RESEARCH REPORT

Induction of the G2/M transition stabilizes haploid embryonic stem cells

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ABSTRACT

The recent successful establishment of mouse parthenogenetic haploid embryonic stem cells (phESCs) and androgenetic haploid ESCs (ahESCs) has stimulated genetic research not only in vitro but also in vivo because of the germline competence of these cell lines. However, it is difficult to maintain the haploid status over time without a frequent sorting of the G1 phase haploid ESCs by fluorescence-activated cell sorting (FACS) at short intervals, because haploid cells tend to readily self-diploidize. To overcome this spontaneous diploid conversion, we developed a phESC culture condition using a small molecular inhibitor of Wee1 kinase to regulate the cell cycle by accelerating the G2/M phase transition and preventing re-entry into extra G1/S phase. Here, we demonstrate that, under this condition, phESCs maintained the haploid status for at least 4 weeks without the need for FACS. This method will greatly enhance the availability of these cells for genetic screening.

KEY WORDS: Parthenogenesis, Haploid embryonic stem cells, Cell cycle, Mouse

INTRODUCTION

The remarkable progress of forward genetics has largely depended on the use of haploid organisms with a single genome, such as bacteria and yeast. This is because a clearly distinguishable phenotype is evident, even in the case of recessive mutations of essential genes, due to the absence of an alternate gene copy to compensate for the modified or missing function. Therefore, researchers have endeavored to establish haploid cell lines from diploid organisms, such as human KBM-7 leukemia cell lines (Carette et al., 2009; Kotecki et al., 1999) and medaka ES cells (Yi et al., 2009).

Since 2011, several groups have successfully established parthenogenetic and androgenetic haploid ES cell lines (phESCs and ahESCs) from mouse haploid preimplantation embryos (Leeb and Wutz, 2011; Elling et al., 2011; Yang et al., 2012; Li et al., 2012) using the 2i condition (Ying et al., 2008). The 2i culture condition provides a serum-free environment that blocks the activity of both ERK and GSK3 by the inhibitors PD0325901 and CHIR99021, respectively, and is used to establish diploid ES cells with high germline transmissibility. The haploid ES cell lines obtained using the 2i condition exhibit stemness and differentiation capacity, as well as germline transmission via chimeric mice (Leeb et al., 2012). However, it is difficult to maintain haploid ES cells in the haploid state over time, as they tend to diploidize spontaneously. Therefore, frequent G1 phase sorting of the haploid ES cells by fluorescence-activated cell sorting (FACS) is an inevitable requirement.

It remains unclear how these so readily diploidize, although the diploid state is congenitally more stable than the haploid state. One conceivable mechanism is that the diploidization occurs due to abnormal cell cycle regulation in the haploid cells; for example, by skipping cell division once and re-entering into an extra G1/S phase. In this study, we attempted to develop culture conditions suitable for the long-term maintenance of haploid ESCs by regulating their cell cycle using an inhibitor of Wee1 kinase in order to induce the occurrence of a normal transition at the G2/M phase.

RESULTS AND DISCUSSION

Derivation of phESCs and effect of the Wee1 inhibitor PD166285 on stabilizing the haploid state of phESCs

We attempted to generate phESCs from the C57BL/6 (B6) strain because of its known advantages for genetic research. The developmental rate of haploid blastocysts from the B6 oocytes was actually lower than those from F1 hybrid mice, such as BDF1 and DBF1 (18%, 35% and 50%, respectively; Table 1). Their morphology at the blastocyst stage was also poor compared with diploid embryos as well as with BDF1 or DBF1 haploid embryos; nevertheless, the morphology was still good until the morula stage (supplementary material Fig. S1A). Therefore, we tried to establish ES cells from embryos at the morula stage or earlier and successfully obtained 64 ES cell lines, including 27 haploid ES cell lines (Table 1, Fig. 1A).

