Identification and propagation of haploid human pluripotent stem cells

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Haploid human pluripotent stem cells (PSCs) integrate haploidy and pluripotency, providing a novel system for functional genomics and developmental research in humans. We have recently derived haploid human embryonic stem cells (ESCs) by parthenogenesis and demonstrated their wide differentiation potential and applicability for genetic screening. Because haploid cells can spontaneously become diploid, their enrichment at an early passage is key for successful derivation. In this protocol, we describe two methodologies, namely metaphase spread analysis and cell sorting, for the identification of haploid human cells within parthenogenetic ESC lines. The cell sorting approach also enables the isolation of haploid cells at low percentages, as well as the maintenance of highly enriched haploid ESC lines throughout passaging. The isolation of essentially pure populations of haploid human ESCs by this protocol requires basic PSC culture expertise and can be achieved within 4–6 weeks.

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INTRODUCTION

Haploid cells offer unique opportunities for functional genomics using loss-of-function screening, as well as for exploring the role of ploidy in shaping genomic architecture and developmental processes. Devising strategies for reaping the benefits of using haploid cells in mammals, and humans in particular, could thus provide valuable tools for biomedical research. In mammals, as in most animals, fertilization of haploid gametes results in a diploid zygote that gives rise to all the cells of the organism[1]. Thus, the fundamental mammalian chromosomal complement is diploid. Although deviation from a diploid karyotype occurs in specific lineages during development[2] and is a hallmark of tumorigenesis[2,3], mammalian haploidy is normally regained only via meiosis and is not propagated through cell division.

The discoveries of an aneuploid chronic myeloid leukemia cell line with a near-haploid karyotype and its haploid derivative have provided powerful means for genetic screening in human cells[4,5]. However, a different experimental paradigm was required for obtaining nontransformed human cells capable of dividing while carrying a haploid genome. Unfertilized mammalian oocytes can be activated experimentally to initiate preimplantation development through parthenogenesis, providing a source of cells with a haploid set of maternally inherited chromosomes[6].

Importantly, although haploidy can persist in parthenogenetic blastocysts, it may be lost in derived ESCs as a result of diploidization[7]. Nevertheless, in recent years, self-renewing haploid parthenogenetic ESCs have been isolated from several mammalian species, including mouse[8,9], rat[10] and macaque monkey[11]. Interestingly, haploid androgenetic ESCs from mice[12,13] and rats[14] have also been generated by replacing the genome of the oocyte with that of a spermatocyte.

We recently derived haploid human ESCs through parthenogenesis[15]. We hypothesized that, as in other species, haploid cells should be present in at least some human parthenogenetic ESC lines originating from haploid oocytes, as long as ploidy is analyzed before complete diploidization. Indeed, low proportions (~1%) of haploid cells were identified within 2 of 14 early-passage human parthenogenetic ESC lines. Despite this rarity, we subsequently established two individual haploid ESC lines by repeated rounds of enrichment using fluorescence-activated cell sorting (FACS), which enables distinction between haploids and diploids according to their DNA content (**Fig. 1**). These cells could be cultured in conventional conditions that are commonly used for culturing diploid human ESCs. To dispose of diploidized cells and to maintain a highly enriched haploid cell population throughout passaging, the same DNA-based FACS strategy was used. In this protocol, we describe the methods for identifying, isolating and maintaining haploid human ESCs from existing human parthenogenetic ESC lines[16–19].

Molecular, cellular and developmental features of haploid human ESCs

Haploid human ESCs feature several molecular and cellular properties that make them highly similar to their diploid counterparts, but also have properties that set them apart[15]. Like conventional diploid human PSCs, haploid human ESCs display a typical morphology and can self-renew while preserving genomic integrity and a stable karyotype. Haploid human ESCs also exhibit a molecular signature characteristic of undifferentiated human PSCs based on the regulation of various pluripotency-specific markers at the levels of DNA methylation, mRNA and protein expression and enzymatic activity[15]. As parthenogenetic cells, haploid human ESCs also exhibit the expected maternal patterns of epigenetic regulation and gene expression at imprinted loci[15,20]. Importantly, isogenic haploid and diploid cells could not be distinguished by their relative global gene expression patterns, indicating robust maintenance of the pluripotent state in spite of different ploidies. However, haploid and diploid cells differ in four main aspects[15]: (i) haploid cells have around half the volume of diploid cells; (ii) their absolute gene expression levels are markedly reduced; (iii) they do not undergo X chromosome inactivation, which is often observed in one of the two X chromosomes in female diploid human PSCs[21,22]; and (iv) they display a relative upregulation of oxidative phosphorylation genes, likely arising from differential mitochondrial abundance. Notably, none of these differences seem to require the modification of

the conventional culture conditions regularly used for growing diploid human PSCs.

A defining hallmark of PSCs is their intrinsic ability to differentiate into any embryonic or adult cell fate[23]. Remarkably, haploid human ESCs maintain this developmental potential, as they can be differentiated into somatic cells of all three embryonic germ layers, including neurons, cardiomyocytes and pancreatic cells, while remaining haploid[15]. Importantly, differentiation of haploid somatic cells is also observed in teratomas *in vivo*. Taken together, these findings indicate that haploid human ESCs qualify as fully pluripotent cells, and that haploidy is compatible with both proliferation and differentiation of human cells.

Applications of haploid human ESCs and comparison with other experimental systems

Haploid human ESCs hold an exceptional applicative potential, owing to the combination of several key advantages that exist only partly in alternative experimental systems, namely haploidy, pluripotency and a stable human genome.

The predominant advantage associated with haploid cells is their utility for loss-of-function forward genetic screens[24,25], which rely on phenotypic selection of perturbed genes underlying a specific biological process. In diploid cells, screening for recessive traits is challenging, as targeting a single allele is insufficient to induce a phenotype. Although global loss-of-function screens can be performed in diploid cells by RNA interference and nuclease-based genome editing[26,27], these approaches require prior knowledge of target sequences and often involve incomplete gene silencing or variable targeting efficiencies. By contrast, in haploid cells, random mutagenesis is applicable, as any single targeting event can be effectively translated into a selectable phenotype. Indeed, haploid genetic screens have been performed in a wide variety of biological systems, from haploid yeast[28] to transformed haploid human cancer cells[4,5]. The discovery of haploid ESCs in nonhuman species has extended haploid genetics beyond the limits of a single cellular identity, by allowing genetic dissection of the developmental processes[29,30] of cells ranging from undifferentiated cells to potentially any type of differentiated cell. Aside from their differentiation potential, the unlimited proliferation capacity of PSCs and their amenability to genetic manipulation enhance their utility for genetic screening. Now that haploid human ESCs have been derived, the advantages of haploid pluripotent cells are applicable in the context of a stable human genome. As a proof-of-principle, we identified the autosomal purine metabolism gene *NUDT5* (ref. 31) as part of the toxicity pathway of the purine analog 6-thioguanine by screening a library of randomly mutagenized haploid human ESCs[15]. These results emphasize that haploid human ESCs readily facilitate genetic screening involving cytotoxic selection, but additional screening assays (e.g., by flow cytometry) should also be applicable.

Haploid human ESCs may be useful for pursuing additional biomedical objectives not involving genetic screening. For example, studying their diploidization may lead to a better understanding of ploidy changes in cancer. Moreover, the surprising ability of human ESCs to differentiate by virtue of a haploid genome may provide insights into ploidy requirements and the effects of aneuploidy during development. As the genomic content of haploid human ESCs is equivalent to that of mature gametes, they may also prove valuable in reproductive applications.

