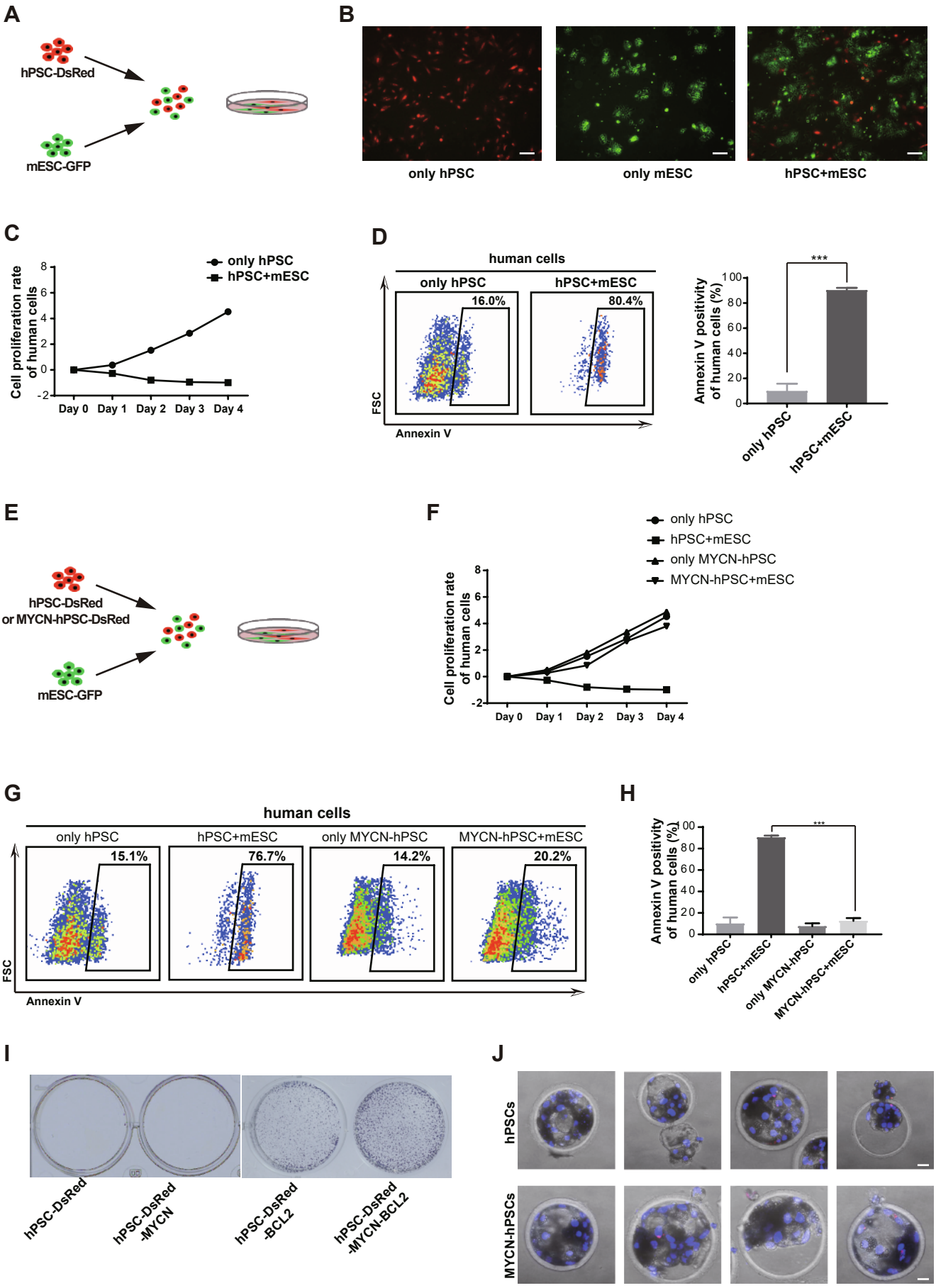
