Germline competency of parthenogenetic embryonic stem cells from immature oocytes of adult mouse ovary

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Parthenogenetic embryonic stem cells (pESCs) have been generated in several mammalian species from parthenogenetic embryos that would otherwise die around mid-gestation. However, previous reports suggest that pESCs derived from in vivo ovulated (IVO) mature oocytes show limited pluripotency, as evidenced by low chimera production, high tissue preference and especially deficiency in germline competence, a critical test for genetic integrity and pluripotency of ESCs. Here, we report efficient generation of germline-competent pESC lines (named as IVM pESCs) from parthenogenetic embryos developed from immature oocytes of adult mouse ovaries following in vitro maturation (IVM) and artificial activation. In contrast, pESCs derived from IVO oocytes show defective germline competence, consistent with previous reports. Further, IVM pESCs resemble more ESCs from fertilized embryos (fESCs) than do IVO pESCs on genome-wide DNA methylation and global protein profiles. In addition, IVM pESCs express higher levels of Blimp1, Lin28 and Stella, relative to fESCs, and in their embryoid bodies following differentiation. This may indicate differences in differentiation potentially to the germline. The mechanisms for acquisition of pluripotency and germline competency of IVM pESCs from immature oocytes remain to be determined.

INTRODUCTION

Parthenogenetic embryonic stem cells (pESCs) can be induced from parthenogenetic ‘embryos’ created by artificial activation of oocytes without fertilization and may provide an alternative to (f)ESCs as a valuable source of autologous stem cells for regenerative medicine (1–3). Parthenogenetic ‘embryos’ developed from artificial activation of oocytes, without paternal genomes, do not survive beyond mid-gestation, because aberrant genomic imprinting and defective placenta tion fail to support subsequent development (4–7). pESCs have been generated from parthenogenetic embryos of mice (2,8–12), monkeys (1,13) and humans (3,14–17), as well as several other mammalian species. pESCs show extensive differentiation capacity in vitro in both mice and primates (18,19) and contribute to a variety of adult tissues in chimeras (9). However, the proliferation and differential potential of pESCs or their derivatives remain controversial (9,19–22). One strict test of stem cell pluripotency routinely applied is transmission of cells through the germline of chimeric
animals (23–25). Notably, most pESC lines reported thus far generated from in vivo ovulated (IVO) mature oocytes exhibit low chimera production and poor germline transmission, even after repeated cross-breeding (2,9–11,26), suggestive of restricted pluripotency. Genomic imprints are established during gametogenesis and play important roles in fetal growth and development (27). Efficacy and safety issues currently limit the use of pESCs in regenerative medicine, presumably due to consequences of aberrant genomic imprinting (28,29).

Limited availability of human mature oocytes further constrains potential application of pESCs to regenerative medicine (14). In vitro fertilization (IVF) clinics, however, routinely produce and discard immature oocytes as by-products of ovarian stimulation and poor germline transmission, even after repeated cross-breeding (2,9–11,26), suggestive of restricted pluripotency. Genomic imprints are established during gametogenesis and play important roles in fetal growth and development (27). Efficacy and safety issues currently limit the use of pESCs in regenerative medicine, presumably due to consequences of aberrant genomic imprinting (28,29).

