Current Biology, Volume *21*

Supplemental Information

Blimp1 Expression Predicts Embryonic Stem Cell Development In Vitro

Li-Fang Chu, M. Azim Surani, Rudolf Jaenisch, Thomas P. Zwaka

Supplemental Inventory

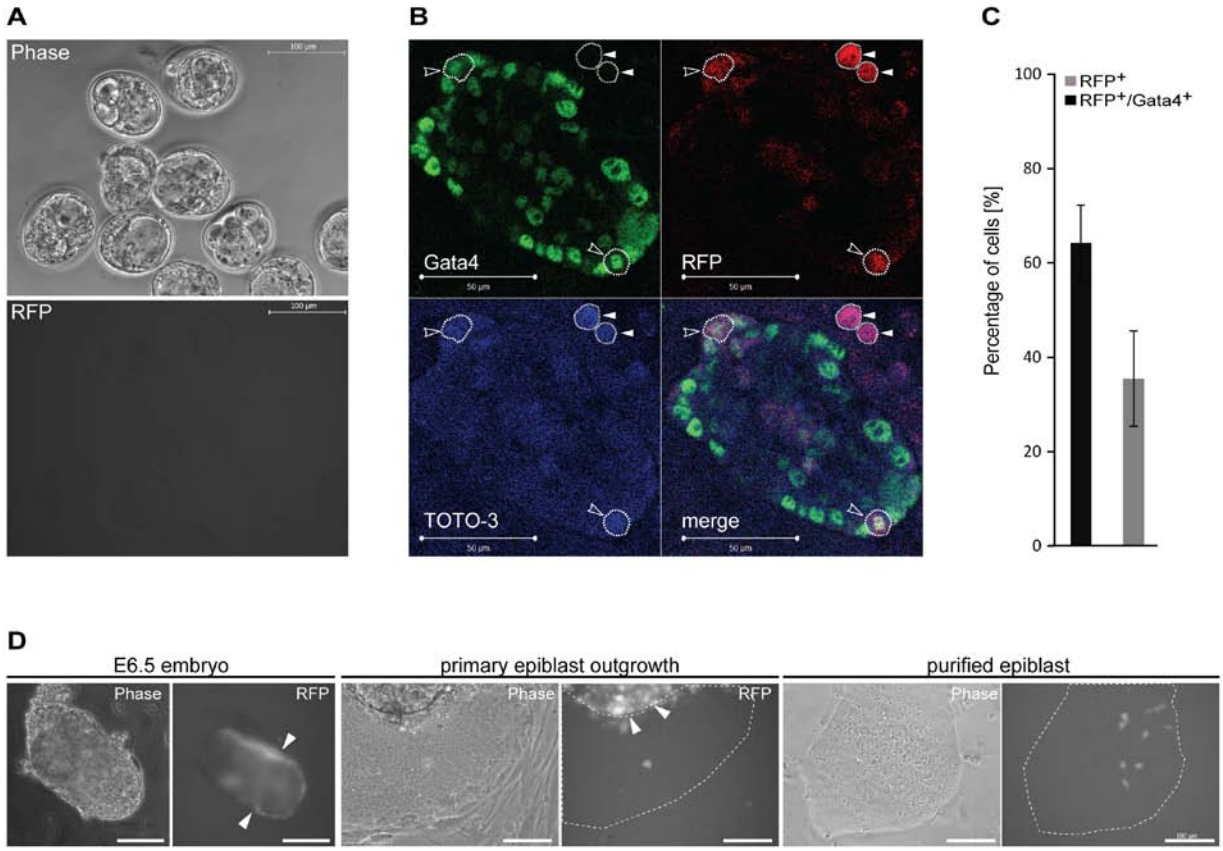

Figure S1. Characterization of *Blimp1-Cre* Expression in the Blastocyst, ICM Outgrowth and Epiblast, Related to Figure 1

(A) Representative images depicting *Blimp1* expression in preimplantation embryos. E3.5 embryos were obtained from the cross of *Blimp1-Cre* X *Rosa26-RFP* lines (examined a total of 171 embryos from five experiments). Scale bars = 100 μm.

(B) Representative images of regions within day-4 ICM outgrowth after immunostaining with anti-RFP and anti-Gata4 antibodies are shown. Solid arrowheads indicate $Gata4^{-}/RFP^{+}$ cells; open arrowheads indicate $RFP^{+}/Gata4^{+}$ cells. Scale bars = 50 μm.

(C) Quantification of $Gata4^{-}/RFP^{+}$ and $Gata4^{+}/RFP^{+}$ cells within day-4 ICM outgrowths. The data represent mean ± SEM. (n=5 per group).

