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Report

Direct Differentiation of Human Pluripotent Stem Cells into Haploid Spermatogenic Cells

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SUMMARY

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have been shown to differentiate into primordial germ cells (PGCs) but not into spermatogonia, haploid spermatocytes, or spermatids. Here, we show that hESCs and hiPSCs differentiate directly into advanced male germ cell lineages, including postmeiotic, spermatid-like cells, in vitro without genetic manipulation. Furthermore, our procedure mirrors spermatogenesis in vivo by differentiating PSCs into UTF1-, PLZF-, and CDH1-positive spermatogonia-like cells; HIWI- and HILI-positive spermatocyte-like cells; and haploid cells expressing acrosin, transition protein 1, and protamine 1 (proteins that are uniquely found in spermatids and/or sperm). These spermatids show uniparental genomic imprints similar to those of human sperm on two loci: *H19* and *IGF2*. These results demonstrate that male PSCs have the ability to differentiate directly into advanced germ cell lineages and may represent a novel strategy for studying spermatogenesis in vitro.

INTRODUCTION

Infertility affects ~15% of couples, with male factors responsible for 40%–60% of cases of infertility (Schlegel, 2009). For men without a genetic cause of infertility, stem cell transplantation represents a possible treatment option (Marques-Mari et al., 2009; Mathews et al., 2009; Yao et al., 2011). Protocols to preserve future fertility in boys who are undergoing cancer therapies and cannot yet bank their own sperm are under development (Hermann et al., 2007; Sadri-Ardekani et al., 2011; Schlatt et al., 2009). However, for adult and prepubescent patients who have been rendered sterile prior to sperm collection, no treatments to restore fertility are currently available.

Mouse embryonic stem cells (ESCs) (Geijsen et al., 2004; Hayashi et al., 2011), nonhuman primate (NHP) ESCs, and human ESCs (hESCs) (Bucay et al., 2009; Fukunaga et al., 2010; Kee et al., 2009; Panula et al., 2011; Park et al., 2009; Teramura et al., 2007; Tilgner et al., 2008; Yamauchi et al., 2009) have been differentiated into primordial germ cells (PGCs), fetal precursors of the spermatogenic lineage. In addition, recent studies suggest that human pluripotent stem cells (hPSCs) can enter meiosis and in some cases produce haploid products (Eguizabal et al., 2011; Kee et al., 2009; Panula et al., 2011). In this study, we developed an in vitro method that achieves two significant endpoints. First, male hESCs and human induced PSCs (hiPSCs) differentiate directly into adult-type spermatogonia. Second, differentiating stem cells give rise to cells that are phenotypically similar to postmeiotic round spermatids. These results highlight the full plasticity of hPSCs by showing their ability to undergo spermatogenesis in vitro, culminating with haploid round spermatid-like cells. These results also contribute to the overall goal of both understanding germ cell development in vitro and ultimately generating gametes that may prove invaluable for understanding infertility mechanisms.

RESULTS

Mouse Spermatogonial Stem Cell Conditions Elevate the Expression of Germ Cell Markers

Previous studies have shown that human testis cells cultured in PSC conditions directly dedifferentiate into PSCs (Conrad et al., 2008; Ko et al., 2009; Kossack et al., 2009). Thus, we examined whether ESCs could directly differentiate into germline stem cells. Our goal was to differentiate PSCs into spermatogonial stem cell (SSC)-like cells, because this spermatogenic lineage has shown an exceptional ability to recolonize sterilized testes and thus restore fertility in certain species, including mice and NHPs (Brinster and Avarbock, 1994; Jahnukainen et al., 2011). One advantage of this strategy is that there are established protocols for culturing and expanding rodent SSCs in vitro (Kanatsu-Shinohara et al., 2003). We cultured H1 (WA01) hESCs and human foreskin fibroblast 1 (HFF1) iPSCs directly in

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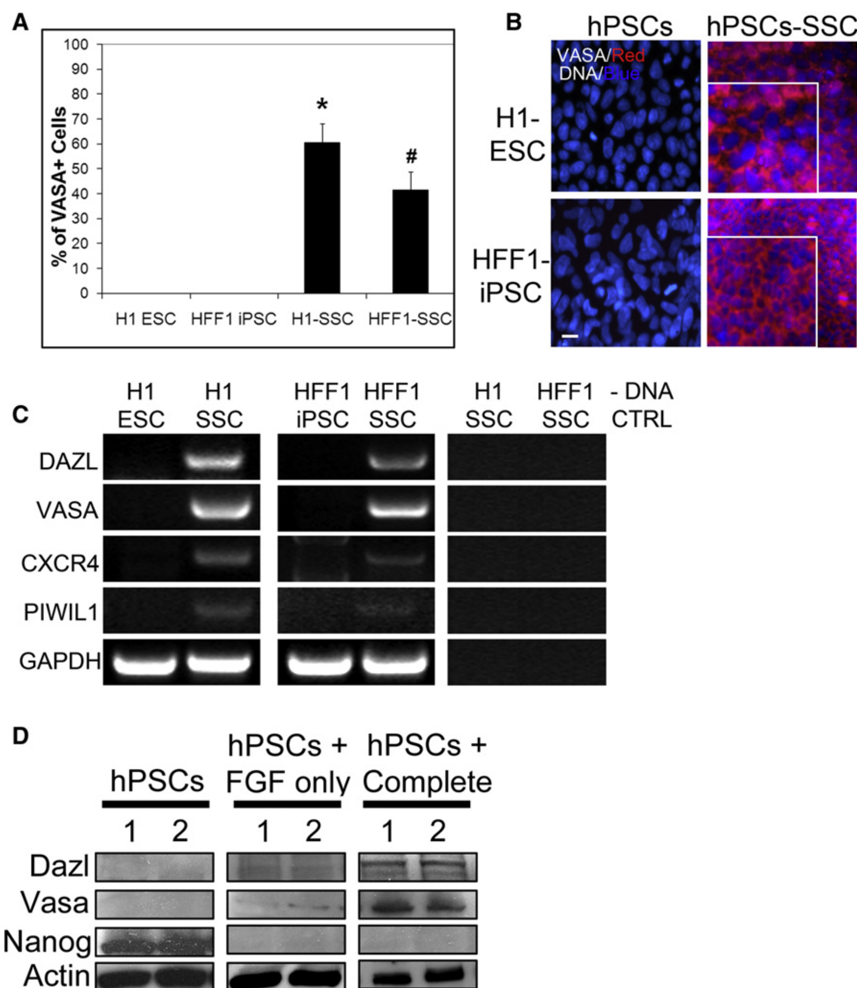