RESULTS
Parthenogenetic ‘embryos’ developed from immature oocytes and derivation of IVM pESC lines
To generate IVM pESCs from immature oocytes, we collected immature oocytes at the GV stage enclosed in compact cumulus complexes (COC) collected from an adult B6C3F1 mouse ovary exhibit cumulus expansion at the meiosis II (MII) stage following IVM for 15 h. IVM oocytes develop to parthenogenetic (PA) blastocysts following activation by strontium chloride (Sr^{2+}). Arrowhead, polar bodies. Scale bar, 50 μm. (B) IVM pESC 1116 (C57BL/6XC3H F1) at passage 8 (P8) showing typical morphology characteristics of undifferentiated pESC BF10P9, with clear, smooth boundary around the colonies with densely packed cells. (C and D) Like fESC BF10 cells, IVM pESCs 1116 express specific ESC markers, Oct4 (pink), SSEA-1 (pink) and alkaline phosphatase activity (AP) (blue, D). Scale bars (B–D), 100 μm. (E) Genotyping by microsatellite analysis of IVM pESC 1116 cell line. Arrow, IVM pESC bands. fESC, fertilized embryonic stem cells; IVM pESC, parthenogenetic ESCs from IVM oocytes.
combination with cytochalasin D that inhibits extrusion of the second polar body to diploidize the genome. Some oocytes were further confirmed at the MII stage as evidenced by chromosomes aligned on MII spindles using immunostaining and fluorescence microscopy, and showing 20 chromosomes by karyotypes. Activated oocytes were cultured in potassium simplex optimized medium added with amino acids (KSOmA) and developed to blastocysts. We compared the developmental capacity of embryos derived from oocytes matured in vitro versus in vivo and found that the rate of cleavage to two cells following activation did not differ in two hybrid F1 mouse strains (B6C3F1 and B6X129F1), although rates of development to blastocyst for IVM oocytes were slightly lower than those of IVO oocytes (Supplementary Material, Table S1). IVM oocytes developed to blastocysts at high rates following artificial activation (86 and 65%, for B6C3F1 and B6X129F1, respectively).

Blastocysts seeded onto mouse embryonic fibroblast (MEF) feeder layers allowed inner cell mass (ICM) outgrowth in the ES medium supplemented with either knockout serum replacement (KSR) or fetal bovine serum (FBS). KSR improved ICM outgrowth and generated primary ES-like colonies with higher efficiency than FBS (Supplementary Material, Table S2). Similar high efficiency was obtained to derive new cell lines outgrowth and generated primary ES-like colonies with higher efficiency by injecting them subcutaneously into immunodeficient nude mice (n = 8). Four weeks after injection, all mice injected with IVM pESCs formed teratomas (Fig. 2D and E), whereas mice injected with MEF, as controls, did not form teratomas (Fig. 2F). Teratomas contained all three embryonic germ layers, including immature neural tissue, neural tube (ectoderm), muscle, cartilage (mesoderm), gland and ciliated columnar epithelium (endoderm) (Fig. 2G–L). The differentiation capacities of IVM pESCs are similar to those of IVO pESCs (12).

### Generation of germline-competent chimeras from IVM pESCs

To further determine developmental pluripotency in vivo, we injected IVM pESCs of non-albino genetic background into host blastocysts of albino background, examined for chimera production and germline transmission, and compared with those of IVO pESCs and fESCs. Regardless of early (passages 5–6), middle (passage 21) and late passage (passage 54) cells, IVM pESCs 1116 of agouti background injected into albino ICR mouse blastocysts produced chimeras with high efficiency, based on coat color (Fig. 3A), compared with fESCs (Fig. 3B; Supplementary Material, Table S3). IVO pESCs also produced chimeras with high efficiency, regardless of passage number. Inbred Balb/c blastocysts serving as hosts for IVM pESCs produced significantly more chimeras than did outbred ICR blastocysts (69 versus 25%, P < 0.01). IVM pESC line 11191 injected into Balb/c blastocysts also resulted in high production of chimeras, comparable to fESC F5 (60 and 58%, respectively).

IVM pESCs contributed to all tissues and organs, as evidenced by microsatellite genotyping analysis (Fig. 3C), a standard test for the identification and contribution of ESCs in chimeras (12,37). Notably, contributions of IVM pESCs to both gonads and somatic tissues of female chimeras exceeded those of male chimeras (Supplementary Material, Table S4). Chimeras produced from IVM pESCs, IVO pESCs or fESCs were mated with albino mice to test their germline competence. Initially, IVM pESC 1116 hosted in ICR blastocysts generated chimeras with germline transmission at efficiency.
of about 12% after several rounds of mating (Table 1). Notably, chimeras produced from IVM pESCs hosted in Balb/c blastocysts exhibited higher rates of germline transmission (Fig. 3D), with efficiency of 20–100% (average of 55%) after only the first or second round of breeding with albino mice. Chimeras generated from IVM pESCs of B6X129F1 background also showed high germline transmission (40%) after the first-round breeding and delivery (Table 1). IVM pESC-4 from Nanog-EGFP mice injected into ICR blastocysts also generated germline-competent chimeras (10%).