(D) Analysis of *Blimp1-Cre* expression in E6.5 embryo, epiblast outgrowth and purified epiblast. Epiblast fragments from E6.5 *Blimp1-Cre; Rosa26-RFP* embryos were cultured for 3 to 5 days. Arrowheads indicate RFP^{+} VE cells within the embryos. Dashes outline areas of epiblast outgrowth or purified epiblast. Scale bars = 100 μm.

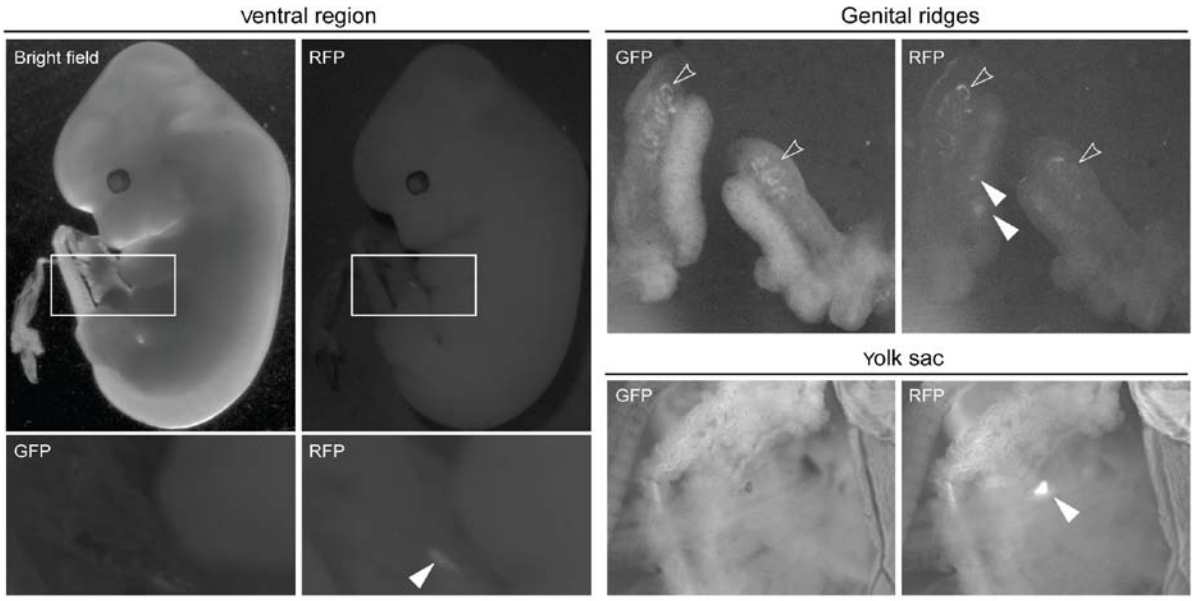

Figure S2. Analysis of E12.5 Host Chimeras after *in utero* Injection into E8.5 Embryos, Related to Figure 2

RFP^+ donor cells can be seen in the recovered embryos, genital ridges or yolk sac. Bottom panels (left) show higher magnification of the boxed areas. All solid arrowheads indicate RFP^+ cells. The GFP channel was used to estimate the autofluorescence level (open arrowheads).

