Complete Meiosis from Human Induced Pluripotent Stem Cells


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Disclosure of potential conflicts of interest is found at the end of this article.

ABSTRACT

Gamete failure-derived infertility affects millions of people worldwide; for many patients, gamete donation by unrelated donors is the only available treatment. Embryonic stem cells (ESCs) can differentiate in vitro into germ-like cells, but they are genetically unrelated to the patient. Using an in vitro protocol that aims at recapitulating development, we have achieved, for the first time, complete differentiation of human induced pluripotent stem cells (hiPSCs) to postmeiotic cells. Unlike previous reports using human ESCs, postmeiotic cells arose without the over-expression of germ-line related transcription factors. Moreover, we consistently obtained haploid cells from hiPSCs of different origin (keratinocytes and cord blood), produced with a different number of transcription factors, and of both genetic sexes, suggesting the independence of our approach from the epigenetic memory of the reprogrammed somatic cells. Our work brings us closer to the production of personalized human gametes in vitro.

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INTRODUCTION

Human germ cells transmit genetic information across generations. During development, primordial germ cells (PGCs) migrate from the base of the allantoids, where they originate, to the genital ridges [1, 2]. After colonizing the primordial gonad, PGCs undergo global DNA demethylation, chromatin modifications, erasure of parental imprinting [3-5], and, in the female, initiation of meiosis. Functional gamete development to sperm and oocytes ends with completion of meiosis years later: after puberty in the spermatozoa of male and after fertilization of the oocyte in the female. As developmental errors can result in infertility and other pathological conditions [6], creation of in vitro models may not only advance clinical applications of infertility treatments, but also aid in in vitro production of donor-derived gametes.

Recent studies indicate that mouse [7-11] and human [12-18] embryonic stem cells (ESCs) can differentiate in vitro into oocyte- or sperm-like cells. However, gametes derived from ESC lines would be unrelated to the patient in need of fertility treatment. Moreover, the issue of identity of a new individual, and that of the biological parent, would likely discourage this route of treatment. On the other hand, induced pluripotent stem cells (iPSCs) overcome both issues, as they are genetically related to the donor individual. Thus, we sought to develop methodologies that would lead to meiosis progression and gamete formation from iPSC.

MATERIALS AND METHODS

Culture of hESCs and hiPSC

Human ESCs (hESCs) and human iPSCs (hiPSCs) were cultured on mitotically inactivated primary human foreskin fibroblasts (hFFs) in hESC medium with knockout (KO)–Dulbecco’s modified Eagle’s medium containing 20% KO serum replacement, nonessential amino acids (1X), penicillin/streptomycin (50 U/ml), GlutaMAX (1X), and basic fibroblast growth factor (bFGF; 8 ng/ml), all from Gibco (Invitrogen, Carlsbad, CA, www.invitrogen.com). Medium was changed every 24 hours. ESCs and iPSCs were passaged mechanically onto new hFF layers every 7 days (Table 1).

Differentiation to Haploid Gamete-like Cells

Cells were maintained in a six-well plate for 3 weeks with hESC media in the absence of bFGF. Subsequently, 1 μM retinoic acid (RA) (Sigma, St. Louis, MO, www.sigmaaldrich.com)
was added to the medium and the culture extended for 3 more weeks. At the end of this period, cells were sorted and the desired fraction was seeded at a concentration of 3.5 × 10^5 cells per well in a six-well plate in the presence of 10 μM Forskolin (FRSK) (Sigma), human recombinant leukemia inhibiting factor (rLIF; 1,000 U/ml) (Sigma), and bFGF (8 ng/ml) and the CYP26 inhibitor R115866 (1 μM) (generously supplied by Johnson & Johnson Pharmaceutical Research and Development) for 2, 3, and 4 more weeks, depending on the timing of the experiment. The medium was changed every 2 days.