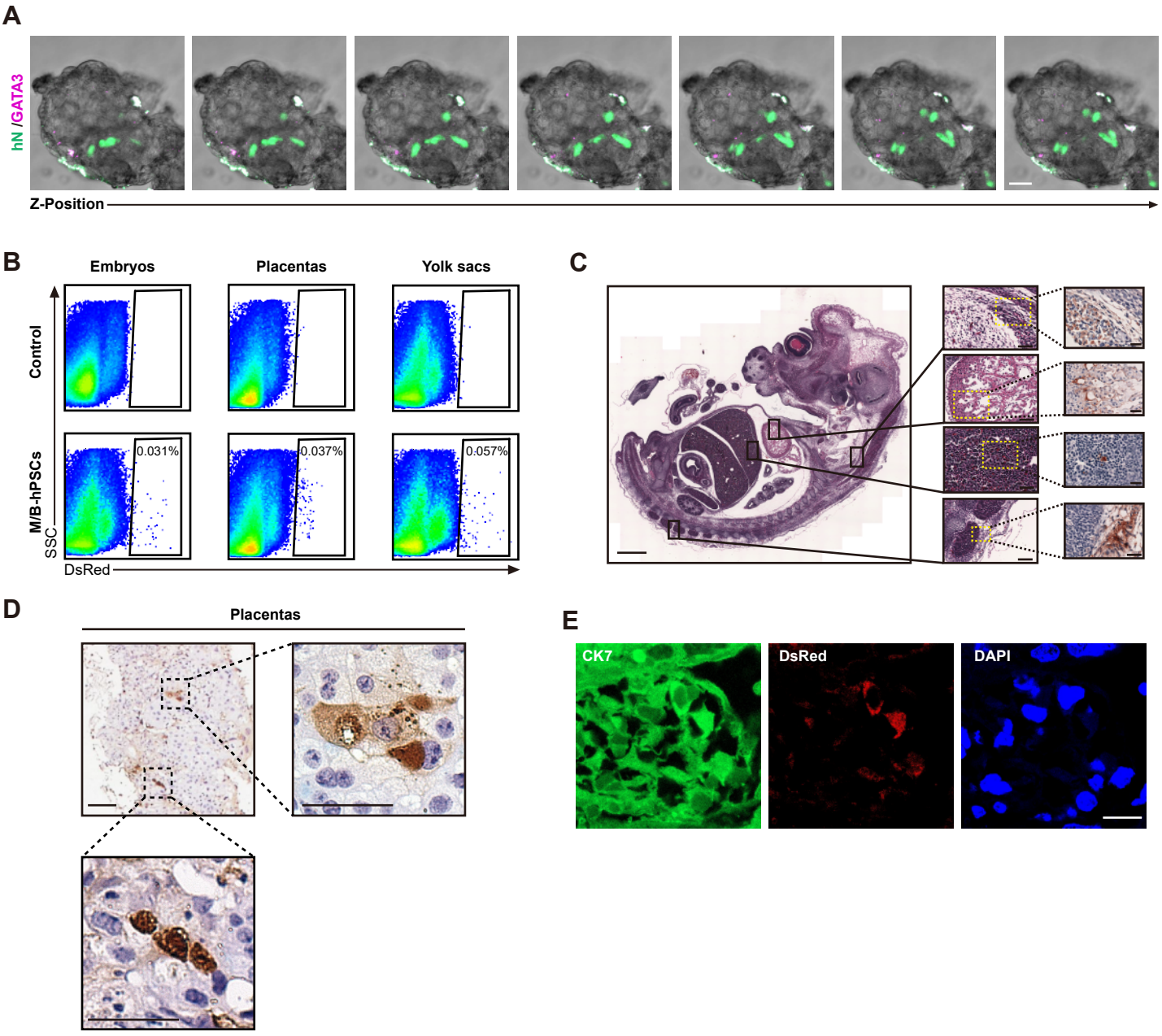
Supplemental figure 1