In contrast, chimeras generated from IVO pESCs C2 or C3 from B6C3F1 background hosted in either ICR or Balb/c blastocysts failed in germline transmission, even after repeated mating. Notably, 30–100% of female chimeras (n = 11) produced from IVM pESCs, but no male IVM pESC chimeras (n = 2) exhibited transmission through the germline. Comparatively, female chimeras generated from fESCs resulted in low germline transmission (0–25%, n = 5), compared with male fESC chimeras (67–78%, n = 12). It is of interest that it is the female chimeras with greater gonad contribution, possibly suggesting a memory for their oocyte heritage. One
reason that female versus male chimeras from IVM pESCs could be fertile might reflect X chromosome constitution. It is generally assumed that the inactivation of X chromosomes is associated with increased expression of \textit{Xist}. We show that \textit{Xist} expression was increased following the differentiation of IVM pESC 1116 (P18), in contrast to IVO pESC C3. The fESCs F1 did not show much change in the expression of \textit{Xist} following differentiation, likely because the male fESCs with XY chromosomes did not exhibit X inactivation (Supplementary Material, Fig. S3). Also, the contribution of IVM pESCs to gonads of the female chimeras might be important for germline competency of IVM pESCs.

Molecular analysis of pluripotency and germline competence of IVM pESCs

Mechanisms underlying the efficient production of pluripotent IVM pESCs from immature oocytes remain elusive, but likely involve the alteration of genome-wide methylation of imprinted and non-imprinted genes, histone modification or microRNA regulation that together influence global gene expression (38). To determine the global DNA methylation levels of IVM pESCs or IVO pESCs, we examined relative DNA methylation profiles throughout the genome using MeDIP enrichment on NimbleGen CpG island plus promoter array and compared them with fESCs. Using the Mann–Whitney non-parametric paired test and taking significance as unadjusted \( P, 0.01 \), only 60 of 22 621 (0.27%) regions of interest (ROIs) were differentially methylated between fESCs and IVM pESCs; and 699 of 22 621 (3.1%) ROIs differentially methylated between fESCs and IVO pESCs (Fig. 4A; Supplementary Material, Tables S8–S11). The ROIs do vary from each other in length. The mean length of the entire 22621 ROIs examined was \( \approx 1.6 \text{ kb} \) and \( \approx 97\% \) of all the ROIs had their lengths within 1 kb of the mean. The genome-wide heat map (Fig. 4A) displayed 100 randomly selected 22 621 ROIs. Some ROIs exhibited preferential differences only in specific chromosomes, e.g. chromosome 16 (Fig. 4B). Observation of the methylation profiles as shown in the plots, however, showed overall similar methylation patterns, suggesting, perhaps, that these differences were generally small. On the basis of the result of the paired test, IVO pESCs appear more different from fESCs than IVM pESCs from fESCs. Of these differentially methylated ROIs, 53 (\( P, 0.05 \)) or 8 (\( P, 0.01 \)) were common to both IVM pESCs versus fESCs and IVO pESCs versus fESCs (Fig. 4C). It remains to be determined whether these 53 differentially methylated ROIs have any meaningful functions in pESCs, compared with fESCs. In the individual comparison between fESC and IVM pESC or between fESC and IVO pESC, the non-parametric Mann–Whitney paired test revealed that none of the key pluripotency factors, \textit{Oct4}, \textit{Esrrb}, \textit{Sox2}, \textit{Klf4}, \textit{Nanog} and \textit{c-Myc}, were differentially methylated at the
unadjusted $P$-value of 0.01, although an ROI on chr3:34547676–34550288, which contains Sox2, was revealed to be differentially methylated (unadjusted $P$-value = 0.02; BH-FDR adjusted $P$-value = 0.15) in the individual comparison between fESC and IVO pESC.