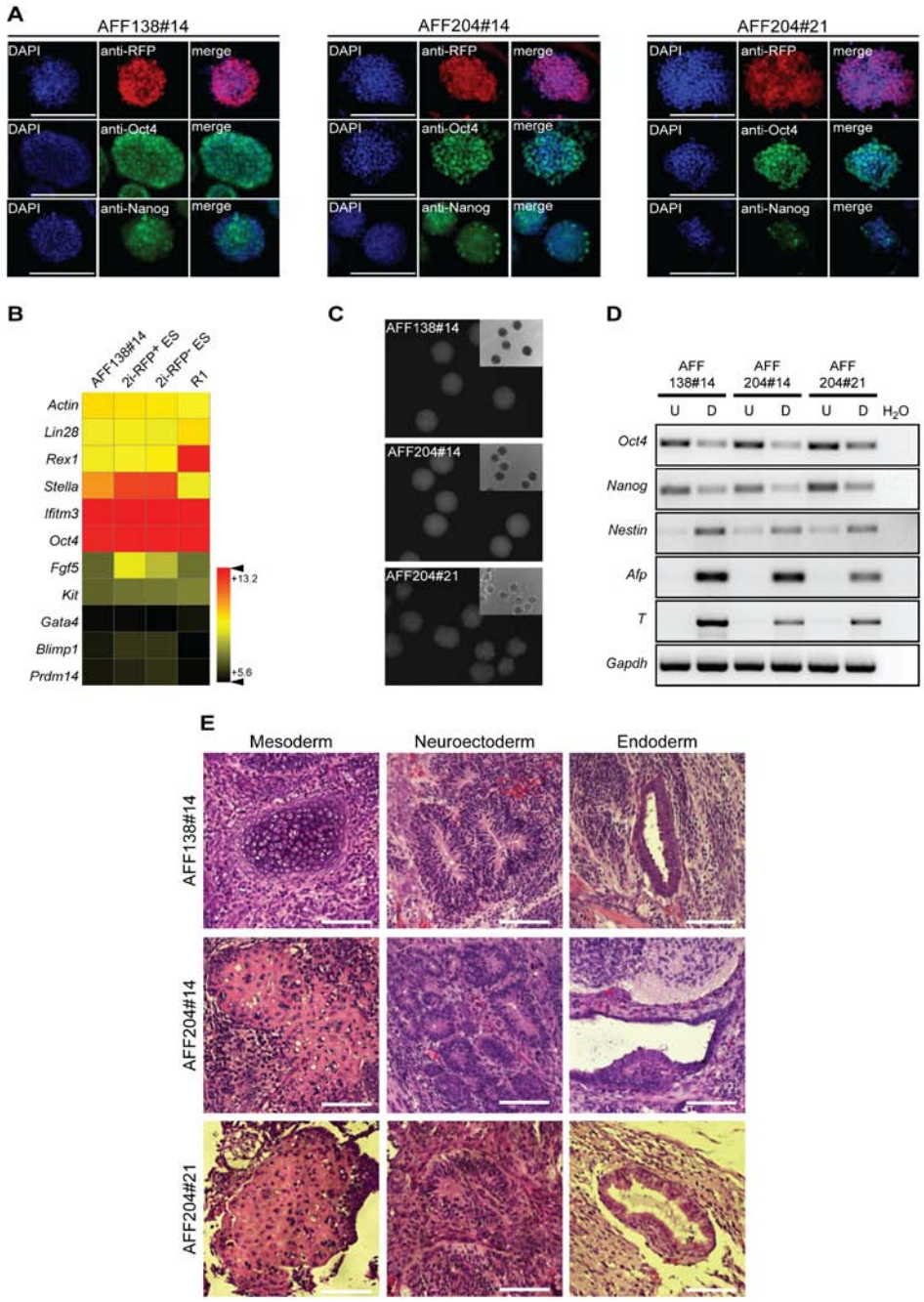

Figure S3. Characterization of Established *Blimp1-Cre; Rosa26-RFP* ES Cell Lines, Related to Figure 3

(A) Immunostaining for Oct4 and Nanog expression in three independent ES cell lines generated from a cross of *Blimp1-Cre* and *Rosa26-RFP* reporter mice.

(B) Gene expression profiling with selected markers in four established ES cell lines by microarray analysis.

(C) Embryoid body (EB) formation (RFP^+). Inserts show the corresponding phase image.

(D) RT-PCR analysis of EBs for markers representing the three germ layers. U, undifferentiated. D, differentiated.

(E) Histological analysis of teratoma formed after injection of the three ES cell lines into SCID mice. Scale bars = 100 µm.

A

ES cell lines	Genotype
Batch#1-1	WT
Batch#1-2	**KO**
Batch#1-3	Het
Batch#1-4	Het
Batch#1-5	WT
Batch#1-6	Het
Batch#2-1	Het
Batch#2-2	**KO**
Batch#2-4	Het
Batch#2-5	WT
Batch#2-7	**KO**
Batch#2-8	Het
Batch#2-9	WT
Batch#2-10	Het
Batch#2-11	WT