In present study, phESCs (which exhibit the haploid karyotype; Fig. 1B, left and inset) established by existing culture condition, also exhibited the previously reported self-diploidization (Fig. 1B, middle and right) (Leeb and Wutz, 2011; Elling et al., 2011), and frequent sorting of the G1 phase phESCs was inevitably required at short intervals to maintain their haploid status (supplementary material Fig. S1B). Therefore, it should be an important issue to elucidate the mechanism of spontaneous diploidization in haploid ES cells.

Polyploid cells in a diploid organism are formed by cell fusion, endoreplication and a variety of defects that result in an abnormal cell cycling (Storchova and Pellman, 2004). During endoreplication, cells skip mitosis (e.g. polytene chromosomes) or omit cytokinesis (e.g. megakaryocytes), and proceed through several rounds of DNA replication, resulting in polyploid cells, which generally do not proliferate further (Edgar and Orr-Weaver,
In this study, we assumed that spontaneous diploidization of haploid cells occurs both in vivo and in vitro as the result of an abnormal cell cycle progression, because haploid ES cells exhibited rapid proliferating ability and evidence from previous reports also suggested that cell fusion and endoreplication cannot be the cause of increasing ploidy in haploid ES cells (Taylor, 2002; Vignery, 2000; Leeb et al., 2012; Lanni and Jacks, 1998; Andreassen et al., 2001).

Table 1. Establishment of parthenogenetic haploid ES cell lines

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Genetic background</th>
<th>Oocyte activated</th>
<th>Embryo status</th>
<th>Number of embryos</th>
<th>ES cell lines obtained</th>
<th>ES cell lines with the haploid contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57BL/6</td>
<td>174</td>
<td>8 cell</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16 cell</td>
<td>20</td>
<td>8</td>
<td>3 (15%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Morula</td>
<td>48</td>
<td>25</td>
<td>13 (27%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Blastocyst</td>
<td>20</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>C57BL/6 (CAG-EGFP)</td>
<td>180</td>
<td>Morula</td>
<td>63</td>
<td>21</td>
<td>7 (16%)</td>
</tr>
<tr>
<td>3</td>
<td>C57BL/6</td>
<td>137</td>
<td>Morula</td>
<td>38</td>
<td>18</td>
<td>7 (18%)</td>
</tr>
<tr>
<td>4</td>
<td>BDF1</td>
<td>120</td>
<td>Morula</td>
<td>72</td>
<td>60</td>
<td>21 (35%)</td>
</tr>
<tr>
<td>5</td>
<td>DBF1</td>
<td>68</td>
<td>Morula</td>
<td>21</td>
<td>12</td>
<td>6 (50%)</td>
</tr>
</tbody>
</table>

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Fig. 1. Spontaneous diploidization of phESCs. (A) Morphology of parthenogenetic haploid morula and phESCs before FACS sorting. (B) Flow cytometry analysis of DNA contents by Hoechst 33342 staining and karyotyping of phESCs using DAPI staining (inset). (C) Hypothetical mechanism of self-diploidization of haploid ES cells. Spontaneous diploidization may occur by inhibition of cyclin B1/Cdc2 complex by Wee1, which inhibits Cdc2 dephosphorylation for inactivation of Cdc2. (D) Flow cytometry analysis of DNA contents. (E) Morphology of the phESCs-B6 cultured with 300 nM PD166285 appeared healthy under a feeder condition. (F) Growth curve of PD166285-treated and untreated phESCs during short-term culture (up to 12 days). Scale bars: 50 μm.
In the normal cell cycle, the cyclin A/B-Cdc2 complex actively promotes cell cycle progression from the G2 to M phase via Cdc2 dephosphorylation by Cdc25, while Wee1 and Myt1 inhibit the activity of this complex via Cdc2 phosphorylation, causing G2 arrest at a DNA damage check point (Perry and Kornbluth, 2007; top panel of Fig. 1C showing the supposed normal cell cycle in haploid cells). We hypothesized that the diploid conversion of phESCs occurs by transient G2 arrest and the abrupt insertion of an extra G1/S phase (Fig. 1C, bottom), because the diploid state is more stable for the cells. Therefore, we attempted to regulate the haploid cell cycle by adding the small molecule Wee1 inhibitor PD166285 to facilitate a normal transition from the G2 to M phase without entry into the extra G1/S phase. We first employed culture conditions of 300, 500 or 1000 nM of PD166285 (supplementary material Fig. S2A) using phESCs-B6 cells harvested after the sorting of G1 phase haploid cells (Fig. 1D). In accordance with a previous report (Hashimoto et al., 2006), phESCs-B6 cultured with 500 or 1000 nM of PD166285 exhibited a growth-retarded morphology, suggesting an anti-proliferative effect of PD166285. However, in the case of treatment with 300 nM PD166285, phESCs-B6 formed more compact colonies than those without PD166285 under the usual culture condition with feeder cells, as well as under a feeder-free...
condition (Fig. 1E; supplementary material Fig. S2A). No difference was observed in the doubling time of phESCs-B6 with and without PD166285 over a period of 12 days (Fig. 1F). As shown in Fig. 2A and supplementary material Fig. S2B, the cells in the 300 nM PD166285 group retained a haploid state for at least 4 weeks (long term culture). In detail, the ratio of the haploid G1 phase (1N) cells in the phESCs-B6 cells with 300 nM PD166285 was evidently higher than that without PD166285, even after 33 days under the feeder-free condition (26.8 and 12.1%, respectively), and the ratio of the diploid G2 phase (4N) cells was lower than that without PD166285 (8.5% and 12.3%).