We analyzed the expression of several key imprinted genes in oocytes and in IVM pESCs and IVO pESCs. Expression of the imprinted genes, $H19$, $Igf2r$ and $S138a4$, was low or undetectable in both IVM and IVO oocytes. Yet, $Impat$ was highly expressed, and $Snprn$ and the DNA methyltransferase

Figure 4. DNA methylation of genome-wide CpG-rich regions (ROIs). (A) Genome-wide methylation heat map of ROIs. ROIs (heat-map rows) were divided in 10 bins (columns) and the average enrichment within each bin was determined. Blue and red represent enrichment and depletion in respect to input DNA. (B) MeDIP enrichment of one of the differentially methylated ROIs identified on chromosome 16 is shown ($P < 0.05$, Mann–Whitney paired test). (C) Differentially methylated ROIs. The 53 differentially methylated ROIs were identified genome-wide ($P < 0.05$, Mann–Whitney paired test). ROIs (heat-map rows) were divided in 10 bins (columns) and the average MeDIP enrichment within each bin was determined. Blue and red represent enrichment and depletion in respect to input DNA.
Dnmt3L were slightly increased in IVM oocytes compared with IVO oocytes (Fig. 5A). Expression levels of IVO oocytes and fESC BF10P6 cells served as controls are arbitrarily designated as 1.0, and comparison is made with controls, respectively. *P < 0.5; **P < 0.01. n = three replicates. (C) Methylation status in the ICR of H19 and DMR1 of Snrpn from IVM oocytes, and IVM pESC 1116P7, IVO pESC C3P9, compared with fESC BF10P6. Genomic DNA was treated with bisulfite, followed by PCR amplification and sequencing. Shown are cytosines for a number of independently sequenced templates (horizontal lines). Percentage indicates methylated cytosines. fESC, fertilized embryonic stem cells; IVO pESC, parthenogenetic ESCs from IVO oocytes; IVM pESC, parthenogenetic ESCs from IVM oocytes.

We further checked the methylation status of specific imprinted genes in IVM pESCs and IVO pESCs and compared those with fESCs, and compared with progenitors oocytes. The ICR of the maternally expressed imprinted gene H19 was hypomethylated in IVM oocytes, and the methylation level was increased in IVM pESCs, relative to IVO pESCs, but still lower than in fESCs. Unfortunately, this gene was not represented on the global methylation array. The differentially methylated regions (DMRs) of paternally expressed imprinted genes Snrpn were hypermethylated in IVM oocytes and showed significantly reduced methylation in IVM pESCs to levels resembling those of fESCs (Fig. 5C). Comparatively, the methylation of DMRs of Snrpn was lower, coincident with its increased expression of IVO pESCs.

Pluripotency of ESCs is characterized by appropriate expression and the regulatory circuitry of key transcription factors Nanog, Oct4, Sox2 and Esrrb (38–41). Consistently, these factors were highly expressed in fESCs than in MEF served as controls. Moreover, IVO pESCs and IVM pESCs expressed high levels of these factors similar to those of fESCs, except for that Nanog was higher in IVO pESCs and Sox2 relatively higher in IVM pESCs than in fESCs (Fig. 6A). Abnormally, high expression of Nanog could suppress normal differentiation of ESCs (42).
Specification of germ cell fate is fundamental in development (43). The germline competence of IVM pESCs might be related to intrinsic specification of germ cells. We tested whether IVM pESCs express genes specific for germ cell specification and progression, compared with those of fESCs and IVO pESCs, and particularly following differentiation into EBs, which is commonly used for the specification and derivation of primordial germ cells (PGCs) in vitro (44,45). Germ cell marker genes, Stella, Blimp1, Lin28, Dazl and Mvh, all were expressed in fESCs, and Blimp1 and Lin28 showed increased expression in EBs 3 days following differentiation (Fig. 6B). IVM pESCs showed increased expression (P < 0.05) of Stella, Blimp1 and Lin28, compared with those of fESCs, and even higher (P < 0.01) expression of Stella and Blimp1 in their EBs than those of EBs differentiated from fESCs (Fig. 6B). Expression of Dazl, Mvh and Lin28 did not differ in their EBs from IVM pESCs and fESCs. In contrast, expression of these germ cell marker genes in IVO pESCs did not increase in their EBs following differentiation induction, but rather Dazl and Mvh were notably decreased in their EBs. Expression of Lin28 was much reduced in the EBs from IVO pESCs compared with that of EBs from fESCs (Fig. 6B).