**Immunofluorescence**

Cells were grown on cover slides or seeded by Cytospin preparation, and fixed with 4% paraformaldehyde (PFA). The following antibodies were used: TRA-1-81 (AB5922, 1:200), both from Chemicon (Temecula, CA, www.chemicon.com); stage-specific embryonic antigen 3 (SEEA-3) (MC-631, 1:2) and SEEA-1 (MC-480, 1:1), both from the Developmental Studies Hybridoma Bank at the University of Iowa; OCT-3/4 (C-10, Santa Cruz, CA, www.scbt.com), sc-5279, 1:100), NANOG (Everest Biotech [Oxfordshire, UK, www.everestbiotech.com]) applying Summit software. The specificity of the staining was verified by matched isotype control mAbs. A total of 10,000 cells per well were subjected to FACS analysis and sorting.

**Flow Cytometry Immunophenotyping**

For surface phenotype and cell sorting, the following fluorochromes were used: fluorescein isothiocyanate (FITC), Alexa-Fluor-488, phycoerythrin (PE), or allophycocyanin (APC)-labeled monoclonal antibodies (mAbs), all from Becton Dickinson Biosciences (Franklin Lakes, NJ, www.bd.com): anti-CD9 FITC (M-L13), anti-CD49f PE (GoH3), anti-SEEA-4 AF488 (MC-813-70), anti-CD90 APC (5E10), and anti-SEEA-1 AF647 (MC-480). The specificity of the staining was verified by matched isotype control mAbs. A total of 10,000 events were collected. Hoechst 33,358 (H258) was included at 1 μg/ml in the final wash to detect live/dead cells. All analyses were performed on a MoFlo cell sorter (DakoCytomation, Glostrup, Denmark, www.dako.com) applying Summit software.

**Fluorescence In Situ Hybridization**

Samples were processed and labeled as described previously [19]. A triple-color fluorescence in situ hybridization (FISH) was used to determine ploidy of the cells using centromeric probes against chromosomes 18, X, and Y (Spectrum Aqua, Spectrum Green, and Texas Red, respectively; Vysis, Abbott Laboratories,Abbott Park, IL, www.abbottmolecular.com). Analyses were carried out using an Olympus BX60 epifluorescence microscope equipped with a triple-band pass filter and specific filters for Aqua, FITC, and Cy3.

**Fluorescence-Activated Cell Sorting (FACS) Analysis for DNA Content**

Single cell suspensions were prepared as described above. Cell were fixed in 4% PFA for 15 minutes at RT, permeabilized in saponin containing buffer for 30 minutes and stained with 4',6-diamidino-2-phenylindole overnight. All samples were subjected to FACS analysis and sorting.

**Promoter Methylation Analysis**

Genomic DNA from hESCs and hiPSCs at various stages of differentiation was extracted from about 1,000,000 cells using QIAamp DNA Mini Kit (Qiagen, Germantown, MD, www.StemCells.com) and standard PCR using primers as described (Table 2 and Supporting Information Table 1).

### Table 1. List of human embryonic stem cell and induced pluripotent stem cell lines used in this study

<table>
<thead>
<tr>
<th>Name of the cell line</th>
<th>Cell type</th>
<th>Culture conditions</th>
<th>Sex</th>
<th>Factors</th>
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<td>Feeders</td>
<td>F</td>
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<tr>
<td>ES[6]</td>
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<td>Feeders</td>
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**Abbreviations:** CBiPSC, Cord blood induced pluripotent stem cell; ESC, embryonic stem cell; F, female; hESC, human embryonic stem cell; KipSC, keratinocyte derived induced pluripotent stem cell; M, male.
RESULTS

CBiPSC and KiPSC lines used in this study were generated and characterized following previously reported protocols [20, 21]. All hiPSC lines expressed the pluripotency-associated transcription factors OCT4, NANOG, SOX2, KLF4, C-MYC, DPPA4, DNMT3B, REX1, SAL2, and LIN28 and surface markers SSEA-3 and TRA-1-81 (Supporting Information Figs. 1, 2, 3A). All iPSC lines were negative for the germ cell marker VASA, both at the RNA and protein level (Supporting Information Figs. 1, 3C). Moreover, the transgenes used to induce the pluripotent state were silenced in all lines (Supporting Information Fig. 3B). All cell lines exhibited a normal karyotype throughout the experimental period (Supporting Information Fig. 4), indicating that the ploidy levels recorded were a product of the differentiation strategy.