Figure 1. Differentiation of hPSCs in SSC Culture Yields Significant Percentages of VASA+ Cells

(A) H1 ESCs and HFF-1 iPSCs cultured in mouse SSC conditions for 10 days and then stained for VASA. The percentage of VASA expression was quantified in the parent PSC lines and the differentiated lines. A representative graphical analysis from five separate trials, with >5,000 cells counted for each condition, is shown. *p < 0.01 comparing H1 ESC with H1 SSC; #p < 0.01 comparing HFF1 iPSC with HFF1 SSC. Error bars shown for each data set represent the standard deviation (SD) of the mean.

(B) Representative images of PSCs and PSCs differentiated in SSC culture conditions for 10 days and stained for VASA. DNA labeled with Hoechst. Scale: 50 μm. Enlarged insets show typical perinuclear localization of VASA.

(C) RT-PCR for germ cell markers *DAZL*, *VASA*, *CXCR4*, and *PIWIL1* in PSCs and their differentiated counterparts. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) is shown as a loading control. No DNA (−DNA) is also shown as a negative control.

(D) Representative western blot analyses showing upregulation of germ cell marker expression and a concomitant loss of the pluripotent marker Nanog in complete SSC culture conditions (with GDNF and FGF). Despite loss of Nanog in FGF-only SSC medium (i.e., without GDNF), germ cell markers were not expressed. Actin is a loading control.

standardized mouse SSC culture conditions. After a 10 day culture, we observed significant increases in VASA+ cells, with ~60% of H1 cells and ~40% of HFF1 cells expressing VASA (Figures 1A and 1B and enlarged insets). These VASA+, germ-like cells showed typical VASA staining patterns as seen in human testis sections (Figure S1A). Day 10 was the optimum time point, because day 7 cultures yielded lower numbers of VASA+ cells and day 15 cultures did not yield an appreciable increase (Figure S1B). In comparison with previous protocols, we observed a 4- to 5-fold increase in VASA+ cells derived from hPSCs, all within 10 days postdifferentiation.

We further analyzed H1 and HFF1 cells cultured in mouse SSC conditions (termed H1 SSC and HFF1 SSC, respectively) for expression of additional germ cell markers. Deleted-in-Azoospermia-like (DAZL) and VASA are two germline-specific, RNA-binding proteins that are important in germ cell development and normal spermatogenesis (Castrillon et al., 2000; Kee et al., 2009). Recently, Kee et al. (2009) showed that some hESCs, mainly female lines, express low levels of *VASA* mRNA. Here, both male hESC and hiPSC lines did not exhibit expression of *VASA* mRNA (Figure 1C). H1 SSC and HFF1 SSC cells showed an increase in all germ cell markers tested, including *CXCR4* and *PIWIL1*, by reverse transcriptase (RT)-PCR, suggesting that this is an efficient way to generate germ cell lineages (Figure 1C). VASA and DAZL protein expression was also elevated in H1 SSCs and HFF1 SSCs compared with the undifferentiated, parent PSC lines (Figure 1D). We also observed that germ cell differentiation was dependent on the growth factor glial-derived neurotrophic factor (GDNF; hPSCs + Complete). Cells differentiated without GDNF (hPSCs + fibroblast growth factor [FGF] only) demonstrated no increase in VASA or DAZL protein expression but did show a loss of the pluripotent marker Nanog, suggesting that both H1 hESCs and HFF1 hiPSCs differentiated (Figure 1D). These results suggest that GDNF containing SSC medium efficiently and rapidly differentiates hPSCs into germ cell lineages.

hPSCs Cultured in Mouse SSC Conditions Express PLZF

CXCR4 is a chemokine receptor that is expressed by spermatogonia and plays a role in SSC maintenance (Payne et al., 2010). Because we detected elevations in *CXCR4* in both H1 SSCs and HFF1 SSCs (Figure 1C), we next evaluated whether H1 SSCs and HFF1 SSCs expressed PLZF, a zinc-finger transcription factor that is a consensus marker of stem and progenitor spermatogonia. PLZF, or ZBTB16, plays a critical role in SSC self-renewal and growth (Buaas et al., 2004; Costoya et al., 2004; Hobbs et al., 2010). A 10 day culture in mouse SSC conditions induced