Epigenetic alternations ultimately change protein expression and action (46). We performed global proteomics analysis of protein expression in IVM and IVO pESCs and compared them with fESCs. High-sensitive fluorescence two-dimensional differential in-gel electrophoresis (2D-DIGE) techniques not only detect levels of protein expression, but also post-translational modifications (47,48). By 2D-DIGE, 1291–1466 protein spots were found in ESC samples from three repeated experiments. IVO pESCs showed 39 differentially expressed proteins/peptides compared with fESCs (Fig. 7A and B), whereas IVM pESCs and fESCs exhibited only 13 differentially expressed protein spots (Fig. 7C and D), when the average ratio value was set to 1.5 and significance defined as P < 0.05 by t-test (Supplementary Material, Table S5). Fewer proteins were down-regulated and changes were smaller in IVM pESCs than in IVO pESCs, compared with fESCs, respectively. Thus, the protein profile of IVM pESCs closely resembles that of fESCs. Differentially expressed protein spots were further characterized by matrix-assisted laser desorption/ionization-time-of-flight and liquid chromatography/mass spectrometry analysis and found to involve in cell signaling, metabolism, cytoskeleton, apoptosis and oxidative stress. Consistent with genome-wide methylation data, protein profiling also detected no clear differences in the expression of imprinted genes between IVM pESCs and fESCs.

**DISCUSSION**

We show that parthenogenetic pluripotent stem cells can be effectively induced from immature oocytes of adult ovaries following IVM and activation. The IVM pESCs exhibit high germline competence, in contrast to conventional (IVO) pESCs derived from ovulated mature oocytes. Chimeras produced from IVM pESCs continued germine transmission, even after several rounds of breeding, and produced fertile offspring, demonstrating that IVM pESC-derived tissues survive long term without visible defects.

Epigenetic modification during IVM of oocytes may play a critical role in the generation of germline-competent IVM pESCs. Production of epigenetically competent oocytes is complex and involves many epigenetic regulators of chromatin remodeling and embryo development (reviewed in 49). GV
oocytes and cytoplasmic factors related to demethylation of histone H3-K9 enhance somatic cell nuclear reprogramming after nuclear transfer (NT) (50,51). Global gene expression profiling by microarray analysis demonstrates that IVM oocytes exhibit immature patterns of gene expression reminiscent of immature GV oocytes, compared with IVO oocytes (52,53). Our data show that IVM oocytes exhibit elevated expression of *Dnmt3L* and particularly *Impact*. *Impact* is imprinted after the oocyte has grown to the final, antral follicle stage (54). DNA methyltransferase 3-like (*Dnmt3L*) is essential for the establishment of maternal methylation imprints during oogenesis (55,56). Overexpression of *DNMT3L* increases nuclear reprogramming (57). Together, oocytes matured *in vitro* may maintain some immature state, which facilitates epigenetic reprogramming and derivation of IVM pESCs.

Full activation of oocytes by Sr²⁺ also may contribute to efficient derivation of pluripotent IVM pESCs. By mimicking sperm-induced Ca²⁺ oscillations during fertilization (58), Sr²⁺ induces Ca²⁺ oscillations, fully activates oocytes and enhances parthenogenetic embryo development, like fertilized embryos (59,60). Sr²⁺-activated cytoplasts were used for

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**Figure 7.** Proteomics analysis of IVM pESCs or IVO pESCs versus fESCs by 2D-DIGE. Shown are representative scanned gel images of whole-cell lysates from: (A) fESC, (B) IVO pESC, (C) fESC and (D) IVM pESC. Separation of proteins was performed in the first dimension (horizontal) by IEF and, subsequently, in the second dimension (vertical) by SDS–PAGE. pH range used for IEF is indicated on top of the figure (3.0–10.0). Mw, molecular weight (kDa). Spots representing protein expressed differentially between IVO pESC and fESC and between IVM pESC and fESC are indicated by arrows with numbers.
somatic cell NT to successfully produce cloned mice (61) and to improve NT-pESCs (10). In addition, an embryo culture condition could affect parthenogenesis prior to isolation of stem cells from ICM of parthenogenetic ‘blastocysts’. Embryos cultured in KSOMAA show global gene expression, patterns of genomic imprinting and embryo development, like in vivo developed embryos (60, 62–64).

