Derivation and differentiation of haploid human embryonic stem cells

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Diploidy is a fundamental genetic feature in mammals, in which haploid cells normally arise only as post-meiotic germ cells that serve to ensure a diploid genome upon fertilization. Gamete manipulation has yielded haploid embryonic stem (ES) cells from several mammalian species1-6, but haploid human ES cells have yet to be reported. Here we generated and analysed a collection of human parthenogenetic ES cell lines originating from haploid oocytes, leading to the successful isolation and maintenance of human ES cell lines with a normal haploid karyotype. Haploid human ES cells exhibited typical pluripotent stem cell characteristics, such as self-renewal capacity and a pluripotency-specific molecular signature. Moreover, we demonstrated the utility of these cells as a platform for loss-of-function genetic screening. Although haploid human ES cells resembled their diploid counterparts, they also displayed distinct properties including differential regulation of X chromosome inactivation and of genes involved in oxidative phosphorylation, alongside reduction in absolute gene expression levels and cell size. Surprisingly, we found that a haploid human genome is compatible not only with the undifferentiated pluripotent state, but also with differentiated somatic fates representing all three embryonic germ layers both in vitro and in vivo, despite a persistent dosage imbalance between the autosomes and X chromosome. We expect that haploid human ES cells will provide novel means for studying human functional genomics and development.

Haploid genetics is useful for delineating genome function. Although haploid genetics has been mostly restricted to unicellular organisms, recent reports of haploid ES cells have extended it into animal species, including mammals1-7. Deriving haploid human ES cells by similar approaches has probably been hindered by the limited availability of human oocytes8. Artificial activation of unfertilized metaphase II (MII) human oocytes results in efficient development to the blastocyst stage9-11 and subsequent derivation of parthenogenetic ES (pES) cells9-11. In mouse parthenogenetic embryos haploidy usually persists at the blastocyst stage12,13, but diploid cells progressively dominate over increasing cell cycles due to spontaneous and irreversible diploidization14-15 (Fig. 1a). By estimation, even if diploidization occurs in 1 out of 10 cell cycles, 1% of ES cells may remain haploid at early passages (Extended Data Fig. 1a).

To explore the feasibility of deriving haploid human ES cells, we generated and analysed a collection of 14 early-passage human pES cell lines for the persistence of haploid cells. We initially used chromosome counting by metaphase spreading as a method for unambiguous and quantitative discovery of rare haploid nuclei. Among ten individual pES cell lines, a low proportion of haploid metaphases was found in one cell line, pES10 (1.3%, Extended Data Table 1). In four additional lines, we also used viable fluorescence-activated cell sorting (FACS) with Hoechst 33342 staining, aiming to isolate cells with a DNA content corresponding to less than two chromosomal copies (2c), leading to the successful enrichment of haploid cells from a second cell line, pES12 (Extended Data Table 2).

Figure 1  Derivation of haploid human ES cells. a, Schematic of putative haploidy in pES cells. b, DNA content profiles of haploid pES10, established by repeated enrichment of 1c cells. Top to bottom, unsorted diploid cells, partially purified (fourth sort) and mostly purified (sixth sort) haploid cells. c, pES10 karyotypes before and after 1c-cell enrichment. p, passage. d, e, DNA FISH (d) and centromere staining (e) in haploid-enriched pES10 cells. Magnifications show haploid nuclei with a single hybridization signal (d) and 23 centromere foci (e), respectively. Scale bars, 10 μm.

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doi:10.1038/nature17408
Two individual haploid-enriched ES cell lines were established from pES10 and pES12 (hereafter referred to as h-pES10 and h-pES12) within five to six rounds of 1c-cell enrichment and expansion (Fig. 1b and Extended Data Fig. 1b). These cell lines were cultured in standard conditions for over 30 passages while including cells with a normal haploid karyotype (Fig. 1c and Extended Data Fig. 1c). However, since diploidization occurred at a rate of 3–9% cells per day (Extended Data Fig. 1a; see Methods), sorting at every three to four passages was required to maintain haploid cells. Visualization of ploidy was further enabled by DNA fluorescence in situ hybridization (FISH) (Fig. 1d and Extended Data Fig. 1d) and quantification of centromere protein foci (Fig. 1e and Extended Data Fig. 1e; see Supplementary Notes and Extended Data Fig. 2). Besides having an intact karyotype, haploid ES cells did not harbour significant copy number variations (CNVs) relative to their unsorted diploid counterparts (Extended Data Fig. 1f), nor common duplications that would result in pseudo-diploidy, indicating that genome integrity was preserved throughout haploid-cell isolation and maintenance.