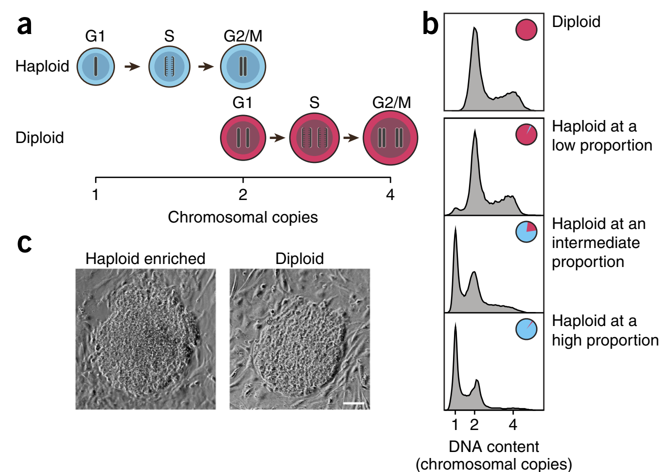

Figure 1 | Use of DNA content to distinguish between haploid and diploid human ESCs. (**a**) The number of chromosomal copies (c) varies according to ploidy and cell cycle stage. In a mixed population, 1c cells are haploids in G1, 2c cells are a mixture of haploids in G2/M and diploids in G1, and 4c cells are diploids in G2/M. (**b**) Flow cytometry plots showing the DNA content profiles of diploid ESCs and ESC samples containing increasing proportions of haploid cells, obtained by repeated rounds of 1c-cell enrichment and expansion (1×10^5 pES10 cells[15] were used to generate each flow cytometry plot). (**c**) Colony morphology of haploid-enriched and diploid human pES10 cells. Scale bar, 50 μm. Experiments involving human ESCs were conducted at the Hebrew University under the guidelines of the Bioethics Advisory Committee of the Israel Academy of Sciences and Humanities, and were also approved by the ESC Research Oversight Committee and the Institutional Review Board at Columbia University Medical Center. **c** adapted with permission from ref. 15, Nature Publishing Group.

Development of the protocol

The identification and maintenance of haploid human ESCs require methodologies that allow discrimination between haploid and diploid cells. Because previous attempts to isolate haploid human PSCs had only yielded diploid cells[19,32], we made two important improvements. First, we analyzed parthenogenetic ESC lines at early passage (passages 3–9). Second, to identify haploid cells within a population of diploid cells, we used both chromosome counting by metaphase spread analysis and viable DNA-based FACS.

Metaphase spreading is a cytogenetic method for investigating the number and structure of chromosomes in dividing cells by microscopic analysis. In this protocol, metaphase spreading is used to produce a quantitative readout of the proportion of rare haploid cells in a parthenogenetic ESC line, as ploidy can be directly assigned to each metaphase by enumerating the chromosomes: 23 chromosomes in a haploid metaphase and 46 chromosomes in a diploid metaphase. DNA-based FACS is performed by viable DNA staining with Hoechst 33342, followed by isolation of cells with a DNA content corresponding to less than two chromosomal copies—that is, potentially haploid cells in G1 (**Fig. 1**). By this approach, haploid ESCs present in the culture can be simultaneously detected and isolated as an initial step in establishing a haploid human ESC line. Upon detection of haploid cells by metaphase spread analysis, isolation is performed as a subsequent step.

Another important consideration is the survival of dissociated haploid human ESCs after sorting. Contrary to their murine counterparts, human PSCs are prone to apoptosis upon single-cell dissociation. However, single ESC survival is strongly increased in

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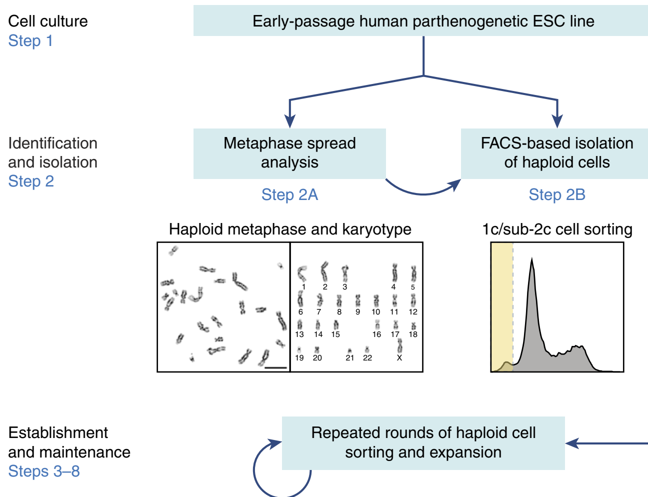

Figure 2 | Schematic overview of the protocol. To identify haploid human ESCs in a human parthenogenetic ESC line, the cells can be subjected to either metaphase spread analysis or DNA-based FACS. Metaphase spread analysis is quicker and more conclusive, and is also useful for karyotype analysis. However, it requires FACS for isolation of haploids subsequent to the determination of ploidy, which is inherently included in the FACS-based detection analysis. After the initial isolation of haploid ESCs, they are further enriched by repeated rounds of sorting and enrichment, allowing the establishment and maintenance of a highly enriched haploid human ESC line. The metaphase (scale bar, 5 μm), karyotype and DNA content profile shown are of early-passage pES10 cells[15]. Experiments involving human ESCs were conducted at the Hebrew University under the guidelines of the Bioethics Advisory Committee of the Israel Academy of Sciences and Humanities, and were also approved by the ESC Research Oversight Committee and the institutional review board at Columbia University Medical Center.

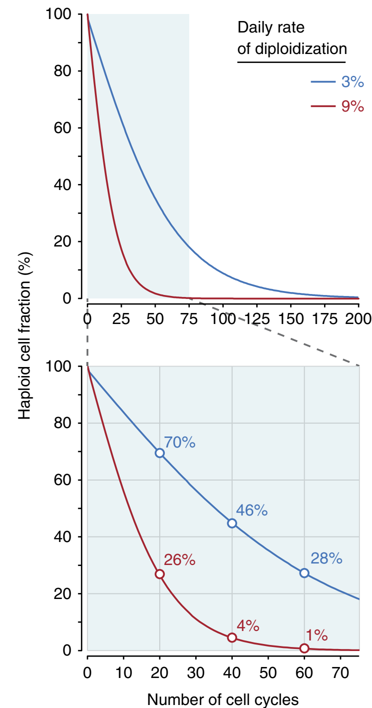

Figure 3 | Determination of the frequency of FACS-based enrichment rounds for maintaining a haploid human ESC line. Computational kinetic models for daily diploidization rates[15] of 3% (blue curve) and 9% (red curve) during the first 75 and 200 cell cycles (lower and upper panels, respectively. The first 75 cell cycles are highlighted by a light blue background. Initial population size = 1×10^6). The remaining fraction of haploid cells for both rates is approximated for every five passages (assuming an average doubling time of ~1 d and passaging every 3–4 d with a split ratio of 1:6).

the presence of the small molecule Y-27632, which acts as a Rho-associated kinase (ROCK) inhibitor[33]. We therefore incorporate the use of Y-27632 during and after FACS, allowing the growth of ESC colonies from single haploid cells within 2–7 d.

Limitations of the protocol

As mentioned above, establishment of new haploid human ESC lines by this protocol involves early-passage human parthenogenetic ESC lines. The derivation of these cell lines requires accessibility to human oocytes and subjecting them to parthenogenesis, which is possible only in certain countries, depending on policy[34]. Parthenogenesis can be performed by a calcium pulse in the presence of a translation inhibitor, which allows second polar body extrusion and the generation of a haploid egg with a single maternal pronucleus. Approximately 25% of these eggs develop to the blastocyst stage, enabling subsequent derivation of parthenogenetic ESC lines. We have isolated haploid ESCs from parthenogenetic cell lines that were originally derived under normoxic conditions in the presence of the ROCK inhibitor Y-27632 and passaged enzymatically after manual picking of the initial cell outgrowth[15]. Most existing human parthenogenetic ESC lines were found to be diploid, and although it is unclear when diploidization occurred, haploid ESC cultures are known to diploidize during passaging. Therefore, new parthenogenetic ESC lines should be analyzed at the earliest passage possible (passages 3–5), and enrichment sorts should be performed repeatedly to establish a human haploid ESC line. Diploidization may also occur during the process of genetic modification, such as in the context of genetic screening, but this is not a major limitation, as modifications would be homozygous if they occur in the haploid state.

