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Fate of iPSCs Derived from Azoospermic and Fertile Men following Xenotransplantation to Murine Seminiferous Tubules

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SUMMARY

Historically, spontaneous deletions and insertions have provided means to probe germline developmental genetics in *Drosophila*, mouse and other species. Here, induced pluripotent stem cell (iPSC) lines were derived from infertile men with deletions that encompass three Y chromosome *azoospermia factor* (*AZF*) regions and are associated with production of few or no sperm but normal somatic development. *AZF*-deleted iPSC lines were compromised in germ cell development in vitro. Undifferentiated iPSCs transplanted directly into murine seminiferous tubules differentiated extensively to germ-cell-like cells (GCLCs) that localized near the basement membrane, demonstrated morphology indistinguishable from fetal germ cells, and expressed germ-cell-specific proteins diagnostic of primordial germ cells. Alternatively, all iPSCs that exited tubules formed primitive tumors. iPSCs with *AZF* deletions produced significantly fewer GCLCs in vivo with distinct defects in gene expression. Findings indicate that xenotransplantation of human iPSCs directs germ cell differentiation in a manner dependent on donor genetic status.

INTRODUCTION

Much progress has been made in recent years in elucidating the molecular genetic requirements of germline formation and differentiation in diverse organisms, including studies focused on murine germline development in vivo and in vitro from mouse embryonic stem cells (mESCs). Studies indicate that a key set of transcriptional regulators, including Prdm1, Prdm14, and Tfap2c, comprises a tripartite transcriptional core that functions in suppression of somatic fate and acquisition of germline fate in vivo (Magnúsdóttir et al., 2013). In vitro studies have similarly demonstrated that induced expression of these factors converts mouse epiblast-like cells (mEpiLCs) to primordial germ-cell-like cells (PGCLCs); moreover, Prdm14 appears to be sufficient for this activity (Nakaki et al., 2013). The resulting PGCLCs are capable of contributing to spermatogenesis and fertile offspring following transplantation to murine seminiferous tubules (Hayashi et al., 2011, 2012). In parallel, in other studies, EpiSCs were shown to have an infinite capacity for generating PGCLCs as long as conditions were maintained to sustain pluripotency and self-renewal in vitro (Hayashi and Surani, 2009).

In contrast to mouse germline development, much less is known of the genetic and molecular requirements to establish the population of PGCs that ultimately gives rise to human sperm and oocytes. Indeed, this is in spite of the fact that infertility is remarkably common, affecting 10%–15% of couples (Skakkebaek et al., 1994). Moreover, genetic causes of infertility are surprisingly prevalent among men, most commonly due to the de novo deletion of one or more *AZF* (*azoospermia factor*) regions of the human Y chromosome (Reijo et al., 1995, 1996; Vogt et al., 1996; Foresta et al., 2000; Kuroda-Kawaguchi et al., 2001; Ferlin et al., 2003; Hopps et al., 2003; Navarro-Costa et al., 2010). Pure sterile phenotypes in men with deletions vary from the complete absence of germ cells and sperm (termed Sertoli cell-only [SCO] syndrome) to production of germ cells that arrest in development (early maturation arrest; EMA) to very low sperm counts (oligospermia) (Skakkebaek et al., 1994; Reijo et al., 1995, 1996). It is not known whether the genes of the Y chromosome *AZF* regions are required for PGC formation, maintenance of germline stem cell populations, and/or commitment to later stages of meiosis and haploid germ cell morphogenesis. Because of the unique nature of Y chromosome gene content in men, studies that probe the function of genes that map to the *AZF* regions must be conducted on a human genome background.

In order to recapitulate human germ cell formation in vivo, we tested the hypothesis that the somatic niche of murine seminiferous tubules can direct formation and maintenance of GCLCs

from undifferentiated human induced pluripotent stem cells (iPSCs). This hypothesis seemed reasonable given the extensive similarity between ESCs/iPSCs and PGCs in terms of pluripotency and gene expression and given that in both undifferentiated human ESCs (hESCs) and human iPSCs, PRDM14, the key inducer of PGC fate in mice, is very highly expressed and only reduced upon somatic differentiation (Chia et al., 2010). Thus, we generated patient-specific iPSCs from infertile men that harbor the most common genetic deletions of the *AZF* regions and then we induced germ cell formation from these iPSCs via xenotransplantation. Our studies offer a strategy for probing the function of naturally occurring mutations in germ cell development that is analogous to strategies used historically in species such as *Drosophila*.

RESULTS

Derivation and Characterization of iPSCs from Azoospermic Men with Y Chromosome Deletions

iPSC lines were derived from dermal fibroblasts from five males; lines were analyzed for Y chromosome deletions, and a deletion map was constructed (Figure 1A and Table S1). We verified that the fertile controls (*AZF1* and *AZF2*) had intact Y chromosomes, whereas all three infertile patients had deletions: one had a complete deletion of the *AZFa* region (*AZFΔa*); the second, a deletion of both the *AZFb* and *AZFc* regions (*AZFΔbc*); and the third, an *AZFc* deletion (*AZFΔc*). The men with *AZFΔa* and *AZFΔc* deletions presented with SCO syndrome and had no germ cells found in their testes upon extensive clinical examination; *AZFΔbc* was severely oligospermic. iPSCs generated from fibroblast cell lines (i*AZF1*, i*AZF2*, i*AZFΔa*, i*AZFΔbc*, and i*AZFΔc*) were isogenic with the parental fibroblast samples; no additional deletions occurred as a consequence of reprogramming. All iPSC lines met classic criteria of pluripotency (Figures 1 and S1). They expressed pluripotency markers at the mRNA and protein levels (Figures S1B and 1B), were karyotypically normal (Figure S1C; except for the presence of Y chromosome deletions as indicated), and differentiated in vitro and in vivo to cells of all three germ layers (Figures 1C and 1D).

In Vitro Differentiation of PGCs from Azoospermic Men and Controls

We and others previously reported germ cell differentiation from both hESCs and iPSCs in vitro (Clark et al., 2004; Kee et al., 2006, 2009; Park et al., 2009; Panula et al., 2011; Gkountela et al., 2013). To assess germ cell development from azoospermic men relative to controls, we introduced a *VASA:GFP* reporter into all iPSCs, differentiated cells, and enriched for presumptive VASA:GFP+ single-germ cells via FACS (fluorescence-activated cell sorting). We observed that the percentage of GFP+ cells across all lines was similar (2%–8%) as determined from two independent clones per line (Figures S2A–S2E) but that germ cells differed in gene expression as a function of genotype. First, to determine similarities and differences between single cells differentiated from each of the four cell lines, we performed hierarchical clustering on single-cell expression (150–160 cells) and constructed a condensed heatmap of 25 single cells (iPGCs) expressing *VASA* and *GFP* mRNAs (Figure S2F). To evaluate germ-cell-specific gene expression of in-vitro-derived PGCs (iPGCs) from each line, we validated the identity of 50 individual cells that expressed the *GFP* transcript (Figure 2A) and queried expression of germ cell genes in a subset of cells for each line. Downregulation of key pluripotency genes and expression of *VASA* and other classical human PGC genes such as *DAZL* is considered indicative of a distinct PGC fate of iPSCs and hESCs following germ cell differentiation in vitro (Kee et al., 2009; Gkountela et al., 2013).

We observed that iPGCs derived from all five lines expressed *PRDM1*, *PRDM14*, and *DAZL* mRNAs (Figure 2A). This was also reflected at the protein level for DAZL in GFP+ cells (Figure 2B). However, the overall number of cells expressing *VASA*, *STELLA*, *IFITM3*, and *NANOS3* genes varied across the lines, with the greatest number observed in i*AZF*1-derived iPGCs compared to *AZF*-deleted iPGCs (Figure 2A). Moreover, we noted that GFP+ cell populations derived from i*AZFΔa* and i*AZFΔbc* lines contained the fewest number of cells expressing at least seven germ-cell-specific genes. At the protein level, iPGCs expressed VASA, STELLA, and DAZL, but we detected fewer cells positive for each protein in i*AZF*Δc, i*AZF*Δbc, and i*AZF*Δa cells relative to controls (Figure 2B). For all iPGCs derived, core pluripotency genes were expressed at very low levels relative to undifferentiated cells (Figure 2A, right). Moreover, linear discrimination analysis of 12 candidate germ cell genes (Tables S2 and S3) in each population of GFP+ single cells revealed that i*AZF1* and i*AZFΔc* iPGCs were distinct from i*AZFΔbc* and i*AZFΔa* iPGCs (Figure 2C). To further quantify these observations, we determined the percentage of VASA+/GFP+ cells expressing between 1 and 12 candidate germ cell genes and observed that i*AZF1* GFP+ cells expressed a minimum of 5–8 germ cell genes in 70% of the cell population (Figure 2D, left column, dashed lines). By comparison, 70% of GFP+ single cells from each of the three *AZF*-deleted lines expressed a range of only one to six germ cell genes with the i*AZFΔa* and i*AZFΔbc* lines expressing the fewest germ-cell-specific genes within single cells. Further analysis of stage-specific gene expression revealed that, independent of genotype, the majority of GFP+ iPGCs expressed at least four to six PGC markers (Figure 2D, right column). In contrast, *AZF*-deleted germ cells, particularly from i*AZFΔbc* and i*AZFΔa*, expressed zero to two spermatogonial markers, whereas iAZF1 expressed two to four spermatogonial genes (Figure 2D). On the basis of germ-cell-specific gene expression at single-cell resolution, these analyses suggest that all lines can differentiate to iPGCs regardless of genetic background. However, when i*AZFΔa* and *iAZFΔbc* cells are differentiated, fewer germ cells are produced relative to control i*AZF*1 cells.