Both h-pES10 and h-pES12 exhibited classical human pluripotent stem cell features, including typical colony morphology and alkaline phosphatase staining. Scale bars, 50 μm. c, Flow cytometry analysis of gated h-pES10 1c cells by co-staining DNA and cell surface markers TRA-1-60* and CLDN6* (ref. 16) in single haploid cells (Fig. 2c).

Haploid cells are valuable for genetic screening because phenotypically selectable mutants can be identified upon disruption of single-copy alleles. To demonstrate the applicability of this principle in haploid human ES cells, we generated a genome-wide mutant library using a gene trap transposon system (Fig. 2f and Extended Data Fig. 4e; see Methods), and screened for resistance to the purine analogue 6-thioguanine (6-TG). Out of six isolated and analysed 6-TG-resistant colonies, three harboured an identical gene trap insertion at the NUDT5 autosomal gene (Fig. 2g). The disruption of this gene was recently confirmed to confer 6-TG resistance in human cells17 (Fig. 2h). Detection of a loss-of-function phenotype due to an autosomal mutation thereby validates that genetic screening is feasible in haploid human ES cells.

The ability of human ES cells to exist as both haploids and diploids led us to investigate whether these two ploidy states differ in certain aspects of gene regulation and cell biology. To analyse haploid and diploid ES cells in the same cell cycle phase, we used FACs to isolate G1-phase haploid cells (1c) and compared them with isogenic G1-phase diploid cells (2c) from unsorted diploid cultures (Fig. 3a and Extended Data Fig. 5a; see Methods). We first aimed to uncover putative ploidy-associated differences by comparing the transcriptomes of haploid and diploid ES cells using RNA sequencing (RNA-seq), considering that observed changes in expression levels would be relative to the total gene expression of each ploidy state, rather than representing absolute differences. On the genome-scale, undifferentiated haploid and diploid ES cells clustered closely and separately from differentiated embryoid bodies (EBs) (Fig. 3b). Nonetheless, we identified 275 relatively upregulated and 290 relatively downregulated genes in haploids compared with diploids (greater than twofold change, false discovery rate (FDR) <0.05; Extended Data Fig. 5b).
Notably, X chromosomal genes were significantly overrepresented among the relatively upregulated gene set (40%, P < 0.001, χ² goodness of fit test) (Fig. 3c), and the expression levels of X chromosomal genes alone clearly distinguished between haploid and diploid ES cells (Fig. 3b). These data correlate with an expected differential status of X chromosome inactivation in haploid and diploid human ES cells: whereas the single-copy X chromosome in haploids is transcriptionally active (X₀), one of the two X chromosomes in diploids is often inactivated (X₀X₀)²⁸. Indeed, haploid human ES cells exhibited a relative increase in X chromosome gene expression and lacked expression of the XIST transcript which drives X chromosome inactivation (Fig. 3d and Extended Data Fig. 5b–d), as in diploid X₀X₀ human ES cells²⁸. X chromosome inactivation is regulated by repressive histone modifications and DNA methylation. H3K27me3 foci were consistently observed in unsorted diploid ES cells, but not in the haploid-enriched counterparts (Fig. 3f). Moreover, methylation analysis showed that the X chromosome DNA methylation signature of haploid ES cells resembles that of diploid male ES cells (X₀Y), whose single-copy X chromosome is largely hypomethylated, as opposed to the composite pattern of a hypomethylated X₀ and a hypermethylated X₀ in diploid female cells (Fig. 3g). Interestingly, recently diploidized ES cells (see Methods) remained X₀X₀ soon after diploidization by all assays mentioned earlier (Fig. 3a, d–g).

Normalization to total gene expression²⁰ resulted in seemingly similar expression levels of autosome genes, but higher levels of X-linked genes in haploid ES cells (Fig. 3d and Extended Data Fig. 5c). However, assuming that the absolute expression of X-linked genes in haploid X₀ and diploid X₀X₀ cells are equivalent, these data suggest a genome-wide autosome gene expression level reduction in haploids (Extended Data Fig. 5e, f). In support of this, total RNA amounts isolated from haploid ES cells were significantly lower than those from equal numbers of diploid cells (Fig. 3h). A decrease in total gene expression implied that physical dimensions may also be altered. Indeed, the average haploid:diploid diameter ratio of G1-sorted ES cells was ~0.8, corresponding to ~0.7 surface area ratio and ~0.6 volume ratio (Fig. 3h and Extended Data Fig. 5g).