Haploid human parthenogenetic ESCs carry only a single maternal copy of the genome. Although human parthenogenetic PSCs are functionally pluripotent based on their ability to give rise to all embryonic lineages[16,17,20], mammalian parthenogenetic development is restricted because of the non-equivalence of parental genomes[35,36]. Therefore, the uniparental origin of haploid cells must be considered in any genetic or developmental analysis. The protocol described here should also be applicable to the isolation of haploid androgenetic ESCs, providing the paternal counterpart of haploid parthenogenetic ESCs. Androgenesis would require introducing a sperm genome carrying an X chromosome into an enucleated oocyte, as a Y-chromosome-carrying sperm would not support preimplantation development. Effectively, this means that only half of the oocytes would potentially give rise to blastocysts.

Overview of the procedure

The following protocol describes the identification of haploid cells in human parthenogenetic ESC lines, the isolation of new haploid human ESC lines and the maintenance of established cell lines, as schematically outlined in **Figure 2**. First, we describe the procedures required for routinely handling human parthenogenetic ESCs in culture, including detailed instructions for thawing frozen cells, expanding cells for downstream analyses and freezing cells for future use (Step 1). Next, we describe the two methodologies for determining the presence of haploid cells within early-passage

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Box 1 | Assessment of the diploidization rate of haploid human ESCs

● TIMING 3–6 weeks for preparing the samples and ~2 h for flow cytometry analysis

The continuous diploidization of haploid human ESCs decreases the percentage of haploids over time, which requires periodic enrichment of haploid cells by DNA-based FACS. To determine the proper frequency of enrichment rounds, the diploidization rate can be assessed by performing the following assay:

1. Choose one six-well plate containing highly enriched haploid human ESCs at 80–90% confluence.
2. Aspirate the medium, wash once with PBS and trypsizine the cells as in Step 1A(ix) of the PROCEDURE. After aspirating the trypsin, use 6 ml of human ESC medium to detach and collect the cells in a 15-ml conical tube.
3. Transfer 1 ml of the cell suspension to a new 15-ml conical tube, and add 11 ml of human ESC medium and Y-27632 to a concentration of 10 μM, and plate the human ESCs on a new MEF-containing six-well plate (prepared as in Step 1A(i–iii) of the PROCEDURE, split ratio of 1:6).
4. Transfer another 1 ml of the cell suspension from step 2 to a new 15-ml conical tube (discard the remaining 4 ml). Centrifuge the tube at 150*g* for 5 min at 4 °C, remove the supernatant, and resuspend the cell pellet using 0.5 ml of cold (4 °C) PBS. In a chemical fume hood, fix the cells by adding 4.5 ml of cold (4 °C) methanol in a dropwise manner while vortexing in shaking mode (at low speed), and store the sample at –20 °C.

■ PAUSE POINT The sample can be stored at –20 °C for 1–2 months.

5. Continue culturing the cells from step 3 until they reach 80–90% confluence, and repeat step (1–4) until fixed cell samples from at least five consecutive passages are obtained. Mark the date and passage number on each sample.
6. Once all samples are ready for analysis, centrifuge them at 150*g* for 5 min at 4 °C, aspirate the supernatant, and resuspend the cell pellets using 0.5 ml of PBS at room temperature.
7. Incubate the samples with RNase A at a final concentration of 200 μg per ml for 30 min at room temperature.
8. Add PI to a final concentration of 50 μg per ml, and incubate for 5 min at room temperature.
9. Filter each sample through a 70-μm cell strainer into a sterile FACS tube and keep the FACS tubes on ice and protected from light.
10. Analyze the samples by flow cytometry and quantify the proportion of the 1c-cell population.
11. Using a statistical software, plot the percentage of 1c cells versus time in days, and fit the data to an exponential function ($y = e^{-\lambda x}$). Calculate the daily diploidization rate using the formula $(1 - e^{-\lambda}) \times 100\%$.

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human parthenogenetic ESC lines, metaphase spread analysis and DNA-based FACS, followed by (or coinciding with) the initial isolation of haploid ESCs (Step 2). After the initial isolation of haploid human ESCs by DNA-based FACS, the same methodology is repeatedly performed to achieve and maintain a high degree of haploid-cell enrichment (Steps 3–8).

Experimental design

Culture of human parthenogenetic ESCs. Both haploid and diploid human ESCs can be grown in conventional culture conditions, including feeder-dependent and feeder-free conditions, as detailed in Step 1A and Step 1B, respectively. In feeder-dependent conditions, cells are cultured on growth-arrested mouse embryonic fibroblasts (MEFs) in standard human ESC medium containing knockout serum replacement (KSR). In feeder-free conditions, the cells are cultured on Matrigel-coated plates in defined medium, such as mTeSR1 medium. Undifferentiated human PSCs typically display a high nuclear-to-cytoplasmic content ratio and grow as dense colonies. Human PSCs are particularly sensitive to perturbations in culture conditions, which might result in spontaneous differentiation, cell death or genomic instability[37,38]. It is therefore critical to continually monitor the cells by assessing their morphology and growth, as well as to ensure that the cultures are not contaminated by bacteria or fungi. Performing karyotype analysis every 10–20 passages is highly recommended to confirm chromosomal stability.

Identification and isolation of haploid cells in early-passage human parthenogenetic ESC lines. Critically, the potential of isolating haploid cells from a given parthenogenetic ESC line decreases with passage number because of irreversible diploidization, and therefore ploidy analysis should be performed at the earliest passage available. Metaphase spread analysis has several advantages over cell sorting for the identification of rare haploid cells. Confirming haploidy by metaphase spreading is less time-consuming (3 d compared with ~2 weeks by FACS), and is mostly unambiguous, as metaphase chromosomes can be easily discerned and counted. By contrast, the haploidy of sorted cells remains inconclusive until confirmed by a follow-up analysis of the presumably haploid-enriched sorted population. This is especially relevant when haploids are indistinguishable from background, in which case sorted events may not be viable cells or may even be diploid cells. In addition, relatively fewer ESCs are required to obtain sufficient numbers of metaphases for analysis (even $<5 \times 10^6$ cells as compared with $\sim 30 \times 10^6$ cells), which means shorter expansion times and analysis at an earlier passage. Hundreds of metaphases can be analyzed per sample, reaching a detection resolution of below 1%. Contrary to cell sorting, metaphase spread analysis is applied to fixed cells and can therefore be performed independently of cell survival considerations. At the same time, however, a fraction of the cells must be sacrificed. In that regard, an advantage of cell sorting is that the identification of haploid ESCs is inherently coupled to their initial isolation, which in metaphase spread analysis must be included afterward.

Further enrichment and maintenance of haploid ESC lines by DNA-based FACS. At the first sort, low numbers of haploid cells may be present in the culture, increasing the chance of inadvertently including diploid cells during sorting. Upon consecutive rounds of FACS-based enrichment and expansion, the haploid proportion increases, allowing the establishment of a highly pure haploid human ESC line within as little as five such rounds[15].

In principle, sorting should be repeated as soon as the sorted haploid cells are sufficiently expanded. The routine maintenance of a haploid human ESC line throughout passaging also relies on occasional enrichment, the frequency of which is directly related to the diploidization rate of the cells, which may be variable. In our experience, diploidization rates can range between 3 and 9% of cells per day and can be modeled to fit an exponential decay curve[15]. On the basis of this range and our model, the haploid population of a fully haploid culture could drop to 26–70% after 20 d in culture, stressing the importance of occasional enrichment (**Fig. 3** and **Box 1**).

Controls for DNA-based FACS of haploid human ESCs. Several controls can be included when performing DNA-based FACS of haploid human ESCs (as described in Steps 2B(i–x) and 4). As a negative control, we recommend allocating a fraction of the cell sample to analysis without Hoechst 33342 staining, which should result in no detectable flow cytometry signal. In addition, it is useful to analyze a diploid human ESC sample as reference for determining the 1c-cell gate. The unstained sample and diploid reference sample controls are especially important when setting up the system for the first time. A sorting purity control is informative for confirming a high degree of haploid-cell enrichment, especially for certain downstream experiments such as comparative analyses and genetic manipulations. However, note that this control is not recommended in the initial isolation of haploid cells, as their quantity may be very low.