Transplantation and Survival of Fetal Germ Cells to Murine Seminiferous Tubules

The gold standard for phenotypic characterization of stem cells is assessment of function in vivo (Hanna et al., 2007; Nelson et al., 2009; Weissman, 2012; Takahashi and Yamanaka, 2013). As previously reported, in transplantation with human spermatogonial stem cells, germ cells migrate to the seminiferous tubule basement membrane and proliferate to form chains and patches of spermatogonia that persist long term but do not appear to initiate or complete meiosis (Nagano et al., 2002; Hermann et al., 2010; Dovey et al., 2013). To date, the fate of human pluripotent stem

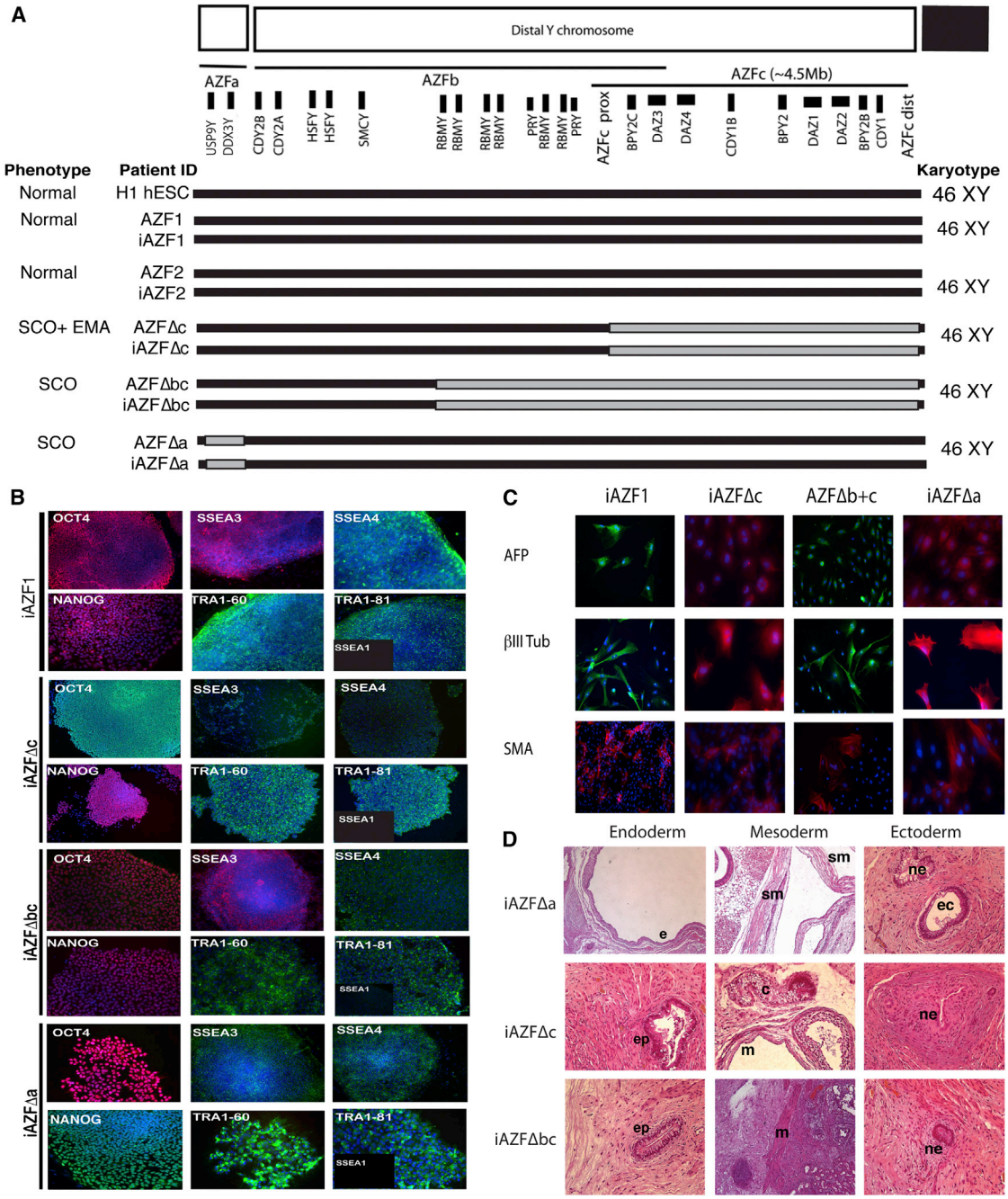


Figure 1. Derivation and Characterization of iPSCs from *AZF*-Intact and *AZF*-Deleted Patient Fibroblasts

(A) Genotype-phenotype map to visualize deletion of *AZFa*, *AZFb*, and *AZFc* regions in hESC lines, patient samples, and patient-derived iPSCs used for this study. A deletion map was constructed for every hESC line, patient fibroblast, and iPSC line by testing for the presence of 20 major STSs by PCR. Vertical black bars (top) represent the STS amplification regions and genes we used to diagnose *AZFa*, *AZFb*, and *AZFc* deletions. Gray boxes represent the deleted regions of the Y chromosome. The fertility phenotype (left; SCO and EMA) and karyotype (right) of each patient are listed. The triangle symbol (Δ)indicates the deletion of an *AZF* region in that cell line.

(B) Immunocytochemistry in all patient-derived iPSC lines for nuclear (OCT4 and NANOG) and cell surface (SSEA1/SSEA3/SSEA4, TRA1–60, and TRA1–81) markers of pluripotency.

(C) In vitro differentiation of four patient-derived lines to cells representative of all three germ layers: AFP, α-fetoprotein; βIII-Tub, β-III-tubulin/Tuj1; SMA, smooth muscle actin.

(D) In vivo teratoma formation of i*AZF*Δbc, i*AZF*Δc, and i*AZF*Δa lines showing evidence of all three germ layers. e, endoderm; ep, gut-like epithelium; m, mesoderm; sm, smooth muscle-like mesoderm; c, cartilage-like; ec, ectoderm; ne, neural ectoderm.

See also Figure S1 and Table S1.

cells in the mouse seminiferous tubule has not been explored. To verify that human germ cells could survive and engraft inside murine seminiferous tubules, we transplanted human fetal testicular cell suspensions (22 weeks old) into busulfan-treated testes of immunodeficient mice (Figure 3A). The use of busulfan treatment eliminates endogenous mouse germ cells from the seminiferous tubules (Figure 3B). Prior to transplantation, we observed that the 22-week-old human fetal testis contained a subset of cells positive for the germ-cell-specific protein VASA (Figure 3Ci). Using whole-mount staining analyses, we immunostained with a primate-specific antibody that is known to recognize all human donor cells only (regardless of germ cell fate) and, consistent with reports on adult testis transplants, observed single-donor cells, small chains (Figure 3Cii), or larger clusters of human donor cells on the basement membrane 2 months posttransplantation. In parallel, we also performed immunohistochemistry in cross-sections of testes transplanted with human fetal testis cells. In order to detect all human donor cells in recipient testes, we assessed expression of NuMA (Figure 3D), a well-characterized protein that exclusively labels human cells and tissues (Abad et al., 2007; Brüning-Richardson et al., 2012; Saadai et al., 2013). We observed significant human germ cell engraftment 8 weeks posttransplantation (Figure 3Ciii).