Supplemental figure 2



Supplemental figure 3



Supplemental figure 4

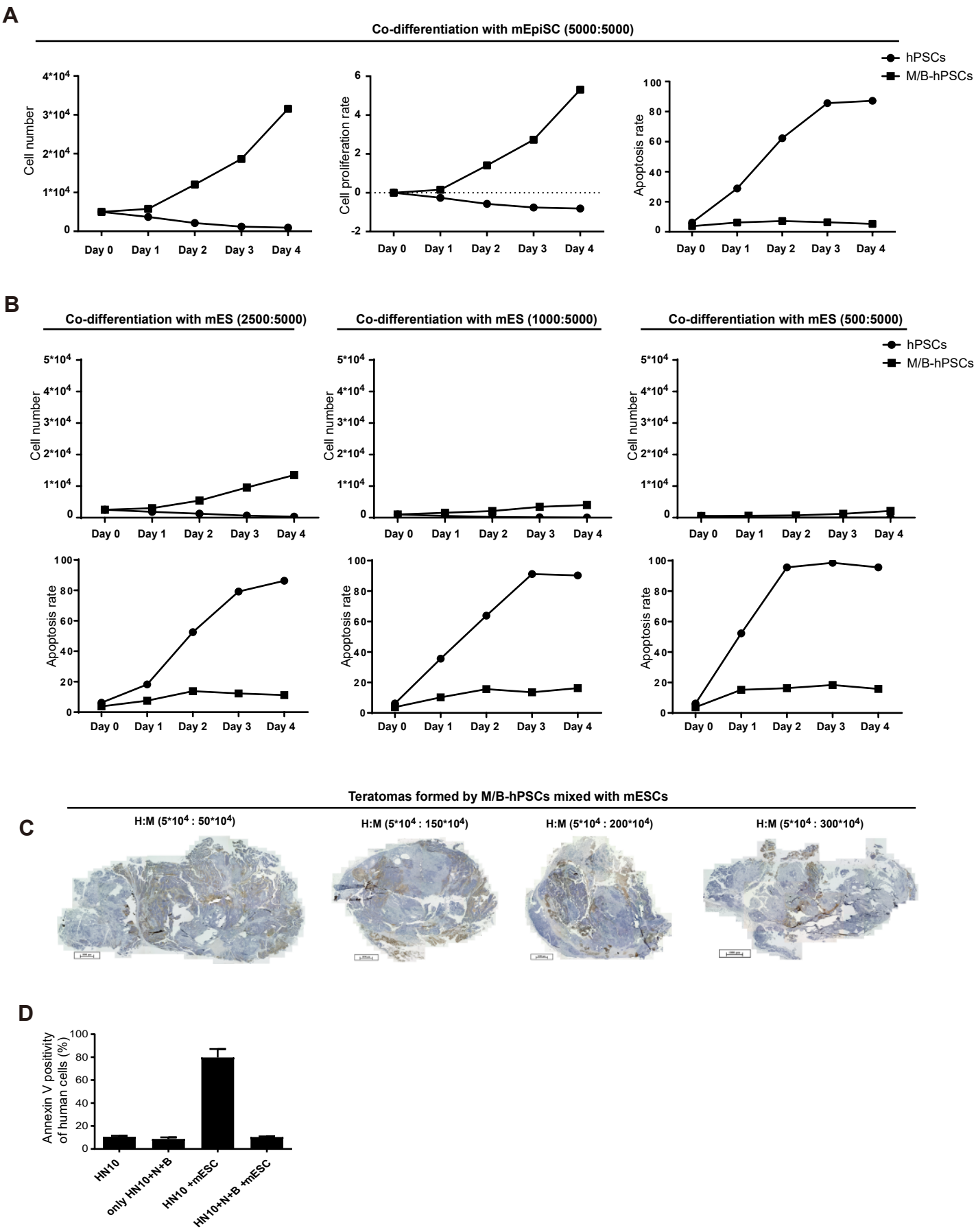


Figure legends

Supplemental figure 1

(A) The morphology of MYCN or MYCN/BCL2 expressed hPSCs. Scale bars, 100 μm. **(B)** RT-qPCR analysis of pluripotency markers and other lineage genes in MYCN or MYCN/BCL2 expressed hPSCs. **(C)** Flow cytometry analysis of the pluripotency markers in MYCN-hPSCs (M-hPSCs) and MYCN/BCL2-hPSCs (M/B-hPSCs) . **(D)** The cell growth rate of hPSCs, M-hPSCs and M/B-hPSCs under normal maintenance culture.**(E)** Karyotype analysis of M-hPSCs and M/B-hPSCs. **(F)** Hematoxylin and eosin (H&E) staining of the teratoma formed by M-hPSCs in the presence or absence of Dox (2mg/ml). Scale bars, 100 μm.