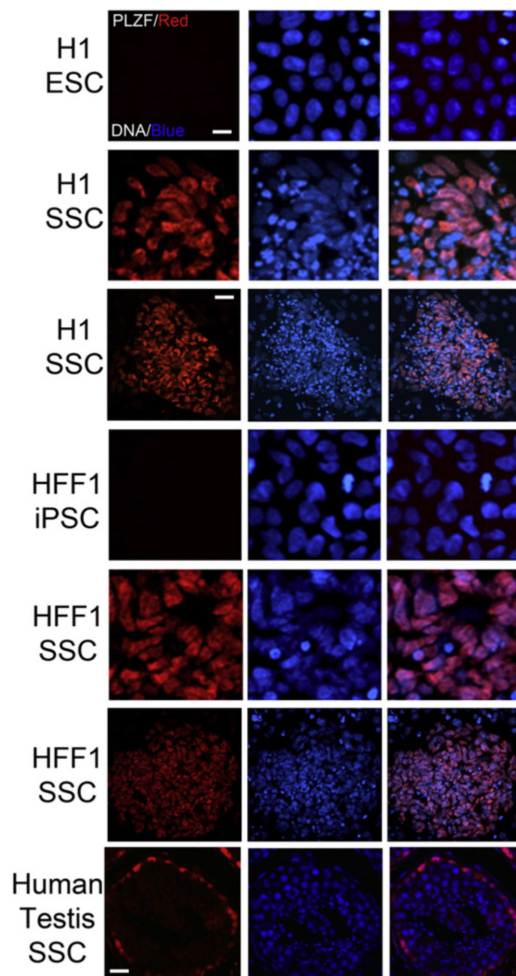


Figure 2. Differentiation of hPSCs in SSC Conditions Results in Expression of the SSC Marker PLZF

Although the parent PSC lines do not express detectable levels of PLZF, 10 day culture in SSC conditions upregulates PLZF (red) expression in both lines. Hoechst (blue): DNA. Scale: 40 μm. Global view (rows 3 and 6) of differentiated colonies shows a large portion of cells expressing PLZF. Scale: 100 μm. The row 7 panel depicts PLZF staining in human testis sections.

expression of PLZF, localized to the nucleus, in both H1 and HFF1 SSCs (Figure 2). This nuclear expression of PLZF mirrors that observed in human testes (Figure 2, row 7). Furthermore, our protocol generates a high percentage of PLZF-positive cells within differentiating colonies (Figure 2, low-magnification views, rows 3 and 6), with ~82% of H1 SSCs and ~78% HFF1 SSCs expressing PLZF (Figure S2A). Unlike other methods, our protocol induces PLZF expression (Figure S2B). This suggests that we are more closely mirroring the early events of in vivo spermatogenesis.

SSC Conditions Yield Postmeiotic, Acrosin-Positive Cells

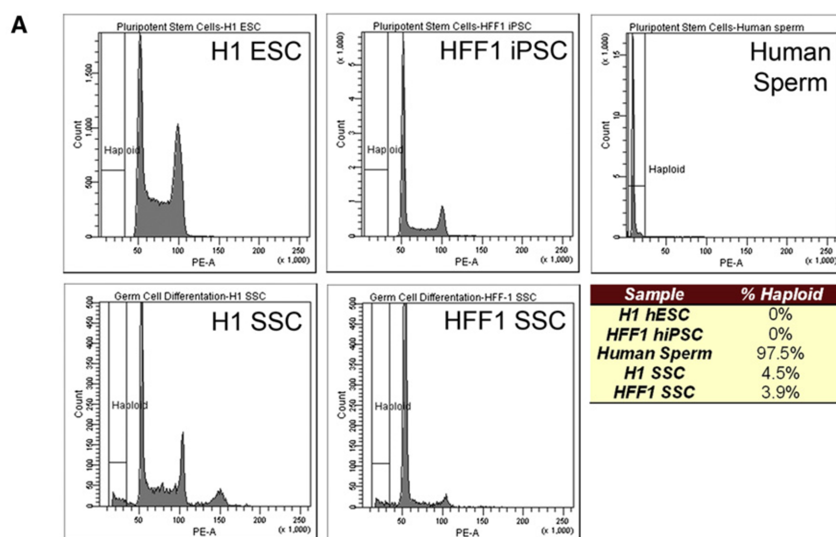
SSCs are defined in part by their ability to produce gametes through a complex combination of division and differentiation. Mouse SSCs can differentiate into haploid cells in vitro (Feng et al., 2002), so we next quantified whether haploid cells were produced in H1 SSCs and HFF1 SSCs. Flow cytometry analyses indicated that a haploid population exists in H1 SSCs (4.5%) and HFF1 SSCs (3.9%) corresponding to haploid peaks observed with human sperm (Figure 3A; Figure S3A). We further confirmed haploidy of isolated cells by fluorescence in situ hybridization (FISH) with a locked nucleic acid (LNA) probe to satellite DNA found on chromosomes 1, 9, 16, and Y (Figure S3C). After fluorescence-activated cell sorting (FACS), the majority of haploid cells isolated from both H1 SSCs and HFF1 SSCs exhibited polar acrosin localization (Figure 3B, enlarged insets; Figure S3B). These results suggest that we are able to generate a small percentage of acrosin-positive, haploid cells in vitro from hPSCs within 10 days of SSC culture. Ten days proved to be optimal because haploid cell production decreased after 20 days (Figure S3D).