The ICMs of parthenogenetic ‘blastocysts’ derived from IVM oocytes seemed to develop slower than did parthenogenetic ‘blastocysts’ from IVO oocytes and fertilized blastocysts. ICM outgrowths were extended to about 10 days for IVM ‘blastocysts’ to achieve primary colonies. ICM outgrowth for shorter duration (5 days), which produce primary colonies for IVO pESCs, could not produce appropriate primary colonies for the isolation of IVM pESCs. Extension of culture time for the isolation process may have allowed sufficient epigenetic reprogramming of somatic cells to iPSCs (65, 66).

Likewise, prolonged in vitro culture alters expression of imprinted genes in embryonic cells (67, 68). Genome-wide methylation and protein expression profiles suggest that epigenetic modification and gene expression in both imprinted and non-imprinted genes of IVM pESCs are similar to those of fESCs. Specifically, expression of Igf2r, Srpcp, Impact, Igf2 and Slec36a4 in IVM pESCs resembles that of fESCs. Appropriate methylation and expression of Igf2r, Srpcp and U2af1-rs1 are associated with pluripotency of IVM pESCs (37). Specification and determination of PGCs requires Blimp1 and its regulation by Lin28 (43, 69, 70). Blimp1 marks Mvh and Dazl are expressed during progression of germ cells, and Dazl regulates the pre-meiotic translation of Mvh (70–72). Stella is constantly expressed in blastocysts, and PGC specification and progression (71). These genes are more commonly used to identify PGCs and germ cells (73). Notably, genes for germ cell development at later stages Mvh and Dazl show expression in the EBs of IVM pESCs similar to those of fESCs. IVM, full activation of oocytes, embryo culture and/or extended ICM outgrowth collectively may have enhanced the efficiency of derivation of germine-competent IVM pESCs. However, the mechanisms underlying pluripotency and germline competency of IVM pESCs from immature oocytes remain to be fully elucidated.

Remarkably, pESCs are able to repair damaged tissues following transplantation (28, 74). Moreover, pESCs from non-human primates restore function of neurons in experimental Parkinson’s disease and more excitingly no teratoma for- 

MATERIALS AND METHODS

Oocyte IVM

All mice were maintained and cared for based on approved animal protocols by the Institutional Animal Care Committee. Collection of immature oocytes from ovaries and IVM were similar to the procedure described previously with minor modification (77). Briefly, mice were primed with 5 IU of pregnant mare serum gonadotrophin (PMSG, Calbiochem). COCs at the GV stage were collected by puncturing follicles in 20 mm Hepes-buffered IVM medium 42–46 h after injection of PMSG. COCs were cultured in 100 μl droplets of IVM medium covered with mineral oil at 37°C in an atmosphere of 6.5% CO2 in humidified air for 15–16 h, when IVM oocytes reached the metaphase II (MII) stage. The IVM medium consisted of 95% MEM (Invitrogen), 5% FBS (Hyclone), 0.24 mm sodium pyruvate, 1.5 IU/ml human chorionic gonadotrophin (hCG) and 1 IU/ml PMSG.

Fertilized embryos and parthenogenetic ‘embryos’

Mice were superovulated by consecutive injections of 5 IU PMSG and 5 IU hCG 44–48 h apart. Successfully mated females 20–21 h after injection of 5 IU hCG were used for collecting zygotes (day 0.5). Freshly ovulated metaphase II (IVO) oocytes were collected from oviduct ampullae 14 h after injection of 5 IU hCG, then removed off cumulus cells by brief exposure to 0.03% hyaluronidase and pipetting. Parthenogenetic ‘embryos’ were produced by the activation of oocytes with SrCl2 and cytochalasin D, as described previously (60). Embryos were cultured in 50 μl droplets of potassium simplex optimized medium (KSOMAA) (78), covered with mineral oil at 37°C in an atmosphere of 6.5% CO2 in humidified air. IVO or IVM parthenogenetic blastocysts were obtained by culture for 96 h of activated IVO or IVM MII oocytes, respectively.