To determine the effectiveness of Wee1 inhibition, a stringent test was performed using 24 independent phESC colonies derived from two phESC lines and culturing them for 33 days with and without PD166285. The number of clones that survived out of the 24 colonies after 33 days of culture were 21 (line 1) and 20 (line 2) with...
PD166285, and 18 (both lines 1 and 2) without PD166285. Among them, 16 colonies (both lines 1 and 2) maintained a haploid karyotype with >20% of 1N cells under the Wee1 inhibiting condition, while only 1 (line 1) and 0 (line 2) colonies were present without PD166285. The average 1N cell ratios were 27.2 and 24.4 versus 12.7 and 11.3, respectively (Fig. 2B). We also tested the effect of another Wee1 inhibitor, MK1775, on maintaining the haploidy of the phESCs during long-term culture (4 weeks). The ratio of the 1N cells treated with 300 nM MK1775 was also higher than that of control (Fig. 2C, top). Importantly, the doubling time during days 11-31 of both the PD166285- and MK1775-treated cells relatively shorter (1.06, 1.12, line 1 and 0.99, 1.13, line 2) than that of control (1.18, line 1 and 1.34, line 2; Fig. 2C, bottom). These results clearly demonstrate that the acceleration of the G2/M transition by means of these Wee1 inhibitors prevented the spontaneous diploidization of the haploid cells and efficiently maintained the haploid status of phESCs-B6 cells, even though further optimization of this procedure is still needed.

Characterization of phESCs treated with PD166285
Most of the cells in the two phESCs-B6 lines had a complete haploid set of 20 chromosomes, with the identified diploid cells below 10% and no abnormal chromosomes detected by G-band analysis (supplementary material Fig. S2C). Detailed array comparative genomic hybridization (aCGH) analysis of GFP-expressing phESCs-B6 revealed that the genome stability of the phESCs was well maintained in this culture system (Fig. 3A).

To compare the gene expression profiles of phESCs-B6 and mESCs-B6 (natural mating ESCs), we performed DNA microarray analysis using G1 phase cells collected by FACS. They exhibited very similar global gene expression profiles (Fig. 3B) and expressed several stem cell marker genes as normal diploid mESCs (supplementary material Fig. S2D), although a slight difference in the gene expression between pharthenogenetic ESCs (both haploid and diploid) and mESCs was detected (supplementary material Fig. S2E). In terms of pluripotency, phESCs also had alkaline phosphatase activity (Fig. 3C, left) in addition to the typical ES cell markers, including SSEA1, Nanog and Oct3/4, as shown by immunostaining analysis (Fig. 3C, right). We further investigated the in vitro differentiation capacity of phESCs-B6 by subcutaneous injection and the formation of a teratoma comprising three germ layers was confirmed (Fig. 3D). These results suggest that phESCs treated with PD166285 have normal capacity of pluripotency as well as differentiation in vitro and in vivo.