hPSC Differentiation in SSC Conditions Generates Cells that Express Markers for Spermatogonia, Premeiotic Spermatocytes, Postmeiotic Spermatocytes, and Round Spermatids

Because differentiation in SSC conditions altered cell-cycle profiles (Figures S4A and S4B) and yielded a small percentage of haploid cells in addition to a large population of PLZF-positive spermatogonia, we next evaluated whether H1 ESCs and HFF1 iPSCs differentiated into intermediate cell types observed in in vivo spermatogenesis. In addition to PLZF, we observed expression of UTF1 and CDH1 (Figure 4A, left column), proteins that are expressed in both spermatogonia and PSCs. In contrast to the PSCs, we observed an increase in protein expression of RET and GFRα1 (Figure 4A, western blots), receptors for GDNF that are found on spermatogonia.

Differentiation of hPSCs in SSC conditions showed an increase in *PIWIL1* RNA expression (Figure 1C). *PIWIL1* (also known as *HIWI*) is essential in spermatogenic progression from SSCs to round spermatids (Deng and Lin, 2002). We examined the expression of three spermatocyte markers for premeiotic spermatocytes/differentiating spermatogonia, meiotic spermatocytes, and postmeiotic spermatocytes. We identified cells in both differentiating H1 ESCs and HFF1 iPSCs expressing premeiotic HILI protein, the meiotic marker synaptonemal complex 3 (SYCP3, which is involved in recombination and segregation of meiotic chromosomes), and postmeiotic HIWI (Figure 4A, center column). Although there were a large number of HILI-positive cells, very few cells expressed SYCP3 or HIWI, suggesting that there is bottleneck prior to meiosis.

We next isolated cells from the haploid peaks by FACS and immunostained these cells for spermatid markers. During spermiogenesis, acrosin expression is turned on and histones are replaced by protamines via transition proteins (Carrell et al., 2007). Haploid cells isolated from H1 and HFF1 SSC cultures express the postmeiotic sperm markers acrosin, protamine 1 (Prot1), and transition protein 1 (TP1; Figure 4, right column). In particular, acrosin staining exhibits polar localization in both cell lines (Figure 4A, row 1). These haploid cells resemble round spermatids by acrosin localization, the nuclear/perinuclear localization



Sample	% Haploid
H1 hESC	0%
HFF1 hiPSC	0%
Human Sperm	97.5%
H1 SSC	4.5%
HFF1 SSC	3.9%

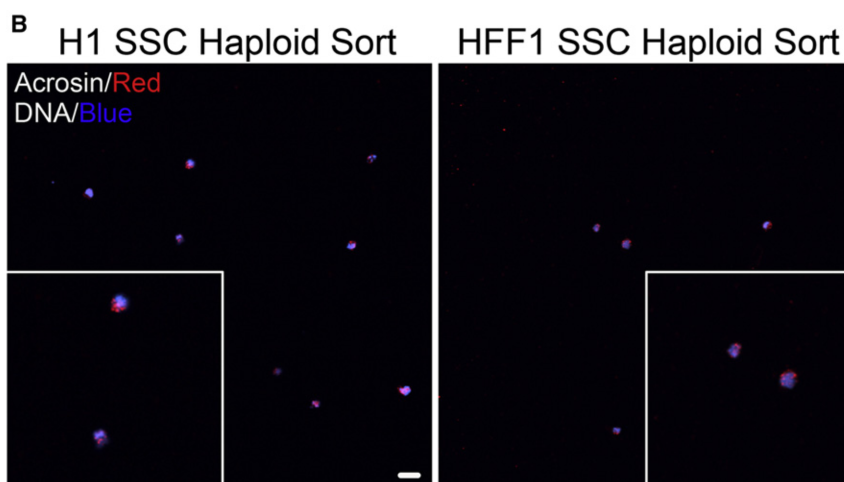


Figure 3. hPSCs Differentiated in SSC Culture Exhibit Haploid Features

(A) FACS ploidy analysis reveals a small haploid peak in hPSCs cultured in SSC culture conditions for 10 days. This peak corresponds to the haploid peak observed in human sperm. The chart below represents the percentage of haploid cells in undifferentiated and SSC-mediated differentiated hPSCs. Data are representative of five cell sorts, with 500,000 cells sorted per experiment.

(B) FACS-isolated haploid cells from H1 SSC (left) and HFF1 SSC (right) were seeded on coverslips and stained with acrosin (red) and Hoechst (DNA, blue). Global view shows several isolated cells with polar acrosin localization. Scale: 50 μm. Insets show zoomed view of acrosin-positive haploid cells.

of TP1, and the perinuclear localization of Prot1 (Figure 4A, right column), which localizes to the perinuclear region of haploid cells and enters the nucleus at the elongated spermatid stage (Carrell et al., 2007). All acrosin-positive cells were also positive for TP1 (Figure S4C). These haploid cells also resemble round spermatids observed in humans and NHPs (Carrell et al., 2007; Moreno et al., 2006; Ramalho-Santos et al., 2002; also see Figure S4D). These results coupled with the above *PIWIL1* expression data suggest that PSCs are able to differentiate directly into postmeiotic, round, spermatid-like cells in vitro.