We subsequently focused on consistent differential regulation within autosomes (see Methods). By transcriptome and methylene analyses, genes relatively downregulated in haploid ES cells were significantly enriched for genes encoding signal-peptide-bearing
That mitochondrial abundance relative to nuclear DNA content is genes across nine cell types (Fig. 4d). Whereas expression levels were G1-sorted haploid ES cells and EB cells, focusing on 18 lineage-specific Data Fig. 6b). We then compared the gene expression profiles of h-pES10-derived and h-pES12-derived EB cells (Fig. 4c and Extended differentiation (Extended Data Fig. 6a). Notably, metaphase spreading was consistent with differentiation of haploid-enriched and diploid ES cells could not be distinguished by their appearance (Fig. 4a), and the morphology of dissociated haploid-cell-derived EB cells was consistent with differentiation (Extended Data Fig. 6a). Notably, metaphase spreading revealed a haploid karyotype (Fig. 4b; 4/4 metaphases), and a largely haploid (~70%) DNA profile was confirmed by flow cytometry in both h-pES10-derived and h-pES12-derived EB cells (Fig. 4c and Extended Data Fig. 6b). We then compared the gene expression profiles of G1-sorted haploid ES cells and EB cells, focusing on 18 lineage-specific genes across nine cell types (Fig. 4d). Whereas expression levels were negligible in undifferentiated ES cells, all tissue-specific genes were expressed in haploid and diploid EB cells (Fig. 4d and Extended Data Fig. 6c). Haploid and diploid EB cells showed insignificant expression of pluripotency-specific genes, consistent with efficient acquisition of differentiated cell fates.

To extend this analysis to more specific cell types, we subjected haploid ES cells to directed differentiation assays. Haploid ES cells differentiated towards a neural fate for ten days remained haploid, while giving rise to NCAM1-positive neural progenitor cells (NPCs), with ~90% efficiency (Extended Data Fig. 7a, b). Sorted haploid NPCs expressed multiple neural-lineage-specific genes but not pluripotency-specific genes (Extended Data Fig. 7c, d). X chromosome inactivation in differentiated diploid female cells results in dosage compensation of 1:2 between the X chromosome and autosomes. As haploid cells do not inactivate their single-copy X chromosome, an X:autosomes dosage imbalance of 1:1 should persist into the differentiated state. Indeed, both haploid NPCs and EB cells showed an XaXi signature contrary to the XaXi signature of diploid EB cells as indicated by whole-genome expression moving median plot. f, g, i, j. Centromere and differentiation marker co-staining in h-pES12-derived neurons (f), cardiomyocytes (g), definitive endoderm cells (i) and pancreatic cells (j). Scale bars, 10 μm.

h, k. DNA content profiles of h-pES12 cells differentiated into cardiomyocytes (h) and PDX1-positive pancreatic cells (k). i, TUJ1 (ectoderm), α-SMA (mesoderm), AFP (endoderm) and OCT4 (pluripotency) staining in an h-pES12-derived teratoma. Scale bars, 50 μm. m. DNA content profile of an h-pES10-derived teratoma. n. Serial h-pES12-derived teratoma sections analysed histologically (left; scale bar, 20 μm) and by DNA FISH (middle; scale bar, 20 μm). Haploid nuclei are shown in magnification (right; scale bar, 5 μm).
with centromeres (Fig. 4f; 47% haploids, n = 104) and FISH analysis (Extended Data Fig. 7e, g; 46% haploids, n = 200). Similarly, haploid cells differentiated into TNNT2-expressing cardiomyocytes (Fig. 4g; 32% haploids, n = 97) during an eleven-day protocol resulting in spontaneously beating clusters (Supplementary Video 1), and 39% (n = 31) of haploid cells sorted from the whole culture (25% 1c-cells) were confirmed as TNNT2-positive (Fig. 4h and Extended Data Fig. 7h). Next, we differentiated haploid-enriched cultures (~70% haploids) to the pancreatic lineage. Analysing two stages of differentiation by centromere foci analysis, we observed robust differentiation (>90%) of both haploids and diploids into FOXA2-positive definitive endoderm cells (Fig. 4i; 56% haploids, n = 112), and differentiation into PDX1-positive pancreatic cells (Fig. 4j; 13% haploid, n = 103), some of which were also positive for NKX6-1. In addition, the persistence of haploid PDX1-positive cells was confirmed by flow cytometry (Fig. 4k; 10% PDX1-positive 1c cells; and Extended Data Fig. 7i, j).