MATERIALS

REAGENTS

- ddH_2O
- Gelatin (MP Biomedicals, cat. no. 960317)
- DMEM with high glucose, GlutaMAX supplement and pyruvate (Thermo Fisher Scientific, cat. no. 10569044)
- FBS (Biological Industries, cat. no. 04-007-1, or Thermo Fisher Scientific, cat. no. 16000044)
- Penicillin–streptomycin solution (penicillin 10,000 units per ml, streptomycin 10 mg per ml; Biological Industries, cat. no. 03-031-1B, or Thermo Fisher Scientific, cat. no. 15140148)
- PBS without $CaCl_2$, $MgCl_2$ (Sigma-Aldrich, cat. no. D8537)
- BSA (Sigma-Aldrich, cat. no. A9418)
- Human basic fibroblast growth factor (bFGF) (R&D Systems, cat. no. 4114-TC)
- Knockout DMEM (Thermo Fisher Scientific, cat. no. 10829018)
- KSR (Thermo Fisher Scientific, cat. no. 10828028)
- L-Glutamine solution (200 mM; Biological Industries, cat. no. 03-020-1A, or Thermo Fisher Scientific, cat. no. 25030081)
- Nonessential amino acid solution (100×; Biological Industries, cat. no. 01-340-1B, or Thermo Fisher Scientific, cat. no. 11140035)
- β-Mercaptoethanol (55 mM; Thermo Fisher Scientific, cat. no. 21985023) **! CAUTION** β-Mercaptoethanol is toxic by ingestion and absorption through the skin, and it can cause irritation if inhaled or if it comes into contact with the eyes.
- DMSO (Sigma-Aldrich, cat. no. D4540) **! CAUTION** DMSO can be absorbed through the skin; avoid inhalation and contact with the skin and eyes.
- Y-27632 2HCl (Selleckchem, cat. no. S1049)
- Ethanol, 70% (vol/vol) (Fisher Scientific, cat. no. 04-355-309)
- Irradiated CF-1 MEFs (GlobalStem, cat. no. GSC-6201G)
- DMEM/F12 (Thermo Fisher Scientific, cat. no. 11320082)
- Matrigel human ESC-qualified matrix (Corning, cat. no. 354277)
- mTeSR1 medium (StemCell Technologies, cat. no. 05850)
- Human parthenogenetic ESC lines, such as pES10 and pES12 cells, described in Sagi *et al.*[15] (available from the authors through a material transfer agreement generated by Yissum (the Research Development Company of the Hebrew University) and the New York Stem Cell Foundation; both cell lines were authenticated by single-nucleotide polymorphism (SNP) genotyping and were free of *Mycoplasma*) **! CAUTION** All relevant national and institutional regulations relating to the use of human parthenogenetic ESCs must be followed. The derivation of new human parthenogenetic ESC lines from human oocytes must conform to the appropriate national laws and institutional regulatory board guidelines, and it requires informed consent from oocyte donors. Experiments involving human ESCs were conducted at the Hebrew University under the guidelines of the Bioethics Advisory Committee of the Israel Academy of Sciences and Humanities, and were also approved by the ESC Research Oversight Committee and the institutional review board at Columbia University Medical Center **! CAUTION** Cell lines should be regularly authenticated and tested for *Mycoplasma contamination*.
- *Mycoplasma* detection kit (Lonza, cat. no. LT07-418)
- Trypsin–EDTA (Biological Industries, cat. no. 03-052-1A, or Thermo Fisher Scientific, cat. no. 25200056)
- Colcemid solution (Biological Industries, cat. no. 12-004-1, or Thermo Fisher Scientific, cat. no. 15212012)
- dH_2O
- KCl (Sigma-Aldrich, cat. no. P9333) **! CAUTION** KCl may cause irritation on contact with the eyes, skin or respiratory tract.
- Sodium citrate (Sigma-Aldrich, cat. no. W302600) **! CAUTION** Sodium citrate may cause irritation on contact with the eyes, skin or respiratory tract.
- Methanol (Sigma-Aldrich, cat. no. 34860) **! CAUTION** Methanol is flammable and toxic if inhaled or ingested, or if it comes into contact with the skin.
- Acetic acid (Sigma-Aldrich, cat. no. 320099) **! CAUTION** Acetic acid is flammable and can cause burns if inhaled or ingested, or if it comes into contact with the skin or eyes.
- Ethanol, 100% (vol/vol) (Sigma-Aldrich, cat. no. 24102)
- Buffer tablets, pH 6.8 (Merck Millipore, cat. no. 111374)
- Earle's Balanced Salt Solution (Biological Industries, cat. no. 02-010-1A, or Thermo Fisher Scientific, cat. no. 24010043)
- Trypsin–EDTA, no phenol red (Thermo Fisher Scientific, cat. no. 15400054)
- Giemsa stain, modified (Sigma-Aldrich, cat. no. GS500) **! CAUTION** Giemsa stain is hazardous if ingested and may cause irritation on contact with the skin and respiratory tract.
- Eukitt mounting medium (O. Kindler)
- Immersion oil (Sigma-Aldrich, cat. no. 56822)
- TrypLE Select (Thermo Fisher Scientific, cat. no. 12563029)
- Hoechst 33342 (Sigma-Aldrich, cat. no. B2261-25MG) **! CAUTION** Hoechst 33342 is toxic if ingested and can cause irritation on contact with the skin and respiratory tract.
- RNase A (Sigma-Aldrich, cat. no. R6513)
- Propidium iodide (PI; Sigma-Aldrich, cat. no. P4170) **! CAUTION** PI is a suspected mutagen and can cause irritation on contact with the eyes, skin and respiratory tract.
- Bleach (Sigma-Aldrich, cat. no. 239305)

EQUIPMENT

- Autoclave
- Chemical fume hood
- Tissue culture hood
- Vacuum aspiration system
- Humidified tissue culture incubator, 37 °C, 5% CO_2 (such as Forma Series II 3110, Thermo Fisher Scientific, cat. no. 3110)
- Water bath (37 °C)
- Refrigerator (4 °C)
- Freezer (−20 °C)
- Freezer (−80 °C)
- Liquid nitrogen storage container (such as CryoPlus, Thermo Fisher Scientific)
- Filter tips (Gilson)
- 1.5-ml Microcentrifuge tubes (Fisher Scientific, cat. no. 05-408-129)
- 1.5-ml Light-protected microcentrifuge tubes (Sigma-Aldrich, cat. no. Z688312)
- 2-ml Pipettes (Greiner Bio-One, cat. no. 710180)
- 5-ml Pipettes (Greiner Bio-One, cat. no. 606180)
- 10-ml Pipettes (Greiner Bio-One, cat. no. 607180)
- 15-ml Conical tubes (Corning, cat. no. 352096)
- 50-ml Conical tubes (Corning, cat. no. 352070)
- Cell culture plates, six wells (Greiner Bio-One, cat. no. 657160)

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- Cell culture centrifuge (Eppendorf)
- Light microscope (such as Zeiss Axioskop 40)
- Sterile cryogenic storage vials (Sigma-Aldrich, cat. no. V7634)
- Isopropanol chamber (e.g., Mr Frosty freezing container; Thermo Fisher Scientific, cat. no. 5100-0001) Fisher Scientific, cat. no. 5100-0001)
- Vortex mixer
- Plastic Pasteur pipettes, 1 ml (Thermo Fisher Scientific, cat. no. 201C)
- Glass bottles, 100 ml (Corning, cat. no. 1395-100)
- Glass microscope slides (Fisher Scientific, cat. no. 12-544-7)
- Delicate task wipers (Kimberly-Clark, cat. no. 34155)
- Hot plate
- Erlenmeyer flask, 125 ml (Fisher Scientific, cat. no. FB500125)
- Drying oven (such as Heraeus B 6030, Thermo Fisher Scientific)
- Coplin staining jars (Fisher Scientific, cat. no. 08-815)
- 0.2-µm Filter
- Coverslips (glass; Fisher Scientific, cat. no. 12-541A)
- 70-µm Cell strainers (Corning, cat. no. 431751)
- Sterile FACS tubes, 5 ml, 12 × 75 mm, round-bottom (Corning, cat. no. 352003)
- Cell sorter with a near-UV or 405-nm laser (such as BD FACSAria III or BD Influx, BD Biosciences)

REAGENT SETUP

Gelatin solution (500 ml), 0.2% (wt/vol) Add 1 g of gelatin to 500 ml of ddH_2O and autoclave it. Gelatin solution can be stored at room temperature (25 °C) for up to 1 year.

