Single-Cell Dynamic Analysis of Mitosis in Haploid Embryonic Stem Cells Shows the Prolonged Metaphase and Its Association with Self-diploidization

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SUMMARY
The recent establishment of mammalian haploid embryonic stem cells (ESCs) provides new possibilities for genetic screening and for understanding genome evolution and function. However, the dynamics of mitosis in haploid ESCs is still unclear. Here, we report that the duration of mitosis in haploid ESCs, especially the metaphase, is significantly longer than that in diploid ESCs. Delaying mitosis by chemicals increased self-diploidization of haploid ESCs, while shortening mitosis stabilized haploid ESCs. Taken together, our study suggests that the delayed mitosis of haploid ESCs is associated with self-diploidization.

INTRODUCTION
Most animal cells are diploid, and haploidy is generally limited to the gametes. However, haploid embryonic stem cells (ESCs) have recently been established from both parthenogenetic and androgenetic embryos of several species (Elling et al., 2011; Leeb and Wutz, 2011; Li et al., 2012; Sagi et al., 2016; Yang et al., 2012, 2013). These haploid ESCs have provided exciting possibilities in many aspects (Elling et al., 2011; Li et al., 2014; Wutz, 2014; Yang et al., 2012). However, the haploid state is not stable and haploid ESCs tend to diploidize spontaneously during continuous cell passage (Elling et al., 2011; Leeb et al., 2012; Li et al., 2012, 2014; Yang et al., 2012). Although suppressing the self-diploidization of haploid ESCs is very much needed, it is still unknown how haploid ESCs undergo self-diploidization.

The cell cycle is the most important process in the growth of organisms, and is tightly linked to cell proliferation, cell-fate decisions, and many other cell functions (Bower et al., 2016; Dalton, 2015; Pauklin and Vallier, 2013). Recent studies have demonstrated that the duration of each cell-cycle phase is important for stem cell self-renewal and differentiation: the G1 phase is associated with cell-fate specification (Dalton, 2013; Pauklin and Vallier, 2013; Singh et al., 2015), while the S and G2 phases actively promote the pluripotent state (Gonzales et al., 2015). Although the cell cycle of diploid cells has been extensively studied, the cell cycle of haploid ESCs is far less understood. Interestingly, a recent study reported that accelerating G2/M transition could partially stabilize mouse haploid ESCs, suggesting an interconnection between the cell cycle and self-diploidization of haploid ESCs (Takahashi et al., 2014). However, whether the M phase itself is associated with the self-diploidization of haploid ESCs is elusive.

RESULTS
The Cell Cycle in Haploid ESCs Was Longer Than That in Diploid ESCs
Although the cell-cycle progression in normal diploid ESCs has been well studied, the dynamics of cell cycles in haploid ESCs is still unknown. Due to the spontaneous diploidization of haploid ESCs, it is difficult to separate haploid ESCs from the bulk cells and examine cell-cycle progression by measuring cellular DNA content with fluorescence-activated cell sorting (FACS). To overcome this problem and directly visualize cell-cycle progression in haploid ESCs, we took advantage of the Fucci (fluorescent ubiquitination-based cell-cycle indicator) technology, which labels G1 phase nuclei in red and S-G2/M phases nuclei in green (Figure 1A; Sakaue-Sawano et al., 2008), and established two Fucci-probe-expressing haploid mouse ESC lines, namely Fucci-HG165 and Fucci-A7. These cell lines made it possible to separate both haploid and diploid populations from the bulk cells for simultaneous cell-cycle analysis (Figure 1B). Using Hoechst 33342 staining followed by FACS analysis, we found that the percentage of G1 phase in haploid ESCs was almost the same as that in diploid mouse ESCs, while the percentage of G2 phase...
was slightly higher in haploid ESCs than in diploid ESCs (Figure 1C). To accurately quantify the proportion of cells in S phase, we performed a double staining with both Hoechst and EdU, and found that haploid ESCs exhibited slightly but not statistically significantly shorter S phase than diploid ESCs (Figures 1D and S1A). Next, we combined the Fucci technology with immunostaining of phospho-histone H3 (Ser28), a specific marker of the M phase, which allowed us to measure the percentages of mitotic cells in haploid and diploid ESCs (Figure 1E). Interestingly, we found that the percentage of mitotic cells was significantly increased in haploid ESCs than in diploid ESCs (Figures 1E and 1F), indicating distinct dynamics of mitosis in haploid and diploid ESCs.