During in vivo germ cell specification, genomic imprints are removed at the primordial germ cell stage and then reestablished during spermatogenesis (Lucifero et al., 2002). In mice, differentiation of PSCs into functional germ cells results in progeny that exhibit epigenetic disease phenotypes (Nayernia et al., 2006; Nolte et al., 2010). It was proposed that this may be due to improper imprinting during gametogenesis (Lucifero and Reik, 2006). To evaluate imprinting statuses on the haploid spermatids differentiated here, we isolated haploid cells by FACS and examined the methylation status of the imprinting control region (ICR) for paternally imprinted (*H19*) and maternally imprinted (*IGF2*) genes. As previously reported, iPSCs showed aberrant imprinting (Pick et al., 2009), but ESCs showed typical somatic cell imprinting on ICRs for *H19* and *IGF2* (Figure 4B). Isolation of haploid cells from H1 SSC cultures showed imprinting patterns similar to those observed in human sperm, with *H19* ICR methylation of ~90% and *IGF2* ICR methylation of ~5% (Figure 4B). Haploid cells from HFF1 SSCs showed levels of *H19* ICR methylation similar to those observed in human sperm (~90%), but *IGF2* methylation (~14%) was slightly elevated above that observed in human sperm (Figure 4B). These results suggest that the haploid products obtained show similar DNA methylation patterns on at least two parent-of-origin genomic imprints.

DISCUSSION

Several studies have shown that hPSCs differentiate in vitro and in vivo into the three germ layers (endoderm, mesoderm, and ectoderm). However, only recently have studies shown that hPSCs exhibit greater plasticity by differentiating into germ cell lineages. Our study shows that male diploid PSCs differentiate into advanced haploid lineages, including round spermatids. Although female PSCs do not differentiate and undergo cell death in our protocol (data not shown), methods have been developed to generate haploid oocyte-like cells from female lines (for review, see Virant-Klun et al., 2011), furthering demonstrating that both male and female PSCs possess the potential to differentiate into any adult cell type, including gametes.

The generation of viable sperm and spermatids in vitro from PSCs and even somatic cells in humans and other primates

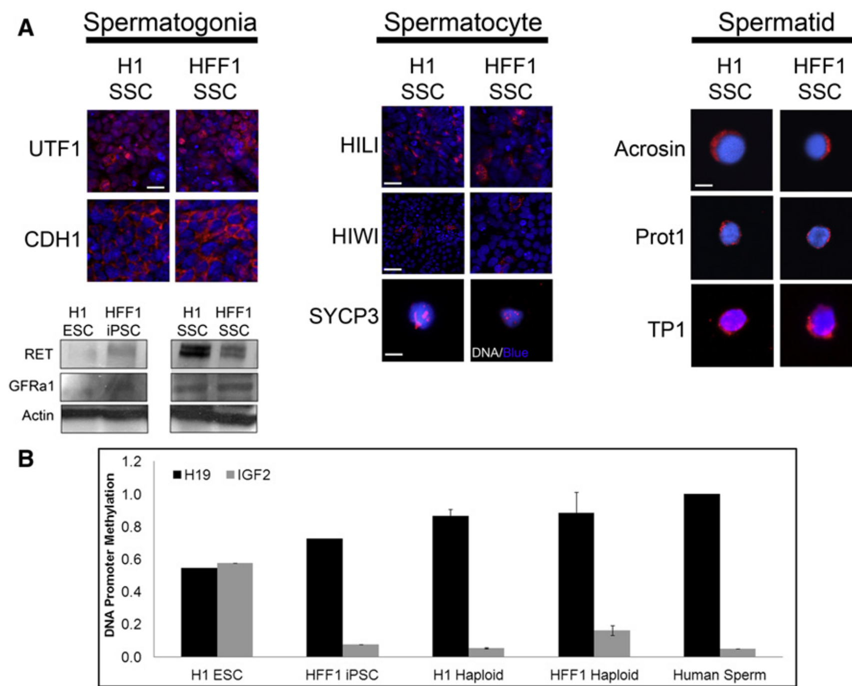


Figure 4. Differentiation of hPSCs in SSC Culture Yields Cells that Express Markers for Spermatogonia, Spermatocytes, and Spermatids

(A) Left: 10 days postdifferentiation, cultures of H1 and HFF1 SSCs express the premeiotic spermatogonial markers UTF1 and CDH1. Scale: 50 μm. Differentiation also induces expression of two membrane receptors, RET and GFRa1. Actin is a loading control. Center: Expression of spermatogonia-to-spermatocyte marker HILI, spermatocyte-to-spermatid marker HIWI, and meiotic marker SYCP3. Scale for HILI: 200 μm; scale for HIWI: 500 μm; and scale for SYCP3: 10 μm. Right: Expression of the postmeiotic spermatid markers acrosin, Prot1, and TP1. Haploid cells were sorted by FACS and immunostained with antibodies directed at the indicated protein. Scale: 10 μm.