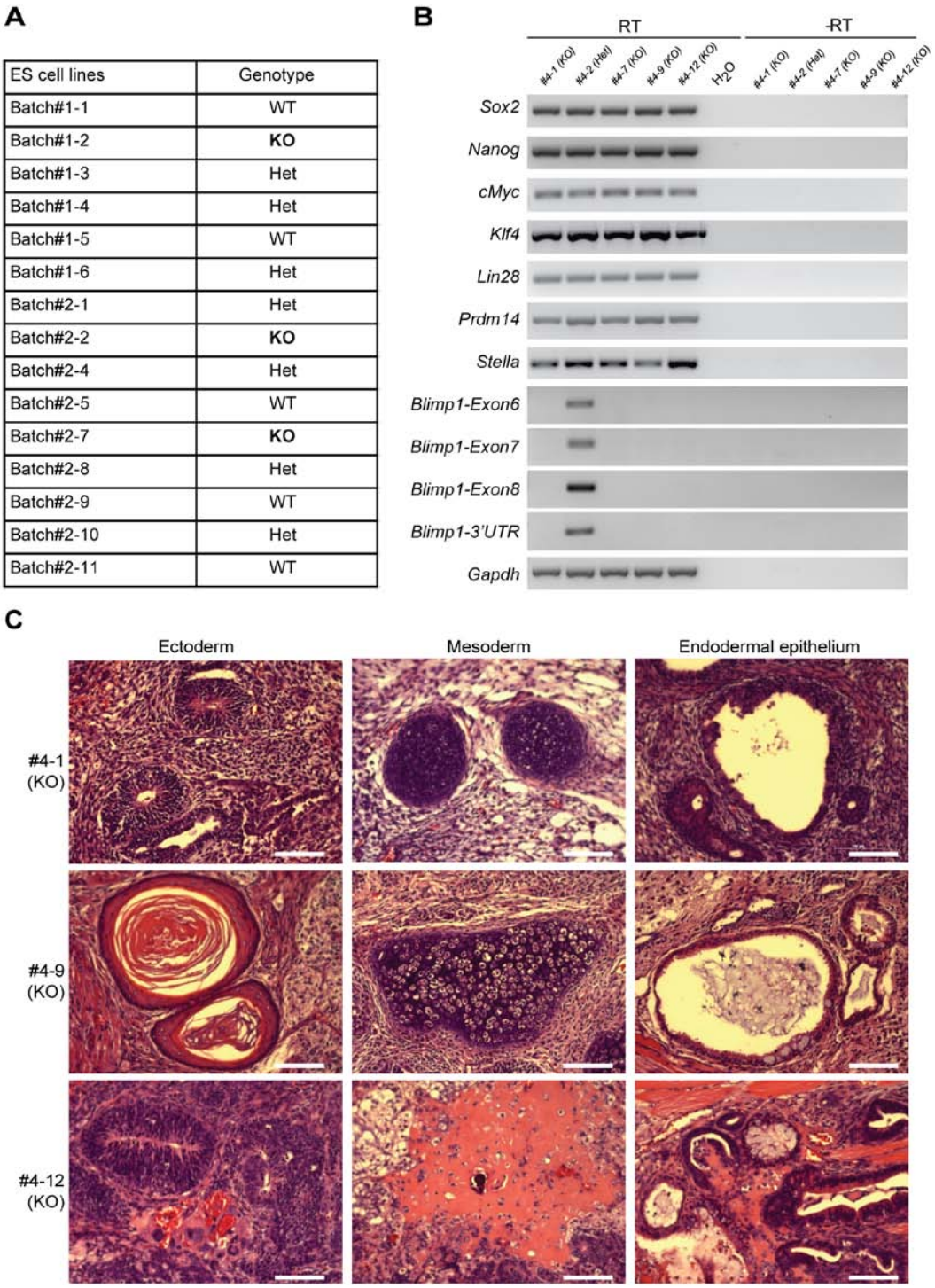

Figure S4. Characterization of Established *Blimp1-KO* ES Cell Lines, Related to Figure 3

(A) A total of three *Blimp1*$^{-/-}$*(KO)* ES cell lines established from derivation experiments. *Blimp1-KO* genotype is shown in bold.

(B) RT-PCR analysis of marker gene expression in established *Blimp1*$^{-/-}$ (KO) and *Blimp1*$^{+/-}$ (Het) ES cell lines.

(C) Histological analysis of teratoma formed after injection of three *Blimp1*$^{-/-}$*(KO)* ES cell lines into SCID mice. Scale bars = 100 μm

Table S1. Summary of *in utero* injection results (Related to Figure 2)

Type of donor cells	No. of recipient embryos injected	No. of embryos recovered on E12.5 (survival)	No. of embryos with chimeric gonads
Whole day-4 ICM outgrowth	74 (2 experiments)	40 (54%)	0 (0%)
Posterior region of E8.5 embryo	99 (2 experiments)	34 (34%)	1 (2%)
RFP^+ day-4 ICM outgrowth	467 (10 experiments)	207 (44%)	4 (2%)
RFP^+ Z/Red ES cells	217 (5 experiments)	74 (34%)	0 (0%)