The Fucci-probe expressing haploid ESCs not only facilitated cell-cycle analysis at the population level, but also made it possible to visualize and analyze cell-cycle dynamics at the single-cell level. Using time-lapse imaging, we analyzed the whole cell cycle of 46 single haploid and diploid ESCs, respectively (Figures 1G, 1H, S1B, and S1C; Movies S1 and S2). Interestingly, while the cell-cycle lengths of our diploid ESCs were consistent with previous reports (Figures 1H and S1D; Ahuja et al., 2016; Coronado et al., 2013; Re et al., 2014; Roccio et al., 2013), haploid ESCs exhibited a significantly prolonged cell cycle (Figures 1H, 1I, S1C, and S1D). Statistical analysis showed that the duration of the G1 phase was almost the same in both haploid and diploid ESCs, but the total length of the S and G2/M phases was about 5 hr longer in haploid ESCs than in diploid cells (Figures 1I, 1J, S1D, and S1E), consistent with a marginal although not statistically significant increase of the S-G2/M proportion in haploid cells (Figures 1J and S1E). Taken together, our results showed that mouse haploid ESCs had unique cell-cycle dynamics compared with diploid ESCs.

**Haploid ESCs Exhibited a Significant Mitotic Delay**

To further examine the mitosis of haploid ESCs, we established two haploid ESC lines stably expressing a fusion protein of histone H2B and red fluorescent protein (H2B-RFP), and evaluated mitosis progression in live cells (Figures 2A, 2B, S2A, and S2B; Movies S3 and S4). Our results showed that diploid ESCs derived by the self-diploidization of haploid ESCs could complete mitosis in 32 min on average, similar to normal diploid ESCs (E14) (Figures 2A and 2C); however, mitosis in haploid ESCs took 60 min on average (Figures 2B and 2C). Similar results were also obtained from another haploid cell line (Figures S2A–S2C). Our observations demonstrated that the duration of mitosis was significantly prolonged in haploid ESCs (Figures 1E and 1F).

Mitosis consists of four basic phases: prophase, metaphase, anaphase, and telophase. We thus further examined haploid ESCs at each phase of mitosis. Although the durations of prophase, anaphase, and telophase were comparable between haploid and diploid ESCs, our observation uncovered a significant delay of prometaphase/metaphase in haploid ESCs (Figure 2D). On average, prometaphase/metaphase was 1.9-fold longer in haploid ESCs than in diploid ESCs (35 versus 19 min) (Figure 2D), and the percentage of prometaphase/metaphase was also significantly increased in haploid ESCs (Figure 2E). More detailed metaphase analysis showed that prometaphase/metaphase duration varied a lot in haploid ESCs, with 64% of cells dividing normally and 36% of cells significantly delayed (prometaphase/metaphase duration >30 min) (Figure 2F). Again, similar results were obtained from another haploid cell line (Figures S2D and S2E). These results showed that haploid ESCs exhibited a significant mitotic delay, especially in the prometaphase/metaphase.