The differentiation protocol that we developed (Fig. 1) consists of a first step in which hiPSCs and hESCs were allowed to differentiate for 3 weeks in monolayer in absence of any growth cytokines. A second step consisted in culturing the cells for 3 further weeks in presence of RA. After these 6 weeks of differentiation, we sorted all cells for a combination of surface markers and cultured the positive fraction (see below) in presence of LIF, bFGF, FRSK, and CYP26 inhibitor for 4 more weeks, reaching a 10 week-long differentiation protocol. During the course of the differentiation protocol, we evaluated the correct recapitulation of the developmental events leading to germline differentiation.

To confirm that our protocol does induce differentiation, we compared by RT-qPCR the expression of pluripotency related genes after 3 weeks of differentiation (Supporting Information Fig. 3D) with undifferentiated cells (Supporting Information Fig. 3A). As an example, OCT4 expression was found to be 18 times higher in pluripotent lines than in differentiated cells. As indirect evidence that germ cell differentiation does not usually occur during spontaneous differentiation, we produced intradermal teratomas with the cell lines used in this study and then performed immunofluorescence for detecting markers of the three germ layers and for germ cells (VASA). We observed expression of the endoderm, mesoderm, and ectoderm markers but not of VASA in those teratomas (Supporting Information Fig. 5C).

RA signaling has been shown to stimulate both PGCs division and entrance into meiosis through the CYP26/Stra8 pathways in genital ridges in female PGCs and at puberty in male germ cells [22, 23]. After 3 weeks of differentiation, we added RA to the culture and extended the differentiation protocol for another 3 weeks. It is interesting to note that when RA was added to the culture from the beginning of the protocol, it did neither increase nor speed up the appearance of PGC markers such as VASA, as assessed by immunofluorescence at 4 and 6 weeks of differentiation (data not shown), indicating that a certain level of cell commitment has to be attained before the RA signaling pathways become active. Three weeks after RA supplementation, 6 weeks of total differentiation, we were able to identify VASA+/SSEA-1+ cells by FACS analysis in some of the samples, indicating possible exit from the PGC state of this population and progression toward premeiotic cells [24]. Interestingly, VASA+ cells were found surrounded by VIMENTIN+ (marker of Sertoli cells), NESTIN+, and 3β-HSD+ (markers of Leydig cells) cells (Fig. 2A), suggesting the possibility of reconstitution of an elementary in vitro testicular niche [25, 26].

Some of these markers are not strictly specific for Sertoli and Leydig cells, but they also detect neural cells and...
cardiomyocytes. To clarify the identities of each cell type, we assayed different combinations of markers by immunofluorescence (Supporting Information Fig. 5A, 5B).

When assessing markers of neural cells (NESTIN and PAX6) in combination with VIMENTIN, we found in the same well a “neural niche” comprised by cells expressing NESTIN and PAX6 (but not VIMENTIN), as well as other areas whose cells did not express PAX6, but they did express NESTIN and VIMENTIN only, confirming the presence of a differentiating population of non-neural origin, and presenting

**Figure 2.** Differentiation towards human germ-like cells at 6 weeks of culture. (A): Immunofluorescence staining for VIMENTIN, VASA, NESTIN, and 3β-HSD in differentiated human embryonic stem cells and human induced pluripotent stem cells before sorting. Human testis is used as a positive control. Scale bar = 50 μm. (B): Polymerase chain reaction for detecting VASA at 6 weeks differentiation in all cell lines used. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole.
testicular markers (NESTIN and VIMENTIN) instead. Also, assaying for a marker of cardiomyocytes (GATA4) in combination with NESTIN and VIMENTIN, we also found noncardiac origin and confirmed the presence of testicular markers.

To evaluate further differentiation, we analyzed the cells after 6 weeks of differentiation. We detected the expression of VASA in all cell lines tested (Fig. 2B), indicating the repeatability of the current differentiation protocol among cell lines.