Table S2. List of oilgos used in this study

Oligos for PCR genotyping

Gene	Oligo Name	Orientation	Oligo Sequences(5'-3')	Tm	Cycle#	Product size (bp)
aMHC-Cre	LFC092_aMHC-Cre5 primer	Forward	ATGACAGACAGATCCCTCCTATCTCC	60	35	350
	LFC093_Cre-3 preimer	Reverse	CTCATCACTCGTTGCATCGAC			
Blimp1-Cre	LFC044_Cre-R199	Reverse	CTGCGGGAAACCATTTCCGGTTATTC	55	35	Blimp1-Cre: 213,WT: 420
	LFC097_Blimp1+1356F	Forward	GCCCACAACACATTTCTGCCTTGA			
	LFC098_Blimp1+1775R	Reverse	TTCAAACTCGGCCTCTGTCCACAA			
Meox2-Cre	LFC080_MoreFor	Forward	GGGACCACCTTCTTTTGGCTTC	55	35	Meox2-Cre: 300, WT: 410
	LFC086_oIMR1871	Reverse	AAGATGTGGAGAGTTCGGGGTAG			
	LFC087_oIMR3671	Reverse	CCAGATCCTCCTCAGAAATCAGC			
Rosa26-tdRFP	LFC076_HL15	Forward	AAGACCGCGAAGAGTTTGTCC	62	35	tdRFP: 301, WT: 209
	LFC077_HL54	Reverse	TAAGCCTGCCCAGAAGACTCC			
	LFC078_HL152	Reverse	AAGGGAGCTGCAGTGGAGTA			
Activated-tdRFP	LFC094_AC-RFP-585F	Forward	AAGCACGTTTCCGACTTGAGTTGC	55	35	actived-RFP: 637
	LFC095_AC-RFP+052R	Reverse	GCACCTTGAAGCGCATGAACTCTT			
Sry-PCR	LFC172_Sry-F	Forward	TGACTGGGATGCAGTAGTTC	53	35	Rapsn: ~590bp Sry: ~270bp
	LFC173_Sry-R	Reverse	TGTGCTAGAGAGAAACCCTG			
	LFC174_Rapsn-F	Forward	AGGACTGGGTGGCTTCCAACTCCCAGACAC			
	LFC175_Rapsn-R	Reverse	AGCTTCTCATTGCTGCGCGCCAGGTTCAGG			
Prdm1-loxp/loxp	LFC227_oIMR7884	Forward	CAATGCTTGTCTAGTGTC	55	35	WT:200; flox:270
	LFC228_oIMR7885	Reverse	AGTAGTTGA ATGGGAGC			
Prdm1-loxp-KO	LFC257-Prdm1-E6-55F	Forward	TTCACTTCAGTTCTCCACAGCCCT	55	35	309bp
	LFC258-Prdm1-E6+254R	Reverse	TGGTCTTGGAAACTAAGCAGCGTC			

Oligos for RT-PCR

Gene	Oligo Name	Orientation	Oligo Sequences(5'-3')	Position	Tm	Product size (bp)
Afp	MD257_Afp-F	Forward	TCGTATTCCAACAGGAGG	across exon 12-13	55	174
	MD258_Afp-R	Reverse	AGGCTTTTGCTTCACCAG			
Blimp1(Prdm1)	LFC009_Blimp1+1965F	Forward	ACACACAGGAGAGAAGCCACATGA	across exon 7-8	55	219
	LFC010_Blimp1+2183R	Reverse	ATGTAGCTCTTGTGACACTGGGCA			
Brachyury(T)	MD255_Brachyury-F	Forward	ATGCCAAAGAAAGAAACGAC	across exon 4- 3'UTR	55	835
	MD256_Brachyury-R	Reverse	AGAGGCTGTAGAACATGATT			
Gapdh	LFC132_GapdhF520	Forward	ACCACAGTCCATGCCATCAC	across exon 4-6	55	452
	LFC133_GapdhR971	Reverse	TCCACCACCCTGTTGCTGTA			
Nanog	JF93_Nanog-F052	Forward	TCGAATTCTGGGAACGCCTCATCA	across exon 1-2	55	258
	JF94_Nanog-R310	Reverse	AGAACACAGTCCGCATCTTCTGCT			
Oct4(Pou5f1)	LFC023_Oct4F	Forward	GGCGTTCTCTTTGGAAAGGTGTTC	across exon 2-5	55	313
	LFC024_Oct4R	Reverse	CTCGAACCACATCCTTCTCT			
Prdm14	LFC126_Prdm14F0825	Forward	TAGAGTGGTCAATGCCAGCGAAGT	across exon 3-5	55	416
	LFC127_Prdm14R1240	Reverse	AGGGCGTGTACTTCAGGTGCTTAT			
Sox2	LFC136_Sox2F1357	Forward	TAGAGCTAGACTCCGGGCGATGA	3'UTR	55	297
	LFC137_Sox2R1653	Reverse	TTGCCTTAAACAAGACCACGAAA			
Dppa3(Stella)	LFC122_Dppa3F023	Forward	TCGACCCAATGAAGGACCCTGAAA	across exon 1-2	55	255
	LFC123_Dppa3R277	Reverse	ACAACAAAGTGCCGACCCTTCTCT			
cMyc	LFC183_cMycF1137	Forward	CAGAGGAGGAACGAGCTGAAGCGC	3'UTR	55	228
	LFC184_cMycR1365	Reverse	TTATGCACCAGAGTTTCGAAGCTGTTCG			
Klf4	LFC185_Klf4F1236	Forward	GCGAACTCACACAGGCGAGAAACC	3'UTR	55	737
	LFC186_Klf4R1973	Reverse	TCGCTTCCTCTTCCTCCGACACA			
Lin28	LFC210_Lin28F0125	Forward	GCATCTGTAAGTGGTTCAACGTGC	across exon 2-3	55	290
	LFC211_Lin28R0415	Reverse	ACCTGTCTCCTTTGGATCTTCGCT			
Blimp1-exon6	LFC287_Blimp1-1361F	Forward	CCAATGGCTTGAGCACCATGAACA	exon 6	55	141
	LFC288_Blimp1-1501R	Reverse	AACTCGGTAGGGAAGCTGGATTGA			
Blimp1-exon7	LFC289_Blimp1-1898F	Forward	CTTTCAAGTGCCAGACCTGCAACA	exon 7	55	91
	LFC290_Blimp1-1988R	Reverse	ACTCATGTGGCTTCTCTCCTGTGT			
Blimp1-exon8	LFC291_Blimp1-2324F	Forward	ACATGGAGGACAGTGTCGATGTGA	exon 8	55	183
	LFC292_Blimp1-2506R	Reverse	TGTGAGGCAACTTCATGAGGGACA			
Blimp1-3'UTR	LFC189_Blimp1F4706	Forward	AGCATGACCTGACATTGACACC	3'UTR	55	162
	LFC190_Blimp1R4867	Reverse	CTCAACACTCTCATGTAAGAGGC			