(B) H1 ESCs, HFF1 iPSCs, fertile human sperm, and haploid cells obtained by FACS from H1 and HFF1 SSC cultures were examined for methylation on ICRs for *H19* (paternally imprinted) and *IGF2* (maternally imprinted). Methylation status was examined using the QIAGEN Epitect Methyl II PCR Array. The graph shows the average percentage of methylation with error bars. Error bars shown represent the SD of the mean for each data set.

has many biomedical justifications even though the endeavor is fraught with experimental and bioethical challenges (Daley, 2007). Furthermore, the stringency with which these in vitro sperm are evaluated varies according the necessary endpoint. The greatest stringency is required for the generation of fully functional sperm or spermatids that would be considered useful and safe for fertilization in assisted reproductive technology (ART) clinics. This objective is justified by the Oncofertility Consortium, which pursues the benevolent goal of preserving fertility in male cancer survivors who have been rendered infertile during their therapies but are also too young or fragile to produce a sperm specimen for cryobanking (Woodruff, 2010). It is also supported by ART practitioners for the potential treatment of men with either diagnosed or idiopathic infertility who cannot provide either sperm or elongated spermatids. Discovering the stages during spermatogenesis at which various forms of idiopathic male infertility arrest would greatly aid in the diagnosis of (and perhaps the development of treatments for) these still mysterious processes. Knowledge about these spermatogenic arrest sites might also contribute to the design of novel contraceptives. In addition, the epigenetic modifications that enable the properly imprinted sperm chromatin and the replacement of nuclear proteins to form the sperm nucleus could be better investigated in these types of cell cultures versus in intact tissues, especially since our protocol seems to generate haploid products with parent-of-origin imprints similar to those observed in fertile human sperm. Anticipated improvements in the efficiency of in vitro spermatogenesis may also help elucidate how mitochondria are modified to create the sperm mitochondria, and how the somatic centrosome is reduced during male meiosis to form the sperm tail's basal body and the sperm centrosome (Schatten, 1994).

Differentiating hPSCs into SSCs is an important step in evaluating the possibility of transplanting patient-specific PSCs or SSCs to restore fertility. The ability to differentiate hPSCs directly into SSCs without having to go through a PGC differentiation protocol is likewise important for decreasing differentiation strategy time frames to increase utilization within a clinical setting. Furthermore, differentiation in SSC conditions yielded several cell types that are observed during spermatogenesis, suggesting that ultimately, patient-specific spermatogenesis can be studied in vitro with only PSCs. We observed the expression of putative markers for spermatogenesis, including markers for undifferentiated spermatogonia, differentiating spermatogonia, premeiotic spermatocytes, meiotic and postmeiotic spermatocytes, and spermatids. In contrast to published results (Bucay et al., 2009; Fukunaga et al., 2010; Kee et al., 2009; Panula et al., 2011; Park et al., 2009; Teramura et al., 2007; Tilgner et al., 2008; Yamauchi et al., 2009), our study not only outlines an approach for generating large numbers of VASA+ cells but also describes a method for rapidly and directly differentiating hPSCs into advanced germ cell lineages, including round spermatids, using only extrinsic factors with no genetic manipulation. Thus, the differentiation method presented here may contribute to the overall objective of using patient-specific hiPSCs to generate germ cell lineages capable of restoring fertility in sterile male patients.

Complete spermatogenesis in vitro has not yet been accomplished in humans. Because this strategy attempts to mimic in vivo spermatogenesis by generating undifferentiated spermatogonia as well as haploid round spermatids, it provides evidence that studying spermatogenesis through the round spermatid stage in vitro is feasible; however, additional work is needed to confirm that an individual cell progresses through all

premeiotic, meiotic, and postmeiotic stages. Although techniques for using round spermatids to fertilize oocytes have not been proven in humans and NHPs, this protocol may allow researchers to study cytoplasmic events during early spermatogenesis from hPSCs to SSCs, as well as to characterize events associated with spermiogenesis to the round spermatid stage.

Our differentiation scheme may also represent a tool for exploring the root causes of male factor infertility. By deriving hiPSCs from infertile men, such as patients with Sertoli-cell-only (SCO) syndrome, followed by direct differentiation with our protocol, we could examine where spermatogenesis arrests, and in the case of SCO patients, identify whether hiPSCs can differentiate into SSCs and whether viability of SSCs is a major concern. Advances such as these would undoubtedly shed new light on the root causes of male factor infertility and clarify whether multiple disorders contribute to a patient's infertility. These potential clinical applications thus underscore the importance of improving our differentiation protocol to increase the efficiency of haploid cell generation as well as progressing further through spermiogenesis. It will be important to evaluate preclinically whether these in-vitro-generated gamete precursors have reproductive capabilities in vivo that will be helpful for infertility patients; however, these cells will be of keen biological importance regardless.