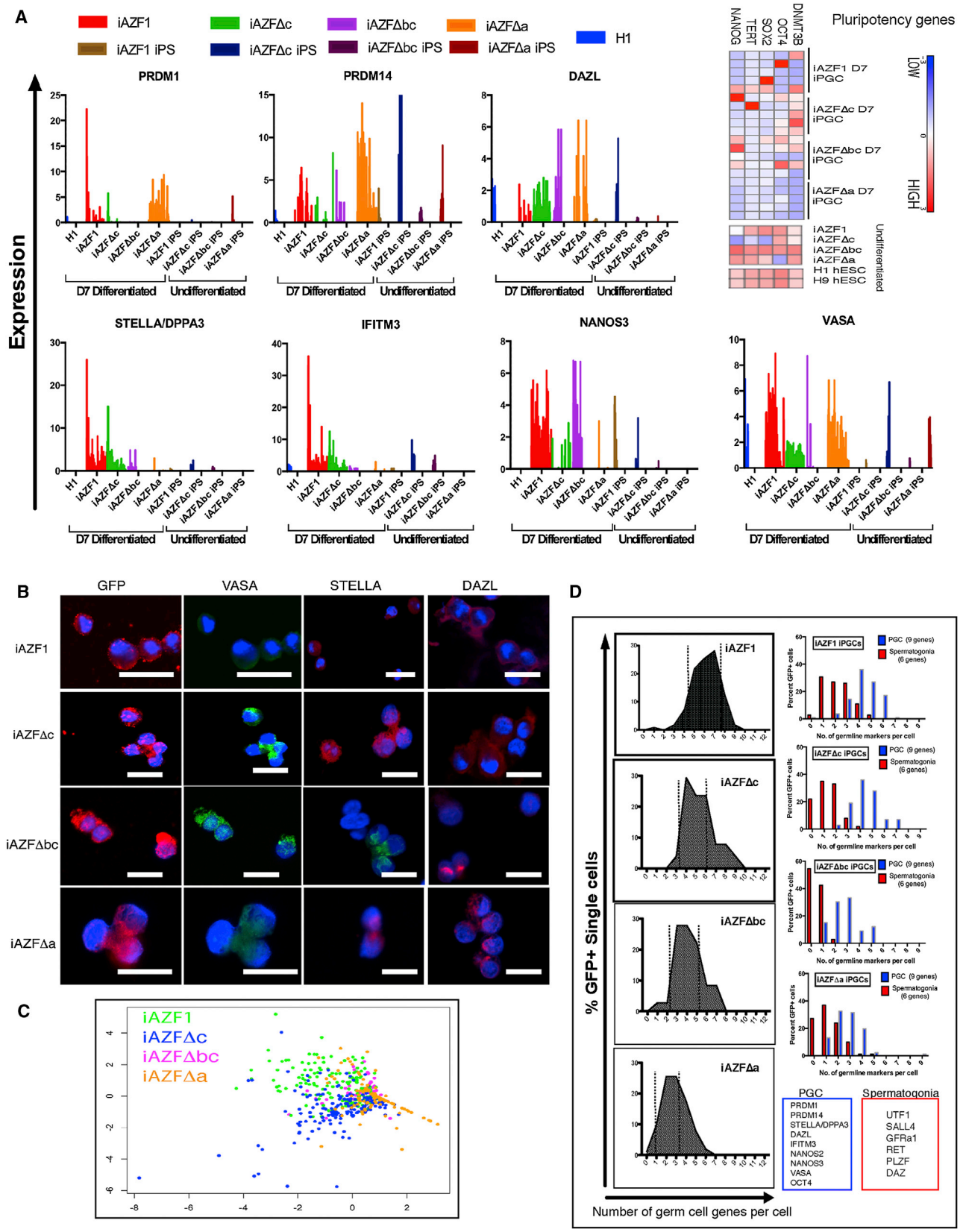
Xenotransplantation of hESCs and Human iPSCs to Seminiferous Tubules Directs Germ Cell Development

As noted above, we hypothesized that human iPSCs, which are distinct from mouse iPSCs (miPSCs), would survive, engraft, and be directed to a germ cell developmental fate if directly injected into the mouse seminiferous tubule, in response to instructive cues from the niche. Thus, we next transplanted undifferentiated male hESCs (H1) and the i*AZF*1 control iPSCs to eight recipient testes each and analyzed results by whole-mount immunohistochemistry as performed previously with human fetal testis transplants (Figure 3A). We observed that all donor-derived cells were detected either as single cells or in clusters of cells; no clear evidence of chain-like structures was observed (Figure S3B). We note that chain formation in human donor cells is a hallmark of spermatogonia but is not a property of PGCs and gonocytes (Nagano et al., 2002; Wu et al., 2009). Thus, we reasoned that engrafted cells might have differentiated to PGCLCs or gonocyte-like cells and tested systematically for the presence of germ-cell-specific proteins on NuMA+ surviving donor cells using serial immunohistochemical analysis across testis cross-sections. We observed that all transplantations, regardless of fertility status, resulted in two fates of donor undifferentiated iPSCs in the testes: (1) differentiation to GCLCs that were spatially located near or at the basement membrane of seminiferous tubules and (2) extensive proliferation of iPSCs distant from the basement membrane most often within the interstitial space. These proliferative cells resembled embryonal carcinoma (EC) and yolk sac tumors (YSTs).

Upon further analysis of H1 and i*AZF*1 xenotransplants, and as expected, we observed that a subset of tubules was occupied with NuMA+ donor cells (Figures S3C–S3I). In tubules containing donor cells, NuMA+ donor cells engrafted both near the basement membrane and in the center of the tubule. A significant number of NuMA+ cells coexpressed the germline protein, VASA (henceforth referred to as NuMA+/VASA+ cells) (Figure 3E, white asterisks). We also observed that a few NuMA+ cells negative for VASA engrafted at the edges of seminiferous tubules (Figure 3E, red asterisk). To exclude the possibility that NuMA+ donor cells could differentiate to Sertoli cells, we costained for GATA4 and SOX9, two nuclear proteins expressed by mouse and human Sertoli cells (Figure S4). We did not observe the localization of SOX9 and GATA4 signals in nuclei of NuMA+ cells (Figures S4A and S4D), but only in cells that resemble human or mouse Sertoli cells at the edge of most tubules (Figures S4B, S4C, and S4E). Based on these results, we defined human GCLCs by the presence of NuMA+ cells that engrafted near the basement membrane of seminiferous tubules, colocalization of germ cell markers, especially VASA, and overall morphological resemblance to human fetal germ cells (Figure 3E). By comparison, staining of human fetal testis cross-sections revealed VASA+ germ cells near the tubule basement membrane (Figure 3Ci). Specifically, in testis xenografts, a large number of NuMA+/VASA+ cells localized to the basal membrane of the tubule either as single cells, rows or in clusters that bear clear resemblance to the arrangement of VASA+ germ cells of the human fetal testis (Figures 3E and 4A–4F).

Male Donor-Derived Cells Engrafted Outside Spermatogonial Tubules Remain Undifferentiated or Differentiate to Primitive Tumors

As noted above, in addition to cells that engrafted at the basement membrane, we observed that donor cells that filled the entire seminiferous tubule or exited the tubule proliferated extensively and did not demonstrate clear differentiation to either GCLCs or somatic cells. Instead, based on histology of xenografts, cells distant from the basement membrane, outside the tubules, resembled the histology of EC or, in some instances, YST cells (Figure S5A; Chaganti and Houldsworth, 2000; Nonaka, 2009). To verify, we stained xenografts for SOX2 and OCT4, protein markers diagnostic, at least in part, for EC formation and undifferentiated cells (Nonaka, 2009). We observed extensive SOX2 and OCT4 expression in NuMA+ nuclei in the interstitial space (Figure S5B). We reasoned that these cells likely resulted from leakage of donor hESCs and iPSCs from tubules into the interstitial spaces of the testis. Notably, donor cells in the interstitial spaces did not form teratomas as assessed by thorough histological analysis, suggesting that they did not receive the appropriate cues to differentiate to somatic lineages or that somatic cells are efficiently removed following differentiation. NuMA+ cells that filled the entire tubular space expressed OCT4 but did not appear to form GCLCs (VASA negative), suggesting that they remained undifferentiated (Figure S5C). We determined the efficiency of interstitial tumor formation across all testis xenografts performed and found that, except for human fetal testis transplantation, hESC and iPSC xenotransplants consistently produced interstitial tumors in more than half of all samples (Figure S5D). Curiously, iAZFΔc iPSCs produced interstitial tumors only 30% of the time, but the tumors were always of EC or YST types. Altogether, these results suggested that the environment inside the seminiferous tubule is permissive for germ cell formation but appears to prevent differentiation of some donor cells to somatic lineages. In contrast, the



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environment outside the tubules promotes somatic differentiation but not teratoma formation in donor cells that exited tubules.