MEF medium (500 ml) To prepare 500 ml of MEF medium, in a tissue culture hood, supplement 447.5 ml of DMEM (high glucose, GlutaMAX supplement, pyruvate) with 50 ml of FBS and 2.5 ml of penicillin–streptomycin solution. MEF medium can be stored at 4 °C for up to 1 month.

Human bFGF solution (5 µg ml^{-1}) In a tissue culture hood, reconstitute 100 µg of human bFGF in 20 ml of PBS with 0.2% (wt/vol) sterile BSA. Divide the solution into aliquots and store them at −20 °C for up to 3 months.

Human ESC medium (500 ml) To prepare 500 ml of human ESC medium, in a tissue culture hood, supplement 411.5 ml of knockout DMEM with 75 ml of KSR, 5 ml of L-glutamine solution (200 mM), 5 ml of nonessential amino acid solution (100×), 2.5 ml of penicillin–streptomycin solution and 1 ml of 55 mM β-mercaptoethanol (final concentration 0.1 mM). Human ESC medium can be stored at 4 °C for up to 1 month. Freshly add human bFGF before use to a final concentration of 10 ng ml^{-1}. ▲ CRITICAL Keep human ESC medium protected from light, as KSR is light-sensitive.

Freezing medium (50 ml) In a tissue culture hood, mix 18 ml of DMEM, 27 ml of FBS and 5 ml of DMSO. Freezing medium can be stored at 4 °C for up to 1 month.

Y-27632 stock solution (10 mM) In a tissue culture hood, dissolve 10 mg of Y-27632 2HCl in 3.123 ml of DMSO. Divide the solution into aliquots and store them at −20 °C for up to 1 year.

Hypotonic solution Dissolve 0.56 g of KCl and 0.5 g of sodium citrate in 200 ml of dH_2O. Hypotonic solution can be stored at room temperature for up to 1 year.

Fixative solution In a chemical fume hood, mix 12 ml of methanol with 4 ml of acetic acid in a glass bottle. Fixative solution should be freshly prepared and kept at −20 °C before use.

Buffer pH 6.8 Dissolve one tablet of buffer pH 6.8 in 1,000 ml of dH_2O. Buffer pH 6.8 can be stored at 5–25 °C and used up to the stated expiry date.

Hoechst 33342 stock solution (25 mg per ml) In a tissue culture hood, dissolve 25 mg of Hoechst 33342 in 1 ml of sterile ddH_2O. Divide the solution into aliquots in light-protected microcentrifuge tubes, and store them at −20 °C for up to 1 year. ▲ CRITICAL Keep Hoechst 33342 protected from light.

FACS medium In a tissue culture hood, add 7.5 ml of KSR to 42.5 ml of PBS without $CaCl_2$ and $MgCl_2$. FACS medium can be stored at 4 °C for up to 1 month.

EQUIPMENT SETUP

Gelatin-coated plates In a tissue culture hood, cover the bottom of the plates with 0.2% (wt/vol) gelatin solution (1 ml per well of a six-well plate), and keep the plates at room temperature for 30 min. Aspirate the solution before use.

Matrigel-coated plates Thaw Matrigel on ice. In a tissue culture hood, dilute Matrigel in cold (4 °C) DMEM/F12 at a ratio of 1:30 (vol/vol) and use the mixture to cover the bottom of the plates (for example, dilute 100 µl of Matrigel in 3 ml of DMEM/F12, and cover the bottom of each well of a six-well plate with 0.5 ml of the mixture). Incubate the plates at 37 °C for 1 h, and aspirate the Matrigel-DMEM/F12 before use. ▲ CRITICAL Matrigel solidifies at room temperature and should therefore be kept cold before coating.

Glass microscope slides for metaphase spreading In a chemical fume hood, submerge glass microscope slides in 100% (vol/vol) ethanol at room temperature for 1 h. Dry the slides by gently wiping them with delicate task wipers on both sides. Write down sample information on the designated area of the slides, and put the slides into dH_2O.

KSR-coated collection tubes In a tissue culture hood, fill sterile 15-ml conical tubes with FACS medium (prepared as described in the Reagent Setup) and store them at 4 °C for at least 1 h before FACS. Before use, aspirate the medium and replace it with 1–2 ml of human ESC medium containing 10 µM Y-27632 (Y-27632 stock solution diluted 1:1,000).

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PROCEDURE

Culture of human parthenogenetic ESCs: thawing, expansion and freezing ● TIMING 1–2 weeks

1| Culture haploid and diploid ESCs in either feeder-dependent conditions (option A) or feeder-free conditions (option B).
▲ CRITICAL STEP Research involving human parthenogenetic ESCs must conform to national laws and institutional regulatory board guidelines. Human PSC cultures should be monitored microscopically on a daily basis. Conventionally, human PSCs grow as dense colonies, with individual cells displaying a high nuclear-to-cytoplasmic content ratio. Culturing the cells in suboptimal conditions may result in varying degrees of spontaneous differentiation and cell death. Routine testing for *Mycoplasma* contamination is highly recommended.
? TROUBLESHOOTING

(A) Culture of human parthenogenetic ESCs in feeder-dependent conditions

(i) Take one frozen vial of irradiated CF-1 MEFs (containing 2 × 10^6 cells) from liquid nitrogen, place it in a 37 °C water bath without submerging the cap, and swirl gently until its contents are almost completely thawed (2–3 min). Immediately dry the vial, spray it with 70% (vol/vol) ethanol and place it in a tissue culture hood. Uncap the vial, add 1 ml of MEF medium using a 2-ml pipette and transfer the MEF cell suspension to a 50-ml conical tube containing 34 ml of warm (37 °C) MEF medium.
▲ CRITICAL STEP Resuspension of MEFs in cell culture medium should be performed rapidly, as 10% (vol/vol) DMSO harms cells at room temperature.

(ii) Take three gelatin-coated six-well plates, prepared as detailed in the Equipment Setup, and place them in the tissue culture hood. Aspirate the gelatin solution.

(iii) Plate the MEFs on the gelatin-coated plates by transferring 2 ml of the MEF cell suspension to each well of a six-well plate, and place the plates inside a humidified tissue culture incubator. To disperse the cells

evenly, gently, yet quickly, rock the plates back and forth and sideways a few times. Proceed to the next step after 3–24 h of incubation.

▲ **CRITICAL STEP** Before use, inspect the MEFs microscopically and ensure that they are firmly attached to the surface of the plates. A characteristic fibroblast morphology should gradually appear within 24 h after plating, but the cells may be used as described below as soon as 3 h after plating, and ideally on the same day of plating. When the cells are fully spread, they should appear 70–80% confluent.

■ **PAUSE POINT** MEF-containing plates can be used within 3–24 h of incubation after plating.

(iv) Take a frozen vial of human ESCs from liquid nitrogen, place it in a 37 °C water bath without submerging the cap, and swirl gently until its contents are almost completely thawed (2–3 min). Immediately dry the vial, spray it with 70% (vol/vol) ethanol and place it in the tissue culture hood. Uncap the vial, add 1 ml of human ESC medium using a 2-ml pipette, and transfer the ESC suspension to a 15-ml conical tube containing 10 ml of human ESC medium.

▲ **CRITICAL STEP** Thawing should be performed rapidly, as DMSO harms cells at room temperature.

(v) Centrifuge the cells at 150*g* for 5 min at room temperature. Remove the supernatant, resuspend the cells using warm (37 °C) human ESC medium to reach a density of ~2.5×10^5 cells ml^{-1}, and add Y-27632 to a final concentration of 10 µM.

(vi) Take the MEF-containing plates from the incubator and place them in the tissue culture hood. Aspirate the MEF medium and wash the cells once with PBS (use 1 ml of PBS for each well of a six-well plate).

(vii) Plate the human ESCs on the MEF-containing plates by transferring 2 ml of the cell suspension to each well of the six-well plates, and place the plates in the incubator. To disperse the cells evenly, gently, yet quickly, rock the plates back and forth and sideways a few times.