**Figure 3.** Characterization of human germ-like cells after enrichment by FACS sorting at 6 weeks of culture. (A): Upper dot-plots: FACS analysis and sorting of CD9+/CD49f+/CD90- / SSEA-4- population (orange) and CD9-+ / CD49f+/CD90+/SSEA-4+ population (red) at 6 weeks of differentiation sample. Lower dot-plots: FACS analysis of different population in human testis as a control; CD9-/CD49f-/CD90+ population (red), CD9+/CD49f+/CD90- population (orange) and CD9+-/CD49f+/CD90- population (green). (B): Immunofluorescence of VASA in CD9+/CD49f+/CD90- and CD9-+/CD49f+/CD90- fractions at 6 weeks of differentiation (see arrowhead representing VASA positive cell) Scale bar = 25 µm. (C): Polymerase chain reaction for the detection of VASA at 6 weeks of differentiation after enrichment by FACS sorting. (D): Representative morphologies of sorted fractions from KiPS1 and KiPS2 cultured at 8 weeks of differentiation (see arrowheads representing round cells). Scale bar = 50 µm. Abbreviations: APC, allophycocyanin; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PE, phycoerythrin; SSEA4, stage-specific embryonic antigen 4.
In primates, CD49f has been shown to be expressed in a subset of early spermatogonia [27, 28], whereas CD9 is a marker of spermatogonia [29, 30] and CD90 expression extends to the early spermatid in human [31]. A combination of CD9, CD49f, CD90, and SSEA-4 staining (Fig. 3A) revealed that the CD9+/CD49f+/+CD90-/SSEA-4 fraction comprised about 45% of all cells (orange population) and, most importantly, contained a small VASA+ population, roughly 2%, which is in line with the final proportion of haploid cells, as shown by immunofluorescence and RT-PCR (Fig. 3B, 3C). As a control, we characterized samples of human testis with the same combination of markers except for SSEA-4 (Fig. 3A, orange population). We identified the same CD9+/CD49f+/+CD90+ population in the testis, about 29%, which comprised germ cells. Taken together, these data indicate that our protocol is conducive to the differentiation of spermatogonia-like cells [32].

Thus, we attempted to further advance differentiation toward meiosis of the CD9+/CD49f+/+CD90+/SSEA-4 fraction by culturing the cells in the presence of human LIF (hLIF), FRSK, bFGF, and R115866, a CYP26 inhibitor, for either 2, 3, and 4 more weeks. The CD9+/+/CD49f+/+CD90+/SSEA-4+ cell fraction was also cultured in parallel. We observed different morphologies between the two fractions (Fig. 3D); specifically, where CD9+/+/CD49f+/+CD90-/SSEA-4- cells displayed a round shape and a high nucleus to cytoplasm ratio (as seen in germline cells), CD9+/+/CD49f+/+CD90+/SSEA-4+ cells were smaller, of variable shape, and tended to aggregate in colonies of ESC-like appearance. As expected, this population expressed SSEA-4, an ESC marker. Conversely, the cells in the CD9+/+/CD49f+/+CD90-/SSEA-4- fraction expressed the male germline marker ACROSIN (Fig. 5D). This population was also negative for the female markers ZP1, ZP3, and FIG 1α (data not shown), indicating a predominant presence of male germ-like cells.

Next, we evaluated the expression of meiosis related markers; we detected SCP3 and H2AX expression in a fraction of CD9+/+/CD49f+/+CD90-/SSEA-4- cells, indicating meiotic competence (Fig. 4A). This was further confirmed by detecting meiotic phase 1 in chromosomal spreads of these fractions (Fig. 4B) and by the expression of premeiotic gene, STRA8 (Fig. 4C). We concluded that cells in the CD9+/+/CD49f+/+CD90-/SSEA-4- fraction enters meiosis by 9 weeks of differentiation. We found that both hESC and hiPSC can initiate meiosis, as assessed by SCP3 and H2AX expression (Fig. 4A, 4B). However, as detailed below, only hiPSCs were able to complete meiosis and generate haploid cells (Fig. 5A–5D and Supporting Information Fig. 7).