TaqMan Gene Expression Assays

Gene	Probe No.	Reporter
Pou5f1	Mm03053917_g1	FAM
Rex1	Mm01194090_g1	FAM
Prdm14	Mm01187285_m1	FAM
Gata4	Mm00484689_m1	FAM
Dppa3	Mm01184198_g1	FAM
Ifitm3	Mm03052142_s1	FAM
Prdm14	Mm01237814_m1	FAM
Lin28	Mm00524077_m1	FAM
Kit	Mm00445212_m1	FAM
Actb	Mm00607939_s1	FAM
Gapdh	4352339E	VIC
18S	4319413E	VIC

Supplemental Experimental Procedures

Mice

Rosa26-RFP reporter mice, α *MHC-Cre* mice, *Blimp1-Cre* mice (the Jackson Laboratory, stock no. 008827), *Meox2-Cre* mice (The Jackson Laboratory, stock no. 003755), W/W^{v} mice (The Jackson Laboratory, stock no. 100410) and FOX Chase SCID Beige mice (Charles River, Strain Code: 250), *Rosa26-DTA* (The Jackson Laboratory, stock no. 006331) and *Prdm1*$^{loxP/loxP}$ mice (The Jackson Laboratory, stock no. 008100) were used in this study (129S5/SvEvBrd[129S5] was the wild-type strain). Oligonucleotides for genotyping are listed in Table S2. All mice were exposed to a 14 hr light cycle with the lights switched on at 6 am and off at 8 pm. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

ES Cell Derivation

ES cells were derived from the blastocysts of 3- to 5-week-old female mice that had been induced to superovulate and then mated with stud males. Noon of the day that a vaginal plug was observed was considered embryonic day 0.5 (E0.5). At E3.5, blastocysts were flushed from the female uterine horns, and each blastocyst was seeded into a single well of a 4-well dish containing irradiated MEF feeder cells and standard ES derivation medium (Knockout DMEM, with 2 mM L-glutamine, 100 μ M nonessential amino acids, 100 μ M β -mercaptoethanol, 2 × 10^{3} U/ml Lif, and 20% Knockout Serum Replacement) at 37° C under 5% CO_2 for 5 days (unless otherwise stated). The cultured cells were dissociated and passaged in Knockout DMEM with 2 mM L-glutamine, 100 μ M nonessential amino acids, 100 μ M β -mercaptoethanol, 1 × 10^{3} U/ml Lif, and 10% FBS, until pluripotent ES cells emerged.

2i Experiments

The ES derivation medium described above was supplemented with 2i inhibitors (3 μ M CHIR99021 and 1 μ M PD0325901, STEMGENT). For ES cell derivation, 2i was added into the medium prior to seeding the blastocysts and throughout the entire ICM outgrowth period. To compare global expression profiles of R1 vs. 2i-derived ES cells, we cultured ES cells for 2 days in standard medium.

In Utero Injection

The embryos of pregnant mice anesthetized with Avertin (working concentration: 1.25% of a 20 mg/ml stock), between E8.5 and E8.75 (noon and 6 pm of E8), were injected with donor cells via a long ventral incision. Briefly, sorted RFP^{+} donor cells were mixed with or without carrier cells (irradiated MEFs) in the sorting catch tube at a ratio of 1:60 to 1:100. Mixed donor and carrier cells were then drawn into a glass micropipette with a closed sharp tip and a side hole that was 40-70 mm in diameter. The pipette was manually inserted into the ventral third of the decidual swelling, and 0.5-1.0 μl of liquid was introduced per injection. Between 500 and 1,000 cells were injected per embryo.