(viii) On the next day and onward, change the medium daily using fresh, warm (37 °C) human ESC medium.

▲ **CRITICAL STEP** It is important to monitor the cells daily under the microscope to assess their growth rate.

(ix) Once the ESC colonies reach 80–90% confluence (usually within 3–7 d after thawing), aspirate the medium and add trypsin–EDTA (0.5 ml to each well of a six-well plate). Keep the cells at room temperature until individual cells start to round up (usually within 1–2 min), and aspirate the trypsin. The cells should remain attached at this point. To detach the cells from the surface, rinse them with warm (37 °C) human ESC medium by repeated pipetting using a 5- or 10-ml pipette (use at least 1 ml of medium per well of a six-well plate). The cells should readily come off the plate and break into small clusters.

▲ **CRITICAL STEP** Dissociation should result in small clusters of cells. Trypsinizing for too long can result in a reduced splitting yield. Aspirating the trypsin before the cells start to round up will make it harder for them to detach from the surface, which may require additional trypsinization time.

(x) Collect the cell suspension in a 15- or 50-ml conical tube. To collect the remaining cells, repeat the rinsing step and add the cells to the same conical tube.

(xi) Adjust the final volume of the medium to conform to the desired split ratio (split ratios of 1:6 to 1:18 are recommended), add Y-27632 to a final concentration of 10 µM and plate the human ESCs on new MEF-containing plates (prepared as in Step 1A(i–iii)). For example, cells from one well of a six-well plate can be split 1:6 by diluting the cell suspension to a final volume of 12 ml of medium with 12 µl of 10 mM Y-27632 and plating 2 ml in each well of a new six-well plate.

(xii) Place the human ESC plates in the incubator. To disperse the cells evenly, gently, but quickly, rock the plates back and forth and sideways a few times.

▲ **CRITICAL STEP** On the next day, small colonies should be evenly distributed across the plate. Accumulation of colonies at the center requires adjustment of the rocking technique.

(xiii) Continue culturing the cells as in Step 1A(viii–xii) until they have been sufficiently expanded for further procedures. Depending on the split ratio, the cells may be ready for resplitting again within 3–7 d. If you wish to freeze the cells, proceed to the next step; otherwise, proceed to Step 2.

(xiv) To freeze human ESCs, follow Step 1A(ix and x). Rather than performing Step 1A(xi), centrifuge the cells at 150*g* for 5 min at room temperature. Remove the supernatant and resuspend the cells using cold (4 °C) freezing medium. We recommend resuspending 1×10^6 to 2×10^6 cells in 1 ml of freezing medium. Quickly transfer the cells in freezing medium to sterile cryogenic storage vials (1 ml per vial), and place the vials in an isopropanol chamber. Keep the chamber at –80 °C overnight, and on the following day transfer the vials to storage in liquid nitrogen.

▲ **CRITICAL STEP** Label the cryogenic storage vials before resuspending the cells in freezing medium. Extended exposure to 10% (vol/vol) DMSO at room temperature harms cells.

■ **PAUSE POINT** Frozen cells can be stored indefinitely in liquid nitrogen.

(B) Culture of human parthenogenetic ESCs in feeder-free conditions

(i) To thaw human ESCs in feeder-free conditions, follow Step 1A(iv–viii), substituting mTeSR1 for human ESC medium (except in Step 1A(iv)), and substituting Matrigel-coated plates (prepared as detailed in the Equipment Setup) for MEF-containing plates. Washing Matrigel-coated plates with PBS is not required before use.

▲ **CRITICAL STEP** Thawing in feeder-free conditions is recommended only for human ESCs that had been adapted to grow well in these conditions before freezing. Human ESCs cultured in feeder-dependent

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conditions before freezing should first be thawed, as detailed in Step 1A(i–viii), and split into feeder-free conditions, as in Step 1B(ii).

(ii) To split human ESCs in feeder-free conditions, follow Step 1A(ix–xiii), substituting mTeSR1 for human ESC medium and Matrigel-coated plates for MEF-containing plates. Freezing can be performed as in Step 1A(xiv).

Identification and isolation of haploid cells in early-passage human parthenogenetic ESC lines

2| Ideally, the analysis of human parthenogenetic ESCs for the presence of haploid cells should be performed at the earliest passage possible, as the haploid fraction decreases with the number of cell cycles. Identification of haploid-cell-containing human parthenogenetic ESC lines can be carried out either by metaphase spread analysis (option A) or directly by attempting to isolate haploid cells by DNA-based FACS (option B) (see the INTRODUCTION for more details).

(A) Identification of haploid ESCs by metaphase spread analysis ● TIMING 3 d

(i) Choose three to six wells of a six-well plate containing early-passage human parthenogenetic ESCs at 60–70% confluence, which were fed with fresh medium the day before.

▲ CRITICAL STEP Optimal growth is important for obtaining sufficient numbers of cells in metaphase.

(ii) Add colcemid solution directly to the medium to a final concentration of 100 ng per ml (for example, add 20 µl of 10 µg per ml colcemid solution to 2 ml of medium in one well of a six-well plate). Incubate the cells for 40 min in a humidified tissue culture incubator.

(iii) Aspirate the medium, wash it once with PBS and trypsinize the cells at room temperature using trypsin–EDTA (0.5 ml in each well of a six-well plate). Once the cells become dissociated, add MEF medium (2 ml of medium per well of a six-well plate), and using a 5- or 10-ml pipette, collect the cell suspension in a 15-ml conical tube.

(iv) Centrifuge the cells at 150*g* for 5 min at room temperature. Aspirate the supernatant down to 1 ml, and gently resuspend the cell pellet in the remaining medium by vortexing in shaking mode (at low speed).

▲ CRITICAL STEP Throughout this procedure, cell pellets should be completely resuspended, and clumping should be avoided. If necessary, dissociate cell clumps by gentle pipetting using a plastic Pasteur pipette.

(v) While shaking the cell suspension, add 5 ml of warm (37 °C) hypotonic solution in a dropwise manner, and incubate the tube in a 37 °C water bath for 20 min.

(vi) In a chemical fume hood, add 2.5 ml of cold (–20 °C) fixative solution in a dropwise manner down the side of the tube while shaking it slowly, and incubate the tube at room temperature for 5 min.

(vii) Centrifuge the cells at 150*g* for 5 min at room temperature, aspirate the supernatant, and resuspend the cell pellet in 2.5 ml of fixative solution, as in Step 2A(vi). Incubate the cells at room temperature for 5 min.

(viii) Repeat Step 2A(vii) at least twice more using 2 ml of fixative solution. Centrifuge the cells at 150*g* for 5 min at room temperature, aspirate the supernatant, and resuspend the cell pellet in 1.5 ml of fixative solution as in Step 2A(vi). Keep the sample at –20 °C overnight or longer.

■ PAUSE POINT Fixed samples can be stored at –20 °C for several months.

(ix) Centrifuge the sample at 150*g* for 5 min at room temperature, and place it in a chemical fume hood. Resuspend the fixed cells in freshly prepared fixative solution (1.5–5 ml, depending on the amount of cell pellet) by pipetting with a plastic Pasteur pipette. The cell suspension should appear cloudy yet homogeneous.

(x) Take 3 glass microscope slides (prepared as described in the Equipment Setup) out of dH_2O and dry the back surface of the slides by gently wiping them with delicate task wipers.

(xi) Using a plastic Pasteur pipette, drip three to four drops of the cell suspension along the front surface of each slide. Spread the drops by blowing three strong, short breaths across the slides. Place the slides on a 60 °C hot plate in a humid environment (which can be set by placing a small water-containing Erlenmeyer flask and damp paper on the hot plate) for 2 min.

(xii) Incubate the slides in a drying oven at 60 °C overnight.

(xiii) On the next day, perform Giemsa staining. Prepare six Coplin staining jars in a 37 °C water bath and place the slides into jars 1–6 for the time indicated, as follows:

Jar number	Contents	Time (s)
1	50 ml of Earle's Balanced Salt Solution	60
2	1 ml of trypsin–EDTA without phenol red and 55 ml of Earle's Balanced Salt Solution	20
3	10 ml of FBS and 40 ml of Earle's Balanced Salt Solution	60
4	50 ml of Earle's Balanced Salt Solution	60
5	2 ml of Giemsa stain (passed through a 0.2-µm filter) and 50 ml of buffer (pH 6.8)	140
6	50 ml of buffer (pH 6.8)	60

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(xiv) Dry the slides at room temperature, and mount each slide with a coverslip using Eukitt mounting medium.
■ **PAUSE POINT** Stained slides can be stored at room temperature for several years.