EXPERIMENTAL PROCEDURES

Mouse SSC Differentiation Medium and FACS

H1 (WA01, WiCell) hESCs and HFF1 (parent fibroblasts from ATCC, iPS derived internally) (Easley et al., 2012) hiPSCs were cultured for 10 days in mouse SSC medium containing (all from Sigma, unless otherwise noted) minimum essential medium (MEM) alpha (Invitrogen), 0.2% bovine serum albumin, 5 μg/ml insulin, 10 μg/ml transferrin, 60 μM putrescine, 2 mM L-glutamine (Invitrogen), 50 μM β-mercaptoethanol, 1 ng/ml human basic FGF (hbFGF; BD Biosciences), 20 ng/ml GDNF (R&D Systems), 30 nM sodium selenite, 2.36 μM palmitic acid, 0.21 μM palmitoleic acid, 0.88 μM stearic acid, 1.02 μM oleic acid, 2.71 μM linoleic acid, 0.43 μM linolenic acid, 10 mM HEPES, and 0.5X penicillin/streptomycin (Invitrogen). To isolate haploid cells by FACS, H1 SSCs and HFF1 SSCs were stained with Vybrant DyeCycle Violet live-cell stain (Invitrogen) in the SSC medium described above but substituting OptiMeM with no phenol red and run on a FACS Aria sorter (BD Biosciences). Haploid cells were then cultured on poly-D-lysine-coated coverslips and fixed with 4% paraformaldehyde prior to immunostaining.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.07.015.

LICENSING INFORMATION

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REFERENCES

Brinster, R.L., and Avarbock, M.R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. Proc. Natl. Acad. Sci. USA *91*, 11303–11307.

Buaas, F.W., Kirsh, A.L., Sharma, M., McLean, D.J., Morris, J.L., Griswold, M.D., de Rooij, D.G., and Braun, R.E. (2004). Plzf is required in adult male germ cells for stem cell self-renewal. Nat. Genet. *36*, 647–652.

Bucay, N., Yebra, M., Cirulli, V., Afrikanova, I., Kaido, T., Hayek, A., and Montgomery, A.M. (2009). A novel approach for the derivation of putative primordial germ cells and sertoli cells from human embryonic stem cells. Stem Cells *27*, 68–77.

Carrell, D.T., Emery, B.R., and Hammoud, S. (2007). Altered protamine expression and diminished spermatogenesis: what is the link? Hum. Reprod. Update *13*, 313–327.

Castrillon, D.H., Quade, B.J., Wang, T.Y., Quigley, C., and Crum, C.P. (2000). The human VASA gene is specifically expressed in the germ cell lineage. Proc. Natl. Acad. Sci. USA *97*, 9585–9590.

Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., Aicher, W., Bühring, H.J., Mattheus, U., Mack, A., et al. (2008). Generation of pluripotent stem cells from adult human testis. Nature *456*, 344–349.

Costoya, J.A., Hobbs, R.M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K.E., Wolgemuth, D.J., and Pandolfi, P.P. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. Nat. Genet. *36*, 653–659.

Daley, G.Q. (2007). Gametes from embryonic stem cells: a cup half empty or half full? Science *316*, 409–410.

Deng, W., and Lin, H. (2002). miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. Dev. Cell *2*, 819–830.

Easley, C.A. 4th, Miki, T., Castro, C.A., Ozolek, J.A., Minervini, C.F., Ben-Yehudah, A., and Schatten, G.P. (2012). Human amniotic epithelial cells are reprogrammed more efficiently by induced pluripotency than adult fibroblasts. Cell Reprogram. *14*, 193–203.

Eguizabal, C., Montserrat, N., Vassena, R., Barragan, M., Garreta, E., Garcia-Quevedo, L., Vidal, F., Giorgetti, A., Veiga, A., and Izpisua Belmonte, J.C. (2011). Complete meiosis from human induced pluripotent stem cells. Stem Cells *29*, 1186–1195.

Feng, L.X., Chen, Y., Dettin, L., Pera, R.A., Herr, J.C., Goldberg, E., and Dym, M. (2002). Generation and in vitro differentiation of a spermatogonial cell line. Science *297*, 392–395.

Fukunaga, N., Teramura, T., Onodera, Y., Takehara, T., Fukuda, K., and Hosoi, Y. (2010). Leukemia inhibitory factor (LIF) enhances germ cell differentiation from primate embryonic stem cells. Cell Reprogram. *12*, 369–376.

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., and Daley, G.Q. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature *427*, 148–154.

Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S., and Saitou, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell *146*, 519–532.

Hermann, B.P., Sukhwani, M., Lin, C.C., Sheng, Y., Tomko, J., Rodriguez, M., Shuttleworth, J.J., McFarland, D., Hobbs, R.M., Pandolfi, P.P., et al. (2007).

Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. Stem Cells *25*, 2330–2338.

Hobbs, R.M., Seandel, M., Falciatori, I., Rafii, S., and Pandolfi, P.P. (2010). Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell *142*, 468–479.

Jahnukainen, K., Ehmcke, J., Quader, M.A., Saiful Huq, M., Epperly, M.W., Hergenrother, S., Nurmio, M., and Schlatt, S. (2011). Testicular recovery after irradiation differs in prepubertal and pubertal non-human primates, and can be enhanced by autologous germ cell transplantation. Hum. Reprod. *26*, 1945–1954.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol. Reprod. *69*, 612–616.

Kee, K., Angeles, V.T., Flores, M., Nguyen, H.N., and Reijo Pera, R.A. (2009). Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. Nature *462*, 222–225.

Ko, K., Tapia, N., Wu, G., Kim, J.B., Bravo, M.J., Sasse, P., Glaser, T., Ruau, D., Han, D.W., Greber, B., et al. (2009). Induction of pluripotency in adult unipotent germline stem cells. Cell Stem Cell *5*, 87–96.