Isolation and culture of ES cells and IVM pESCs

Intact blastocysts 96 h cultured from zygotes or activated oocytes were seeded on feeder layers of mitomycin C-treated MEF, prepared on 0.1% gelatin-treated four-well dishes and cultured in the ESC medium consisting of Knockout DMEM (Invitrogen) and 20% FBS (Hyclone) or KSR (Invitrogen), supplemented with 1000 U/ml mouse ESGRO leukemia inhibitory factor (LIF, Chemicon), 0.1 mm NEAA, 1 mm l-glutamine, 0.1 mm β-mercaptoethanol, 50 IU/ml penicillin, 50 IU/ml streptomycin and 50 μM MAP kinase inhibitor PD98059 (Cell Signaling) (12). Half of the medium was changed daily, beginning from the second day when blastocysts were seeded. Approximately 5 days after seeding of fertilized blastocysts and IVO blastocysts, or 10 days after seeding of IVM blastocysts, ICM outgrowths were mechanically removed and disaggregated into small clumps and reseeded on fresh feeder cells. Stable ES-like cells were routinely obtained after two or three passages. ESC or IVM pESC lines were passaged and cultured in the ESC medium supplemented with FBS but without PD98059 following brief digestion with 0.25% trypsin–EDTA (GIBCO). For storage, cell lines were kept in freezing medium consisting of 40% ES medium, 50% FBS and 10% DMSO and stored frozen in liquid nitrogen.
Karyotype analysis and alkaline phosphatase assay

Metaphase chromosomes were prepared by exposing cells to 0.4 μg/ml nocodazole for 2 h, followed by hypotonic treatment with 75 mM KCl solution, fixed with methanol:glacial acetic acid (3:1) and spread onto clean slides. Spreads were stained with Giemsa and ~40 metaphase spreads examined from each cell culture. Alkaline phosphatase assay was performed using the Vector blue kit from Vector Laboratories.

Immunocytochemistry and fluorescence microscopy

ESCs or pESCs were cultured in tissue culture grade four-well plates for 2 days. Cells were fixed in freshly prepared paraformaldehyde [4% w/v in phosphate-buffered saline (PBS)], rinsed with blocking buffer (3% goat serum in PBS), permeabilized with 0.1% Triton X-100 in blocking buffer, washed and left in blocking solution for 1 h. Cells were incubated overnight at 4°C with mouse monoclonal antibody against Oct4 (Santa Cruz), SSEA-1 (DSHB, MC-480), GFAP (Invitrogen), MF20 (kindly provided by D.A. Fischman, Cornell University) or AFP antibody (DAKO), diluted at 1:100 in blocking solution. Primary antibodies were omitted for controls. After washing, cells were incubated with appropriate secondary antibodies conjugated to Alexa Fluor 568 (or 488) (Molecular Probes), washed and counterstained with 0.2 μg/ml Hoechst 33342 or DAPI in Vectashield mounting medium. Fluorescence was imaged under a Leica fluorescence microscope.

Teratoma formation test

Approximately 2 × 10⁶ cells were injected subcutaneously into 4-week-old immunodeficient nude mice to evaluate teratoma formation. Four weeks after injection, the resultant teratomas were excised, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned for histological examination.

Chimera generation, germline transmission and DNA microsatellite polymorphism analysis

Approximately 10–15 ESCs/pESCs were injected into blastocoeals of expanded host blastocysts (3.5 dpc) of different strains using a Piezo injector (12). Injected blastocysts were transferred into uterine horns of 2.5 dpc surrogate mice 1–4 h following ESC injection. Chimeras initially were identified by coat color. The contribution of ESCs to various tissues from chimeras was confirmed and estimated by standard DNA microsatellite genotyping analysis (12,24), using D8Mit4, D16Mit4 and D18Mit17 primers. Genomic DNA was extracted from tissues collected from chimeras, recipient tissues and donor ESCs/pESCs. By screening over 36 microsatellite markers from MGI (http://www.informatics.jax.org), some located on different chromosomes were identified to be polymorphic in our experiments, and therefore used for genotyping the chimeras. Microsatellite analysis was performed by polymerase chain reaction (PCR) amplification with primers designed from conserved sequences flanking each marker and subsequent electrophoresis using 15% polyacrylamide gels. The gels were silver-stained and scanned, and the genotype was determined. Ratios of contribution of stem cells in chimeras were estimated by quantification of the density of the bands after subtraction of the background noise using Bio-Rad Quantity One software. Some chimeras were randomly selected for breeding with albino strains, and further examined for transmission through the germline.