Transplantation of iPSCs into Murine Seminiferous Tubules Reveals Differences in Germ Cell Differentiation between Control and *AZF*-Deleted Lines

We predicted that *AZF*-deleted iPSCs would form and/or maintain fewer GCLCs than *AZF*-intact iPSC lines. From our immunohistochemical analysis, we observed that transplanted human fetal testis cells, H1 hESCs, i*AZF1*, i*AZF2*, and i*AZFΔc* iPSCs were usually localized in NuMA+/VASA+ clusters containing at least three or more cells (Figures 3E and 4A–4D, arrows and white asterisks). In contrast, i*AZFΔbc* and i*AZFΔa* iPSCs gave rise to significantly fewer clusters of NuMA+/VASA+ cells (Figures 4E and 4F, arrows and white asterisks). To quantify this observation for each donor sample, we counted NuMA+/VASA+ cells and tubules across entire cross-sections (20× magnification) at three to four different depths of cross-sections and in at least four biological replicates per sample. Using this strategy, we extracted the average percentage of positive seminiferous tubules (Figure 4G). Although human fetal donor cells always produced the highest percentage of positive tubules (>30%), both i*AZF1* and i*AZF2* lines produced significantly higher tubule occupancy over *AZF*-deleted iPSCs (Figure 4G). We next determined the average number of NuMA+/VASA+ cells in each positively stained tubule (Figure 4H). We determined that, on average, >30 NuMA+/VASA+ cells per tubule were observed with human fetal donor cells and approximately 20–25 NuMA+/VASA+ cells per tubule with human i*AZF1* and i*AZF2* donor cells. These values were significantly higher than those observed in the case of i*AZFΔa*, i*AZFΔbc*, and i*AZFΔc* donor cells, where we observed four to eight NuMA+/VASA+ cells per tubule (Figure 4H). To determine the relative germ-cell-forming potential of each donor cell population, we multiplied the tubule occupancy (per 100 tubules) by NuMA+/VASA+ cells per tubule (Figure 4I). Our calculations reveal an approximate 50- to 100-fold reduction in formation of GCLCs from *AZF*-deleted iPSCs relative to *AZF*-intact iPSCs.

To further validate in vivo GCLC formation from donor cells, we examined expression of the germ-cell-specific proteins DAZL, PLZF, UTF1, STELLA, and DAZ in donor-derived GCLCs from xenotransplants (Figures 5 and S6). Based on previous reports, we predicted that STELLA, DAZL, and VASA would label iPSCs that had differentiated to PGCLCs, whereas UTF1, PLZF, and DAZ proteins would label iPSCs that had entered the pool of gonocyte-like or prospermatogonia-like cells and would overlap with expression of VASA (Buaas et al., 2004; Anderson et al., 2007; Kristensen et al., 2008; Culty, 2009; Phillips et al., 2010; Wongtrakoongate et al., 2013). Fetal testes at 22 weeks of gestation are expected to contain gonocytes and undifferentiated spermatogonia (Culty, 2009). Interestingly, fetal germ cells expressed DAZL, STELLA, and UTF1 simultaneously (Figure S6A). NuMA+/VASA+ cells derived from human fetal testis donor cells expressed the PGC proteins STELLA and DAZL in a similar nuclear and cytoplasmic pattern, respectively, to endogenous germ cells in the human fetal testis (Figure 5A, panel 1, and Figure S6A). Similarly, NuMA+/VASA+ cells derived from all *AZF*-intact and *AZF*-deleted donor lines expressed STELLA and DAZL proteins (Figures 5B–5F, panel 1). We further observed that expression of UTF1, PLZF, and DAZ proteins varied between donor cell lines dependent on genotype. In the control human fetal testis, UTF1, PLZF, and DAZ proteins were expressed in a few GCLCs in a subset of seminiferous tubules (Figure S6A). In a similar fashion to the fetal testis, UTF1 was localized in several DAZL+ donor-derived germ cells near the edges of tubules in all samples except i*AZFΔbc* and i*AZFΔa*, where we could not detect UTF1 signals (Figures 5A–5D, panel 2, and Figure S6). In addition, the prospermatogonial protein PLZF was detected in only a handful of VASA+ cells in *iAZF1* xenografts, but not in other samples (Figure 5C, panel 2). We further observed that none of the three *AZF*-deleted lines expressed the Y chromosome-encoded protein DAZ. In contrast, we detected cytoplasmic expression of DAZ proteins in NuMA+ cells of *AZF*-intact donor cells (H1, i*AZF*1, and i*AZF*2) and donor cells from the human fetal testis (Figures 5G and S6B). The pattern of DAZ expression closely corresponded to that of endogenous fetal germ cells in the human testis (Figure S6A). Collectively, results indicate that the human fetal testis donor cells and all patient-derived iPSCs are capable of forming PGCLCs but that, in general, *AZF*-deleted iPSCs form fewer GCLCs with altered expression of germ-cell-specific proteins (Figure 5H).

Epigenetic Analysis of Donor-Derived GCLCs from *AZF*-Intact iPSCs

In order to evaluate if epigenetic reprogramming, characteristic of endogenous germ cells, occurs in donor iPSC xenografts, we performed immunohistochemistry for 5-methylcytosine (5-mC)

Figure 2. Derivation and Characterization of iPGCs from *AZF*-Intact and *AZF*-Deleted iPSCs In Vitro

(A) Single-cell gene expression of VASA-GFP+ iPGCs derived from undifferentiated *AZF*-intact iPSCs (i*AZF*1), hESCs (H1), and *AZF*-deleted iPSCs (i*AZF*Δc, i*AZF*Δbc, and i*AZF*Δa). The relative expression of PGC genes *PRDM1*, *PRDM14*, *DAZL*, *STELLA* (*DPPA3*), *IFITM3*, *NANOS3*, and *VASA* was measured after normalization in all cells positive for *GFP* mRNA and plotted individually. Expression levels in undifferentiated single cells from each iPSC line are also shown in each graph. Each vertical bar represents 1 individual cell, and at least 25 cells are represented for differentiated samples and 20 cells for undifferentiated samples. See legend colors for sample identification. A heat index of pluripotency gene expression in five differentiated single cells and in undifferentiated iPSCs is shown (right).

(B) Immunocytochemistry of GFP+ sorted cells for GFP and PGC proteins VASA, STELLA, and DAZL. Nuclei are counterstained with DAPI (blue). Scale bars represent 25 μm.

(C) Two-component linear discrimination analysis of all single-cell RT-PCR measurements for 27 germ cell genes from all iPSC lines tested. Each single dot represents one single-cell measurement.

(D) Percentage of GFP+ sorted single cells coexpressing 12 additional germ-cell-associated genes (see Table S3) as plotted by the number of markers within each individual cell. Dashed, vertical lines indicate the segment of the population of single cells from each line relative to the middle 70%–80% of control, i*AZF*1 population. Coexpression of nine PGC (blue) and six gonocytic/spermatogonial genes (red) was quantified in GFP+ iPGCs and plotted by the number of respective markers within each cell.

See also Figure S2 and Tables S2 and S3.