Finally, both haploid-enriched human ES cell lines gave rise to teratomas comprising cell types of ectodermal, mesodermal and endodermal origins (Fig. 4l and Extended Data Fig. 8a, b), meeting the most stringent criterion for human pluripotency in vivo. Importantly, no residual undifferentiated OCT4-positive cells were detected (Fig. 4l and Extended Data Fig. 8b). Upon dissection, DNA content analysis revealed that a considerable population of h-PE10-derived teratoma cells remained haploid (Fig. 4m). Combined analysis of serial sections from an independent, h-PE12-derived teratoma, by histology and FISH confirmed the existence of in vivo differentiated haploid human ES cells capable of contributing to an organized tissue structure while responding to developmental signals (Fig. 4n). Haploid cells were identified in all analysed teratomas (n = 4), albeit with variable proportions, which may be influenced by the initial amount of haploid cells and/or the duration of teratoma formation.

Haploid mammalian cells have proven invaluable for loss-of-function screens2. Using a genome-wide library of gene-trapped haploid human ES cells, we demonstrated their potential for biomedically relevant functional genomics by forward genetic screening (Supplementary Discussion). Whereas previous studies on non-human haploid ES cells mostly emphasized the similarity between haploids and diploids, here we also pointed to several transcriptional, epigenetic and physical properties that set them apart (Supplementary Discussion). Interestingly, we did not observe global transcriptional compensation in haploid cells, indicating that it is not required for cellular viability as long as an autosomal balance is preserved. In contrast, autosomal imbalance appears intolerable based on the strict absence of human autosomal monosomes in in vitro fertilization ES cells21. Remarkably, we found that a haploid human karyotype is not a barrier for ES cell differentiation. As observed in the mouse2, haploid human ES cells gave rise to NPCs while remaining haploid. However, while mouse studies showed that haploid cells are lost upon further differentiation2,14, we observed specification of human haploid cells into somatic cell fates of all three embryonic germ layers, despite persistent dosage imbalance between the X chromosome and autosomes (Supplementary Discussion).

Throughout evolution, mammalian genomes have been solidified by diploidy-dependent adaptations such as parental imprinting, which restrict the development of haploid uniparental embryos. Nonetheless, haploid cells are capable of directing development in certain animal species22. The surprising differentiation potential of haploid human genomes suggests that diploidy-dependent adaptations, rather than haploidy, pose the predominant barriers for uniparental development in humans. The discovery of haploid human ES cells should thus provide novel means to delineate basic aspects of human genetics and development.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 30 July 2015; accepted 8 February 2016.
Published online 16 March 2016.


Supplementary Information is available in the online version of the paper.

Acknowledgements We thank all members of the Benvenisty and Egli laboratories for input and support. We thank Y. Avior and W. Breuer for their assistance with experimental procedures. I.S. is supported by the Adams Fellowships Program for Doctoral Students, G.C. is supported by the A*STAR International Fellowship, U.W. is a Clore Fellow, D.E. is a NYSF-Robertson Investigator, and N.B. is the Herbert Cohn Chair in Cancer Research. This work was partially supported by the Rosetrees Trust and by The Azrieli Foundation (to N.B.), by the Russell Berrie Foundation Program in Cellular Therapies of Diabetes, by the New York State Stem Cell Science (NYSTEM) IIRP Award number C026184, and by the New York Stem Cell Foundation (to D.E.).

Author Contributions I.S., D.E. and N.B. designed the study and wrote the manuscript with input from all authors. I.S. isolated and characterized haploid human ES cell lines, performed differentiation experiments and analysed the data. G.C. developed and performed the centromere quantification analysis and carried out neuronal differentiation. T.G.-L. assisted in tissue culture and carried out neuronal analyses and tissue sectioning. I.S., M.P., U.W. and O.Y. were involved in the genetic screening. M.P. and U.W. assisted with teratoma assays, I.S. assisted with pancreatic differentiation. M.W.S. was involved in all aspects of oocyte donation and research. D.E. derived human pES cell lines from haploid oocytes. D.E. and N.B. supervised the study.

Author Information All high-throughput data have been deposited at the Gene Expression Omnibus (GEO) under accession number GSE71458. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.E. (de2220@cumc.columbia.edu) or N.B. (nissimb@cc.nyu.edu).