In vitro differentiation

ESCs/pESCs were trypsinized from six-well plates and injected for 50 min once or 30 min twice to remove feeder cells. Suspensions were centrifuged and ES cells transferred to a 100 cm Petri dish with the ESC medium without LIF for 3 days, then large and round-shaped EBs were placed into four-well plates, with three EBs per well, and cultured for 18 days, when cardiac-like muscles beat rhythmically. Some cells from day 3 EBs were collected for RNA extraction and real-time PCR analysis of germ cell marker genes. Fixation, immunostaining and microscopy for characterization of three embryonic germ layers were performed as described above.

Genome-wide methylation analysis by MeDIP and microarray

Genome-wide methylation was profiled by MeDIP coupled with NimbleGen microarray. MeDIP was performed according to a NimbleGen protocol, with slight modification from a previous study (79). Briefly, the DNA from pESCs and ESCs were digested with MseI, and the fragment size was verified on 2% agarose gel as 200 bp–1 kb. A monomolecular mouse anti-5-methylcytidine (Eurogentec) was used to attach the pooled beads:antibody:DNA were then digested with protease K and purified with phenol/chloroform/isoamyl alcohol. Each of the enriched immunoprecipitated (IP) DNAs (30 ng) obtained above and their corresponding input control (Input) were amplified with WGA2 (Sigma) and purified with QIAquick spin PCR kit (Qiagen).

The IPs were labeled as cy5, and inputs as cy3. Probe-level MeDIP enrichment was determined by the log2(cy5/cy3). Ratios were normalized within and between arrays using the R/Bioconductor limma library (http://www.bioconductor.org). Probe-level normalized enrichment was assigned to ROIs based on the array design provided by NimbleGen. ROIs are designed to span across CpG-rich regions, including gene promoter and distal CpG islands. For the selection of differential methylated ROIs, the Mann–Whitney non-parametric paired test was used. As a result, 53 ROIs with P < 0.05 on the comparison of fESCs with both IVM pESCs replicated experiments and the comparison of two replicates of fESCs and IVO pESCs were identified. Heat maps were created using the GPlot (http://www.astro.rug.nl/~gpsy/gp/plot/profile2dset.html). ROI-specific plots were created by custom R scripts. On average, about 15 probes made up each ROI. Using the midpoints of the genomic locations of all probes within an ROI, the ROI was subdivided into 10 bins and the MeDIP enrichment of probes within each bin was averaged. Each row on the heat map therefore represented
an ROI divided in 10 bins (columns), each representing the average MeDIP enrichment within it.

Re-defining ROIs on the different platforms: the original experiment was designed to compare IVM pESCs and fESCs on the 380 K platform, and the ROIs were defined using MM8 annotation. The new experiment compared IVO pESCs and fESCs on a 700 K platform using MM9-annotated ROIs. The MM8-annotated ROIs and probes were converted to MM9 using the UCSC genome lift-over tool. Originally, the experiment on the 380 K platform included 23 425 ROIs (357 296 probes); after the genome lift-over, 22 621 ROIs (340 491 probes) were retained for analysis of IVM pESCs versus fESCs. Using the MM9-annotated 22 621 ROIs, the probes on the 700 K platform were mapped to appropriate ROIs based on genomic location. The same 22 621 ROIs (340 519 probes) were used in the comparison analysis of IVO pESCs versus fESCs.

Real-time PCR
Total RNA was isolated from oocytes (50 per sample), fESCs/pESCs using RNeasy micro kit (Qiagen). Extracted RNA was quantified using a spectrophotometer (Eppendorf), and samples from which more than 1 µg of RNA was extracted were subjected to cDNA synthesis using Reverse Transcript System. Total RNA and PCR products were separated were subjected to cDNA synthesis using Reverse Transcrip-

DNA methylation by bisulfite sequencing
Genomic DNA was extracted from samples of ESCs/pESCs or oocytes using QIAamp DNA Micro Kit or DNeasy Tissue Kit (Qiagen) according to the manufacturer’s instructions. Bisulfite treatment of DNA was performed with the EpiTect Bisul-

REFERENCES