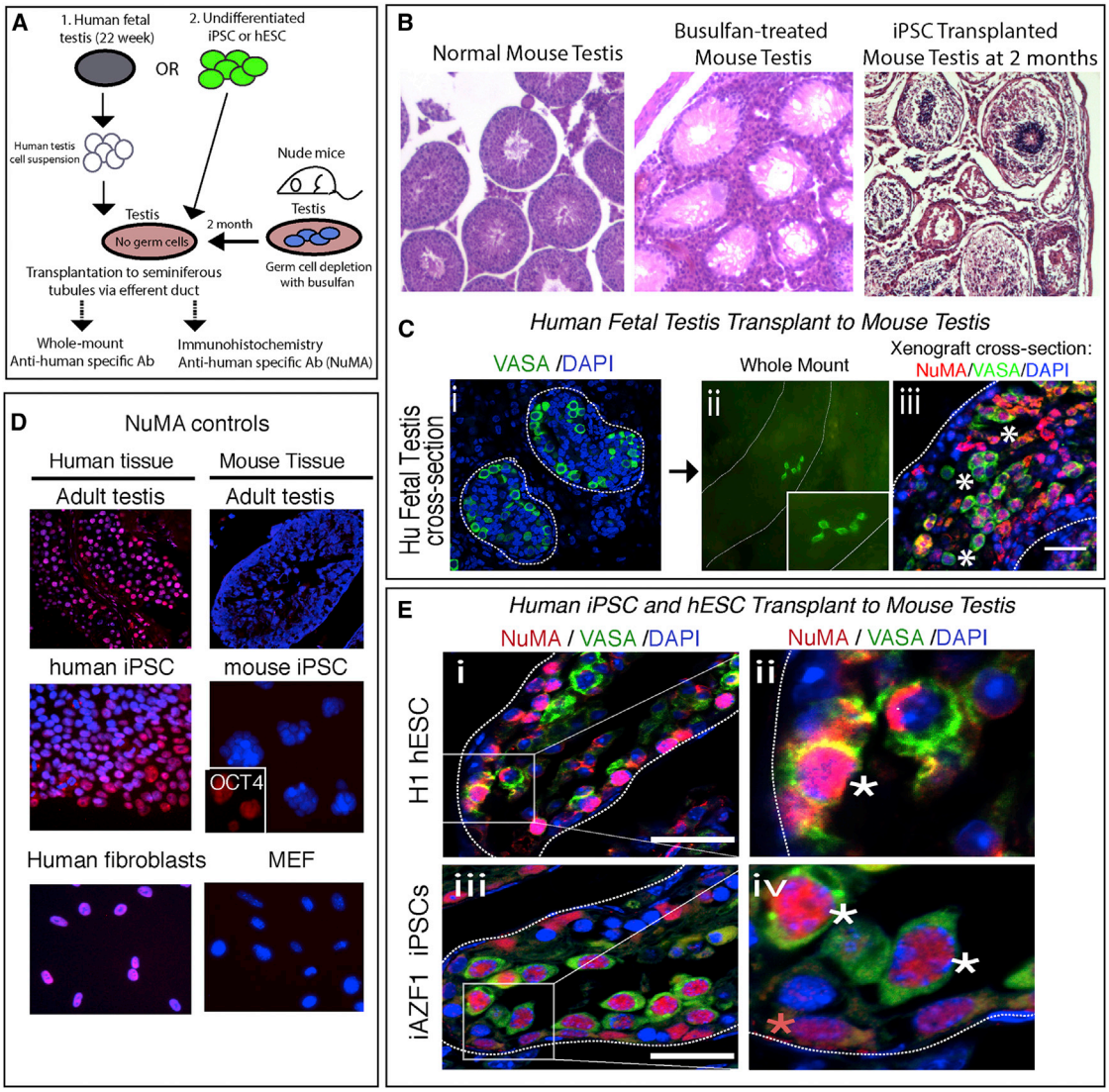


Figure 3. Survival and Germ Cell Differentiation of Fetal Germ Cells, hESCs, and iPSCs after Transplantation to Mouse Spermatogonial Tubules

(A) Transplantations were performed by independently injecting 22-week-old human fetal testicular cells, H1 hESCs, or human iPSCs directly into seminiferous tubules of busulfan-treated mouse testes (pink oval) that were depleted of endogenous germ cells. Testis xenografts were analyzed by (1) whole-mount histology using a human cell-specific antibody, or (2) immunohistochemistry using a human cell-specific antibody, NuMA, to detect donor cells.

(B) Hematoxylin and eosin histology of normal mouse recipient testes before busulfan treatment, after busulfan-mediated germ cell depletion, and 2 months posttransplantation of iPSCs.

(C) Human (Hu) fetal testis (22 weeks old) with cytoplasmic VASA expression (green) in germ cells (i). Nuclei are counterstained with DAPI. Human fetal testis was enzymatically dissociated, and fetal germ cells were transplanted and then detected after 2 months by (ii) whole-mount staining or (iii) cross-sectional immunohistochemistry for NuMA+/VASA+ cells (white asterisks).

(D) Human and mouse immunostaining controls for NuMA antibody specificity.

(E) Immunohistochemical analysis of testis xenografts derived from undifferentiated (i and ii) H1 hESC and (iii and iv) i*AZF1* iPSCs. All images are merged from NuMA (red), VASA (green), and DAPI-stained nuclei. White rectangles indicate regions of interest of positive colocalization of NuMA and VASA and are magnified in (ii) and (iv). Red asterisks indicate NuMA+ donor cells adjacent to the basement membrane, whereas white asterisks represent NuMA+/VASA+ donor cells near the basement membrane.

In all panels, dashed white lines indicate the outer edges of spermatogonial tubules. Scale bars represent 50 μm. See also Figure S3.

as a marker of global CpG methylation. We compared 5-mC status in endogenous germ cells of both human fetal and adult testes (Figures 6A and 6B) and in all recipient mouse testis xenografts (Figures 6C–6G). We observed that a majority of endogenous VASA+ germ cells in fetal testes were 5-mC positive, with a subset of VASA+ cells 5-mC negative (Figure 6A, yellow arrowheads and white dotted circles, respectively). Intriguingly, NuMA+/VASA+ cells in all xenografts derived from human fetal testis

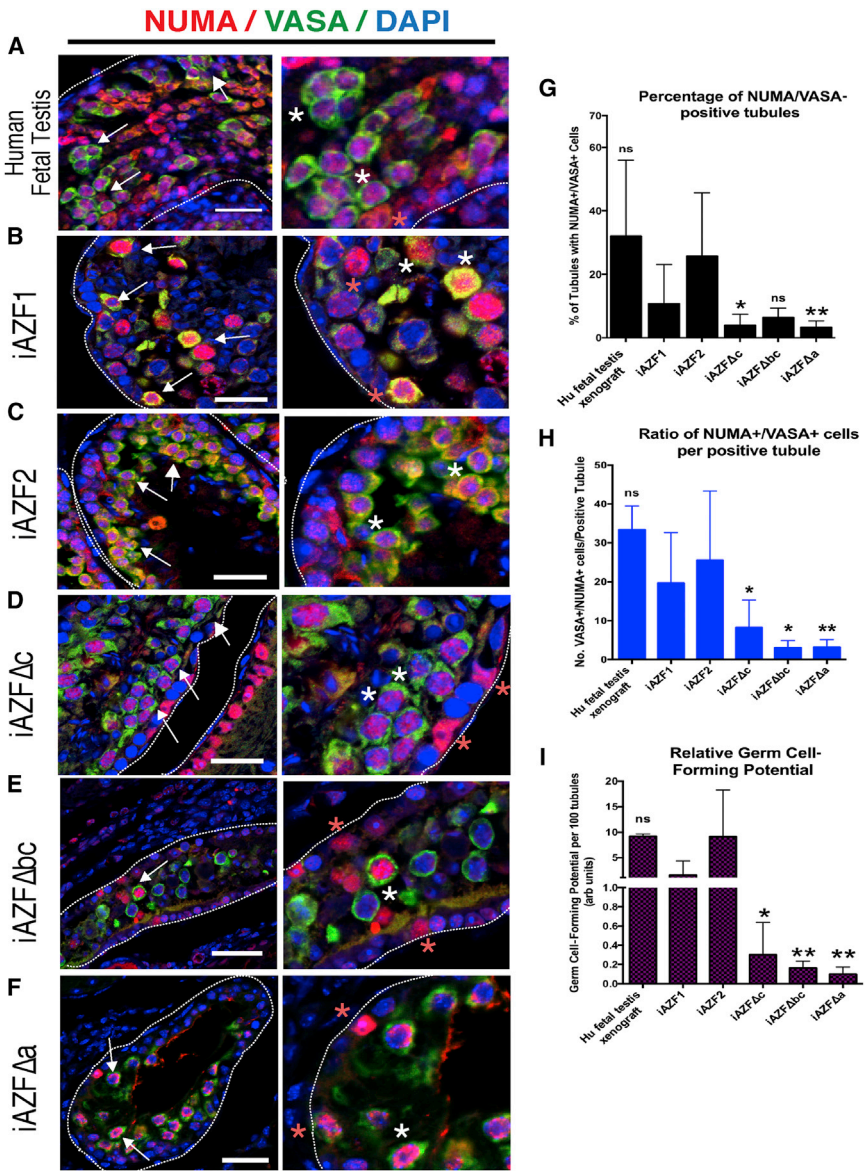


Figure 4. Differences in GCLC-Forming Potential between Patient-Derived iPSC Donor Cells in Mouse Spermatogonial Tubules

(A–F) Mouse testis xenografts were analyzed by immunohistochemistry using the human cell-specific antibody NuMA and an antibody recognizing the pan-germ cell marker VASA, marking PGCs, gonocytes, and spermatogonia. Cross-sections of testis xenografts derived from (A) 22-week-old human fetal testis cells, (B) i*AZF*1, (C) i*AZF*2, (D) i*AZFΔc*, (E) i*AZFΔbc*, and (F) i*AZFΔa* cell lines. All images are merged from NuMA (red), VASA (green), and DAPI-stained nuclei. Arrows indicate cells or clusters with positive colocalization of NuMA and VASA. Red asterisks indicate NuMA+ donor cells adjacent to the basement membrane, whereas white asterisks represent NuMA+ donor cells near the basement membrane. In all panels, dashed white lines indicate the outer edges of spermatogonial tubules. Scale bars represent 50 μm.