Furthermore, we assessed the ability of our differentiation protocol to epigenetically reprogram the differentiating cells. We selected six imprinted genes, two of which are normally expressed from the maternally inherited allele: pleckstrin homology-like domain, family A, member 2 (PHLDA2) [33] and cyclin-dependent kinase inhibitor 1C (CDKN1C) [33], and four that are expressed from the paternally inherited one: mesoderm specific transcript homolog (MEST) [34], insulin like growth factor 2 (IGF2) [35], neuronatin (NNAT) [36], and small nuclear ribonucleoprotein polypeptide N (SNRPN) [37]; (Supporting Information Fig. 6). The expression level of all genes after 10 weeks of differentiation was similar to that of spermatogonia-like cells in human testis [27, 28].

Figure 4. Meiotic progression of germ-like cells at 9 weeks of culture. (A): Immunofluorescence of meiotic spreads at 9 weeks of differentiation. Detection of VASA, SCP3, and H2AX proteins in human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) lines. (B): Upper pictures: Prostate I morphology after Leishmann staining of CD9+/+/CD49f+/+ (arrowheads); lower pictures: representative morphology of the nucleus in the CD9+/+/CD49f+/+ fraction. Scale bar = 10 μm. (C): Polymerase chain reaction detecting the expression of VASA, Stra8, and S18 in some cell lines used in this study. (D): CpGs in the H19 promoter DMR were analyzed by bisulphite sequencing in undifferentiated hiPSC and hESC and after 6 and 10 weeks of differentiation. Methylated CpGs are represented as filled circles; unmethylated CpGs are represented as open circles. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; SCP3, synaptosomal complex protein 3.
of mature spermatozoa, whereas the expression at 6 weeks was consistently different. We also assayed the expression level of the stem cell specific gene telomerase reverse transcriptase (TERT) and the gene controlling X inactivation (XIST). In both cases, the expression level of hiPSCs after 10 weeks of differentiation was comparable with spermatozoa, while it remained higher at 6 weeks of differentiation (Supporting Information Fig. 6). Furthermore, we evaluated the DNA methylation status of the promoter of the maternally expressed, paternally imprinted gene H19 (Fig. 4D). Four of six cell lines tested (one hESC and five hiPSC) increased the methylation status of H19 from an average 48.5% (range 43–51) to

Figure 5. Haploid formation of germ-like cells at 10 weeks culture. (A): DNA content analysis of differentiation process of KIPS2: (i) undifferentiated, (ii) 10 weeks, (iii) 10 weeks + R115866, (iv) control of human sperm. (B): Fluorescent in situ hybridization against chromosomes X and Y in a female (KIPS4) and male (KIPS2) samples of differentiated human induced pluripotent stem cells over 10 weeks in presence of inhibitor. Scale bar = 10 µm. (C): Percentages of 1n, 2n, 4n, and aneuploid cells in all samples analyzed at 10 weeks of differentiation. (D): Immunofluorescence of haploid marker, Acrosin, and Vasa in 10 weeks of differentiation sample (unsorted and sorted haploid population) in presence of inhibitor (see arrowheads showing a positive cell). Human testis was used as a positive control. Scale bar = 50 µm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole.
an average 75.8% (range 53–100). One hiPSC line decreased in H19 methylation level from 72% to 53%. Moreover, the culture of the cells in presence of the CYP26 inhibitor, which acts at a stage of differentiation posterior to imprinting re-establishment, did not affect imprinting re-establishment.