(xv) Use the ×20 objective of a light microscope to find metaphases, and the ×100 objective (with immersion oil) to count the number of chromosomes per metaphase. The metaphases can also be used for karyotype analysis by a qualified cytogeneticist.
▲ **CRITICAL STEP** We recommend analyzing at least 200–300 metaphases per parthenogenetic ESC line, as the proportion of haploid cells may be very low (even below 1%).
? TROUBLESHOOTING

(xvi) To isolate haploid cells from human parthenogenetic ESC lines in which haploid metaphases have been identified, follow Step 2B (see ANTICIPATED RESULTS).

(B) Isolation of haploid ESCs by DNA-based FACS ● TIMING 75 min for preparing the cells for sorting, ~2 h for sorting and ~2 weeks for expansion

(i) Before performing this procedure, prepare one MEF-containing well of a six-well plate as in Step 1A(i–iii) (at least 3 h in advance), as well as a KSR-coated collection tube, as described in the Equipment Setup (at least 1 h in advance). Assemble at least three six-well plates containing early-passage human parthenogenetic ESCs at 80–90% confluence, which have been fed with fresh medium the day before. As reference, also choose one to two wells of a six-well plate of growing diploid human ESC culture at 80–90% confluence.
▲ **CRITICAL STEP** Large amounts of early-passage parthenogenetic ESCs are required here, as the initial proportion of 1c cells may be very low at the time of analysis (even below 1%), and their efficient enrichment depends on a minimal number of sorted cells.

(ii) Aspirate the medium and wash the cells once with PBS without $CaCl_2$ and $MgCl_2$ (1 ml in each well of a six-well plate). Dissociate the cells by adding warm (37 °C) TrypLE Select (0.5 ml to each well of a six-well plate) and incubating in a humidified incubator at 37 °C for 10–15 min.
▲ **CRITICAL STEP** During this step, monitor the cells under the microscope and gently pipette several times to ensure dissociation of cell clumps into single cells.

(iii) Once dissociation is completed, add human ESC medium (2 ml to each well of a six-well plate). Collect the early-passage parthenogenetic ESC single-cell suspensions in a single 50-ml conical tube. Collect the diploid ESC single-cell suspension in a 15-ml conical tube. Rinse the plates with additional human ESC medium to collect any remaining cells.

(iv) Centrifuge the cells at 150*g* for 2 min at room temperature, and discard the supernatant. Resuspend the early-passage parthenogenetic ESCs pellet in 10 ml of human ESC medium for each six-well plate initially used in Step 2B(i). Resuspend the diploid ESC pellet in 10 ml of human ESC medium.

(v) Allocate 5% of the early-passage parthenogenetic ESC suspension to a separate 15-ml tube to be used as an unstained control, and compensate for the volume removed with the same volume of human ESC medium. To this 'unstained control' tube, add human ESC medium to obtain a final volume of 10 ml.

(vi) Divide the remaining early-passage parthenogenetic ESC suspension into 15-ml tubes with 10 ml per tube (for example, if three plates were initially used in Step 2B(i), resuspend the cell pellet in 30 ml of medium and divide the cell suspension into three 15-ml tubes).

(vii) Add 4 μl of 25 mg per ml Hoechst 33342 stock solution to each 15-ml conical tube containing 10 ml of early-passage parthenogenetic ESC suspension or diploid ESC suspension (final concentration 10 μg per ml). Do not add Hoechst 33342 to the 'unstained control' tube. Incubate all tubes in the incubator for 30 min while gently inverting them twice every 10 min.

(viii) Centrifuge the cells at 150*g* for 2 min at room temperature. Discard the supernatant and resuspend the cell pellets in 1–2 ml of FACS medium containing 10 μM Y-27632 per 15-ml tube.

(ix) Combine the contents of the 15-ml conical tubes containing Hoechst 33342-stained early-passage parthenogenetic ESCs in a single tube and pipette gently. Filter the cells through a 70-μm cell strainer into sterile FACS tubes (divide the cell suspension to 1.5–2 ml per FACS tube) and keep the FACS tubes on ice. Filter the Hoechst-33342-stained diploid ESCs and the 'unstained control' cells into separate sterile FACS tubes.

(x) Run the samples in a cell sorter using the proper settings for Hoechst 33342 excitation and for human ESC sorting (we use a 405-nm laser with a 450/40 filter, with either an 85- or a 100-μm nozzle, PBS as the sheath fluid and a flow rate of below 6,000 events per s). Before running the samples intended for sorting, run the 'unstained control' (a Hoechst 33342 signal should be undetectable) and the diploid ESC sample (to be used as reference for setting the proper gates for sorting). Define gates as illustrated in **Figure 4** to selectively sort the 1c-cell population of the early-passage parthenogenetic ESCs. Note that a clear 1c-cell peak might not be visible when analyzing a putatively haploid-containing parthenogenetic ESC line for the first time, in which case gating can be more permissive and the

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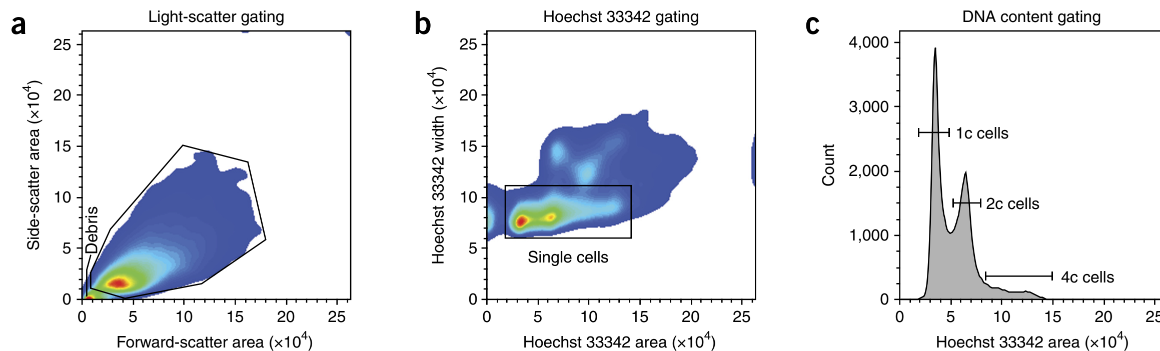

Figure 4 | Gating of haploid cells in DNA-based FACS with Hoechst 33342 staining. (**a**) Using the forward- and side-scatter area channels, a gate is defined that excludes low-signal events (probably debris, here ~10% of the events). (**b**) Gated events are visualized by Hoechst 33342 area and width, and a second gate is defined on the single-cell population, as shown (here 85% of the gated events). (**c**) In a Hoechst 33342 area histogram view, 1c-, 2c- and 4c-cell peaks (here 45, 43 and 12%) are differentiated, and the 1c-cell population is sorted to enrich for haploid cells in G1. **a** and **b** are plotted in pseudocolor, ranging from blue to red for increasing density. The flow cytometry plots are of haploid-enriched pES12 cells[15] (1.2×10^5 cells were counted). Experiments involving human ESCs were conducted at the Hebrew University under the guidelines of the Bioethics Advisory Committee of the Israel Academy of Sciences and Humanities, and were also approved by the ESC Research Oversight Committee and the institutional review board at Columbia University Medical Center.

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sub-2c-cell population should be sorted. During the sort, cool the sample to 4 °C and direct sorted cells into a KSR-coated collection tube.

▲ CRITICAL STEP Sorting in contaminant-free conditions is critical. Before sorting, run a sample of 70% (vol/vol) ethanol for at least 1 min, followed by back-flush for ~30 s. Running bleach for at least 1 min before ethanol is also recommended. The flow rate may be adjusted to minimize the duration of the sort, which preferably should not exceed 1–1.5 h. Running separate samples consecutively may also be considered.