**Visualization of Haploid ESC Self-diploidization**

Haploid ESCs exhibited a tendency of rapid, spontaneous, and irreversible diploidization, which made it difficult to maintain their haploid state (Leeb and Wutz, 2011; Li et al., 2012; Yang et al., 2012). It is therefore important to understand how and when self-diploidization happens. Diploidization has been reported primarily to be the result of endoreduplication of the haploid genome instead of cell fusion (Leeb et al., 2012). One recent study suggested that the cell cycle is also related to diploidization (Takahashi et al., 2014). Indeed, mitosis failures have been reported...
to be a major reason for generating polyploid cells from diploid cells (Ganem et al., 2007; Stegemann et al., 2008). Therefore, we hypothesized that self-diploidization might be related to the prolonged mitosis in haploid ESCs. To test this hypothesis, we directly visualized the self-diploidization events of haploid ESCs using live-cell imaging. Interestingly, we found that some haploid ESCs failed to divide into daughter cells during mitosis (Figures 3A, 3B, S2F, and S2G), a phenomenon that we had almost never seen in diploid ESCs. Moreover, we observed two types of mitosis failures in haploid ESCs: some haploid ESCs entered mitosis normally and exhibited a typical prophase, but stayed at prometaphase/metaphase for an abnormally long time and finally failed to divide into two cells (type I) (Figures 3A and S2G); some other haploid ESCs only underwent nuclear division but not cytoplasmic division and thus bore two nuclei, which then fused together at the metaphase of the next cycle of mitosis, when the cell formed one spindle and finally divide into two diploid cells (type II) (Figure 3B). We also analyzed the prometaphase/metaphase duration of those haploid ESCs that were undergoing diploidization and found that all haploid cells undergoing diploidization had an extremely longer prometaphase/metaphase length than other haploid ESCs (Figure 3C). These observations suggested that self-diploidization happened during mitosis and was a result of mitosis failure.

Pharmacologically Induced Mitotic Delay Increased the Self-diploidization Rate of Haploid ESCs

To further examine whether prolonged mitosis could promote diploidization, we treated haploid ESCs with S-trityl-L-cysteine (STLC) and nocodazole (Figures S3A and S3B), two widely used mitotic inhibitors that reversibly pause the prometaphase and metaphase by inhibiting EG5-mediated centrosome separation or depolymerizing microtubules, respectively. After being treated with these inhibitors or DMSO for 5 hr, cells were subsequently cultured in drug-free medium and analyzed by time-lapse live imaging for 24 hr. We found that haploid ESCs treated with either STLC or nocodazole exhibited a substantial delay in mitosis (Figures 3D and 3E), especially in the prometaphase/metaphase (Figures 3F and 3G). In contrast, these two chemicals did not appear to impact prophase, anaphase, or telophase in haploid ESCs (Figure 3F). We then performed long-term experiments by repeating the daily 5-hr treatment for 10 days and determined the self-diploidization rate of haploid ESCs by FACS analysis. Compared with control, both STLC- and nocodazole-treated haploid ESCs consisted of significantly less 1N cells, indicating less haploid cells and more diploid cells (Figures 3H and 3I). Since the growth rate and apoptosis did not show significant difference between haploid and diploid cells upon inhibitor treatments (Figures S3A and S3B), our data suggested that chemical-induced mitosis delay could promote self-diploidization of haploid ESCs. Interestingly, we found that STLC and nocodazole also prolonged mitosis of diploid ESCs and promoted tetraploidization (Figures S3C–S3F), suggesting a general role of prolonged mitosis in generating polyploid cells.

Recently, we have identified by small-molecule screening a chemical cocktail RDF (R, Repsox, an inhibitor of the TGF-β pathway; D, DMH1, an inhibitor of the BMP4 pathway; F, Forskolin, an adenylate cyclase activator) that could stabilize haploid ESCs (data not shown). As shown in Figures 4A and 4B, RDF treatment led to a significant increase in the proportion of 1N cells in the haploid ESC culture, suggesting a reduced self-diploidization rate. To investigate whether RDF inhibited diploidization by shortening the mitosis duration, we analyzed the mitosis dynamics of RDF-treated haploid ESCs. As expected, these cells exhibited a shorter mitosis duration than control cells (Figure 4C). Further analysis showed that prometaphase/metaphase, but not the other phases, was shortened upon RDF treatment (Figures 4D and 4E). We then tested whether PD166285, a chemical reported to stabilize haploid ESCs by promoting G2/M transition (Takahashi et al., 2014), could shorten mitosis as well. However, PD166285 treatment did not shorten mitosis of haploid ESCs (Figures S3G and S3H), consistent with a former report in diploid cells (Araujo et al., 2016). Taken together, our results indicated that either shortening mitosis or promoting G2/M transition could reduce diploidization. While PD166285 repressed diploidization via promoting G2/M transition, RDF functioned through shortening mitosis.