(G) Percentage of tubules positive for NuMA+/VASA+ cells was calculated across multiple cross-sections (relative to total number of tubules).

(H) For each positive tubule, the ratio of NuMA+/VASA+ cells per tubule was determined.

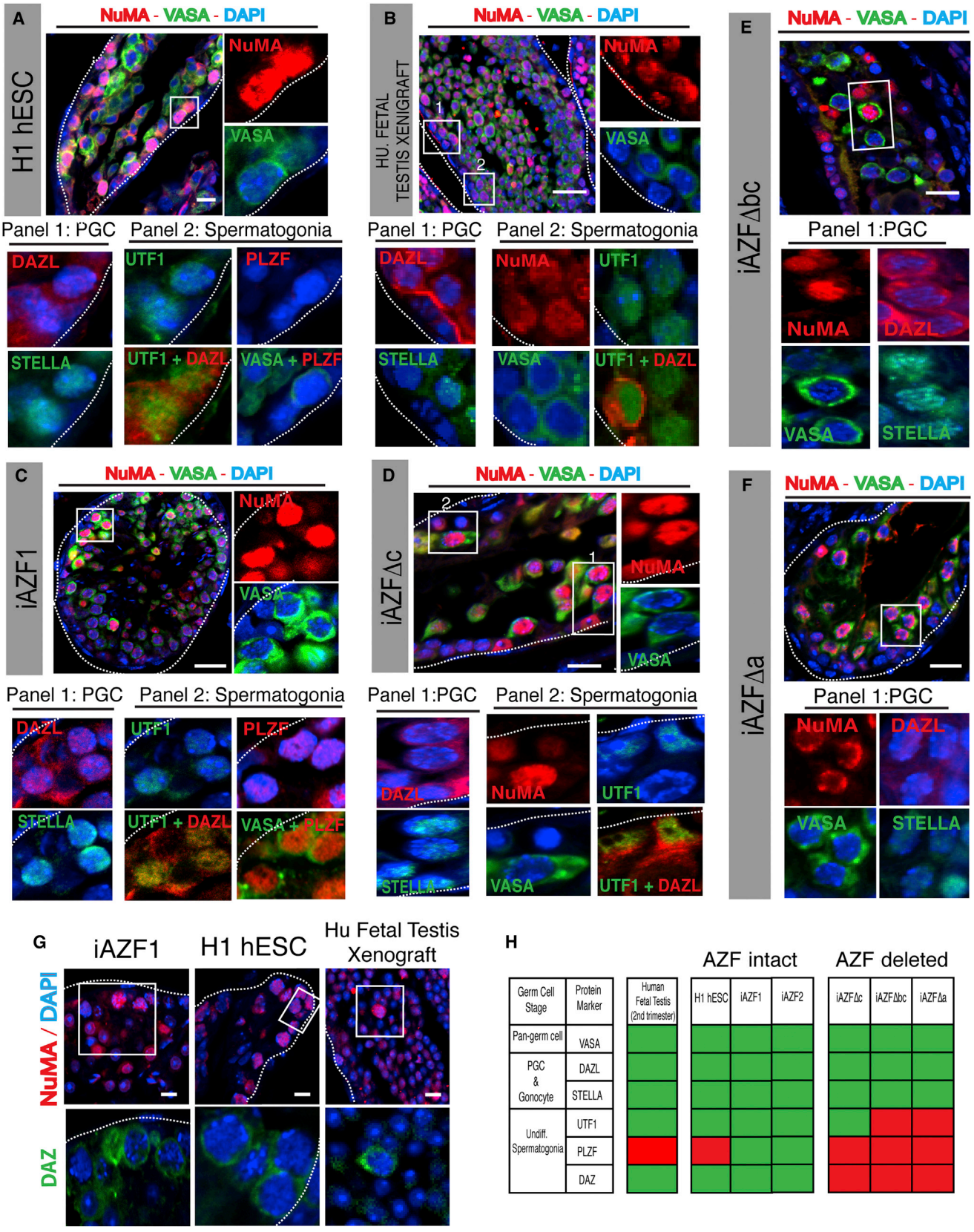
(I) Relative germ-cell-forming potential calculated by multiplying the fraction of positively stained tubules with the number of VASA/NUMA-costained cells for each sample. Data are represented as mean ± SD of replicates. In each graph, significant differences in percentages/ratios between controls (i*AZF*1 or i*AZF*2) and *AZF*-deleted lines were determined by one-way ANOVA. *$p < 0.05$; **$p < 0.001$; ns, nonsignificant.

See also Figure S3.

donor cells or iPSC lines exhibited similar numbers of cells negative or positive for 5-mC (Figures 6C–6G, yellow arrowheads and white dotted circles, respectively). The 5-mC reduction was confined to germ cells within tubules (i.e., NuMA+ donor cells outside tubules were all 5-mC positive) (Figure 6H). Additionally, undifferentiated iPSC donor cells in culture exhibited uniform levels of 5-mC prior to transplantation (Figure 6H, bottom). In addition to immunohistochemical analysis, we quantified the pattern of 5-mC signals in NuMA+/VASA+ cells within tubules and observed a fairly uniform percentage (15%–20%) of 5-mC-negative cells in all xenografts, including those derived from human fetal testis donor cells. This observation mirrored the approximate 25% of endogenous fetal testis germ cells that were 5-mC negative (Figure 6I). Meanwhile, endogenous adult testis germ cells were highly methylated (only 3%–5% 5-mC negative). Collectively, our results suggest that human donor cells undergo global DNA demethylation upon initiation of germ cell differentiation inside the mouse seminiferous tubule.

DISCUSSION

A major emphasis in stem cell biology and regenerative medicine is focused on iPSC-derived cell transplantation to restore somatic cellular and tissue function (Wu and Hochedlinger, 2011; Takahashi and Yamanaka, 2013). Much less focus has been directed at the use of human iPSCs to derive germ cells for potential cell replacement therapies, despite elegant studies in the mouse that demonstrate the ability to completely reconstitute mouse germline development from ESCs and iPSCs (Hayashi et al., 2011, 2012; Grabole et al., 2013; Nakaki et al., 2013). Both mouse and human studies have demonstrated requirements for key germ-cell-specific genes and dependence of germ cell development on interaction with the niche (Nicholas et al., 2009; Hayashi et al., 2011; Kee et al., 2009; Panula et al., 2011; Easley et al., 2012; Medrano et al., 2012; Gkountela et al., 2013). Yet, there are difficulties in interpreting the human data given the low numbers of GCLCs formed in vitro, the extensive overlap of gene expression shared between ESCs and PGCs



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such that fewer than a dozen genes may differ (Clark et al., 2004; Zwaka and Thomson, 2005; Johnson et al., 2008; Sabour et al., 2011), and the asynchrony in development of germ cells in vitro.

In this study, we observed that transplantation of normal and *AZF*-deleted iPSCs into the environment of the seminiferous tubule resulted in GCLC formation, whereas iPSCs outside the tubules failed to differentiate to GCLCs. Based on these results, we reason that iPSC and hESC donor cells inside seminiferous tubules may receive signals that permit them to engraft near the basement membrane. Our findings are supported by the observation that human spermatogonia transplanted to mouse testes "home" to the basement membrane but cannot differentiate to more mature stages (Nagano et al., 2002; Wu et al., 2009; Takashima et al., 2011). We suggest that cell-to-cell contacts between cells of the basement membrane niche, especially Sertoli cells, and donor cells enable exchange of instructive signals. Furthermore, we suggest that this crosstalk favors acquisition of a PGC- or gonocyte-like fate in human iPSCs with suppression of extensive proliferation and somatic cell differentiation (Resnick et al., 1992; Johnson et al., 2008; Kanatsu-Shinohara et al., 2012). In a similar fashion, undifferentiated murine spermatogonia self-renew or differentiate based on Sertoli cell density and factors secreted by Sertoli cells (Phillips et al., 2010; Oatley et al., 2011). These Sertoli cell-produced factors include paracrine growth factors such as transforming growth factor (TGF) and stem cell factor (SCF), which have been implicated in the survival and/or differentiation of PGCs (Mullaney and Skinner, 1991; Ewen and Koopman, 2010). In mice, the transition to epiblast stem cells and overexpression of Prdm14 facilitates germ cell differentiation posttransplantation (Nakaki et al., 2013). In contrast, both undifferentiated hESCs and human iPSCs are believed to be "epiblast-like" and moreover express high levels of PRDM14 endogenously as shown in this manuscript and other reports by Brons et al. (2007) and Tesar et al. (2007).