? TROUBLESHOOTING

(xi) Centrifuge the sorted cells at 150*g* for 5 min at room temperature. Gently resuspend the cell pellet in 2 ml of fresh, warm (37 °C) human ESC medium and add Y-27632 to a final concentration of 10 µM.

(xii) Plate the sorted cells on one well of a MEF-containing six-well plate as in Step 1A(vi and vii).

(xiii) On the next day and onward, change the medium daily using fresh, warm (37 °C) human ESC medium. Small cell colonies initiated from single cells usually become apparent within 2–3 d after plating.

▲ CRITICAL STEP If no colonies appear within 7 d, discard the plate. Human parthenogenetic ESC cell lines in which haploid cells are undetectable at the analyzed passage by both metaphase spread analysis and DNA-based FACS can be considered diploid (see ANTICIPATED RESULTS).

? TROUBLESHOOTING

(xiv) Split the colonies as in Step 1A(ix–xii) once they reach 80–90% confluence, and continue culturing the cells as in Step 1A(viii–xii) until they are sufficiently expanded to repeat the DNA-based FACS procedure (Step 2B), or perform metaphase spread analysis (Step 2A) for validating the enrichment of haploid cells.

Further enrichment and maintenance of haploid ESC lines by DNA-based FACS ● TIMING 75 min for preparing the cells for sorting, ~2 h for sorting and 1–2 weeks for expansion

▲ CRITICAL Repeated rounds of haploid-cell enrichment by DNA-based FACS should be performed on haploid-enriched human parthenogenetic lines every few passages according to the estimated rate of diploidization (**Fig. 3** and **Box 1**).

3| Follow Step 2B(i–x), starting with haploid-containing parthenogenetic ESCs identified in Step 2A or B, until the colonies reach 80–90% confluence. The initial amount of cells needed in this procedure may vary from that contained in a few single wells of a six-well plate up to that contained in several plates, according to the amount of cells required for downstream procedures and the estimated proportion of haploids in the culture. In general, fewer cells are required as the culture becomes more highly enriched with haploids. Instead of performing Step 2B(xi), proceed to Step 4.

4| As an optional sorting purity control, mix the collection tube by inverting it twice, transfer 100 µl of the sorted cell suspension to a separate FACS tube, and add 400 µl of FACS medium. Briefly vortex the sample and run it on the FACS machine as in Step 2B(x). A clearly defined 1c-cell peak should appear within the borders of the gate defined during sorting.

5| Centrifuge the sorted cells at 150*g* for 5 min at room temperature, and resuspend the cell pellet in fresh, warm (37 °C) human ESC medium with 10 µM Y-27632 according to the estimated number of sorted cells (use 4 ml of medium to resuspend 1×10^6 to 2×10^6 sorted cells).

6| Plate the sorted cells on MEF-containing six-well plates as in Step 1A(vi and vii).

7| On the next day and onward, change the medium daily using fresh, warm (37 °C) human ESC medium. Cell colonies initiated from single cells usually become apparent within 2–3 d after plating.

8| Once the colonies reach 80–90% confluence, split them as in Step 1A(ix–xii) and continue culturing the cells as in Step 1A(viii–xiii), or transfer the cells to feeder-free conditions as in Step 1B(ii).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Human ESCs display retarded growth, spontaneous differentiation or cell death	Low MEF quality or density	Plate MEFs at a higher density. Do not use MEF plates more than 24 h after plating
		Insufficient medium replacement	Replace the medium on a daily basis. Use larger volumes when the cell colonies become exceedingly confluent
		Suboptimal activity of medium components	Prepare new medium, and do not use medium stored at 4 °C for >1 month. Note that the quality of KSR and freshly added bFGF is especially critical, and that it may vary across batches
		Bacterial or fungal contamination	Routinely test for contaminations (e.g., by *Mycoplasma*) and discard contaminated cultures
2A(xv)	Insufficient number of metaphases prevents the determination of ploidy	The amount of dividing cells analyzed is too low	Repeat Step 2A(ix) using a smaller volume of fixative solution, or prepare additional slides in Step 2A(x)
	Nuclei are too crowded to discern individual metaphases	The density of cells per slide is to high	Repeat Step 2A(ix) using a larger volume of fixative solution
	Metaphase chromosomes are overlapping	Metaphase chromosomes are not sufficiently spread	In Step 2A(xi), increase the hot plate temperature to 80 °C
2B(x)	DNA content profiles are ambiguous	Cell clumping obscures the results	Briefly vortex the cells before running the sample
		Sorting settings are not properly defined	Consulting with a flow cytometry technician is advisable; certain settings can be adjusted (e.g., reduction of the flow rate may result in a clearer cell cycle profile)
2B(xiii)	Colonies do not appear within 7 d after sorting	Haploid cells may be absent in the analyzed human parthenogenetic ESC line	Discard the culture and confirm diploidy by metaphase spread analysis
		Haploid cells are too scarce to be isolated	Several steps can be modified as follows—Step 2B(i): use more than three six-well plates of human parthenogenetic ESCs at the earliest passage possible; Step 2B(x): reduce the duration of sorting by dividing the sample into several samples and running them consecutively; Step 2B(xii): plate the sorted cells at a higher density on one well of a MEF-containing 12-well plate

● **TIMING**

Step 1, culture of human parthenogenetic ESCs: thawing, expansion and freezing: 1–2 weeks, depending on the initial amount of cells, the culture growth rate and the amount of cells required for downstream procedures

Step 2A, identification of haploid ESCs by metaphase spread analysis: 3d

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Step 2B, isolation of haploid ESCs by DNA-based FACS: 75 min for preparing the cells for sorting, ~2 h for sorting and ~2 weeks for expansion
Steps 3–8, further enrichment and maintenance of haploid ESC lines by DNA-based FACS: 1–2 weeks, depending on the amount of sorted cells, the culture growth rate and the amount of cells required for downstream procedures
Box 1, assessment of the diploidization rate of haploid human ESCs: 3–6 weeks for preparing the samples and ~2 h for flow cytometry analysis.

ANTICIPATED RESULTS

This protocol describes a method for the identification and propagation of haploid human ESCs, taking advantage of the pre-existing haploidy of human oocytes and its persistence throughout preimplantation parthenogenetic development and ESC derivation[15].

The most crucial part of this protocol is the accurate determination of ploidy within early-passage human parthenogenetic ESC lines (Step 2). It should be emphasized that even though every human parthenogenetic ESC line is a potential source of haploid cells, these might be present at a rather low percentage or may be completely absent, depending on diploidization events during preimplantation development and during the establishment of an ESC line. We advise using metaphase spreading (Step 2A) as the primary assay for ploidy analysis, as it allows rapid and high-resolution detection of haploid ESCs in a given culture. Isolation of haploids by DNA-based FACS (Step 2B) is unlikely to succeed if haploid cells are undetectable after analyzing hundreds of metaphases. Furthermore, failure to isolate haploid ESCs by FACS should not be taken as proof of the absence of haploids, unless later confirmed by metaphase spread analysis, as it may result from technical factors related to sorting live ESCs. In case there is evidence for the presence of haploid cells in a culture, sorting efficiency may be increased by modifications to the protocol (Troubleshooting **Table 1**).

Upon the successful identification of haploid human ESCs, a gradually increasing purity of haploids can be achieved by repeated FACS-based enrichment (Steps 3–8). In general, we recommend performing haploid-cell enrichment before any analysis of haploid human ESCs.

Diploid human parthenogenetic PSCs behave as normal PSCs with respect to their efficient self-renewal in conventional culture conditions, sustainment of the pluripotent state and capacity to differentiate into all three embryonic lineages *in vitro* and *in vivo*[16,17,20]. Importantly, these defining characteristics of human pluripotency are also found in haploid human parthenogenetic ESCs, and may be confirmed by multiple assays, as described elsewhere[39]. Several assays can also be used to ascertain pluripotency in single haploid cells, by combining the analysis of lineage-specific markers with methods that can indicate a haploid genome, including flow cytometry with DNA staining, chromosome quantification using DNA fluorescence *in situ* hybridization or centromere foci immunofluorescence staining[15].

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