Figure 2. Single-Cell Cell-Cycle Analysis Shows a Longer Mitosis in Haploid ESCs

(A and B) Time-lapse images of dividing diploid (A) and haploid (B) ESCs expressing H2B-RFP and tubulin-EGFP. The arrowheads show the dividing cells.

(C) Duration of mitosis in haploid and diploid ESCs (n > 20 single cells per group; 4 independent experiments).

(D and E) Duration (D) and percentages (E) of each mitosis stage in haploid and diploid ESCs (n > 20 single cells per group; 4 independent experiments).

(F) Distribution of prometaphase + metaphase duration of haploid and diploid ESCs (n > 20 single cells per group; 4 independent experiments).

Data are shown as mean ± SEM, *p < 0.05.
Overexpression of Aurora B Shortened Mitosis and Reduced Self-diploidization of Haploid ESCs

To better understand the difference between the mitosis of haploid and diploid ESCs and its relationship with self-diploidization, we set experiments to analyze the gene expression in the mitotic cells of haploid and diploid ESCs. We first sorted mitotic cells positive for phosphorylated histone H3 (Ser28) from both haploid and diploid ESCs, and extracted RNA from these cells using a novel method (Figures S4A and S4B) that allows extraction of high-quality RNA from fixed and stained cells (Hrvatin et al., 2014). Then we analyzed expression levels of several cell-cycle-related genes by qPCR. Compared with diploid mitotic ESCs, the haploid mitotic ESCs expressed a significantly lower level of Aurora B (Aurkb), an important regulator of mitosis progression; however, expression levels of Aurora A (Aurka), Cyclin B (Ccnb), and Polo-like kinase 1 (Plk1) were comparable in both haploid and diploid mitotic ESCs (Figure 4F).

Aurora B is required for the phosphorylation of histone H3, and thus important for chromosome condensation (Goto et al., 2002; Hsu et al., 2000). It is also required for correct chromosome alignment and segregation during metaphase, and therefore regulates kinetochore functions (Afonso et al., 2016; Cheng et al., 2011; Lampson and Cheeseman, 2011). In addition, Aurora B plays critical roles in spindle checkpoint and cytokinesis (Ruchaud et al., 2007; Steigemann et al., 2008). Consistently, Aurora B deficiency or inactivation was involved in cytokinesis failure and tetraploidization in diploid cells (Giet et al., 2005; Steigemann et al., 2008). Given the important roles of Aurora B in mitosis, we overexpressed Aurora B in haploid ESCs and performed mitosis analysis (Figures S4C and S4D). The results showed that the duration of mitosis, especially prometaphase/metaphase, was shortened and the self-diploidization rate was decreased in Aurora B-overexpressing cells (Figures 4G–4J), while the growth rate or apoptosis were not significantly affected (Figures S3A and S3B). In contrast, overexpressing Aurora A did not affect the self-diploidization of haploid ESCs (Figures 4I and 4J). Taken together, our data suggested that Aurora B was an important regulator of self-diploidization, and that shortening mitosis by overexpressing Aurora B could restrain haploid ESCs from self-diploidization.