Kossack, N., Meneses, J., Shefi, S., Nguyen, H.N., Chavez, S., Nicholas, C., Gromoll, J., Turek, P.J., and Reijo-Pera, R.A. (2009). Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells *27*, 138–149.

Lucifero, D., and Reik, W. (2006). Artificial sperm and epigenetic reprogramming. Nat. Biotechnol. *24*, 1097–1098.

Lucifero, D., Mertineit, C., Clarke, H.J., Bestor, T.H., and Trasler, J.M. (2002). Methylation dynamics of imprinted genes in mouse germ cells. Genomics *79*, 530–538.

Marques-Mari, A.I., Lacham-Kaplan, O., Medrano, J.V., Pellicer, A., and Simón, C. (2009). Differentiation of germ cells and gametes from stem cells. Hum. Reprod. Update *15*, 379–390.

Mathews, D.J., Donovan, P.J., Harris, J., Lovell-Badge, R., Savulescu, J., and Faden, R. (2009). Pluripotent stem cell-derived gametes: truth and (potential) consequences. Cell Stem Cell *5*, 11–14.

Moreno, R.D., Palomino, J., and Schatten, G. (2006). Assembly of spermatid acrosome depends on microtubule organization during mammalian spermiogenesis. Dev. Biol. *293*, 218–227.

Nayernia, K., Nolte, J., Michelmann, H.W., Lee, J.H., Rathsack, K., Drusenheimer, N., Dev, A., Wulf, G., Ehrmann, I.E., Elliott, D.J., et al. (2006). In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev. Cell *11*, 125–132.

Nolte, J., Michelmann, H.W., Wolf, M., Wulf, G., Nayernia, K., Meinhardt, A., Zechner, U., and Engel, W. (2010). PSCDGs of mouse multipotent adult germline stem cells can enter and progress through meiosis to form haploid male germ cells in vitro. Differentiation *80*, 184–194.

Panula, S., Medrano, J.V., Kee, K., Bergström, R., Nguyen, H.N., Byers, B., Wilson, K.D., Wu, J.C., Simon, C., Hovatta, O., and Reijo Pera, R.A. (2011). Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells. Hum. Mol. Genet. *20*, 752–762.

Park, T.S., Galic, Z., Conway, A.E., Lindgren, A., van Handel, B.J., Magnusson, M., Richter, L., Teitell, M.A., Mikkola, H.K., Lowry, W.E., et al. (2009). Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells. Stem Cells *27*, 783–795.

Payne, C.J., Gallagher, S.J., Foreman, O., Dannenberg, J.H., Depinho, R.A., and Braun, R.E. (2010). Sin3a is required by sertoli cells to establish a niche for undifferentiated spermatogonia, germ cell tumors, and spermatid elongation. Stem Cells *28*, 1424–1434.

Pick, M., Stelzer, Y., Bar-Nur, O., Mayshar, Y., Eden, A., and Benvenisty, N. (2009). Clone- and gene-specific aberrations of parental imprinting in human induced pluripotent stem cells. Stem Cells *27*, 2686–2690.

Ramalho-Santos, J., Schatten, G., and Moreno, R.D. (2002). Control of membrane fusion during spermiogenesis and the acrosome reaction. Biol. Reprod. *67*, 1043–1051.

Sadri-Ardekani, H., Akhondi, M.A., van der Veen, F., Repping, S., and van Pelt, A.M. (2011). In vitro propagation of human prepubertal spermatogonial stem cells. JAMA *305*, 2416–2418.

Schatten, G. (1994). The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev. Biol. *165*, 299–335.

Schlatt, S., Ehmcke, J., and Jahnukainen, K. (2009). Testicular stem cells for fertility preservation: preclinical studies on male germ cell transplantation and testicular grafting. Pediatr. Blood Cancer *53*, 274–280.

Schlegel, P.N. (2009). Evaluation of male infertility. Minerva Ginecol. *61*, 261–283.

Teramura, T., Takehara, T., Kawata, N., Fujinami, N., Mitani, T., Takenoshita, M., Matsumoto, K., Saeki, K., Iritani, A., Sagawa, N., and Hosoi, Y. (2007). Primate embryonic stem cells proceed to early gametogenesis in vitro. Cloning Stem Cells *9*, 144–156.

Tilgner, K., Atkinson, S.P., Golebiewska, A., Stojkovic, M., Lako, M., and Armstrong, L. (2008). Isolation of primordial germ cells from differentiating human embryonic stem cells. Stem Cells *26*, 3075–3085.

Virant-Klun, I., Stimpfel, M., and Skutella, T. (2011). Ovarian pluripotent/multipotent stem cells and in vitro oogenesis in mammals. Histol. Histopathol. *26*, 1071–1082.

Woodruff, T.K. (2010). The Oncofertility Consortium—addressing fertility in young people with cancer. Nat. Rev. Clin. Oncol. *7*, 466–475.

Yamauchi, K., Hasegawa, K., Chuma, S., Nakatsuji, N., and Suemori, H. (2009). In vitro germ cell differentiation from cynomolgus monkey embryonic stem cells. PLoS ONE *4*, e5338.

Yao, L., Yu, X., Hui, N., and Liu, S. (2011). Application of iPS in assisted reproductive technology: sperm from somatic cells? Stem Cell Rev. *7*, 714–721.