Supplemental figure 2

(A) Schematic overview of co-differentiation of DsRed labeled hPSCs with mESCs *in vitro*. hPSCs-DsRed and mESCs-GFP are initially mixed in 1:1 ratio at single cell for further differentiation. **(B-C)** Analysis of survival of hPSCs-DsRed or mESCs-GFP in co-differentiation in vitro. Dissociated hPSCs-DsRed or mESCs-GFP cells are cultured in differentiation condition either alone or mixed with each other in 1:1 ratio for 4 days. Scale bars, 50 μm. **(D)** Flow cytometric analysis of Annexin V^+ human cells from hPSC+mESC co-differentiation condition for 4 days. Error bars represent mean+SEM of three independent replicates. ***$p < 0.001$. **(E)** Schematic overview of co-differentiation of DsRed labeled hPSCs or MYCN-hPSCs with mESCs *in vitro*. hPSCs-DsRed and mESCs-GFP are initially mixed in 1:1 ratio at single cell for further differentiation. **(F-H)** Analysis of human cell proliferation rate and Annexin V^+ cells on the mixture of hPSCs-DsRed or MYCN-hPSCs-DsRed and mESCs cultured in differentiation condition for 4 days. Error bars represent mean+SEM of three independent replicates. ***$p < 0.001$. **(I)** Cloning efficiency is analyzed by alkaline phosphatase staining on colonies formed by the individualized cell of the indicated cell lines. 2000

individualized were plated in each well of 12-well-plate and cultured for 7 days. **(J)** Analysis the survival of Dsred labeled hPSCs in pre-implantation pig embryos. $DsRed^+$ hPSCs (3-5 cells for pig) were injected into 8-cell (8C) stage embryos and cultured for 3 days. Scale bars, 20 μm.

Supplemental figure 3

(A) Z-Stack confocal microscope images showing the integrated of M/B-hPSCs co-staining with hN (human cell nucleus specific antibody) and GATA3 (early extra-embryonic lineages marker) in cultured chimeric embryos. scale bars, 20μm. **(B)** Analyses of $DsRed^+$ human cells in E10.5 mouse chimeras by flow cytometry. $DsRed^+$ human cells were detected in embryonic part, placentas and yolk sacs. **(C)** Analyses of human cells at different embryonic regions in E10.5 mouse chimera by HE staining and Immunohistochemical staining using human specific marker Stem121. Scale bars, 500 μm (D-left), 20 μm (D-middle), 5 μm (D-right). **(D)** Analyses of human cells in placenta by Immunohistochemical staining using human specific marker Stem121. Scale bars, 100 μm. **(E)** Representative placenta confocal images showing $DsRed^+$ human cells can contribute to trophoblastic lineages in chimeric E10.5 placentas. The placentas were stained with anti-CK7 (trophoblastic lineages marker). Scale bars, 20 μm.

Supplemental figure 4

(A) Analysis the cell growth rate and apoptosis rate of hPSCs or M/B-hPSCs during co-differented with primed mPSCs (mEpiSCs) in 1:1 ratio for 4 days. **(B)** Analysis the cell counts and apoptosis rate of hPSCs or M/B-hPSCs during co-differented with mESCs in different ratio. **(C)** Teratomas formed by M/B-hPSCs mixed with mESCs in different ratio. **(D)** Co-differentaiton assay mixed another M/B-hPSCs cell line (HN10+N+B) with mESCs in 1:1 ratio for 4 days.