Finally, we asked whether the CD9+/CD49+/+/CD90−/SSEA-4− fraction contained haploid cells. DNA content analysis by FACS showed putative presence of haploid cells, indicative of meiosis completion, by 10 weeks (Fig. 5A and Supporting Information Fig. 7); FACS positive samples were further evaluated by FISH with centromeric probes on chromosomes 18, X, and Y, and the haploidy was confirmed (Fig. 5B). Using FISH, which we considered the most stringent assay of ploidies, after performing several controls (Supporting Information Table 3), we consistently observed between 0.4% and 2.3% of haploid cells per sample derived from hiPSC (Fig. 5C). The heterogeneity of results could be due to either the variable differentiation ability of each individual hiPSC line, the effect of uncontrolled variables in the protocol, or differences of sensibility among techniques (DNA content vs. FISH). Also, we performed a RT-qPCR for detecting the silencing of the transgenes, and we found that most of the lines had silenced them at 6 weeks of differentiation; however, three of eight lines had slightly reactivated the Oct4 transgene (Supporting Information Fig. 3E). Also, we observed that haploid cells appeared only when the inhibitor R115866 and the rest of the growth factors (FRSK, FGF2, and hLIF) were added to the differentiation medium (Supporting Information Table 3). Finally, we verified that the haploid cells were masculine by detecting ACROSIN by immunofluorescence at 10 weeks of differentiation (Fig. 5D).

**DISCUSSION**

Gamete differentiation in the human species is a complex process that starts in the early embryo when PGCs arise and ends as much as 40 years later with the completion of meiosis after fertilization of the female oocyte. Along the way, the developing gamete undergoes a finely orchestrated series of events, which renders them the most specialized cells in the body and the only pluripotent one. Perhaps for this reason, in vitro differentiation of ESCs and iPSCs to germ cells has been the most challenging and the least successful.

We reasoned that hiPSCs could be induced to reach the meiotic stage of differentiation by recapitulating known steps of gamete development. Thus, we set out to transition the cells from a pluripotent state to a committed one. This was initially achieved by culturing the cells without bFGF for 3 weeks, releasing them from the pluripotency pool and allowing unguided differentiation. Direct differentiation onto mono-layers of human fibroblasts rather than by embryoid bodies ensured more consistent differentiation results, as shown by a more widespread decrease of pluripotency markers under this condition [14]. At the end of this period, the expression of the pluripotency associated markers SSEA-4 and OCT4 were downregulated [14, 18] (Supporting Information Figs. 3, 8). SSEA-4 is one of the last surface markers to be lost during hiPSC and hESC differentiation [38] and is, therefore, a good indicator of a permanent exit from the pluripotent program.

We speculate that during the initial 3 weeks of unguided differentiation, some of the cells in the pluripotent population will stochastically start down the meiotic differentiation path, but this initial “inclination” would be lost without the RA signaling cascade being activated. Our hypothesis gains strength by the observation that adding RA to the differentiation protocol from the very beginning does neither speed up nor increase the appearance of this initial population.

LIF is produced in the male gonad by the peritubular cells and promotes survival and proliferation of rat gonocytes [39]. FRSK induces proliferation in germ cells by activation of cyclic adenosine monophosphate [40] and is involved in meiosis induction [41]. bFGF is produced by Sertoli cells, where its role is to help balancing self-renewal and differentiation of spermatogonial stem cells [42]. In various animal models, the expression of bFGF is involved in the autocrine and paracrine regulation of proliferation and differentiation of spermatogonia and spermatocytes via its receptors [43]. The CYP26 inhibitor R115866 acts by suppressing the inhibitory effect of CYP26 on the meiosis regulator gene STRA8 [24]. Specifically, CYP26 is part of a regulatory pathway that regulates the timing of meiotic initiation by increasing RA degradation and consequent STRA8 inactivation in the developing gonad. This results in blocking germ cells from entering meiosis. As the effect of R115866 on this pathway has been shown in different biological contexts [44, 45], we speculated that it could improve meiotic entry in our system.