BioMark Real-Time PCR Analysis

The 48.48 Dynamic array IFC was used for the BioMark real-time PCR analysis. Briefly, individual 20X TaqMan gene expression assays (Applied Biosystems) were mixed with 2X assay loading reagent (Fluidigm, PN 85000736) to prepare a 10X assay mix, 5.0 μ l of which was loaded into each assay inlet of the array. 2X TaqMan Universal PCR Master Mix (Applied Biosystems, PN 4304437) was combined with 20x GE sample loading reagent (Fluidigm, PN 85000746) to make up the sample pre-mix. 2.75 μ l o which was mixed with single-cell cDNA and a 5.0 μ l of the sample mix was then loaded into each sample inlet of the array. The TaqMan gene expression assay is outlined in Table S2. The loaded array chips were primed and run with Data Collection software (Fluidigm) on the BioMark system. The data were analyzed by real-time PCR analysis software (Fluidigm).

ES Cell Lines

RFP^{+} Z/Red ES cells were generated by transient transfection of Z/Red ES cells [1] with a Cre-expression plasmid. The resultant Z/Red ES cells were then purified by sorting for the RFP^{+} population (purity of 99%) and maintaining the cells under standard ES culture conditions.

RNA Isolation and RT-PCR

RNA was isolated with the RNeasy kit (Qiagen) according to the manufacturer's protocol. Total RNA (50 ng) was reverse transcribed with the ImPromII reverse transcription system (Promega) with random primers used to generate cDNA. Total cDNA was diluted two-fold and used at 2 μ l per 50 μ l of PCR reaction. PCR reactions were performed with GoTaq Green Master Mix (Promega) under the following cycle conditions: denaturation for 3 min at 94° C, followed by variable numbers of cycles of 30 sec at 94° C, 30 sec at 55° C, 30 sec at 72° C, and a final extension for 15 min at 72° C. Sequences for gene-specific primers, cycle numbers and PCR product size are listed in Table S2. To quantify the relative expression level of a particular gene, we analyzed PCR signals from three independent PCR reactions after agarose gel electrophoresis and ethidium bromide staining with the AlphaEraseFC (FluorChem) software version 5.0.1 (Alpha Innotech).

Gonadal Explant and Coculture

Cocultures were as previously described. Briefly, female genital ridges were collected from E13.5 embryos. The sex of the embryos was determined morphologically and further confirmed by genotyping for the presence of Sry (Table S2). From 6 to 8 gonads were cut into 4 to 6 small pieces that were placed in the center of a Transwell (Falcon 3095) in DMEM supplemented with 20% FBS, 2 mM L-glutamine, and penicillin/streptomycin (all from Invitrogen). In addition, 10 to 12 gonads were dissociated in 0.25% trypsin-EDTA (10 min at 37° C), mixed with FACS-sorted RFP^+ cells, and centrifuged at 200 g for 5 min. After centrifugation, the cell pellet was resuspended and transferred into the Transwell prepared on the same day with small pieces of gonads.

EB Differentiation and Teratoma Formation Assay

Embryoid bodies (EBs) were formed in hanging drops (600 cells in 20 μ l of mES medium without Lif) that were cultured for 4 days. EBs were then washed off from the culture dish lid and plated onto gelatinized plate to allow them to attach and form monolayer outgrowths. Four to six days later, total RNA was isolated with the RNeasy Kit (QIAGEN) according to the manufacturer's protocol. To induce teratoma formation, we injected 5×10^5 cells of each cell line into the quadriceps muscle of the hind legs of immunodeficient FOX Chase SCID Beige mice. A replica injection was made for each cell line. Teratomas were harvested after 14-25 days postinjection; the tumor tissue was fixed in 10% formalin overnight, transferred to 70% ethanol and embedded in paraffin. Sections were analyzed by hematoxylin and eosin (H&E) staining.

Alkaline Phosphatase Staining

For alkaline phosphatase staining, cells were washed once in PBS, fixed for 30 min in 2% paraformaldehyde at room temperature, washed once in PBS and stained in the dark with the AlkPhosIII kit (Vector Laboratories) as described in the manufacturer's protocol.