Our findings also bear some resemblance to reports in flies where the somatic cyst cells, which are immediately adjacent to germline stem cells, induce germline differentiation, whereas perturbation of the niche:germ cell environment leads to extensive proliferation resembling germ cell tumors (Karnis, 2012; Lim and Fuller, 2012). Based on our results and previously published studies, we posit that undifferentiated human iPSCs inside murine seminiferous tubules receive critical molecular cues from Sertoli cells and, potentially, from peri-tubular cells that assist in localization and physical interaction, accessibility to signaling cues, and ultimately direct germline differentiation. Moreover, if the niche is "overbooked" and iPSCs leak out of tubules or are not able to contact Sertoli cells, we suggest that these cells will proliferate extensively to form EC outside the tubule or remain undifferentiated inside the tubule. Indeed, it is remarkable that in sharp contrast to studies with miPSCs, human iPSCs and hESCs never formed teratomas in our studies, perhaps due to their transient interaction with the intratubular environment.

Historically, it has not been possible to address when or how germ cells are depleted in men with spontaneous deletions; heterogeneity of clinical phenotypes associated with the same genetic deletions may be linked to depletion rate or time of presentation (Reijo et al., 1995, 1996). Moreover, it was not known whether *AZF* deletions might disrupt early germ cell development or maintenance. Here, we observed that iPSCs derived from men with *AZFa* and *AZFbc* deletions formed fewer and poor-quality PGCLCs both in vitro and in vivo. Regardless of genotype, however, we determined that human donor-derived GCLCs are induced to undergo global DNA demethylation from somatic methylation levels in a manner that is characteristic of the development of male PGCs to gonocytes (Wermann et al., 2010; Seisenberger et al., 2012). Importantly, our results indicate that there is a clear defect linked to expression of later genes such as *DAZ*, *PLZF*, and *UTF1* that coexpress with or follow expression of PGC markers (Culty, 2009); in particular, those with *AZF* deletions did not express Y chromosome *DAZ* and the genes *UTF1* and *PLZF* in any germ cells. Thus, our results of germ cell differentiation are well correlated with clinical outcomes but also provide some interesting insights. However, iPSCs formed PGCLCs, suggesting that men with these deletions likely form germ cells early in development but that these cells are depleted prior to clinical presentation. This concept is consistent with many mouse models of infertility in which initial populations of germ cells are not maintained (Modi et al., 2003; Rucker et al., 2000). Indeed, in mice, both XX and XY embryos form PGCs, which migrate to the gonad and begin differentiation to sperm or oocytes, independent of germ cell sex chromosome composition, depending on the sexual identity of the gonad proper (Gill et al., 2011). Human genetic data similarly indicate that the XO genotype (Turner syndrome) is compatible with formation of fetal germ cells, but they are depleted by birth or thereafter (Modi et al., 2003; Karnis, 2012).

Our results have been summarized into a working model for the efficiency and fate of human iPSCs during human germ cell formation in the mouse testes (Figure 7). Further studies should refine existing methods by performing transplantations with varying cell numbers, examining genetic complementation and hierarchy, and altering the xenotransplant recipient to a

Figure 5. Donor-Derived GCLCs from H1, Human Fetal Testis, and i*AZF*1 Exhibit Multiple Markers of PGCs and Undifferentiated Spermatogonia, whereas *AZF*-Deleted Donor Cells Only Express PGC Markers

(A–F) Mouse testis xenografts derived from (A) H1 hESCs, (B) 22-week-old human fetal testicular cells, (C) i*AZF*1, and (D–F) *AZF*-deleted human iPSCs were stained in adjacent, serial cross-sections for the PGC markers DAZL and STELLA and for the gonocyte/spermatogonial markers UTF1, PLZF, and DAZ in NuMA+VASA+ regions. For each xenograft, low-magnification regions of the seminiferous tubule containing NuMA+VASA+ cells are shown, and boxed regions of interest are enlarged and shown to the right. In NuMA+/VASA+ donor cells, immunostaining for germ cell proteins is shown as follows: Panel 1, positive immunostaining for DAZL (red) and STELLA (green); and Panel 2, UTF1 (green), UTF1+DAZL, PLZF (green), or PLZF+VASA.

(G) Mouse testis xenografts derived from H1 hESCs, i*AZF*1, and human fetal testicular cells showing NuMA+ cells that coexpressed DAZ in boxed regions and enlarged in lower panels. In all panels, dashed white lines always indicate the outer edges of spermatogonial tubules. Wherever shown, nuclei are counterstained with DAPI (blue). Scale bar represents 50 μm.

(H) Summary of positive (green) and negative (red) germ cell protein expression across all xenografts tested.

See also Figure S4.

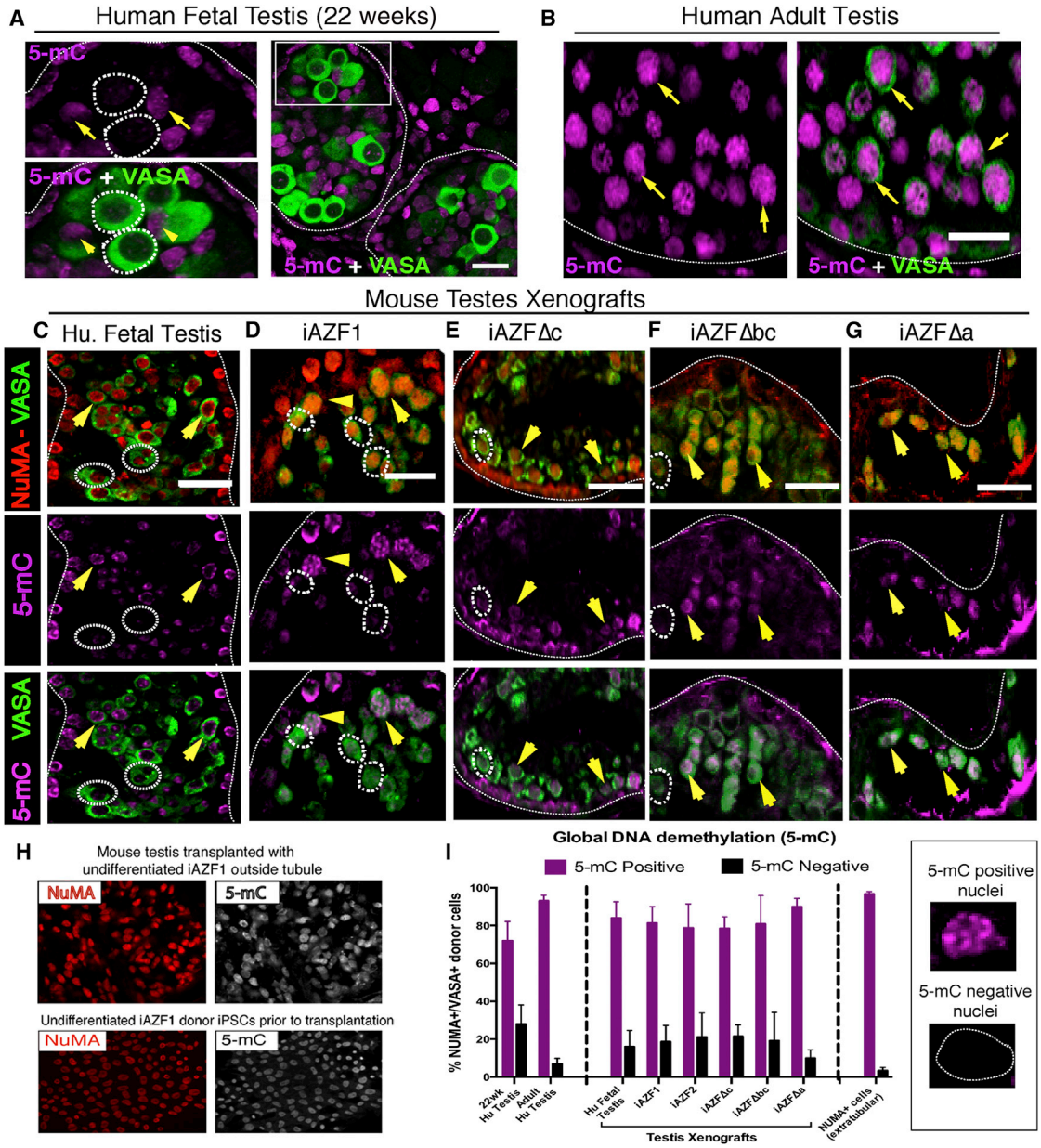

Figure 6. Global DNA Methylation Properties of Xenotransplanted iPSCs

Human fetal (22 weeks) testis (A), human adult testes (B), and mouse testis (C–G) xenografts derived from human fetal testicular cells and iPSCs were stained for VASA and 5-mC in NuMA+ regions.