Epigenetic properties and germline competence of phESCs treated with PD166285
DNA methylation analyses of the differentially methylated regions (DMRs) in several of the imprinted regions were performed by COBRA and bisulfite sequencing. The paternally imprinted regions, such as IG and H19 DMRs were unmethylated in phESCs-B6, while methylation of maternally imprinted regions, such as Snrpn and Igf2r DMRs remained, confirming their parthenogenetic origin. However, maternal DMRs were not fully methylated, especially in the later passages (around passage 60), they were completely unmethylated (Fig. 3E, supplementary material Fig. S3A). Furthermore, we analyzed phESCs-B6 cells in passage three and found that even these cells were unable to maintain a complete maternal imprinting status, suggesting methylation was perturbed at the initial stage of the establishment of haploid cells (supplementary material Fig. S3B). It has also been reported that diploid parthenogenetic ES cell lines from B6C3F1 hybrid mouse are not able to maintain a maternally imprinted status (Li et al., 2009) and that the in vitro culture environment was capable of introducing alterations into the imprinting status of mouse diploid ES cell lines (Humphreys et al., 2001), especially in the case of prolonged passage (Dean et al., 1998). For future applications of these phESCs to artificial reproductive technology (ART), the problem of the imprinting issue needs to be overcome, as in the previous report for semi-cloning experiment using phESCs (Wan et al., 2013).

Next, to evaluate the ability of the phESCs-B6GFP cells treated with PD166285 to physically contribute to mice, we injected several dozen G1 phase phESCs-B6GFP (GFP-positive) cells into C57BL/6 (GFP-negative) blastocysts (Fig. 3F, left). GFP-positive embryos at embryonic day (E) 14.5 were smaller than GFP-negative embryos, but no morphological abnormalities were found in these tissues (Fig. 3F, middle). Through FACS analysis, ~50% of the entire body of the fetus exhibited chimerism, with the brain having the highest degree but no chimerism detected in the liver, indicating a different proportion of GFP-positive cells in each tissue and embryo (Fig. 3F, right). GFP-positive cells were also observed in the female gonads (Fig. 3F, middle), suggesting probable germline transmission of phESCs-B6GFP cells. Expectedly, one chimeric female mouse successfully delivered healthy, GFP-positive mice after mating with a GFP-negative male (Fig. 3G), demonstrating the germline competence of the phESCs-B6GFP cells. We next tried to introduce genetic modifications into phESCs-B6GFP by infecting them with CSII-EF-HRasV12-IRES2-hKO1 (humanized Kusabira-Orange 1) and CSII-EF-IRES2-hKO1 lentivirus. It was revealed that both were successfully introduced while retaining a haploid state under Wee1 inhibition (Fig. 3H), although HRasV12 may affect haploidy negatively at long-term culture.

In summary, phESCs were established from C57BL/6 mice and the culture conditions were modified so as to maintain the haploid status over a long period of time. These results should enable a more useful application of haploid ESCs for forward and reverse genetic screening. Furthermore, our findings will provide a new insight on cell cycle regulation in haploid ES cells.

MATERIALS AND METHODS

Animals
All mouse experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (TMDU).

Derivation of phESCs and cell culture
Oocytes (C57BL/6NCr and C57BL/6CAG-EGFP) were activated using 5 mM SrCl2 and 2 mM EGTA (Kishigami and Wakayama, 2007), cultured in vitro until the morula stage, and the morula embryos were put on feeder staining with Hoechst 33342 (37°C, 30 min) on a FACS AriaII (BD), as described previously (Leeb and Wutz, 2011). After staining, haploid cells were cultured in ES medium containing 300 nM PD166285 and MK1775, respectively. The analysis of the DNA content was performed after fixation with 70% ethanol and staining with propidium iodide (PI) on a FACS Calibur (BD). For the purpose of carrying out a stringent test, 24 colonies derived from each of the two phESC lines were isolated and cultured for 33 days with or without PD166285 under a feeder-free condition. For karyotype analysis, colcemid-treated (0.1 μg/ml) phESCs were resuspended 0.075 M KCl, and fixed in methanol-acetic acid (3:1). G-banding analysis was performed at Nihon Gene Research Laboratories.