DISCUSSION

Control of cell-cycle progression has been linked to the regulation of self-renewal and cell-fate determination in ESCs (Pauklin and Vallier, 2013; Singh et al., 2015; White and Dalton, 2005). It has been reported that the length of the G1 phase defines the capacity of multipotent stem cells to differentiate in vivo, and that the S and G2 phases actively promote the pluripotent state (Gonzales et al., 2015). However, the interconnection between the M phase and cell-fate determination has remained enigmatic. In this study, we showed that the prolonged metaphase of M phase was associated with the self-diploidization of haploid ESCs, although it is still difficult to determine the direct causality between prolonged mitosis and self-diploidization. By manipulating mitosis progression with pharmacological and genetic approaches, we successfully reduced the self-diploidization rate of haploid ESCs. Although we cannot exclude the possibility that the chemicals we used to manipulate mitosis progression might suppress haploid ESC self-diploidization through other mechanisms due to their broad effects on cells, our results have clearly shown that cell-fate determination is associated with the M phase.

Haploid ESCs have been established in many species, including fish, mouse, rat, monkey, and human. Mouse haploid ESCs tend to rapidly lose the haploid karyotype during differentiation into germ layers, but are relatively stable during extra-embryonic differentiation (Leeb et al., 2012). While our study was based on mouse haploid ESCs, it would be interesting to use a similar strategy to do comparative studies on the mitosis dynamics of haploid ESCs derived from other species.

Cells divide and reproduce in two ways, mitosis and meiosis. During mitosis, diploid cells replicate their chromosomes to produce cells with doubled DNA content (4N) and then divide into daughter cells (2N); while in meiosis, cells with replicated chromosomes (4N) undergo...
A

Control RDF

B

Percentage of 1N %

C

Duration (min)

D

Mitotic cells (%)

E

Duration (min)

F

Aurka

G

Duration (min)

H

Duration (min)

I

J

(legend on next page)
two continuous cell divisions and generate haploid gametes (1N) (Duesbery and Vande Woude, 2002). It is well known that oocytes arrest at the metaphase of the second meiotic division (metaphase II) and await fertilization to complete the meiotic process (Masui and Markert, 1971). Interestingly, we found in our single-cell level observation that the delayed metaphase of haploid ESCs resembled the metaphase II arrest in oocytes, suggesting some common features between the mitosis of haploid ESCs and the meiosis of oocytes. Indeed, compared with M phase diploid ESCs, we found that M phase haploid ESCs expressed a significantly higher level of Mos (Figure S4E), which is the key component of a signal pathway that activates the cytostatic factor to ensure the metaphase II arrest in oocytes (Duesbery and Vande Woude, 2002; Schmidt et al., 2006; Yew et al., 1993). Therefore, we speculated on a similar molecular mechanism between metaphase II arrest of meiosis in oocytes and the delayed metaphase of mitosis in haploid ESCs, and propose that in vitro study of haploid ESCs might provide hints for revealing mechanisms underlying meiosis, which still lacks an in vitro system for study.

Finally, although we observed a prometaphase/metaphase delay in haploid ESCs and demonstrated its association with self-diploidization, the detailed mechanism underlying such an association is still unclear. It is possible that all cells bear an internal clock that monitors the metaphase duration. In this scenario, when a delayed metaphase is detected, it triggers certain signals similar to those in meiotic cells, which promote the cells to double their DNA content by preventing nuclear division or cell division. Identifying such molecular mechanisms is still a high priority in future studies of haploid ESCs.

**EXPERIMENTAL PROCEDURES**

**Live Imaging and Quantification**

Live imaging was performed using the Olympus FV1200MPE or FV10i confocal microscope with a 60×/1.3 silicon oil UPLSAPO60XS. For the analysis of Fucci-tagged cells, images were taken every 30 min with z stacks for more than 24 hr, and images were analyzed by Olympus FV10-ASW 4.2 software. For the analysis of H2B-RFP cells, images were taken every 3 or 4 min with z stacks for the indicated times and mitotic cells were assessed by the morphology of chromosome.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.03.025.

**AUTHOR CONTRIBUTIONS**

A.G., L.S., and G.P. conceived and designed the research; A.G., J.Y., and H.L. performed the experiments; A.G., H.W., and L.S. analyzed the data; A.G., S. H., L.S., and G.P. wrote the manuscript.

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