Supplemental Information

Blimp1 Expression Predicts Embryonic Stem Cell Development In Vitro

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

Supplemental Inventory

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2. Supplemental Experimental Procedures

3. Supplemental References
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 (RFP³). 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.
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>
<thead>
<tr>
<th>Type of donor cells</th>
<th>No. of recipient embryos injected</th>
<th>No. of embryos recovered on E12.5 (survival)</th>
<th>No. of embryos with chimeric gonads</th>
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<tr>
<td>Whole day-4 ICM outgrowth</td>
<td>74 (2 experiments)</td>
<td>40 (54%)</td>
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<tr>
<td>RFP&lt;sup&gt;+&lt;/sup&gt; day-4 ICM outgrowth</td>
<td>467 (10 experiments)</td>
<td>207 (44%)</td>
<td>4 (2%)</td>
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<td>RFP&lt;sup&gt;+&lt;/sup&gt;/Red ES cells</td>
<td>217 (5 experiments)</td>
<td>74 (34%)</td>
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**Table S2. List of oligos used in this study**

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**Table S3. Summary of in utero injection results (Related to Figure 2)**

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Supplemental Experimental Procedures

Mice
Rosa26-RFP reporter mice, αMHC-Cre mice, Blimp1-Cre mice (the Jackson Laboratory, stock no. 008827), Meox2-Cre mice (The Jackson Laboratory, stock no. 003755), W/Wv 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 Prdm1loxP/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 μM nonessential amino acids, 100 μM β-mercaptoethanol, 2 × 10^3 U/ml Lif, and 20% Knockout Serum Replacement) at 37°C under 5% CO2 for 5 days (unless otherwise stated). The cultured cells were dissociated and passaged in Knockout DMEM with 2 mM L-glutamine, 100 μM nonessential amino acids, 100 μM 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 μM CHIR99021 and 1 μM 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 μl 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 μl of which was mixed with single-cell cDNA and a 5.0 μl 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 μl per 50 μl 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 μl 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 × 105 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-μm 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 μg 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 μl 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 μl were subsequently used in qPCR reactions with individual TaqMan Gene Expression assays and 1x TaqMan Universal PCR Master Mix (Applied Biosystems) in a total volume of 20.0 μl.

**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 μM nonessential amino acids (Invitrogen), 100 μM 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**