Array CGH analysis
Genomic DNA was isolated from purified G1 phase phESCs (approximately 2×10^6 cells) by FACS. As a reference for the array used for CGH analysis, the C57BL/6NCr liver genome was used. The Cy3-labeled reference and
Cy5-labeled ES genomes were hybridized to an In situ oligo DNA microarray kit (4×180K, Agilent).

**Gene expression analysis by microarray**
The cells in the G1 phase (approximately 2×10⁵ cells) were collected from phESCs and pdESCs by FACs, and total RNA was isolated by Trizol (Invitrogen). Microarray analysis was performed using a whole mouse genome DNA microarray kit (4×44K ver.2, Agilent).

**Alkaline phosphatase activity and immunocytochemistry of stem cell markers**
Alkaline phosphatase activity was detected using a BCIP/NBT alkaline phosphatase substrate kit (VECTOR). For immunofluorescence staining, the cells were incubated with goat anti-mouse Oct3/4, rabbit anti-mouse Nanog and purified mouse anti-SSEA1 (MC480) antibody at 4°C overnight (1/100; Santa Cruz sc-8629, sc-33760 and BD 560079, respectively). Alexa Fluor 594-conjugated donkey anti-goat IgG, Alexa Fluor 686-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-mouse IgM were used as the secondary antibodies (1/750; Invitrogen A10105, A10106 and A21238, respectively).

**Teratoma formation**
phESCs (2×10⁵) were injected subcutaneously into nude mice. After 30 days, the dissected teratoma was processed for paraffin sectioning after fixation and stained with Hematoxylin and Eosin (HE).

**DNA methylation analysis**
Genomic DNA from G1 phase phESCs was treated with sodium bisulfite. For COBRA, amplified bisulfite PCR products using specific primer sets (supplementary material Table S1) were digested with restriction enzymes, including PvuII, Acil, HhaI and TaqI. Bisulfite PCR products were subcloned and sequenced.

**Injection of phESCs into diploid blastocysts**
Eight-cell host embryos (C57BL/6) at 2.5 dpc were cultured in KSM medium until the blastocyst stage. Only G1 phase phESCs were injected into host blastocysts and blastocysts were transferred into each uterine horn at 2.5 dpc in pseudopregnant ICR. Pregnant recipients were dissected on day 14.5. Analysis of chimerism was performed using a FACS AriaII.

**Lentiviral infection of phESCs**
Lentivirus particles were produced by transient transfection of a CSII-EF-HRasV12-IRES2-hKO1 and a CSII-EF-IRES2-hKO1 vector (gifts from Drs T. Wakayama, Yamanashi University) into 293T cells using Fugene HD, and Lentivirus infection of phESCs by transient transfection of specific primer sets (VECTOR). Fluor 594-conjugated donkey anti-goat IgG, Alexa Fluor 568-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-mouse IgM were used as the secondary antibodies (1/750; Invitrogen A10105, A10106 and A21238, respectively).

**Accession numbers**
The NCBI accession numbers for the gene expression and CGH reported in this paper are GSE55446 and GSM1132971.

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**Competing interests**
The authors declare no competing financial interests.

**Author contributions**
F.I. and T.K.-I. conceived of the study and S.T., J.L., T.K., T.K.-I. and F.I. participated in the experimental design and data analysis. S.T., J.L. and A.M. performed most of the analyses. M.K. and M.K.-A. performed chimera mouse analysis. S.T., J.L., T.K., T.K.-I. and F.I. wrote the manuscript. All of the authors read and approved the final manuscript.

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**Supplementary material**
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110726/-/DC1

**References**