Supplemental Table 1 primers used in qRT-PCR

Q-H-ß- actin-F	CT CCAT CCT GGCCT CGCT GT
Q-H-ß- actin -R	GCT GT CACCTT CACCGTT CC
Q-M-ß- actin -F	CGTT GACAT CCGT AAAGACC
Q-M-ß- actin -R	AACAGT CCGCCT AGAAGCAC
Q-H-MYCN-F	ACCCGGACGAAGAT GACTT CT
Q-H-MYCN-R	CAGCT CGTT CT CAAGCAGCAT
Q-M-MYCN-F	CCT CACT CCT AAT CCGGT CAT
Q-M-MYCN-R	GTGCTGT AGTTTTT CGTT CACTG
Q-H-OCT4-F	CCT CACTT CACT GCACT GT A
Q-H-OCT4-R	CAGGTTTT CTTT CCCT AGCT
Q-H-SOX2-F	CCCAGCAGACTT CACAT GT
Q-H-SOX2-R	CCT CCCATTT CCCT CGTTTT
Q-H-NANOG-F	T GAACCT CAGCT ACAAACAG
Q-H-NANOG-R	T GGT GGT AGGAAGAGT AAAG
Q-H-PAX6-F	AT GT GT GAGT AAAATT CT GGGCA
Q-H-PAX6-R	GCTT ACAACTT CT GGAGT CGCT A
Q-H-T-F	T AT GAGCCT CGAAT CCACAT AGT
Q-H-T-R	CCT CGTT CT GAT AAGCAGT CAC
Q-H-MSX1-F	T CCGCAAACACAAGACGA
Q-H-MSX1-R	ACT GCTT CT GGCGGAACTT
Q-H-SOX17-F	CGCACGGAATTT GAACAGT A
Q-H-SOX17-R	GGAT CAGGGACCT GT CACAC
Q-H-FOXA2-F	ACT ACCCCGGCT ACGGTT C
Q-H-FOXA2-R	AGGCCCGTTTT GTT CGT GA
Q-H-hmtDNA-F	AAT ATT AAACACAAACT ACCACCT ACCT
Q-H-hmtDNA-R	T GGTT CT CAGGGTTT GTT AT AA
Q-H-UCNE-F	AACAAT GGGTT CAGCT GCTT
Q-H-UCNE-R	CCCAGGCGT ATTTTT GTT CT
Q-H-MYC -F	GGCTCCTGGCAAAAGGTCA
Q-H-MYC -R	CTGCGTAGTTGTGCTGATGT

Supplemental Table 2 Antibodies

Antibodies	**Vendor**	**Cat#**	**Dilution**
Anti-human CD34- PerCP-Cy5.5	BD Biosciences	347203	1:100
Anti-human OCT4	BD biosciences	560307	1:100
Anti-human SSEA4	Invitrogen	414000	1:100
Anti-human TRA-1-60	Santa Cruz Biotechnology	sc-21705	1:100
Anti-human TRA-1-85-APC	RD	FAB3195A	1:100
Anti-human TRA-1-81	Santa Cruz Biotechnology	sc-21706	1:100
Anti-human CDX2	Thermo	MA5-14494	1:100
Anti-human stem121	TAKARA	Y40410	1:100
Anti-human CD45	BD Biosciences	560973	1:100
DAPI	Thermo	62248	1:5000
Anti-human GATA3	CST	5852s	1:200
Anti-Human CK7	Zsbio	ZM-0071	1:100

Supplemental Experimental Procedures

Animal experiments

ICR mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. *Flk-1*$^{+/EGFP}$ mice were purchased from Jackson Lab (Bar Harbor, ME, USA). Female mouse at 4–6 weeks of age were selected as donors or surrogate mother. Two days before mating the donors were injected with 7.5 U pregnant mare's serum gonadotropin (PMSG), and 48 h later the donors were injected with 7.5 U hCG and mated with the male mouse. The embryos at 8 cell stage or later morula to early blastocyst stage were obtained, respectively. Ten cells were injected into the embryos for following experiments. For in vitro chimerism assay, the embryo culture medium—KSOM (Millipore) was added with dox (2 μg per mL) for inducible MYCN or MYCN- BCl2 expression. The injected embryos were cultured in medium until blastocyst stage for further analysis. For in vivo chimera assay, the 10–20 embryos were transplanted into the uterus of per pseudopregnant mouse and the surrogate mice with embryos injected hPSCs were fed with the dox-containing water (2 mg per mL) until E10.5. The mice were euthanized, and embryos, vitelline membrane and placenta were obtained for subsequent analysis.

New Zealand rabbits were obtained from Huadong Xinhua experimental animal farms, District of Guangzhou. Female rabbits were injected with 100 IU PMSG, and 72-120 hours later further injected with 100 IU hCG and mated with the male rabbits. Embryos at 8 cell stage were harvested and injected with 7 indicated cells for in vitro assay, and cultured in EBSS (Thermo) Medium until blastocyst stage.