Immunofluorescence Staining and Microscopy

Cultured cells were washed twice with BD Perm/wash buffer and then fixed with BD Cytofix at 4° C for 20 min. Cells were then blocked with BD perm/wash buffer for 30 min at room temperature followed by staining with primary antibody diluted in BD perm/wash buffer at 4° C overnight. The cells were incubated with AlexaFluor secondary antibodies (Invitrogen, 1:1000 dilutions) for 1 h at room temperature. Cells were then washed three times with BD perm/wash buffer and mounted on glass slides with DAPI (Vectors Labs) for imaging with a Leica DMI6000B equipped with an N2.1 filter cube (excitation, 537.5/45 nm band-pass filter; emission, 570-nm long-pass filter) for detecting RFP signals. Fluorescent imaging of embryonic tissues was performed with a Zeiss Lumar fluorescent stereomicroscope equipped with a Rhodamine filter set (excitation, 545 _ 12 nm band pass filter; emission: 580 nm long-pass filter) for detecting RFP signals. Cryosections of embryonic tissues were blocked with 5% BSA at room temperature for 1 h, followed by staining with primary antibody diluted in 2% BSA at 4° C overnight. The next day, sections were first washed three times in PBS before staining with AlexaFluor secondary antibodies (Invitrogen, 1:1000) for 1 h at room temperature. Sections were then washed three times in PBS and mounted with DAPI (Vectors Labs) before imaging. Primary antibodies and dilutions used in this study were: anti-Gata4 (Santa Cruz, sc-1237, 1:100); anti-Oct4 (Santa Cruz, sc-9081, 1:200); anti-Nanog (Abcam, ab21603, 1:200); anti-Mvh (Abcam, ab13840, 1:1000); anti-RFP (MBL, PM005, 1:1000). Confocal imaging was performed using the

Zeiss LSM 510 microscopy and LSM Image Browser software. TO-PRO-3 (Invitrogen, 1:1000) was used as a nuclear counterstain.

FACS Analysis and Cell Sorting

Single cell suspensions were obtained from trypsinized cells filtered through a 40-μ m cell strainer (Becton Dickinson). Samples expressing the RFP reporter gene were analyzed and sorted on the FACSAria II instrument (Becton Dickinson) which was equipped with a yellow laser (excitation wavelength, 561 nm) and appropriate filters (emission, 610/20 nm-band-pass filter and 600-nm long-pass filter).

Microarray Analysis

For ICM/ES cell gene expression profiling, RNA were isolated from ICM outgrowths or ES cell lines as described above. A total of 0.5-1 μ g of RNA from each sample was used for hybridization to Illumina MouseWG-6 v2.0 Expression BeadChip according to the manufacturer's instructions. The sample amplification and hybridization were performed by the Texas Children Cancer Center's (TCCC) Genomics and Proteomics Core Laboratory (GPL). Raw data were normalized by quantile normalization method and analyzed by DNASTAR ArrayStar software. The complete microarray expression data set has been deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov.ezproxyhost.library.tmc.edu/geo/) with the series accession number GSE31868.

Single-Cell Gene Expression Profiling

Single cells were sorted directly into 96-well plates prepared with a cell lysis/RT mix provided with the CellsDirect One-Step qRT-PCR kit (Invitrogen) to perform single-cell RT and target-specific preamplification according to the BioMark Advanced Development protocol. Briefly, single cells were sorted into individual wells containing 9 μ l of 0.2x TaqMan assay (Applied Biosystems) mixed with CellsDirect Reaction Mix and SuperScript III RT/Platinum Taq Mix. Reverse transcription (RT) was performed at 50° C for 15 min. To inactivate the RT enzyme and activate the Taq Polymerase the samples were incubated at 95° C for 2 min followed by 18 cycles of 15 sec at 95° C and 4 min at 60° C to accomplish target specific cDNA amplification. The cDNA was diluted 1:5 and 2 μ l were subsequently used in qPCR reactions with individual TaqMan Gene Expression assays and 1x TaqMan Universial PCR Master Mix (Applied Biosystems) in a total volume of 20.0 μ l.

Primary Epiblast Outgrowth and Isolated Epiblast

Fragments of post-implantation epiblasts were dissected from E6.5 embryos (*Blimp1-Cre; Rosa26-RFP*) and permitted growing on MEF cells for 3 to 6 days in medium containing Knockout DMEM (Invitrogen) with 2 mM L-glutamine (Invitrogen), 100 μ M nonessential amino acids (Invitrogen), 100 μ M β -mercaptoethanol (Sigma) and 20% Knockout Serum Replacement (Invitrogen) with 10 ng/ml Fgf2 (R&D Systems). Purified epiblast were isolated free of surrounding visceral endoderm then plated directly onto MEFs [2, 3].

Supplemental References

1. Vintersten, K., Monetti, C., Gertsenstein, M., Zhang, P., Laszlo, L., Biechele, S., and Nagy, A. (2004). Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. Genesis *40*, 241-246.
2. Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196-199.
3. Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., et al. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191-195.