(A) Cross-section of a human fetal testis (22 weeks) with positive immunostaining for 5-mC alone or VASA and 5-mC together. Enlarged panels on the left represent the region enclosed within the white rectangle of the right panel.

(B) Cross-section of a human adult testis with positive immunostaining for 5-mC alone or VASA and 5-mC together.

(C–G) Cross-sections of mouse testes transplanted with 22-week-old human fetal testicular cells (C) and undifferentiated iPSC donor cells from each line (D–G). For each xenografted line, immunostaining for NuMA+/VASA+ cells (top), 5-mC alone (middle), and 5-mC together with VASA (bottom) is shown. White dotted circles indicate NuMA+/VASA+ cells with complete loss of 5-mC signal; yellow arrowheads indicate NuMA+/VASA+ positive cells with positive 5-mC signals.

(H) Positive 5-mC signals in NuMA+ cells outside tubules (top) and in undifferentiated iPSCs prior to transplantation (bottom).

(I) Quantification of 5-mC positive and negative signals in human testes (left columns) and in NuMA+/VASA+ cells of testis xenografts (middle columns). 5-mC patterns in extratubular NuMA+ cells are shown (right column).

In all panels, dotted lines indicate edges of spermatogonial tubules. Scale bars represent 50 μm. See also Figure S6.

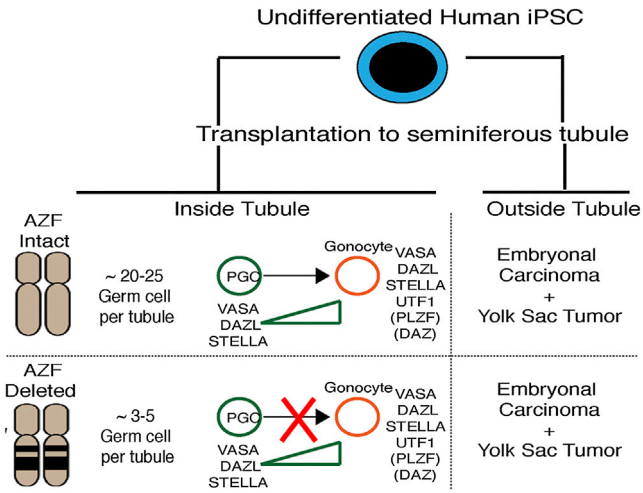

Figure 7. Schematic Illustration of Major Findings of This Study

iPSCs were derived from patients with *AZF*-intact and *AZF*-deleted Y chromosomes using the *OCT4*, *SOX2*, *KLF4*, and *CMYC* cocktail (OSKM). When transplanted, undifferentiated male iPSCs specifically differentiate to PGC-like and gonocyte-like germ cells inside the mouse spermatogonial tubule environment, presumably in the Sertoli cell niche. However, outside the tubule, all patient-derived iPSCs and hESCs remain undifferentiated as primitive tumors. *AZF*-deleted iPSCs appear to have a lower potential to make germ cells in vivo as compared to *AZF*-intact iPSCs and appear to be restricted to forming PGCLCs. See also Figure S5.

nonhuman primate in order to promote full reconstitution of spermatogenesis and overcome innate barriers in xenotransplantation of human cells to the mouse.

EXPERIMENTAL PROCEDURES

Derivation and Culture of iPSC Lines from Primary Human Skin Cells

The human skin-derived primary cell lines (*AZF1*, *AZF2*, *AZFΔa*, *AZFΔbc*, and *AZFΔc*) used in this study were obtained from a 4 mm adult skin punch biopsy and cultured as described by Byrne et al. (2009). *AZF2* iPSCs were derived from normal human fetal foreskin fibroblasts (Stemgent) as previously described (Durruthy Durruthy et al., 2014). Fibroblasts were allowed to expand to 80%–90% confluency before passaging and cryopreservation. Deletion mapping analysis of all patient-derived fibroblasts was determined by sequence-tagged site (STS) PCR analysis with the Y Chromosome Deletion Analysis Kit (Promega) according to manufacturer's instructions. Dermal fibroblasts were infected with a polycistronic lentiviral-reprogramming vector as previously described for reprogramming by Sommer et al. (2010) and Sebastiano et al. (2011). Briefly, 10^5 fibroblasts were seeded in mouse embryonic fibroblast (MEF) medium and infected. After 6 days, cells were transferred onto inactivated MEFs. After replacement with hESC medium, the cells were grown for up to 8 weeks until hESC-like colonies started to emerge. IPSC colonies were manually picked, expanded on MEFs, and after approximately 15–20 passages, transferred to feeder-free culture conditions in mTeSR1 medium (STEMCELL Technologies).

Transduction, Differentiation, and FACS of iPSCs and hESCs with VASA:GFP Reporter Lentivirus

Undifferentiated iPSCs and hESCs were transduced, selected, and differentiated on Matrigel-coated plates with a VASA:GFP lentivirus as previously described by Kee et al. (2009) and Medrano et al. (2012). As controls, transduced cells and nontransduced cells were also used in all differentiation experiments. Differentiation medium was supplemented with 50 ng/ml bone morphogenetic proteins 4 and 8 (BMP4 and BMP8, respectively), 10 nM retinoic acid (RA), and 2 ng/ml human recombinant leukemia inhibitory factor (hLIF) and changed every 3.5 days. Cells were sorted for GFP+ and GFP− populations on a Becton Dickinson FACSArias II instrument.

Xenotransplantation Assay

Human cell lines were transplanted into the testes of busulfan-treated, immune-deficient nude mice (NCr nu/nu; Taconic) as previously described for primate and human spermatogonia by Hermann et al. (2010). Briefly, immunodeficient nude mice were treated with a single dose of busulfan (40 mg/kg; Sigma-Aldrich) at 6 weeks of age to eliminate endogenous spermatogenesis. Xenotransplantation was then performed 5 weeks after busulfan treatment by injecting 7–8 μl cell suspensions containing 10% trypan blue (Invitrogen) into the seminiferous tubules of each recipient testes via cannulation of the efferent ducts. At 8 weeks after transplantation, recipient mouse testes were harvested for histology and immunohistochemical analyses.

Immunohistochemistry

Formalin-fixed human testes or mouse testes xenotransplants were paraffin embedded and sectioned into serial cross-sections of 5–10 μm thickness each (AML Laboratories). Testis sections were deparaffinized in xylene, rehydrated through an ethanol-graded series. For all samples, antigen retrieval was performed by boiling the sections in 0.01 M sodium citrate buffer (pH 6.0) for 20 min, followed by incubation at room temperature for 30 min. For DNA methylation analysis, sections were incubated in 4 N HCl for 20 min prior to blocking. A 10% solution of normal donkey serum (Jackson ImmunoResearch) in PBS was used as a blocking buffer. Sections were incubated with the following primary antibodies diluted in blocking solution (1.0% Normal Donkey Serum, 0.1% Triton X-100, and sterile PBS) overnight at 4°C: VASA (1:200) and GFRα1 (1:250) (R&D Systems); NuMA (1:200), STELLA (1:200), DAZL (1:200), and DAZ (1:250) (Abcam); UTF1 (1:200; Millipore); PLZF (1:250; Chemicon); OCT4 (1:500), SOX2 (1:500), and GATA4 (1:250) (Santa Cruz Biotechnology); SOX9 (1:250; Sigma Aldrich); 5-mC (1:1500; Eurogenetec); and 5-hMC (1:1500; Active Motif). The sections were washed and labeled with Alexa dye-conjugated secondary antibodies. Sections were mounted in ProLong Gold Antifade mounting media containing DAPI (Life Technologies). Negative controls included incubation with rabbit immunoglobulin G antibodies and omission of the primary antibody for all samples. Quantification of sections for NuMA/VASA double staining was determined manually from three to five independent 20× fields taken from three different testis tissue depths and from at least three separate biological replicates. Quantification of positive and negative 5-mC signals was determined by counting at least 150 NuMA+/VASA+ cells inside seminiferous tubules across five 20× fields of view from two independent biological replicates. For human testis cross-sections, 5-mC patterns were quantified in only VASA+ cells inside tubules. Data for statistical analysis follow a normal distribution.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.067.

AUTHOR CONTRIBUTIONS

C.R. performed the majority of experiments and wrote the first draft of the manuscript with assistance from J.D.-D. for transplantation analysis and single-cell gene expression analysis. J.E.A. derived and characterized iPSCs, and M.S. helped with differentiation experiments and performed transplantations. P.J.T. coordinated patient recruitment and consents. R.A.R.P. initiated the research project and provided oversight. K.E.O. and R.A.R.P. assisted with planning and execution of transplantation experiments. C.R. and R.A.R.P. wrote the manuscript with valuable insights provided by other coauthors.

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