Pigs were purchased from a local slaughterhouse and cumulus oocyte complexes (COCs) were aspirated from antral follicles. After 44 hours in vitro maturation, the oocytes were activated with 2 successive DC pulses of 120 V per mm for 30 μseconds using an electrofusion instrument (CF-150B, BLS, Hungary). The activated oocytes were cultured in PZM-3 medium for partheno-development. After 48 hours or 4 days of culture, embryos at 8 cell stage were selected respectively for microinjection. 3-5 cells were injected to 8 cell stage embryos. The injected embryos were cultured in PZM3 medium (ENZO) until blastocyst stage for further analysis.

The interspecies chimerism experiments using hPSCs have been approved by the Ethnical Committee on Animal Experiments at Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. Also, all the experiments were performed according to ISSCR guidelines.

Single-cell cloning efficiency

For single-cell cloning assay of primed hPSCs, cells were plated onto 12-well plate at a density of 2000 cells per well, after dissociating into single cells with Accutase and centrifuged at 300 × g at room temperature for 3 min. Seven days later, cells were fixed in 4% PFA for 2 min. After washing with PBS, cells were stained with alkaline phosphatase staining solution (Beyotime) for 10−15 min.

Immunofluorescence

The injected blastocyst embryos and frozen sections of E10.5 mouse chimeric embryos and placenta were incubated with antibodies (Stem121, CDX2, and OCT4) overnight at 4 °C, washed with PBS and incubated with specific secondary antibody. Nuclei were stained with DAPI. Stained embryos and sections were observed using a LSM800 confocal microscope (Carl Zeiss).

Flow cytometry and cell sorting

The embryonic tissue were digested with collagenase and washed with PBS, and directly detected by flow cytometry. We randomly selected and pooled 10 mouse embryos together to sort the cells. The embryonic tissues were digested with collagenase and washed with PBS, centrifuged to remove the supernatant, then washed with PBS and stained with human cells specific antibody TRA-1-85 and human CD34 antibody (direct-labeled antibody) at 4°C for 30 minutes, finally, the live TRA-1-85^{+}DsRed^{+} cells were obtained by flow cytometry sorting.

Western blot assays

Cells were lysed on ice with 200 μL of RIPA buffer (Beyotime) for 15 min and separated by

12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked in 5% nonfat milk for 1 h and incubated overnight at 4 ℃ with the appropriate diluted primary antibodies or anti-flag/GAPDH antibody. Subsequently, the membranes were incubated with HRP-conjugated secondary antibody for 2 h at room temperature and HRP was detected by ECL (Advanste) and visualized by SmatChemi Image Analysis System (SAGECREATION).

Quantitative real-time PCR

Total RNA was extracted with the RaPure Total RNA Micro Kit (Magen). Two-microgram RNA was reversing transcribed into cDNA and amplified with SYBR Green PCR Master Mix (Bio-Rad).

Teratoma formation

The teratoma formation experiments were approved by the Ethical Committee on Animal Experiments at Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. $1x10^6$-$6x10^6$ Cells were digested by Accuatse (Sigma) for 5 min at 37 °C and resuspended in 30% matrigel (Corning) in DMEM/F12 (Hyclone), and then injected subcutaneously into NOD/SCID immunodeficient mice, obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Teratomas were detected after 4 weeks and fixed in 4% PFA. After paraffin embedding and sectioning, sections were stained with hematoxylin/eosin.

Histopathological observation

After formalin fixation, embryo samples were embedded in paraffin, sectioned at 3-mm thickness, and stained with hematoxylin and eosin (H&E) for histopathological examination.

Immunohistochemistry

Embryo tissue samples were fixed in 4% formalin, embedded in paraffin and sectioned at

3μm thickness. Embryo tissue samples were fixed in 4% formalin, embedded in paraffin and sectioned at 3μm thickness. Lung sections were treated with xylene to remove the paraffin and then were rehydrated. Prior to staining heat-induced antigen retrieval was performed by placing the slides into 0.01M citrate buffer solution (pH6.0), and subjected to microwave heating three times for 5min. Then the sections were incubated with 3% H_2O_2 for 10min at room temperature and washed three times with PBS, followed by incubation with serum for 30min. Samples were incubated with the antibodies (Stem121) overnight at 4°C. After washing with PBS, the slices were incubated with secondary antibodies for 0.5h at 37°C. After staining with 3, 3'-diaminobenzidine (DAB), the sections were observed under optical microscope.