It is interesting to note that omitting R115866 from the differentiation cocktail inhibited meiotic progression, as evidenced by the consistent lack of haploid cells in control experiments, as detected by both chromosomal spreads and FACS analysis (Supporting Information Table 3). During human development, PGCs migrate along the dorsal mesentery and into the genital ridges, and their differentiation lasts about 6–8 weeks [2], while a premeiotic to postmeiotic semi-niferous epithelium cycle (the time needed for a dividing spermatogonia to reach the haploid sperm stage) is about 7 weeks long [46]. Our differentiation protocol is broadly consistent with this timing and faithfully recapitulates sequential steps of development. We were able to direct the differentiation of hiPSC to germ-like cells without the overexpression of any developmentally related genes. However, we could not detect haploid cells even after 10 weeks of differentiation when using hESCs in any of the experiments. As germine differentiation has been achieved in hESCs and hiPSCs by subsequent forced overexpression of DAZL, DAZ, and BOULE [17, 47], one can speculate that hiPSCs are somehow epigenetically predisposed to differentiation along the germ line path as Panaula et al. [47] suggested in their recent publication. One reason might be the epigenetic state of hiPSCs, which has been shown to be different from that of hESCs in some imprinted loci, which might confer hiPSCs an advantage in differentiating along the germline [48]. Another possible reason might be some predisposing epigenetic memory from the original somatic cell population, although we tested hiPSCs from different somatic sources and all were able to differentiate to haploid cells in vitro. Finally, the reactivation of transgene OCT4, a gene involved in early PGCs biology, might play a role in this phenomenon, but in our study OCT4 was slightly reactivated in three of eight cell lines, thus dispensable and not the main cause of haploid cells formation. Further studies using nonintegrative or excisable reprogramming strategies will be needed to dissect the reason for the apparent facility of hiPSCs to differentiate into germ-like cells.

One of the most crucial points on the development of a germ-line cell is the establishment of its imprinting mark to a monoparental state. The imprinted genes are mostly involved in fetal and placental development. To assess the state of imprinted genes reprogrammed to a monoparental state, we selected six imprinted genes, two of which are normally expressed from the maternally inherited allele and four are expressed from the paternally inherited one. We reasoned that, if our differentiation protocol was directing the cells toward a sperm-like phenotype, the expression
level of imprinted genes after 10 weeks of differentiation should have been similar to that of mature spermatozoa. We indeed found a strong tendency in all six transcripts to sperm-like level of expression at 10 weeks of differentiation, while the expression at 6 weeks was consistently different. We also assayed the expression level of the gene TERT and the gene controlling TERT.

Overall, there is a strong tendency to re-imprint in a monoparental, paternal way for most of the assayed hESC and hiPSC lines, but the process is not complete. Furthermore, the presence of the CYP26 inhibitor, which should only push meiosis completion, thus act at a stage of differentiation which posterior to imprinting re-establishment, did not affect imprinting re-establishment, in accordance to prediction.

CONCLUSION

Infertility affects millions of individuals each year, and with the current tendency to postpone childbearing age in both males and females, there is an increasing need for patient specific gametes able to differentiate properly into a new individual. We show here, for the first time, complete and robust meiotic competence of hiPSC-derived cells, and although further assays will be needed to assess the developmental ability of meiotic cells, our work paves the way for the production of in vitro gametes in the human species.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

5 Monk M, Inoue K, Ono R et al. Erasing genomic imprinting memory in H19 methylation level from 72% to 53%, thus displaying faulty imprinting re-establishment. Finally, another hiPSC line displayed a strong methylation mark (94%) since the beginning of culture and did not change significantly. This last instance is analogous to what was reported by Park et al. [18].

Overall, there is a strong tendency to re-imprint in a monoparental, paternal way for most of the assayed hESC and hiPSC lines, but the process is not complete. Furthermore, the presence of the CYP26 inhibitor, which should only push meiosis completion, thus act at a stage of differentiation which posterior to imprinting re-establishment, did not affect imprinting re-establishment, in accordance to prediction.

Infertility affects millions of individuals each year, and with the current tendency to postpone childbearing age in both males and females, there is an increasing need for patient specific gametes able to differentiate properly into a new individual. We show here, for the first time, complete and robust meiotic competence of hiPSC-derived cells, and although further assays will be needed to assess the developmental ability of meiotic cells, our work paves the way for the production of in vitro gametes in the human species.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES