Spring 2018 – Systems Biology of Reproduction
Discussion Outline – Gametogenesis / Stem Cells / Cloning
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
March 22, 2018
Week 11

Gametogenesis / Stem Cells / Cloning

Primary Papers:

1. Ball, et al. (2016) BMC Genomics 17:628
2. Valli, et al. (2014) Fert & Steril 101:3-13
3. Zeng, et al. (2015) Biol Reprod 92(4):89, 1-9

Discussion

Student 1: Reference 1 above

- What regulatory factor was identified?
- How was spermatogonial stem cell renewal identified?
- What insights into spermatogonial stem cells was provided?

Student 2: Reference 2 above

- What are the technologies reviewed?
- What are the limitations of organ cultures?
- What clinical applications for germ cell transplantation exist?

Student 3: Reference 3 above

- What developmental stage of germ cells are derived from stem cells?
- List the different methods to generate germ cells from stem cells.
- Why are these artificial germ cells relevant and what use do they have?

Ball et al. BMC Genomics (2016) 17:628
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BMC Genomics

RESEARCH ARTICLE Open Access

Regulatory complexity revealed by integrated cytological and RNA-seq analyses of meiotic substages in mouse spermatocytes

Robyn L. Ball[1†], Yasuhiro Fujiwara[1†], Fengyun Sun[1], Jianjun Hu[1], Matthew A. Hibbs[1,2], Mary Ann Handel[1*] and Gregory W. Carter[1*]

Abstract

Background: The continuous and non-synchronous nature of postnatal male germ-cell development has impeded stage-specific resolution of molecular events of mammalian meiotic prophase in the testis. Here the juvenile onset of spermatogenesis in mice is analyzed by combining cytological and transcriptomic data in a novel computational analysis that allows decomposition of the transcriptional programs of spermatogonia and meiotic prophase substages.

Results: Germ cells from testes of individual mice were obtained at two-day intervals from 8 to 18 days post-partum (dpp), prepared as surface-spread chromatin and immunolabeled for meiotic stage-specific protein markers (STRA8, SYCP3, phosphorylated H2AFX, and HISTH1T). Eight stages were discriminated cytologically by combinatorial antibody labeling, and RNA-seq was performed on the same samples. Independent principal component analyses of cytological and transcriptomic data yielded similar patterns for both data types, providing strong evidence for substage-specific gene expression signatures. A novel permutation-based maximum covariance analysis (PMCA) was developed to map co-expressed transcripts to one or more of the eight meiotic prophase substages, thereby linking distinct molecular programs to cytologically defined cell states. Expression of meiosis-specific genes is not substage-limited, suggesting regulation of substage transitions at other levels.

Conclusions: This integrated analysis provides a general method for resolving complex cell populations. Here it revealed not only features of meiotic substage-specific gene expression, but also a network of substage-specific transcription factors and relationships to potential target genes.

Keywords: Meiosis, Spermatogenesis, Maximum covariance analysis, Mouse, Transcriptome, RNA-seq

Background

Spermatogenesis is a complex developmental process with a unique cell division, meiosis, as a major defining event. The entire process includes maintenance of a small population of spermatogonial stem-cells, mitotic divisions of differentiating spermatogonia, meiotic prophase and ensuing divisions of spermatocytes, and post-meiotic differentiation of spermatids, by a process known as spermiogenesis. In mammalian testes, spermatogenesis occurs within seminiferous tubules, where all germ cells associate with one kind of somatic cell, the Sertoli cell, which provides the appropriate niche and microenvironment for the spermatogenic process. The adult testis is characterized by presence of all of the cells types in the spermatogenic lineage, with waves of differentiation throughout the testis propelled by retinoic acid signaling [1]. In mice, the first wave of spermatogenesis is initiated by spermatogonia shortly after birth, producing a sequential and orderly appearance of each of the more differentiated stages at regular intervals though the first four weeks of life. Although semi-synchronous with respect to the advancing wave of the most differentiated cells, the juvenile onset of

* Correspondence: maryann.handel@jax.org; greg.carter@jax.org
†Equal contributors
[1]The Jackson Laboratory, Bar Harbor, ME, USA
Full list of author information is available at the end of the article

spermatogenesis also includes regular and asynchronous initiation of subsequent waves of spermatogenic differentiation. This asynchronous and continuous process of spermatogenesis has made it difficult to achieve molecular characterization of specific cell types in the lineage. This has been particularly the case with respect to analysis of the defining process of gametogenesis, meiosis, which occurs in spermatocytes. The complex events of meiosis I prophase include recombination, homologous chromosome pairing, and synapsis, taking place as spermatocytes progress through the leptotene, zygotene, pachytene and diplotene substages. These events culminate in the first meiotic division, a reductive division in which homologous chromosomes are separated, producing secondary spermatocytes that rapidly undergo the second, equational, meiotic division to produce haploid round spermatids. Because of the genetic importance of meiotic recombination for the production of chromosomally normal gametes and offspring, there has been great interest in elucidating the molecular hallmarks and their underlying transcriptional signatures that define the meiotic spermatocyte substages.

Toward the goal of achieving a molecular understanding of spermatogenesis, considerable effort has been devoted to separation of specific germ-cell differentiation substages from the histologically complex seminiferous epithelium. A widely used approach, commonly known as the "STA-PUT" sedimentation process [2–4], involves enzymatic dissociation of germ and somatic cells and enrichment for specific stages by cell-size-based sedimentation at unit gravity on a bovine albumin gradient. Reasonably good enrichment of the most uniquely sized cells (large pachytene spermatocytes and small round spermatids) can be obtained from testes of adult mice. However, many cells of interest (spermatogonia, early meiotic prophase spermatocytes) are not retrieved from adults because of their anatomical position bounded by Sertoli cell tight junctions. While these early cell types can be retrieved from juvenile testes, the amount of biological material required is daunting and enrichment is not robust. Isolation of specific cell types by fluorescence-activated cell sorting, FACS [5–8] is promising and becoming more widely applied; however sample sizes are small. Finally, rather than cell separation, total testis germ-cell populations can be collected in a developmental continuum during the first two to three weeks of juvenile development of mouse testes, to take advantage of the first wave of spermatogenesis. In this way, molecular features have been defined with respect to the sequential appearance of more advanced cell stages of spermatogenesis. This approach is useful primarily for correlating the appearance of a molecular entity to the developmental appearance of a specific cell type. However, the degree of resolution has been suboptimal, because gene or protein expression has not been related to absolute frequencies of cell stages, a challenge we tackle in this study.

Together, these methods for enriching or inferring spermatogenic (and meiotic) substages have contributed to studies over the past decade on the developmental transcriptome of mammalian spermatogenesis, as recently reviewed [9]. Most such studies have taken advantage of microarrays querying known coding sequences [6, 10–12], and thus sequence-biased, but also provided interesting views of alternative splicing and other features of the spermatogenic transcriptome [13], and identified previously unknown potential targets for contraception and fertility [14]. More recently, methods for unbiased high-throughput deep sequencing of the transcriptome by RNA-seq have been employed [5, 15–19]. These studies have revealed unappreciated regulation of piRNAs [17], and global whole-genome views of the male germ-cell transcriptome [5, 18, 19]. However, the challenge for all transcriptomic analyses, particularly RNA-seq approaches, has been to computationally deconstruct the entire testis or germ-cell transcriptome into substage-specific transcriptomes. This is an important goal, given both the abundance and complexity of RNA species in the testis, particularly with respect to the imputed contribution of both coding and non-coding RNA from spermatocytes and spermatids [19]. In one example of such a computational approach, frequencies of specific germ-cell stages throughout the first wave of spermatogenesis previously published in another analysis [4] were used to estimate cell type-specific expression patterns in a separate data set [18]. While coming closer to the goal of substage-specific transcriptomes, this study relied on a low sample size and on integrating non-contemporaneous data. Other approaches [5, 6], employed cell sorting by FACS and subsequent validation of purity by meiotic markers. While these studies have yielded important insights into global gene expression switches, they rely on FACS, not always available or practical for small samples, such as from infertile mutant models.

Here we have tackled the problem of deconvolving transcriptomes of complex germ-cell populations into stage-specific transcriptomes by computationally integrating highly detailed and combinatorial cytological staging of the same cell samples as subjected to RNA-seq analysis. Deep and accurate cell stage phenotyping was conducted using antibodies to STRA8, SYCP3, phosphorylated H2AFX, and HISTH1T, all exhibiting meiotic substage specificity of expression and/or localization. By collecting highly enriched germ-cell populations from testes at two-day intervals through the first wave of spermatogenesis, and by multiple sampling of individual mice (30 total) for both cytological composition and RNA composition by high-throughput sequencing, we developed an unusually deep data set that portrays variation in cell substage

composition and transcript abundance. To decompose these data into substage-specific transcriptome patterns in an unbiased way, we chose a nonparametric solution with minimal assumptions about data structure, thus developing a novel permutation-based maximum covariance analysis. This method enabled us to assign co-expressed transcripts to one or more meiotic substages, thereby linking distinct molecular programs to cytologically defined cell states. Moreover, to better understand the regulation of each germ-cell substage transcriptome, we integrated transcription factor information to identify some of the key molecular regulators driving these cell stages. This approach provides a model for deconvolving transcriptomes of complex cell populations with well-defined cytological attributes. Together, these data provide an unprecedented view of the complexity of meiotic transcription programs and their coordinate regulation.

Results

Experimental design

Germ cells were obtained from individual mice at two day intervals from 8 dpp to 18 dpp ($N = 5$ biological replicates at each age; $N = 30$ samples total) and divided into two aliquots, one for cytology and one for RNA-seq (Fig. 1a). For the C57BL/6J (B6) strain used in this study, this developmental window captures the initial differentiation of spermatogonia into early leptonema, through the stages of pachynema and diplonema. For each sample,

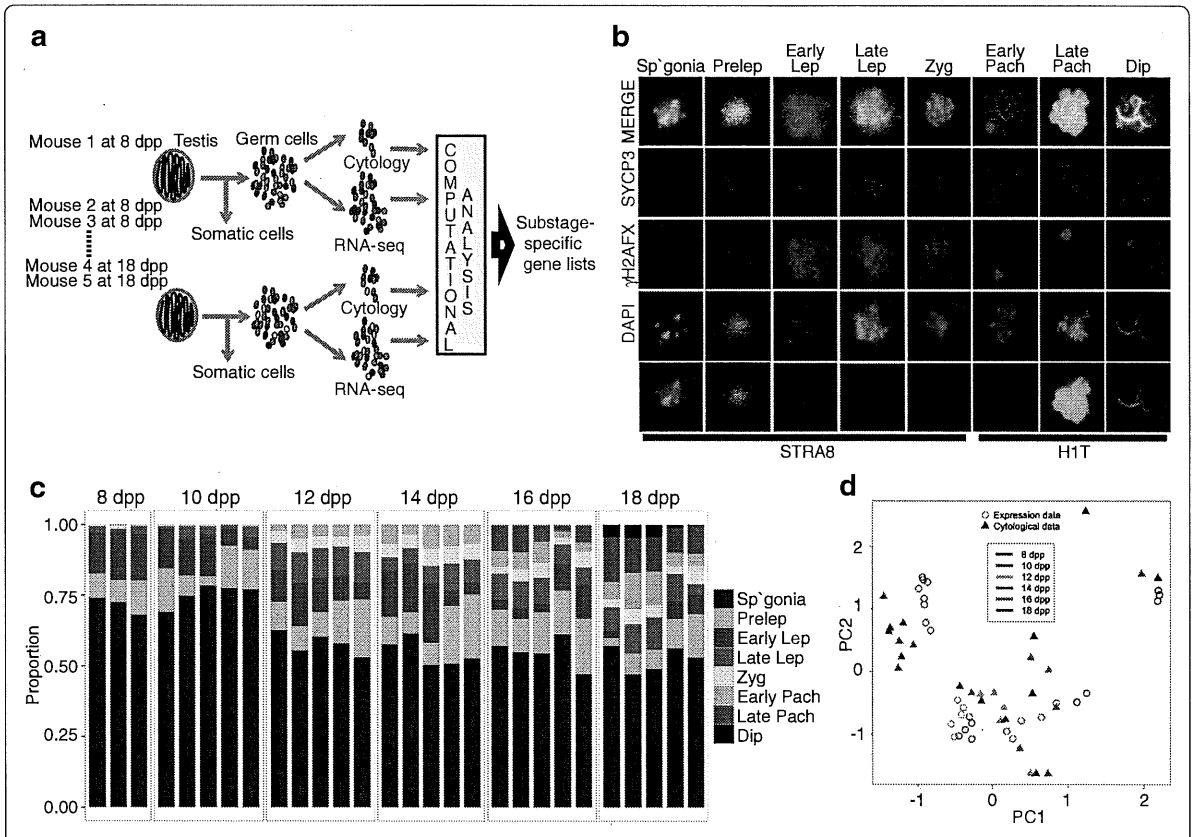

Fig. 1 Decomposition of meiotic cells and gene expression. **a** Experimental design. Germ cells were isolated from whole testes from each of five juvenile male mice at 8, 10, 12, 14, 16 and 18 dpp. Each sample was analyzed for both cytology and gene expression by RNA-seq. PMCA was developed to identify the meiotic substage-specific transcriptomes. **b** Cytological classification and cellular populations. Isolated germ cells were immunolabeled for stage-specific maker proteins: STRA8, expressed in differentiating spermatogonia; SYCP3, a component of synaptonemal complex shows meiotic substage-specific labeling morphology; phosphorylated histone H2AFX (γH2AFX), marking DSBs throughout early prophase nuclei and restricted to the XY chromosomes during pachynema and diplonema; and histone HISTH1T, expressed in post-mid-pachytene spermatocytes. Nuclei were counter-stained with DAPI. **c** Meiotic cell composition during the first wave of spermatogenesis. The contribution by specific germ-cell stages for each developmental time point and mouse is shown, with colors representing specific meiotic substages. Abbreviations: Sp'gonia = spermatogonia, Prelep = pre-leptonema, Early Lep = early leptonema, Lep = leptonema, Late Lep = late leptonema, Zyg = zygonema, Early Pach = early pachynema, Late Pach = late pachynema, Dip = diplonema. **d** Concordance of expression and cytological data. Principal component 1 (PC1) versus principal component 2 (PC2) from independent PCA of cytological and RNA-seq data. Colors correspond to time points and icon shape corresponds to data type as indicated

we determined the relative numbers of cells of each substage by cytological criteria (N = about 400 germ cells per mouse, for a total of 11,990 germ cells scored cytologically by criteria described below); by scoring germ cells from each individual mouse, we captured variability between mice. The purity of germ cells in each sample, computed as germ-cell count divided by total DAPI-stained cell count, was greater than 90 % for all samples. Upon inspection of both cytological and RNA-seq data, two germ-cell samples collected at 8 dpp revealed poor concordance with age-matched samples, likely due to insufficient cell numbers for successful RNA-seq library preparation (Additional file 1: Table S1); thus these samples were omitted from all subsequent analyses (final N = 28).

Cytological deconvolution of the meiotic substage composition of germ cells during the first wave of spermatogenesis

Germ-cell substage frequencies in each cytological preparation were determined by combinatorial immunolabeling with antibodies to meiotic substage marker proteins, scoring 400 germ cells per mouse. Marker proteins assessed were STRA8, a meiosis-initiating factor [20] present in differentiated spermatogonia and some leptotene spermatocytes; SYCP3, a synaptonemal complex (SC) protein present in the chromosomal axes of leptotene and zygotene spermatocytes, in the lateral elements of the mature SC in pachytene spermatocytes, and disassembling in diplotene spermatocytes [21, 22]; the phosphorylated form of histone H2AFX (PH2AFX, also known as γH2AX), which localizes to chromatin surrounding DNA double-strand breaks (DSBs) in characteristic patterns that discriminate early meiotic prophase from the pachytene and diplotene stages [22, 23]; and the testis germ cell-specific histone H1 variant HIST1H1T (herein referred to by its common designation of H1T), which is a marker of the mid-to-late pachytene stage [22]. The combinatorial labeling patterns for each marker protein allowed categorization of germ cells of each sample into eight substages, achieving a higher degree of meiotic substage discrimination than previous transcriptome analyses (Table 1 and Fig. 1b).

Together, these data provide a comprehensive picture of postnatal spermatogenic progress through meiotic prophase of the first wave of differentiating cells for comparison to data previously obtained by histological analyses [4]. At every time point in the current data, greater than 50 % of the retrieved germ cells were spermatogonia (Fig. 1c), reflecting continually initiating separate waves of spermatogenesis. Representation of these cells decreased over time (Fig. 1c), likely due to initiation of additional waves of meiotic cells, increasing numbers of post-spermatogonial spermatocytes, and establishment of cell junctions that impede cell retrieval. Throughout this period, due in part to initiation of subsequent waves of spermatogenesis, the average contribution to the total germ-cell population by preleptotene and leptotene spermatocytes remained relatively constant (Fig. 1c), thus reducing the power to identify transcripts specific to just these substages. The first appreciable numbers of H1T-negative early pachytene spermatocytes were observed at 12 dpp, and H1T-positive late-pachytene cells were abundant by 16-18 dpp, consistent with previously published data [24]. By 18 dpp, less than 10 % of the cells had progressed to the diplotene stage, and only about 0.1 % to metaphase. Overall substage frequencies were similar at 8 and 10 dpp, and also at 12 and 14 dpp (Fig. 1c). For computational analyses (below), we combined substages that exhibited similar frequency patterns across the developmental time span, specifically late leptotene and zygotene substages, and late pachytene and diplotene substages.

High concordance of gene expression and cytological data

To assess the feasibility of associating gene expression with cytologically-defined substages, we performed independent principal component analyses (PCA) on each data set. The high concordance between the cytological frequency of cell substages and RNA expression data (Fig. 1d) suggested that changes in gene expression in pooled germ cells can be explained by variation in cytological proportions of the

Table 1 Immunolabeling criteria for cell types and meiotic substages

Substages	SYCP3	γH2AX	STRA8	H1T
Spermatogonia[a]	None	Negative	Positive	Negative
Preleptonema	Patches	Negative	Positive	Negative
Early Leptonema	Patches	Positive	Positive	Negative
Late Leptonema	Fine foci	Positive	Weakly positive	Negative
Zygonema	Partially paired	Patches	Negative	Negative
Early Pachynema	Paired	Restricted to XY body	Negative	Negative
Late Pachynema	Paired	Restricted to XY body	Negative	Positive
Diplonema	Partially dissociated	Restricted to XY body	Negative	Positive

[a]Spermatogonia are a separate mitotic stage, not a meiotic substage

differentiation states during spermatogenesis. Although cells in different substages may contribute different amounts of RNA to each sample, this analysis demonstrates an overall quantitative agreement between the two data types. This supported the validity of combining these two datasets to identify meiotic substage-specific transcriptional programs.

Meiotic substage-specific gene expression derived by covariance analysis

To identify signatures for meiotic substage-specific gene expression, we developed a novel permutation-based maximum covariance analysis (PMCA), which maps groups of co-expressed genes to combinatorial cytological marker-based staging based antibodies to STRA8, SYCP3, phosphorylated H2AFX, and HISTH1T. This statistical approach demonstrates *concordance* of distinct cellular programs to each meiotic substage based on the similarity of "cyto-pattern" and "gene-expression pattern" across samples. In brief, the concordant patterns are derived from the most preeminent features of covariance between cytological and transcriptome data across all samples (Methods). In this RNA-seq dataset, a total of 15,025 Ensembl genes (http://www.ensembl.org) and 5267 NONCODE lncRNA genes [25] were detected (at a Transcripts per Million (TPM) ≥ 3) in at least one sample of the isolated germ cells. We also detected piRNA precursors transcripts that had previously been identified [17] with appropriate substages, although they are not the focus in the current study. Through PMCA, we identified 1235 spermatogonia genes and 6052 meiosis substage-specific genes. Expression of many transcripts could not be assigned to distinct substages and instead, were shared across several consecutive substages; 131 genes were shared among late leptotene, zygotene, and early pachytene stages while 106 genes were shared among early pachytene, late pachytene, and diplotene stages (Fig. 2; Additional file 2: Figure S1; Table 2; Additional file 1: Table S2). Notably, increased numbers of expressed genes were observed at 16 dpp when cells first reach the late pachytene stage (Table 2; Additional file 1: Table S2).

In addition to identifying genes concordant with specific substages, PMCA also identified genes that were *negatively concordant* with specific substages. The negatively concordant genes have patterns of expression that are opposite to the cytological patterns; thus negatively concordant defines gene expression that is lower when the cytological proportion of a specific substage is higher and vice versa (Fig. 2; Additional file 2: Figure S2; Table 2;

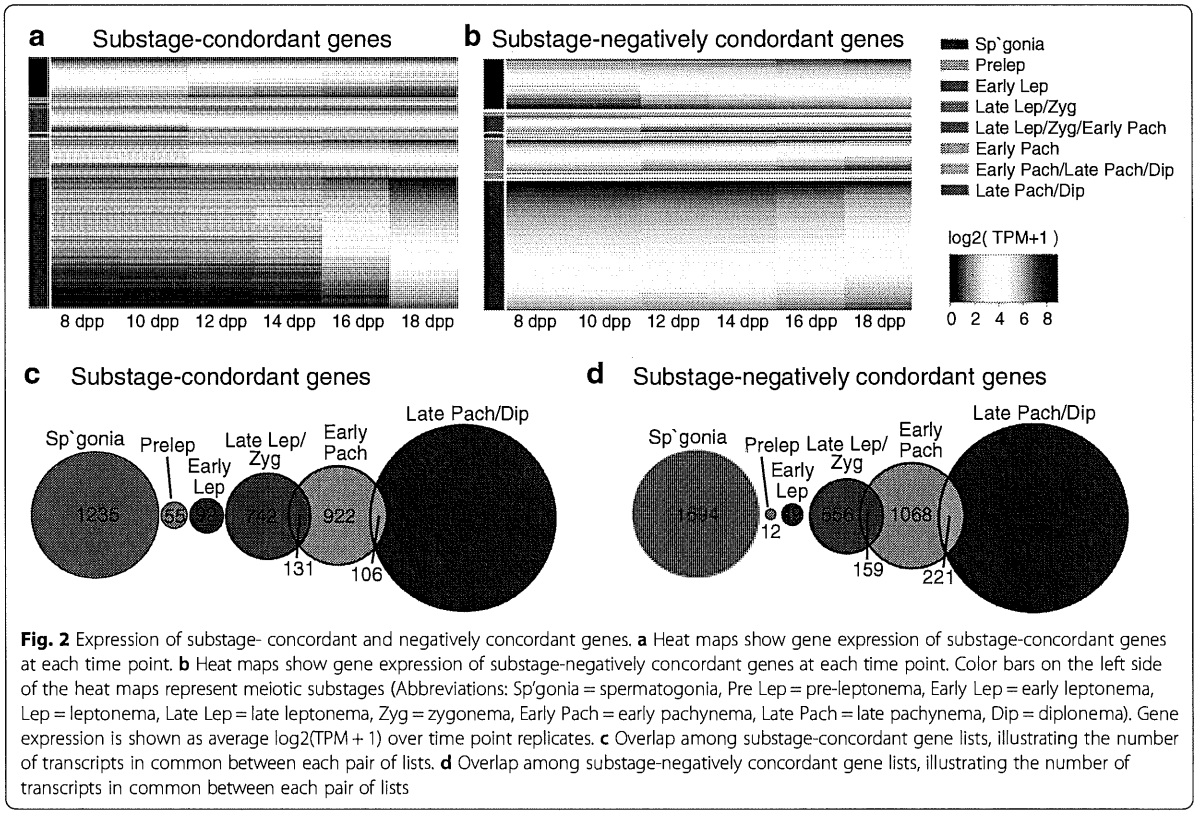

Fig. 2 Expression of substage- concordant and negatively concordant genes. **a** Heat maps show gene expression of substage-concordant genes at each time point. **b** Heat maps show gene expression of substage-negatively concordant genes at each time point. Color bars on the left side of the heat maps represent meiotic substages (Abbreviations: Sp'gonia = spermatogonia, Pre Lep = pre-leptonema, Early Lep = early leptonema, Lep = leptonema, Late Lep = late leptonema, Zyg = zygonema, Early Pach = early pachynema, Late Pach = late pachynema, Dip = diplonema). Gene expression is shown as average log2(TPM + 1) over time point replicates. **c** Overlap among substage-concordant gene lists, illustrating the number of transcripts in common between each pair of lists. **d** Overlap among substage-negatively concordant gene lists, illustrating the number of transcripts in common between each pair of lists

Table 2 Substage-concordant and negatively concordant genes

Substage	Number of genes concordant	Number of genes negatively concordant
Spermatogonia[a]	1235	1694
Preleptonema	55	12
Early Leptonema	92	49
Late Leptonema/Zygonema	742	556
Late Leptonema/Zygonema/ Early Pachynema	131	159
Early Pachynema	922	1068
Early Pachynema/Late Pachynema/Diplonema	106	221
Late Pachynema/Diplonema	4004	4366

[a]Spermatogonia are a separate mitotic stage, not a meiotic substage

Additional file 1: Table S2). Genes detected as negatively concordant with one stage are often detected as concordant with another substage. For example, genes negatively concordant with spermatogonia are genes expressed across the meiotic cell substages, but not enriched in spermatogonia. Not surprisingly, the 1694 genes negatively concordant with spermatogonia were enriched for known meiotic genes. Indeed, among all genes expressed, many of those with known meiotic function and/or Gene Ontology annotation for meiotic function were not concordant with any single meiotic substage, but were expressed across meiotic substages.

Validation of substage-specific gene expression patterns

We bolstered and validated these computationally derived results in three distinctly different ways. First, gene expression analyses by qRT-PCR supported the RNA-seq expression data. Second, the validity of the PMCA procedure was queried by analysis of genes expressed in a highly enriched cell population of mid- to late-pachytene spermatocytes retrieved from adult testes by unit gravity sedimentation. Finally, we used our data to confirm a known pattern of male germ-cell specific gene expression, X-chromosome silencing during meiotic prophase.

We compared transcript expression patterns detected by RNA-seq to independent qRT-PCR assays. We tested representative genes among those increasing, decreasing, or showing little change in expression across the sequential time points, as well as genes known to be highly expressed in pachynema. Germ cells of 10 and 16 dpp males were subjected to qRT-PCR to determine expression of each tested gene relative to a reference housekeeping gene (*Actb*); data are shown in Additional file 2: Figure S3. The qRT-PCR assays reflected RNA-seq findings. For example, in 16 dpp samples we found elevated expression of pachytene-enriched genes and of genes shown by RNA-seq to increase in expression throughout the juvenile period tested (Additional file 2: Figure S3). Overall, patterns of gene expression are mostly concordant between the two different quantification methods and between sample sets, although, as expected, RNA-seq provides finer resolution and higher information content.

We further validated the PMCA-derived meiotic substage transcriptomes by comparison to highly enriched adult pachytene spermatocytes obtained by sedimentation at unit gravity [2–4, 26], which sorts cells based on size, not cytology or DNA content ($N = 4$ samples, each from germ cells pooled from testes of 6 mice at 9 weeks of age). The purity of late pachytene/diplotene spermatocytes in this preparation was 90 % based on cytological immunostaining with antibodies to stage-specific proteins phosphorylated H2AFX, SYCP3, and HIST1H1T. RNA isolated from the size-enriched pachytene spermatocytes was subjected to RNA-seq, allowing us to compare genes expressed in the pachytene spermatocytes to the meiotic substage-specific gene lists derived by PMCA. Cross-referencing from this dataset, there was a highly significant enrichment of pachynema-expressed genes among the gene lists from later meiotic substages (hypergeometric tests, all $p < 2.86 \times 10^{-18}$), but there was no enrichment for pachytene genes in the gene lists for spermatogonia or the early substages of meiosis (Table 3). In fact, 99 % of the genes in the late pachytene/diplotene list were also found in the enriched adult pachytene spermatocyte samples.

Finally, we queried the meiotic substage-specific patterns of gene expression determined by PMCA by confirming meiotic sex-chromosome inactivation (MSCI), a well-known feature of meiotic gene expression. In spermatocytes, most of the axial length of X and Y chromosomes, which are non-homologous, is not paired or synapsed. By the onset of the pachytene stage of meiotic prophase, the unpaired regions of the sex chromosomes become transcriptionally inactivated [11, 27, 28]. We

Table 3 Representation of enriched pachytene spermatocyte genes in meiotic substages

Substage	Number represented/ total concordant	*p*-value
Spermatogonia[a]	614/1235	1
Preleptonema	17/55	1
Early Leptonema	28/92	1
Late Leptonema/Zygonema	550/742	1.2×10^{-09}
Late Leptonema/Zygonema/ Early Pachynema	97/131	9.0×10^{-03}
Early Pachynema	809/922	2.0×10^{-62}
Early Pachynema/Late Pachynema/Diplonema	102/106	1.3×10^{-15}
Late Pachynema/Diplonema	3955/4004	$<1.0 \times 10^{-62}$

[a]Spermatogonia are a separate mitotic stage, not a meiotic substage

assessed expression of X-linked genes from our RNA-seq data; overall, 788 X-linked genes (and 24 Y-linked genes) were detected in the substage-associated gene lists. Of these, only 190 genes were detected in the highly enriched adult pachytene spermatocytes, providing evidence of robust MSCI. These 190 transcripts, which do not show any specific regional localization on the X chromosome, might reflect ongoing transcription and escape from MSCI, but, perhaps more likely, may represent stable transcripts persisting after inactivation. In the developmental transcriptome, the strongest X-linked gene signals are those represented in the negatively concordant gene lists (Table 4), that is, genes with expression pattern opposite to the cytologically determined frequencies of each substage. This strong signal is particularly apparent for genes in the late-pachytene negatively concordant list, which exhibit relatively constant expression from 8 to 14 dpp but then sharply decrease in expression at 16 and 18 dpp; this is evidence for pachytene substage MSCI. In addition to MSCI being revealed by the negatively concordant gene lists, there is also diminished representation of X-linked genes in gene lists concordant with later stages of meiotic prophase (Table 4). Overall, 730 X-linked genes appear to be down-regulated by pachynema to late pachynema, or even earlier (Fig. 3a) and only 58 X-linked genes with early expression are detected at later stages (Fig. 3b).

In conclusion, evidence from both MSCI and the transcriptome of highly enriched adult pachytene spermatocytes validates the PMCA-derived substage- transcriptome signatures derived by PMCA. Moreover, as discussed below, we find good concordance among our stage-specific gene lists and functions with those derived by other studies.

Enrichment analysis of meiotic substage gene expression

The PMCA-derived meiosis substage-specific gene lists, coupled with Gene Ontology (GO) analysis [29], can provide insight into meiotic and spermatogenic function. Genes assigned to each substage were ranked by their similarity score, a measure of how closely the gene's expression pattern follows the substage's cytological pattern. Using ranked lists, we performed a ranked GO analysis in GOrilla [30]; these ranked lists provide approximations of meiotic substage gene ontology. Significantly, many meiosis-specific genes are not prominently represented among the meiosis substage-specific gene lists derived by PMCA. However, GO terms for genes negatively concordant with spermatogonia were predominantly meiotic terms (16 out of 31 GO terms for process, all $p < 9.97 \times 10^{-4}$), confirming that meiotic genes tend to be expressed across multiple meiotic substages and not unique to a particular meiotic substage (Additional file 1: Table S3). Thus meiotic substage transitions are probably not acutely regulated at the level of transcription of many of the known meiotic genes, although among late leptonema/zygonema-associated GO terms, 8 out of 10 GO "biological process" terms were associated with meiosis regulation (all $p < 8.6 \times 10^{-4}$), and similar GO terms were also identified in the early pachynema gene list (13 out of 33 GO terms for process, all $p < 5.56 \times 10^{-4}$) (Additional file 1: Table S4). We extracted 447 genes for *M. musculus* that are associated with any GO terms containing "meiosis" or "meiotic" (www.mousemine.org), and of these genes we considered the 404 genes which are expressed in our developmental time series (those genes not expressed in our time series might include female meiosis-related genes). We found that 229 (57 %) genes were not concordant with any specific substage, while 26 (6 %) were concordant with late leptonema and zygonema, 43 (11 %) were concordant with early pachynema, and 92 (23 %) were concordant with late pachynema and diplonema. Of meiotic terms that were negatively concordant with substages, 67 (17 %) were negatively concordant with spermatogonia and 97 (24 %) were negatively concordant with late pachynema and diplonema (Additional file 1: Table S5). GO terms for genes expressed in late meiotic prophase were significantly enriched for spermatogenesis, spermiogenesis and fertilization, reflecting transcription of mRNAs to be stored for later translation during the haploid phase

Table 4 Substage-specific X-linked gene analysis

Substage	X-linked Concordant	Y-linked Concordant	X-linked Negatively Concordant	Y-linked Negatively Concordant
Spermatogonia[a]	28	0	55	2
Preleptonema	2	0	0	0
Early Leptonema	2	0	1	0
Late Leptonema/Zygonema	34	2	6	0
Late Leptonema/Zygonema/Early Pachynema	3	0	1	0
Early Pachynema	21	1	41	0
Early Pachynema/Late Pachynema/Diplonema	0	0	13	0
Late Pachynema/Diplonema	14	3	329	9

[a]Spermatogonia are a separate mitotic stage, not a meiotic substage

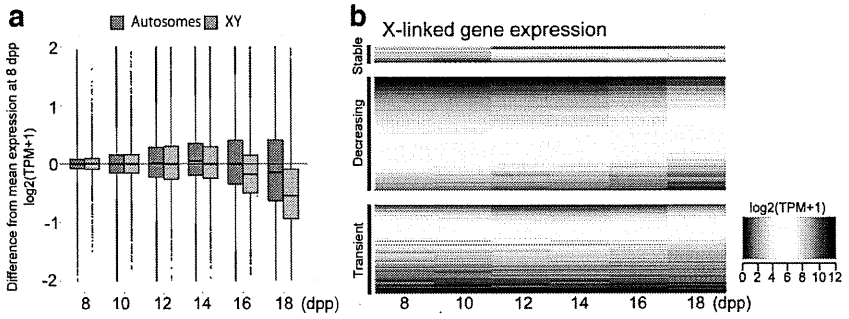

Fig. 3 X-linked gene expression during the first wave of spermatogenesis. **a** Boxplots of the difference from mean gene expression at 8 dpp are shown for each time point, with genes on the autosomes in purple and those on the XY chromosomes in green. Gene expression is log2(TPM + 1). **b** Heatmap of X-linked gene expression at each time point. Genes are clustered based on the pattern of expression: an increase of expression at 12 dpp and then stable throughout (Stable), steadily decreasing over time (Decreasing), or low expression from 8-10 dpp and high expression from 12-14 dpp followed by low expression at 16-18 dpp (Transient). Gene expression is shown as average log2(TPM + 1) over time-point replicates

of spermatogenesis [31]. For example, early pachynema gene lists are enriched for GO terms associated with spermiogenesis (5 out of 33 GO terms for process, all $p < 7.39 \times 10^{-4}$), and the majority of late meiotic prophase- or late-pachynema/diplonema-associated genes were associated with spermiogenesis or fertilization-related GO terms (11 out of 16 GO terms for process, all $p < 7.11 \times 10^{-4}$) (Additional file 1: Table S4). Although genes concordant with early leptonema had transcription-related GO terms in 11 out of 32 GO terms for process (all $p < 1.00 \times 10^{-10}$), we found that many genes negatively concordant with leptotene through zygotene substages have GO terms for transcription and related processes (Additional file 1: Table S3), possibly reflecting the cytologically diminished incorporation of RNA precursors during the earliest meiotic prophase substages [32]. Also among the genes lists for these early stages we found extracellular membrane- or molecular transport-related GO terms prominently represented (Additional file 1: Table S4), which may related to the fact that these cells transit through the Sertoli cell junctions that create the blood-testis barrier [33].

Additionally we performed feature enrichment analysis with the hypergeometric test on the substage-specific gene lists. Interestingly, protein-coding genes are enriched in gene lists concordant with the late-leptotene/zygotene stages ($p = 4.52 \times 10^{-18}$) and the early pachytene stage ($p = 1.19 \times 10^{-8}$). Moreover, they are enriched in the set of genes that is negatively-concordant with late-pachytene and diplotene substages ($p = 5.94 \times 10^{-167}$). However, the genes concordant with late-pachytene and diplotene substages are not depleted in protein-coding gene, which comprise the expected majority of this gene set. This suggests that while certain protein-coding genes are abundant in the Late Pach/Dip substage, large numbers of other protein-coding genes are downregulated in this stage, perhaps reflecting cessation of mRNA transcription in preparation for the meiotic division stage, or, alternatively, post-transcriptional regulation by PIWI-interacting RNAs (piRNAs). Recent studies show that piRNAs play important roles in genome stability by suppressing harmful transposons as well as by regulating mRNAs [34], and future analyses could integrate piRNA expression with these data.

Transcription factor analysis

Because our results point to a large and diverse meiotic germ-cell transcriptome, also noted by others [19], we inferred the underlying regulatory networks accounting for these patterns, using the iRegulon bioinformatic approach to identify transcription factors (TFs) potentially regulating substage-specific genes (Methods). TFs were identified for each substage-specific gene list with high normalized enrichment scores (NES ≥ 4), corresponding to an estimated false discovery rate of less than 0.01 [35]. We then determined which TF genes were unique to, or shared among, the substages. As can be seen in Additional file 1: Table S6 and Additional file 2: Figure S4, mRNA transcripts for some TFs are substage-specific. For example, *Pou5f1* transcript is specific to the preleptotene stage while *Zbtb33* transcript is specific to the pachytene/diplotene substages. Other TF transcripts, similar to meiosis-specific genes in general, are shared across substages but may be specific to early or late meiosis (Additional file 2: Figure S4); *Zfp143* transcript is shared across late meiosis substages while *Tpbl1* transcript is common to the preleptotene (in the "Jazf1 + 3" cluster) and late leptotene/zygotene/early pachytene (in the "Mybl* + 9" cluster) substages. We also found that some TFs, for example MYBl1, also have target genes that are negatively concordant with substages (Additional file 2: Figure S5; Additional file 1: Table S7). We used the MGI bioinformatics Batch Query tool to determine that 160 of the 181 TFs in this analysis were

previously found to be highly expressed specifically and/or significantly in the testis or male germ cells and 39 are annotated to male-reproduction-related phenotypes (Additional file 1: Table S6).

To gain a deeper insight into the regulatory patterns of meiosis, we selected the highest scoring TF genes for further analysis, based on iRegulon's NES score which corresponds to a low false discovery rate. Of these highest scoring TFs, half of them are annotated to meiosis-related functions: ETV4, E2F1, GATA2, RFX4, and ZFP143. We included four other well-known meiosis TFs: RARG, MYBL1, ETV5, and TBPL1. We compared the mRNA expression pattern of these TF genes with the expression patterns of their target genes in order to identify putative regulatory relationships, an analysis that is based on the assumption that a relevant TF protein appears more or less contemporaneously with its transcript (i.e., no translational delay). In cases where the expression pattern of the TF gene had the opposite expression pattern of its target genes, we infer that the TF acts as a repressor on these targets; but in cases where the expression patterns of the TF gene and its target genes were concordant, we postulate that the TF enhances expression of its targets. For TFs with genes that did not change over our time course, such as the known candidate NRF1, we could not infer a relationship to target genes and excluded them from further analysis.

Following these assumptions, as illustrated in Figs. 4 and 5, we suggest that OVOL2 and YY1 act as repressors of target genes in early leptonema and ETV5, ETV6, and ZFP143 act as repressors on target genes after initiation of meiosis, beginning with the late leptotene and zygotene substages. Evidence suggests that ZBTB33, GATA2, and ETV4 also act as repressors on gene targets in pachynema (Fig. 4 and Additional file 2: Figure S6); while RFX4 appears to be an enhancer of target genes in late pachytene and diplotene substages (Additional file 2: Figure S6), but may repress its target genes in preleptonema, although this relationship is less clear. Interestingly, these underlying assumptions based on relative expression levels suggest that MYBL1, TBPL1, and E2F1 act as both enhancers and repressors of their target genes. MYBL1 and TBPL1 appear to repress target genes in early meiosis while enhancing target genes in late meiosis, while E2F1 has the opposite pattern and may repress target genes in late meiosis while enhancing target genes in early leptonema (Additional file 2: Figure S6). Similar regulatory switching has been shown to result from changes in protein co-factors [36] and post-translational modifications [37]. Moreover, genes for some of the TFs described above are also themselves target genes associated with specific substages. By considering these relationships, we inferred candidate regulatory interactions among TFs; for example, ZFP143 and ETV5 suppress *Mybl1* gene expression, while MYBL1 enhances expression of *Rfx4*, *Ovol2*, *Yy1*, and *Tbpl1* genes (Fig. 5).

Overall, 41 % of the genes associated with specific meiotic substages (2483 of 6052 genes) are predicted targets of one or more of these eight meiosis-related TFs. This noteworthy observation suggests that this concise regulatory network can account for a substantial portion of the meiotic program of transcription.

Discussion

Here we identify a male meiotic germ-cell transcriptome using a novel analysis based on a dense dataset of cytological substage-specific signatures. The high concordance between RNA-seq expression data and cytological proportions of isolated germ cells across all samples we analyzed allowed us to develop a novel PMCA to identify the substage-specific transcriptomes for meiotic prophase. This computational method does not require the use of FACS or sedimentation sorting of cells and can be applied to other complex cell populations for which there are well-defined cytological criteria.

Cytological deconvolution of RNA-seq data from the first wave of spermatogenesis

In mammalian males, spermatogenesis is continuous and asynchronous, ensuring daily capacity to deliver sperm backed up by a testis comprised of abundant numbers of germ cells at all stages of spermatogenesis. While advantageous to reproductively active males, this biological imperative has frustrated attempts to isolate stage-purified spermatogenic cells. Methods for stratification of cell samples by either sedimentation [3, 4] or flow cytometry [7, 8] enrich specific spermatogenesis cell stages, while the juvenile first wave of spermatogenesis (used in this study) provides a leading edge of differentiating cells against a background of less differentiated cells. In the laboratory mouse, meiosis is initiated at about 8 dpp in a subset of spermatogonia by retinoic acid stimulation of STRA8 [1, 20]; however, initiation of subsequent waves of meiosis make the cellular population increasingly heterogeneous (Fig. 1c), resulting in a complex histology [4]. The cell-stage frequencies presented here (Fig. 1c) are based on a powerful combinatorial application of antibodies recognizing well-characterized and highly stage-specific marker proteins. We find a continuous and relatively constant background of the earliest spermatogenic cells, namely, spermatogonia and the preleptonema and leptonema (Fig. 1c), and, not surprisingly, these "background" spermatogenic cells are the ones most refractory for computational assignment of a substage-specific transcriptome. On the other hand, pachytene spermatocytes are not present at the earliest juvenile stages, and appear in a discrete time period; consequently, it was possible to assign a robust gene list to this stage.

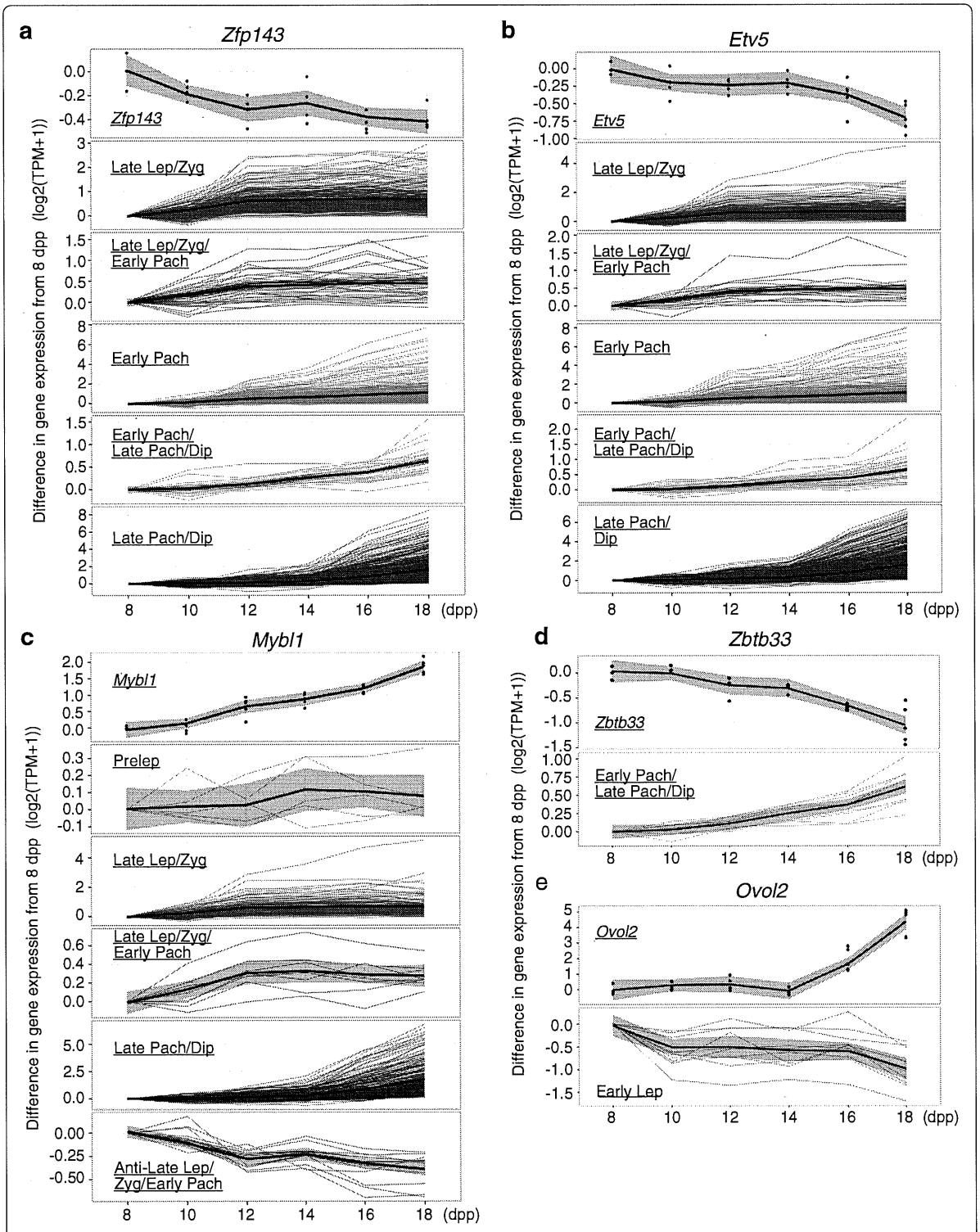

Fig. 4 Expression of TFs and target genes for each substage. The difference from mean gene expression at 8 dpp of the TF and its target genes at each time point. To illustrate the overall pattern, a smoothed line was fit to the substage-specific gene expression. Color of lines represents the substage of target genes. Gene expression is average log2(TPM + 1), averaged over time point replicates. Expression is plotted for (**a**) *Zpf143*, (**b**) *Etv5*, (**c**) *Mybl1*, and (**d**) *Zbtb33*

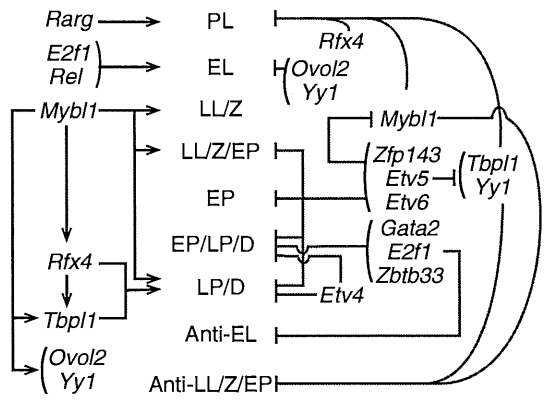

Fig. 5 Regulation of substage-concordant gene expression during meiotic prophase. Analyses suggest that substage-concordant gene expression is regulated both positively and negatively by a network of TFs. TFs are shown by their gene names, and the meiotic substages of predicted TF target genes are shown in the center. Black arrows represent a TF acting as an enhancer on its target genes and red bars represent a TF acting as a suppressor on its target genes. (Abbreviations: PL = pre-leptonema, EL = early leptonema, LL/Z = late leptonema and zygonema, LL/Z/EP = late leptonema, zygonema, and early pachynema, EP = early pachynema, EP/LP/D = early and late pachynema and diplonema), LP/D = late pachynema and diplonema, Anti-EL = genes negatively concordant with early leptonema, Anti-LL/Z/EP = genes negatively concordant with late leptonema, zygonema, and early pachynema)

Gene assignments to specific substages were guided by the covariance of transcriptomic data and the cytological data. Assignment of expressed transcripts to cellular subpopulations in heterogeneous samples has long been a computational challenge [18, 38–40]. In this study, we exploited the advantage of paired RNA-seq and cytological data to develop the novel PMCA approach that isolates changes of gene expression specific to each meiotic substage. Maximum covariance analysis (MCA) was first developed in the meteorological sciences [41] and was popularized in the climatological sciences in the 1990s [42, 43]. More recently, an MCA approach has been used in a bioinformatics context to clarify relationships between gene and protein expression [44]. While MCA is often an effective tool for detecting common signals in two sets of variables, it can be limited by a tendency to fit spurious patterns when faced with increased sampling variation [45, 46]. Current methods [47] employ a parametric smoothing model using principal component regression, which requires a normality assumption or rely on GO analysis [44]. We developed a novel PMCA method that not only overcomes the spurious pattern identification liability but that does so without the need for any parametric assumptions or reliance on GO analysis. Our PMCA approach is broadly applicable to multi-dimensional data derived from a common set of samples.

Substage specificity of meiotic gene expression and regulation

Historically, meiotic prophase substages are characterized by the morphology of chromatin, and correlated genetic mechanisms have been revealed in the past decades [48]. It is known that there is widespread transcription of protein-coding genes in the testis [19], and indeed, we found that among germ cells alone, more than 15,000 genes are transcribed, suggesting that a significant portion of testis transcriptional complexity is due to germ cells. Using PMCA, we successfully identified genes with expression patterns that matched the "cyto-patterns" of stage-specific cell frequencies determined by antibody labeling (Fig. 1c). Some genes were shared among substages; for example, late leptotene and zygotene shared 131 genes with early pachytene and early pachytene shared 106 genes with late pachytene and diplotene. Due to the strong temporal signal of contribution of the late pachytene germ cells, which do not appear until 16 dpp and greatly increase in representation by 18 dpp, we identified over 4000 late pachytene genes. However, since the proportion of preleptotene and early leptotene cells did not vary greatly over time, we were unable to deconvolve stage-specific transcriptomes for the earliest meiotic substages as confidently as for the later substages.

We compared these substage-specific gene lists to those developed in other recent analyses of the germ-cell testis transcriptome. The striking increase in number of genes unique to the pachytene stage was also observed by Soumillon, et al. [19], where, in fact, greater numbers of genes were considered expressed than in this study. However, we have used a more stringent cutoff (TPM ≥ 3) to eliminate from the data transcripts expressed at low levels. When we relaxed this requirement and compared the number of genes expressed with a cutoff of TPM > 0, we found the number of expressed transcripts to be similar to that reported by Soumillion, et al. [19]. We also compared the substage-specific gene lists to the relevant clusters identified by Margolin, et al. [18]. In their study, gene lists were temporally clustered from single samples taken at 6, 10, 12, 14, 16, 18, 20, and 38 dpp. While our study was based on multiple replicates at each time point as well as fine-grained cytological analysis, our PMCA gene lists substantially overlapped with their derived gene list clusters (Additional file 1: Table S8). In all, 4013 of the 4077 genes in clusters identified by Margolin, et al. [18] were also expressed in our dataset.

We also compared our results with those derived from flow-cytometry based methods of sorting by DNA content [5, 6]. For both of these studies, we found overall concordance in genes assigned to meiotic substages (Additional file 1: Table S9). These similarities were especially pronounced for late pachytene and diplotene substages due to the large numbers of genes in these

sets. However, we also observed a broader trend of alignment between early and late substages. Furthermore, our use of multiple markers to cytologically characterize cells paired with our PMCA analysis allowed for more precise substage assignment than possible by cell sorting. For instance, our spermatogonia and pre-leptotene sets were the top two strongest overlaps with the "secondary spermatocyte" (2C) cell fraction in da Cruz, et al. [5] (hypergeometric test $p = 4.5 \times 10^{-4}$ and $p = 1 \times 10^{-3}$, respectively), but split these assignments to provide improved substage resolution. Similarly, our joint late-leptotene and zygotene genes and early pachytene genes had the two greatest overlaps with the combined "leptotene and zygotene" and "pachytene spermatocytes" (LZ + PS) genes (hypergeometric test $p = 8.7 \times 10^{-7}$ and $p = 1.4 \times 10^{-3}$, respectively), and further partition these transcripts into more precise substages. Analysis of Fallahi et al. [6] data was limited by different experimental platforms (microarrays versus RNA-seq), different assay timing (adult versus juvenile mice), and generally low numbers of uniquely assigned genes, but also revealed significant overlaps in late substages (Additional file 1: Table S9). By assessing functional concordance by similarity in GO annotations, we determined that our early leptotene and Fallahi et al. [6] preleptotene sets share a common enrichment in RNA transcription genes. Finally, the most prominent divergences between our data and these previous results were in the spermatogonia fraction. We note that cell sorting techniques are susceptible to inclusion of somatic-cells in this fraction, whereas our spermatogonia genes were significantly negatively-concordant with many standard meiosis genes (Additional file 1: Table S3). In sum, these comparisons suggest that our transcript sets encompass and substantially expand these previous findings.

In this study, we characterized each meiotic substage using the list of substage-specific genes from PMCA analysis. Some meiosis genes were associated to particular substages. For example, *Spata22* [49] and *Msh4/5* [50, 51] are both highly associated with late leptotene/zygotene substages, and both are required for recombination. However, many canonical meiosis genes were found throughout all meiotic prophase substages; these genes include *Rad51*, *Rec8*, and *Syce2*. This may well reflect a lack of acute transcriptional regulation for these important transcripts. Rather than transcription at the precise meiotic substage of use, quality and efficiency of meiosis may be ensured by having transcripts present and available for translation throughout meiotic prophase, with substage transitions regulated post-transcriptionally and/or post-translationally. Further, although we confirmed that spermatogonia negatively concordant genes are enriched with meiotic genes, we found several meiotic genes in the gene list concordant with spermatogonia, including *Fign*, which was reported to be required for meiotic recombination [52], suggesting that some of the meiotic program is set up prior to meiotic entry.

Not surprisingly, this developmental transcriptome analysis also revealed that many genes expressed during meiosis do not have known functions directly contributing to meiosis. These may instead be part of a parallel program of spermatogenic gene expression. For example, many of the genes expressed in early leptonema are associated with transcription or RNA metabolism, as well as with cellular processes such as cell-cell interactions, which are of considerable importance in the biology of the seminiferous tubule. PMCA also revealed that late pachytene/diplotene-expressed genes are significantly associated with GO terms associated with spermiogenic processes, correlating well with findings of other studies [5, 6]. During post-meiotic stages of nuclear condensation, transcription is globally repressed [28, 53], underlying the biological rationale for prior transcription to support the active protein synthesis during spermiogenesis. In addition, the late pachytene/diplotene-associated gene lists are significantly enriched with non-protein-coding RNAs, including lincRNAs (long intergenic non-coding RNA) and piRNA precursors.

Meiotic regulatory networks

Analysis of predicted TF targets [35] generated a regulatory network that potentially governs the meiotic and spermatogenic programs of gene expression identified by this study (Fig. 5). The protein ZFP143 was central in our network, and is required for embryo development in zebrafish [54] and human [55, 56]. The human ortholog, ZNF143, is ubiquitously expressed [57], and binds to the promoter region of target genes where it is required for formation of chromatin loops by interacting with POLII and RAD21 [58]. Because RAD21 is differentially expressed after the pachytene stage of meiosis [59], it is possible that ZFP143 is crucial for spermatogenic processes by regulating the transcription of genes during pachytene/diplotene substage, an idea that should be tested experimentally since our analysis indicates that ZFP143 may target genes strongly associated with genes in these substages. Reinforcing the validity of our approach, our regulatory network also involved MYBL1 (also known as A-MYB). Our results support previous findings that a subset of late pachynema/diplonema-expressed genes, many involved with the spermatogenic and spermiogenic processes, are associated with MYBL1 [60, 61]. MYBL1 regulates transcription of cell cycle-related genes. Germ cells with a homozygous mutation in the *Mybl1* gene exhibit defects in meiotic chromosome synapsis [60]. Moreover, our results suggest that MYBL1 also is in a network associated with the transcription of genes encoding proteins required for piRNA biogenesis, including *Tdrd1*, *Tdrd6* and *Tdrd7*, consistent with previous reports [62]. Going beyond

our core regulatory network, we also identified several other TFs, whose target genes include the Tdrd family and Piwi genes, potentially involved in piRNA processing: ATF3, ELF1, ELK3, ELK4, FLI1, NFYA, NFYB, NRF1, PBX3, RFX2, RFX7, SP1, YY1, ZBTB33, ZFP143 and ZFP42. This analysis not only revealed a core network for transcriptional regulation of meiotic progression (Fig. 5 and Additional file 2: Figures S4 and S5) but also suggested that a significant proportion of the genes expressed in the meiotic transcriptome may be controlled by a concise entourage of transcription factors.

Conclusions

This study has untangled in part the complexity and parallel process of spermatogenesis and meiosis by focusing on associating gene expression with highly specific cytological signatures defining meiotic prophase substages. This unique and powerful approach to deconvolving transcriptomes of complex cell populations is applicable for discovery of transcriptional signals in other such complex cell populations or heterogeneous tissues.

Methods

Experimental design and mice

All C57BL/6J mice used for this study were obtained from The Jackson Laboratory (Bar Harbor, USA). All animal procedures were in accordance with the National Institute of Health guide and U.S. Department of Agriculture standards for animal care and use and were approved by the Animal Care and Use Committee at The Jackson Laboratory (Protocol #05004). Mice were euthanized at 8, 10, 12, 14, 16 and 18 days post partum (dpp) to follow the leading edge of meiotic progression during the first wave of spermatogenesis. For each time point, 5 biological replicates were sampled and germ cells were isolated from the pooled two testes of each mouse. A portion of germ cell sample was used for cytological analysis and the rest of cells were used for RNA-seq analysis. Both cytological and RNA-seq analyses were performed on all 30 samples (Fig. 1a).

Cytological methods

Isolation of mixed testicular germ cells

The procedure was as previously described with some modifications [26]. Briefly, seminiferous tubules were transferred into 20 ml DMEM (Gibco, Life Technologies, Carlsbad, CA, USA) containing 0.5 mg of Liberase TL Research Grade (05401020001, Roche, Basel, Switzerland) and incubated for 20 min at 32 °C. To remove interstitial cells, tubules were washed three times with the same media. In the final wash media, the tubules were pipetted several times to form fragments, which were digested with 0.5 mg of Liberase and 10 μg of DNase in 20 ml DMEM for 13 min at 32 °C in shaking water bath. The isolated cells were further digested by pipetting for 3 min, and germ cells were isolated by filtering through Nitex mesh (53-70 μm pore size). The crude germ cells were washed three times by centrifugation for 7 min at 500 g using 10 ml of the media containing 10 μg of DNase. The cells were resuspended in 1 ml of ice-cold PBS, and cell concentration determined. 1.25 x 10^5 cells (about 10 % of total) were used for cytological scoring, with the remainder used for RNA-seq (see below).

Isolation of enriched populations of adult pachytene germ cells

Each enriched population (4 biological replicates) of pachytene/diplotene spermatocytes was obtained from the testes of six 9-week-old mice by sedimentation at unit gravity [3]. Mixed germ-cell suspensions were prepared as described above, and after the three 0.5 % BSA/KRB washes, cells were separated by cellular sedimentation at unit gravity in a 2–4 % BSA gradient generated over 2.5 h in a STA-PUT apparatus (ProScience Inc., GlassShop, Toronto, ON, Canada). Following the sedimentation process, 10 ml fractions were collected and examined using light microscopy and differential interference contrast Nomarski optics. Cells were identified based on morphological criteria and size [3]. Fractions containing pachytene/diplotene spermatocytes of average purity ~90 % were pooled. For every cell separation, and for each population of cells collected, an aliquot of cells was snap-frozen for subsequent RNA extraction as described below.

Chromatin spread preparation

Germ-cell suspensions prepared as described above were fixed in 2 % PFA containing 0.03 % SDS and mixed with an equal volume of 0.04 % Photo-Flo (Kodak, Rochester, NY, USA). The cell suspension was applied to wells of 12-well Shandon slides, and incubated in a humid chamber for 1 h at RT. After fixation, the cells were briefly air-dried, and subjected to further fixation: 2 % PFA with SDS for 3 min and 2 % PFA without SDS for 3 min. The slides were then washed three times with 0.04 % Photo-Flo. Air-dried slides were stored at -20 °C for further use.

Immunostaining of spread chromatin

Spread chromatin preparations were incubated with 10 % ADB blocking solution (ADB: PBS containing 2 % BSA and 0.05 % Triton-X 100) for 10 min, the same blocking solution with SDS for 10 min, and the blocking solution without SDS. Immunolabeling was performed with rat polyclonal anti-SYCP3 (1:1000 dilution, Handel lab), mouse monoclonal anti-phosphorylated histone H2AX (1:200 dilution; 05-636, Millipore, Billerica, MA, USA), rabbit polyclonal anti-STRA8 (1:1000 dilution; ab49405, Abcam, Cambridge, England) and guinea pig polyclonal

anti-H1t antibodies (1:500 dilution; Handel lab). Subsequently, secondary antibodies conjugated with Alexa 647, 594 or 488 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) were used at 1:500 dilution. Nuclei were stained with DAPI (0.5 μg/mL) for 10 min, and the slides were mounted with glycerol. Images were observed using a Zeiss AxioImager.Z2 epifluorescence microscope equipped with a Zeiss AxioCam MRm CCD camera (Carl Zeiss, Jena, Germany).

RNA methods

Isolation of RNA and sequencing library preparation

Isolated germ-cell samples were resuspended in QIAzol Lysis Reagent according to the manufacturer's instructions, and total RNAs were purified from homogenized cells using Qiagen RNeasy Mini Kit (74104). The quality of the isolated RNA was assessed using an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA) and RNA 6000 Nano LabChip assay (5067-1511, Agilent Technologies).

The mRNA sequencing libraries were prepared using the Illumina TruSeq methodology. mRNAs were purified from total RNA using biotin tagged poly dT oligonucleotides and streptavidin coated magnetic beads. The mRNAs were then fragmented and double stranded cDNA was generated by random priming. The library was then analyzed for quality using an Agilent 2100 Bioanalyzer instrument (Agilent Technologies) and DNA 1000 LabChip assay.

RNA sequencing

Short 100 bp paired-end reads were generated and sequenced using an Illumina® HiSeq (Illumina, San Diego, CA, USA). Sequenced reads were filtered to keep reads for which 70 % of the base pair quality score was > 20, and the 3′ end was trimmed if the base pair quality score was < 20. Two technical replicates for each paired-end were run in different lanes and then merged.

RNA extraction and quantitative real-time quantitative RT-PCR

For real-time RT-PCR, total RNA was isolated from isolated germ cells or enriched germ cells (see above) using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and 1 μg RNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacture's instruction. The real-time RT-PCR was performed by the Applied Biosystems 7500 Real-Time PCR system (Foster City, CA. USA) using the QuntiTect SYBR Green RT-PCR kit (Qiagen). Transcript levels were normalized to the levels of *Actb* by the standard curve method [63], and are presented as the mean normalized expression in 10 μg RNA. Data are represented as mean ± estimated standard deviation. Gene-specific primers are listed in Additional file 1: Table S10.

Computational methods

Alignment and expression

All RNA-seq samples were aligned with Bowtie 1.0.0 [64] and expression levels were estimated by RSEM 1.2.8 [65]. A Bowtie index was prepared for alignment to a combined (mm10) transcriptome of Ensembl Genome Reference Consortium, build 38, release 75 [66], NONCODE v4 lncRNA [25], and piRNA precursor transcripts [17]. The 214 piRNA precursors were obtained from Dr. Christian Roy. Both the NONCODE lncRNA and piRNA precursors were converted to mm10 coordinates using liftOver [67]. For this study, we used log2(TPM + 1) as the expression level, where TPM is transcripts per million, defined by RSEM [65]. A gene was deemed expressed if TPM ≥ 3 for at least one of the 28 samples.

Principal component analysis

To test the concordance of the expression and cytological data, independent Principal Component Analyses (PCA) were performed on each dataset using prcomp(x, scale = TRUE) in the rgl package [68] in R [69]. To produce Fig. 1d, the first and second principal components for both datasets were scaled to have the same range.

Permutation-based Maximum Covariance Analysis (PMCA)

We developed a novel permutation-based maximum covariance analysis (PMCA) that not only overcomes the spurious pattern identification liability of traditional maximum covariance analysis but also does not require any parametric assumptions about the data. Instead, we implemented a permutation procedure that assures the patterns are valid within a given false positive rate (FPR). To begin, let

$$X_{6\times 28}\ \&\ Y_{20368\times 28} \tag{0.1}$$

be the cytological and gene expression data, respectively. Then mean center each row, where $\tilde{X}, \tilde{Y}$ are mean centered (Eq. 1.2.)

$$\tilde{x}_{is} = x_{is} - \frac{1}{28}\sum_{s=1}^{28} x_{is},\ \tilde{y}_{ks} = y_{ks} - \frac{1}{28}\sum_{s=1}^{28} y_{ks} \tag{0.2}$$

$$i = 1, 2, ..., 6,\ s = 1, 2, ..., 28,\ k = 1, 2, ..., 20368$$

We compute the covariance matrix and the SVD of the covariance matrix in Eq. 0.3.

$$C_{6\times 20368} = \frac{1}{28}\tilde{X}\tilde{Y}^T = U\Sigma V^T \tag{0.3}$$

Because we are interested in mapping the genes of Y onto substages of X we consider $P_{x(6\times 28)} = U^T\tilde{X}$, the

principal components of the covariance matrix that correspond to the substages, and calculate the homogeneous and heterogeneous regressions:

$$Z_{x(6\times6)} = XP^T{}_x,\ Z_{y(20368\times6)} = YP_x^T \tag{0.4}$$

To allow for direct comparison, each row of Z_x, Z_y is divided by its respective root mean square, Eq. 0.5.

$$Z_{x(ij)} = z_{x(ij)} \Big/ \sqrt{\sum_{j=1}^{6} z_{x(ij)}^2 / 5} \tag{0.5}$$

$$Z_{y(kj)} = z_{y(kj)} \Big/ \sqrt{\sum_{j=1}^{6} z_{y(kj)}^2 / 5}$$

For each substage cyto-pattern (the row of Z_x corresponding to the substage), we find the genes that have a similar gene expression pattern across the columns of Z_y. Through computation of the SVD, we lose a degree of freedom so the maximum number of patterns is 5; there are 6 substages. For each substage cyto-pattern, we call a gene pattern similar if it is within a certain window of the substage's cyto-pattern.

To determine the optimal window width, we devised a permutation method that iterates through varying window widths and chooses the optimal width based on the estimated false positive rate (FPR). We stipulated that the estimated FPR be less that 0.05 by the third component ($j = 3$). Because the gene lists get finer, and more specific, as we progress through the components, the estimated FPR for cyto-patterns 4 and 5 are less that the specified 0.05. For the substage-specific gene lists described in this paper, the estimated FPR < 0.005. A detailed explanation of the PMCA optimal width selection is provided in Additional file 3.

Bioinformatic analysis

Gene Ontology (GO) analysis

GO enrichment analysis was performed using GOrilla, Gene Ontology enRIchment anaLysis and visuaLizAtion tool [30] with ranked gene lists. Gene lists were ranked for each substage based on a score that measures how closely each gene pattern follows the substage cytopattern. GO terms was established by the GO Consortium [70] and used to group genes according to their biological or molecular functions. A total of 13,363 of the 15,025 genes expressed in our time series have at least one annotation in GO.

Transcription factor analyses

TFs for substage-concordant and negatively concordant genes and the TF target genes were identified using iRegulon, Version 1.3 [35] in Cytoscape, version 3.1.0 [71] with the substage-concordant and negatively concordant gene lists.

Additional files

Additional file 1: This file contains a mini-website of Tables S1-S10. The same mini-website is available at http://carterdev.jax.org/dtx/a2/index.html. Interactive expression plots. **Table S1.** Number and proportion of isolated cells for cytological analysis and RNA-seq. **Table S2.** Gene lists and gene expression values for each of the substages, separated by worksheets. An additional worksheet provides results for X-linked genes. Abbreviations for each substage are as follows: Sp'gonia for spermatogonia, PL for preleptotene, EL for early leptotene, LL + Z for late leptotene and zygotene, LL + Z + EP for those genes found in late leptotene, zygotene, and early pachytene, EP for early pachytene, EP + LP + D for early pachytene, late pachytene, and diplotene, and LP + D for late pachytene and diplotene. Sheet names prefixed with "Anti-"correspond to genes that are negatively concordant with the substage, (e.g., Anti-PL corresponds to genes that are negatively concordant with preleptotene. Genes are ranked by how closely the expression pattern follows the substage pattern (rank = 1 is best). Gene ids and Gene names for piRNA precursors and NONCODE lncRNAs have prefix "pi-" and "NON", respectively. Expression is given in log2(TPM + 1). **Table S3.** GO analysis for substage-negatively concordant genes. Abbreviations for each substage are as follows: Sp'gonia for spermatogonia, EL for early leptotene, LL-Z for late leptotene and zygotene, EP for early pachytene, and LP-D for late pachytene and diplotene. **Table S4.** GO analysis for substage-concordant genes. Abbreviations for each substage are as follows: Sp'gonia for spermatogonia, EL for early leptotene, LL-Z for late leptotene and zygotene, EP for early pachytene, and LP-D for late pachytene and diplotene. **Table S5.** Analysis of genes annotated to meiosis. **Table S6.** TFs for substage-concordant genes. The column names are explained as follows: "NES" is short for Normalized Enrichment Score in iRegulon, "ngenes" is the number of substage-concordant genes associated with the TF, "name" used in the arc-diagram (* indicates a family of genes, e.g., Rfx* for Rfx1, Rfx2, Rfx3, Rfx4). Bolded TFs are highly expressed in the testis and those highlighted in red are associated with male reproduction. **Table S7.** TFs for substage-negatively concordant genes. The column names are explained as follows: "NES" is short for Normalized Enrichment Score in iRegulon, "ngenes" is the number of substage negatively-concordant genes associated with the TF, "name" used in the arc-diagram (* indicates a family of genes, e.g., Rfx* for Rfx1, Rfx2, Rfx3, Rfx4). **Table S8.** Comparison of substage-concordant gene lists with derived clusters in Margolin, et al. [14]. **Table S9.** Comparison of substage-concordant gene lists with sets derived from FACS-sorted cells [5, 6]. **Table S10.** Primers for qRT-PCR. (ZIP 6513 kb)

Additional file 2: This file contains a mini-website of Figures S1-S6 and a link to interactive expression plots. The same mini-website is available at http://carterdev.jax.org/dtx/a1/index.html. **Figure S1.** Expression of substage-concordant genes. (A-H) Heat maps show gene expression of substage-concordant genes at each time point. Gene expression is shown as average log2(TPM + 1) of time point replicates. **Figure S2.** Expression of substage-negatively concordant genes. (A-H) Heat maps show gene expression of substage-negatively concordant genes at each time point. Gene expression is shown as average log2(TPM + 1) of time point replicates. **Figure S3.** Validation of RNA-seq expression pattern by qRT-PCR. Box plots (top) show gene expression detected by RNA-seq at 10 dpp and 16 dpp. Bar graphs (bottom) show gene expression detected by qRT-PCR using isolated germ cells from pooled testes from 3 mice at 10 dpp and 16 dpp. Gene expression for RNA-seq is shown as log2(TPM + 1). Gene expression for qRT-PCR is shown as fold increase relative to *Actb* expression. **Figure S4.** TF arc diagram illustrating the most significant TFs across substages for the concordant gene lists. Node color represents the substage. Node size is related to significance (larger = more significant but all are highly significant with a iRegulon NES > = 4). Width of arc corresponds to how many TFs are shared between nodes (wider = more shared TFs). A node is connected to another node if a node's TFs are a subset of the other node's TFs. Color of the arc (degree) is related to how many connections the node has. Degree equals the number of connections. The name of each node is an abbreviation, where an asterisk indicates multiple members of the TF family are included in the node (e.g., Rfx* for

Rfx1, Rfx2, Rfx3, Rfx4) and when a node name is followed by + integer, as in "Mef2* + 1", it indicates that there are TFs in the Mef2 family plus one other TF. All TFs associated with each node are listed in Additional file 1: Table S6. The number in parenthesis next to the node name indicates the number of substage-concordant genes associated with the TF cluster. **Figure S5.** TF arc diagram illustrating the most significant TFs across substages for the negatively concordant gene lists. Node color represents the substage. Node size is related to significance (larger = more significant but all are highly significant with a iRegulon NES > = 4). Width of arc corresponds to how many TFs are shared between nodes (wider = more shared TFs). A node is connected to another node if a node's TFs are a subset of the other node's TFs. Color of the arc (degree) is related to how many connections the node has. Degree equals the number of connections. The name of each node is an abbreviation, where an asterisk indicates multiple members of the TF family are included in the node (e.g., Rfx* for Rfx1, Rfx2, Rfx3, Rfx4) and when a node name is followed by + integer, as in "Mef2* + 1", it indicates that there are TFs in the Mef2 family plus one other TF. All TFs associated with each node are listed in Additional file 1: Table S7. The number in parenthesis next to the node name indicatea the number of substage-negatively-concordant genes associated with the TF cluster. **Figure S6.** Expression of TF and its target genes for each substage. The difference of mean gene expression at 8 dpp of the TF and its target genes at each time point. To illustrate the overall pattern, a smoothed line was fit to the substage-specific gene expression. Color of lines represents the substage of target genes. Gene expression is shown as average log2(TPM + 1) across time point replicates. (ZIP 13261 kb)

Additional file 3: Details of permutation-based maximum covariance analysis (PMCA). (PDF 150 kb)

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Availability of data and materials
Transcriptome data are available in Gene Expression Omnibus under accession number GSE72833. All other data are available as Additional Files as detailed below.

Authors' contributions
The project was designed by GWC, MAH and MH with YF and RLB; the research was conducted by YF and RLB with contributions from FS, JH and MH. YF, RLB, GWC and MAH analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]The Jackson Laboratory, Bar Harbor, ME, USA. [2]Department of Computer Science, Trinity University, San Antonio, TX, USA.

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Germline stem cells: toward the regeneration of spermatogenesis

Hanna Valli, B.S.,[a,b] Bart T. Phillips, Ph.D.,[a,b] Gunapala Shetty, Ph.D.,[c] James A. Byrne, Ph.D.,[d,e] Amander T. Clark, Ph.D.,[e] Marvin L. Meistrich, Ph.D.,[c] and Kyle E. Orwig, Ph.D.[a,b]

[a] Department of Obstetrics, Gynecology and Reproductive Sciences, Molecular Genetics and Developmental Biology Graduate Program, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; [b] Magee-Womens Research Institute, Pittsburgh, Pennsylvania; [c] Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas; [d] Department of Molecular and Medical Pharmacology, Center for Health Sciences, Los Angeles, California; and [e] Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, California

Improved therapies for cancer and other conditions have resulted in a growing population of long-term survivors. Infertility is an unfortunate side effect of some cancer therapies that impacts the quality of life of survivors who are in their reproductive or prereproductive years. Some of these patients have the opportunity to preserve their fertility using standard technologies that include sperm, egg, or embryo banking, followed by IVF and/or ET. However, these options are not available to all patients, especially the prepubertal patients who are not yet producing mature gametes. For these patients, there are several stem cell technologies in the research pipeline that may give rise to new fertility options and allow infertile patients to have their own biological children. We will review the role of stem cells in normal spermatogenesis as well as experimental stem cell–based techniques that may have potential to generate or regenerate spermatogenesis and sperm. We will present these technologies in the context of the fertility preservation paradigm, but we anticipate that they will have broad implications for the assisted reproduction field. (Fertil Steril® 2014;101:3–13. ©2014 by American Society for Reproductive Medicine.)

Key Words: Male fertility, male infertility, regenerative medicine, spermatogonial stem cells, stem cells

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High-dose chemotherapy, whole-body radiation, or radiation to the gonads can cause permanent infertility (1). This is a significant human health concern because over 75,000 people under the age of 40 in the United States are diagnosed with cancer each year and most are cured (2). Thus, cancer patients can look beyond their diagnosis and treatment to quality of life after cancer. Parenthood is important to cancer survivors, and distress over infertility can have long-term psychological and relationship implications (3). Therefore, the American Society for Clinical Oncology (ASCO) (4) and the American Society for Reproductive Medicine Ethics Committee (5) recommend that the reproductive risks of gonadotoxic therapies and options for preserving fertility be discussed with patients before initiating treatment. While adoption and third-party reproduction provide alternative family-building options, the available data indicate that most cancer survivors prefer to have their own biological children (4).

Postpubertal adolescent and adult males have the option to cryopreserve sperm before oncological treatment (Fig. 1, *top*). This is a simple and established method for preserving fertility potential and allows men to father their own genetic children. Nearly 17,000 men between the ages of 15 and 44 are diagnosed with cancer each year in the United States, and nearly 2,385 survivors will receive a treatment that puts them at high risk of azoospermia (2, 6). Unfortunately, only about 24% of men in this age range cryopreserve semen before their oncological treatment (7). Therefore, we calculate that each year in the United States, over 1,800 adult cancer survivors will be infertile with azoospermia and have limited options to have their

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Current address (B.T.P.): Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC 27709.
Reprint requests: Kyle E. Orwig, Ph.D., Magee-Womens Research Institute, 204 Craft Avenue, Pittsburgh, PA 15213 (E-mail: orwigke@upmc.edu).

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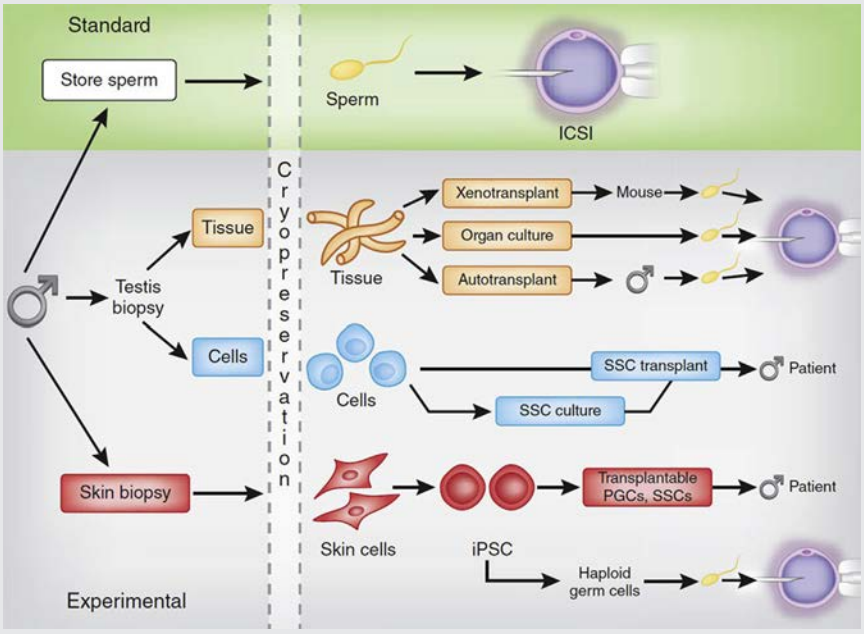
FIGURE 1



Standard and experimental options for preserving male fertility. *Top*, sperm obtained by ejaculation or surgical retrieval from the testes or epididymides are competent to fertilize oocytes using assisted reproductive techniques including IUI, IVF, or IVF ICSI that are standard in most fertility clinics. These options are not available to prepubertal boys who are not producing sperm or to adult azoospermic men. *Bottom*, testis tissue obtained via biopsy from prepubertal boys contains spermatogonial stem cells (SSCs) that can produce sperm in the context of the intact tissue by xenotransplant, organ culture, or autologous transplantation back into the individual (*orange boxes*). Sperm retrieved from cultured or transplanted tissue can be used for ICSI. Cells in suspension obtained from biopsied testicular tissue can be transplanted back into the endogenous seminiferous tubules of the patient (*blue boxes*). SSCs in the suspension can regenerate spermatogenesis and, in some cases, fertility. For infertile individuals who did not preserve germ cells before gonadotoxic therapy, iPSCs may be produced from their somatic cells (e.g., skin or blood) to differentiate into transplantable germ cells (primordial germ cells [PGCs] or SSCs) or haploid germ cells that can be used for ICSI (*red boxes*). Excerpted with permission from Clark AT, Phillips BT, Orwig KE, Nat Med 2000;1564–5.

Valli. Stem cell therapies for male factor infertility. Fertil Steril 2014.

own biological children because they did not save a semen sample. In some cases, sperm can be recovered surgically from small focal areas of spermatogenesis in the testes using the testicular sperm extraction method and can be used to fertilize oocytes by intracytoplasmic sperm injection (ICSI) (8).

There are no options to preserve the fertility of prepubertal boys, who are not yet making sperm. This is a significant problem because about 5,131 boys under the age of 15 in the United States are expected to develop cancer each year and 83% are expected to survive (2). A report from the Childhood Cancer Survivor Study indicates that the cytotoxic therapies for cancer reduce the number of young men subsequently able to have children by 44% (6, 9). Based on these statistics, we calculate that each year in the United States, 1,874 young male cancer patients will become sterile owing to their treatment. In addition to cancer survivors, over 500 patients under the age of 20 receive hematopoietic stem cell transplants each year in the United States for nonmalignant conditions (e.g., bone marrow failure, blood and immune deficiencies, autoimmune disorders) (10). Myeloablative conditioning therapy before bone marrow transplantation is associated with a high risk of infertility (4, 9, 11, 12). The ASCO report notes that "Impaired future fertility is difficult for children to understand, but potentially traumatic to them as adults" (4). The available data indicate that greater than 80% of parents consented to fertility preservation procedures on behalf of their children before initiation of gonadotoxic therapies (13, 14).

The summed incidence of chemotherapy or radiation-induced male factor infertility that cannot be treated with existing reproductive therapies is approximately 4,000 individuals each year in the United States. Therefore, responsible development of novel therapies to help these patients have biological children has a significant potential impact.

Promising results in animal models and human cell lines (Fig. 1, *bottom*) have generated enthusiasm that stem cells might be used or manipulated to preserve and/or restore the fertility of patients who are not producing sperm and have no other options to protect their future fertility before receiving gonadotoxic chemotherapy or radiation treatments (14–34). We will review the methods of spermatogonial stem cell (SSC) transplantation (Fig. 1, *blue boxes*), testicular tissue grafting, testicular tissue organ culture (Fig. 1, *orange boxes*), and induced pluripotent stem cell differentiation into gametes or transplantable male germ line stem cells (Fig. 1, *red boxes*). Table 1 summarizes published reports detailing the progress of each method. Enthusiasm for these experimental stem cell technologies is tempered by concerns about feasibility and safety, particularly for the vulnerable prepubertal

TABLE 1

Literature reporting progress in stem cell technology development.

Stem cell technologies	(q)Real time polymerase chain reaction	Histology	Immunocytochemistry	Immunohistochemistry	Flow/flow cytometry/ magnetic cell sorting	Xenotransplantation	Homologous transplantation	Autologous transplantation	Sperm[a]	Fertilization	Progeny
SSC transplant											
Rodents		(30, 31, 48–51, 57, 58, 118, 119)		(71, 120)		(71, 118–120)	(30, 31, 48–51, 57, 58, 70, 121)		(30, 31, 48, 50, 51, 57, 58, 71, 118–121)	(31, 48, 50, 51, 57, 58, 71, 120, 121)	(31, 48, 50, 51, 57, 58, 71, 120, 121)
Large animal[b]		(54–56)		(56, 64, 72)		(64, 72)	(52–56)	(56)	(52–55)	(52, 55)	(52, 55)
Nonhuman primate		(59, 60)		(122–125)		(122–125)	(29)	(29, 59–61)	(29, 59)	(29)	
Human				(21, 38, 78, 89, 109, 115, 117)		(21, 38, 78, 89, 109, 115, 117)		(67)			
SSC culture											
Rodents		(57, 58)	(70, 83)	(71)	(58, 70, 71, 121)	(71)	(57, 58, 70, 71, 121)		(57, 58, 71, 83, 121)	(57, 58, 71, 121)	(57, 58, 71, 121)
Large animal	(73)		(72–75)	(72)		(72)					
Nonhuman primate	(76)		(76)								
Human	(21, 38, 79, 81)		(77, 79, 81, 82, 126)	(21, 38, 78, 80)	(77, 80–82, 126)	(21, 38, 79)					
Testicular grafting											
Rodents		(92, 93, 95)				(95)	(92, 93, 95)		(92, 93)	(92, 93)	(93)
Large animal		(92)				(92)			(92)	(92)	
Nonhuman primate		(94–98)		(97, 98)		(94–96)		(97, 98)	(94, 98)	(94)	
Human		(99, 101–104)		(100, 101, 103, 104)		(99–104)					
Testicular organ culture											
Rodents		(27)					(27)		(27)	(27)	(27)
Large animal											
Nonhuman primate											
Human											
Pluripotent-derived male germ cells											
Rodents			(28)		(28)		(28)		(28)	(28)	(28)
Large animal											
Nonhuman primate	(32, 33)		(32, 33)								
Human	(22–24, 26)		(22–26)		(22, 23, 25, 26)						

[a] Sperm and spermatogenesis.
[b] Farm animals and companion animals.

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patient population (14, 35–37). Questions about feasibility stem from the early stage of technology development, uncertainty about optimal freezing conditions, concerns that a small testicular biopsy will contain few stem cells, and the lack of culture conditions to expand human germ line stem cells. Questions about safety are associated with the risks of surgery to obtain testicular tissue and the potential for malignant contamination in transplanted cells or tissue. Nonetheless, academic centers around the world are already cryopreserving testicular tissues for prepubertal boys and men in anticipation that stem cells in that tissue can be used safely and effectively to restore future fertility (13, 16, 21, 38–41).

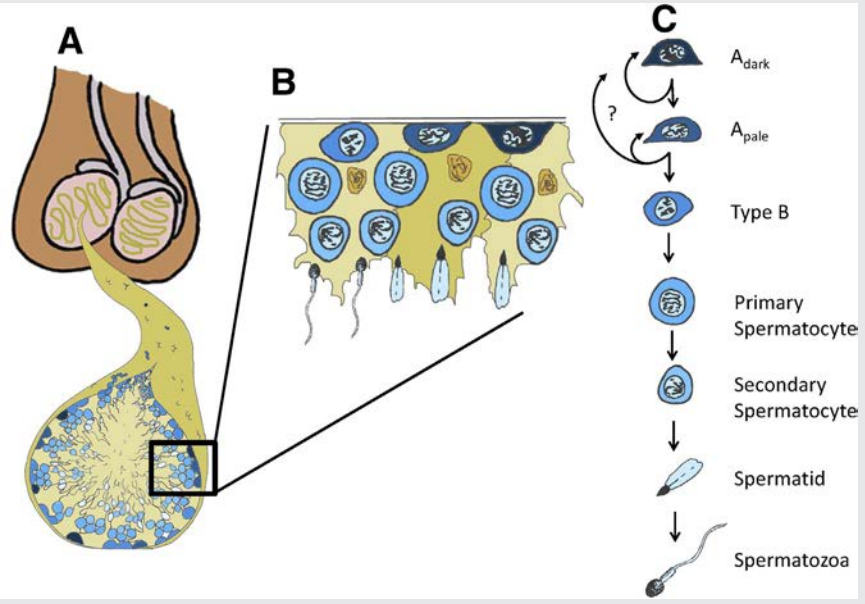
SSCs AND SPERMATOGENESIS

SSCs are the adult tissue stem cells in the testes that balance self-renewing and differentiating divisions to maintain continuous sperm production throughout the postpubertal life of men. SSCs are rare cells located on the basement membrane of seminiferous tubules (Fig. 2A and B). These stem cells give rise to undifferentiated progenitor spermatogonia that undergo several transit-amplifying mitotic divisions, followed by two meiotic divisions and morphological differentiation to produce sperm. Spermatogenesis is an extraordinarily productive system that produces approximately 40 million sperm per gram of tissue per day in mice (42) and 5.5 million sperm per gram of tissue per day in men (43). The difference in sperm production in mice and men can be explained in part by differences in the number of transit-amplifying mitotic divisions that precede meiosis (44). In humans, the SSC pool is composed of A_{dark} and A_{pale} spermatogonia. A_{dark} are relatively quiescent, while A_{pale} are mitotically active and undergo one to two transit-amplifying divisions before giving rise to differentiating type B spermatogonia and then primary spermatocytes, which enter meiosis and migrate off the basement membrane of the seminiferous tubule (Fig. 2B and C). Owing to its highly proliferative nature, the spermatogenic system is an unintended target of chemo- and radiotherapies. The relative effects of various chemo- and radiotherapy regimens on male fertility are detailed in several previous reports (9, 11, 19, 45, 46). The extent that the fertility of a patient is affected depends on the dose, type, and frequency of treatment (1). There has been significant progress in minimizing the unwanted side effects of the cancer treatment by adjusting and modifying the therapeutic regimens without compromising the efficiency of oncological treatments (47). Nonetheless, some cancer treatments, like whole-body radiation, radiation to the gonads, and alkylating chemotherapy, can be particularly gonadotoxic and result in prolonged or permanent azoospermia. In the following sections, we will review experimental stem cell–based approaches that may have application for preserving and/or restoring male fertility.

SSC TRANSPLANTATION

Brinster and colleagues pioneered the technique for SSC transplantation in mice in 1994, demonstrating that donor SSCs could engraft the seminiferous tubules of

FIGURE 2



Human SSCs and spermatogenesis. (A) Testes are composed of seminiferous tubules that start and end at the rete testis. (B) Cut-out of the basement membrane of the seminiferous epithelium. (B and C) The basement membrane of the seminiferous epithelium contains undifferentiated (A_{dark} and A_{pale}) spermatogonia and differentiating type B spermatogonia. Type B spermatogonia give rise to primary spermatocytes that enter meiosis and migrate off the basement membrane. Subsequent meiotic divisions and morphogenesis give rise to secondary spermatocytes, spermatids, and the terminally differentiated spermatozoa that are released into the lumen of the seminiferous tubules.

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chemotherapy-treated recipient mice and regenerate spermatogenesis and fertility leading to the production of viable progeny through normal breeding (31, 31). The SSC transplantation technique has become the experimental gold standard for quantifying stem cell activity and may have application for treating male factor infertility. Homologous species SSC transplantation has now been reported in mice, rats, pigs, goats, bulls, sheep, dogs, and monkeys, including the production of donor-derived progeny in mice, rats, goats, and sheep (29, 48–61). SSCs from donors of all ages, newborn to adult, can regenerate spermatogenesis (49, 62), and SSCs can be cryopreserved and retain spermatogenic function upon thawing and transplantation (29, 63, 64). We recently demonstrated that SSCs from the testes of prepubertal and adult rhesus macaques could be frozen, thawed, and transplanted to regenerate spermatogenesis and produce fertilization-competent sperm (29, 59). Progress in these previous studies suggests that it should be feasible for prepubertal boys or adult men to cryopreserve testicular tissue containing SSCs before treatment and have these cells reintroduced into their testes at a later date to regenerate spermatogenesis.

Radford and colleagues initially introduced the autologous SSC transplantation technique to the human clinic in 1999 (65). In Manchester, United Kingdom, testicular tissue from 12 male non-Hodgkin's lymphoma patients was cryopreserved as a cell suspension before the initiation of chemotherapy. At later dates, seven of the patients had the cells injected back into their testes (66, 67). To our knowledge, there have been no follow-up reports on the fertility status of those patients so the outcome of the experiment is unknown. Even if the men in that study fathered children, it would be difficult to demonstrate unequivocally (in the absence of a unique genetic marker) that those offspring resulted from sperm produced by transplanted stem cells rather than from surviving endogenous stem cells. There have been no subsequent reports of SSC transplantation in humans since the 1999 study. Nonetheless, this bold, pioneering study demonstrated that patients are willing to pursue experimental stem cell approaches to achieve fertility (65, 67). To date, published reports indicate that testicular tissue or cells have been cryopreserved for more than 150 prepubertal and adult male patients worldwide, and this is likely an underestimate of actual cases (13, 14, 21, 38–41, 65, 67).

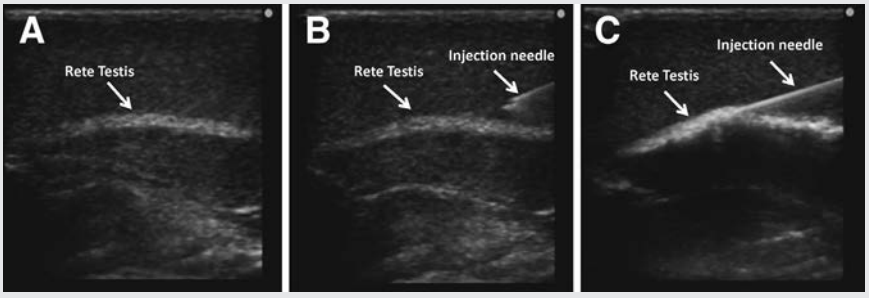
For SSC transplantation in rodents, the testes are typically accessed via a midventral abdominal incision. Testicular cells (including SSCs) are injected using a pulled glass capillary pipette inserted via the efferent ducts into the rete testis space, which can be visualized on the surface of the testis and is contiguous with all seminiferous tubules (68). Testis anatomy in larger animals, including nonhuman primates and humans, is different than that in rodents, with the rete testis being centrally located in the testes. Schlatt and colleagues (69) demonstrated that ultrasound can be used to visualize the rete testis and guide an injection needle into the rete testis space. Ultrasound-guided rete testis injection has now been used for SSC transplantation in several large animals species, including nonhuman primates (29, 52–56, 59–61). For this approach, an injection needle is simply inserted under ultrasound guidance through the scrotal skin and testicular parenchyma into the rete testis space (Fig. 3 depicts an example from nonhuman primates) (29).

Clinical translation of the SSC transplantation technique appears imminent considering successes in several large animal models (Table 1) and that many patients have already cryopreserved testicular tissue or cells. However, several questions and/or challenges remain. First, the small amount of tissue that can be obtained from testicular biopsies (especially from prepubertal boys) may contain a relatively small number of stem cells. Second, in the cancer survivor paradigm, it is essential to eliminate the risk of reintroducing malignant cells. The sections below describe approaches that may circumvent these two challenges. A third challenge for translating new stem cell technologies to the clinic is the limited availability of reagents and experimental tools for studying humans SSCs.

SSC CULTURE

In rodents, SSCs can be greatly expanded in culture and maintain competence to generate spermatogenesis and restore fertility upon transplantation (57, 58, 70, 71). Several groups

FIGURE 3



Ultrasound-guided rete testis injections. For SSC transplantation into larger animals, including nonhuman primates, ultrasound is used to visualize the rete testis (echo-dense structure) (A). (B and C) The injection needle is inserted under ultrasound guidance through the scrotal skin into the rete testis space, which is continuous with the seminiferous tubules. (C) Positive pressure is applied so the cells are slowly injected into the rete testis space and seminiferous tubules.

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have reported extending SSC culture to large animal species (72–76). This approach could theoretically be employed to amplify the small number of stem cells that can be obtained from a biopsy and also to demonstrate the absence of malignant contamination. A number of laboratories have reported culturing human SSCs (21, 38, 77–82), including three from the testes of prepubertal patients (38, 78, 80). These human tissue studies are promising but challenged by the inability to evaluate the full spermatogenic potential of cultured cells by homologous species transplantation into human testes. To date, each laboratory has used a different approach to culture human SSCs and different methods to assess outcomes. These published human SSC culture results need to be replicated in other laboratories and evaluated using robust markers of human spermatogonia and by xenotransplantation to nude mice (see section below on experimental methods to track human germ line stem cells).

In a recent study, Elhija and colleagues reported that testicular cells from 7-day-old immature mice could be expanded in a three-dimensional soft agar culture system and differentiated to produce postmeiotic germ cells, including morphologically normal sperm (83). Additional studies are needed to confirm that the resulting sperm are functionally competent to fertilize mouse oocytes. If a similar approach is successful in humans, this would eliminate the need to put cells back into the patient and thus the risk of reintroducing malignant cells into a cancer survivor.

CELL-SORTING STRATEGIES TO ISOLATE SSCS AND REMOVE MALIGNANT CONTAMINATION

There are legitimate concerns about malignant contamination of preserved testicular tissue, especially from leukemia patients (84, 85). Several groups have demonstrated that it is feasible to isolate germ cells and remove malignant contamination from heterogeneous testicular cell suspensions using a fluorescence-activated cell sorter (FACS) and combinations of germ cell and/or cancer cell markers (86–90). Despite these encouraging results, caution is warranted because even low levels of contamination can lead to cancer and current assays may lack the sensitivity to detect very rare malignant cells (85, 91). Therefore, alternative methods for decontamination and screening as well as methods that do not require transplantation should be considered in some cases.

TESTICULAR TISSUE GRAFTING

In contrast to SSC transplantation, which involves disaggregation of SSCs from their cognate niches, testicular tissue grafting and testicular tissue organ culture (next section) maintain the integrity of the stem cell/niche unit. Honaramooz and coworkers demonstrated that testicular tissues obtained from newborn mice, pigs, and goats could produce complete spermatogenesis when grafted under the skin of nude mice (92) and later reported the production of offspring from sperm obtained after ectopic (under the skin) grafting of mouse testicular tissue (93). Testis tissue from prepubertal rhesus macaques also produced complete spermatogenesis with fertilization-competent sperm after ectopic xenografting into nude mice (94). Survival and spermatogenesis from adult testicular tissue grafts have been less successful than immature grafts (95).

Cryopreservation is an essential component of the fertility preservation paradigm. Jahnukainen and coworkers evaluated several cryopreservation strategies with immature monkey tissues and found that freezing in 1.4 M DMSO provided good graft survival and spermatogenic induction up to the spermatocyte stage (96). However, haploid germ cells were not produced in any grafts from that study, fresh or frozen. Wistuba and colleagues performed autologous testicular grafting in two studies in marmoset monkeys (97, 98) and reported that complete spermatogenesis was obtained in orthotopic (in the scrotum) but not ectopic grafts. Frozen and thawed tissues were also grafted in that study, but these were only transplanted ectopically and did not produce spermatogenesis (98). Therefore, the question of whether frozen and thawed grafts can produce fertilization-competent haploid germ cells still needs to be addressed.

Several groups have reported xenografting of human testicular tissue to nude mice, and so far none have observed complete spermatogenesis or haploid gametes (99–104). In general, testicular tissues xenografted from prepubertal or adolescent boys survived long term (4–12 months) (100, 101, 103, 104), with a calculated 3.7% spermatogonial recovery rate after 6 months in one study (104) and three studies reporting spermatocytes as the most advanced stage of germ cell development between 6 and 12 months after transplantation (101, 103, 104). Some of those tissues were already postpubertal and contained meiotic or postmeiotic cells at the time of transplant, so it is difficult to exclude the possibility that the spermatocytes or occasional spermatids that were observed had persisted from the time of transplant (101, 104). Importantly, the study by Sato and colleagues (103) observed primary spermatocytes 1 year after xenografting of testicular tissue from a 3-month-old boy, which clearly did not contain spermatocytes before grafting. In contrast to prepubertal/adolescent tissues, xenografted adult testicular tissues regressed over time (99, 102). Although the human-to-mouse xenografting results are somewhat discouraging, the results of the monkey studies cited above suggest that autologous transplantation may be an option if suitable cryopreservation conditions are developed. Similar to SSC transplantation, autologous grafting will be problematic in cases where malignant contamination of the testicular tissue is suspected. Xenografting of human testicular tissue into animals could circumvent this problem but is associated with additional concerns about xenobiotics and has been unsuccessful to date.

TESTICULAR TISSUE ORGAN CULTURE

Sato and colleagues (27, 105) demonstrated that haploid male germ cells from mice could be generated in testicular tissue organ culture, using a gas-liquid interface method that was originally devised to keep differentiated organs alive in vitro (106). Testis tissue was obtained from 2.5- to 3.5-day-old mice that contained only undifferentiated germ cells,

similar to prepubertal patients. Testicular tissue was minced into fragments (1–3 mm diameter) that were placed on an agarose gel that was half soaked in medium, such that the tissue was exposed to air and absorbed nutrients through the agarose. Haploid round spermatids or sperm were retrieved after 23 or 42 days in culture, respectively, and used to successfully fertilize mouse oocytes in vitro by round spermatid injection and ICSI. The resulting embryos were transferred to recipient females and generated live offspring with normal development to adulthood and normal fertility (27). The investigators also maintained frozen and thawed testicular tissue pieces in organ culture and were able to generate sperm, but the fertilization potential of that sperm was not tested. If these results in mice can be translated to humans, testicular organ culture would circumvent the need to put tissues or cells back into the patient and may be a safe option for patients with malignancies that contaminate the testes.

GERM CELLS DERIVED FROM PLURIPOTENT STEM CELLS

For males who did not preserve sperm or SSCs before gonadotoxic treatment, generation of transplantable germ cells (primordial germ cells or SSCs) or haploid gametes from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) has been explored. The iPSC technology is especially exciting because it would theoretically allow patients with no germ cells in their testes to father genetically related children. The derivation of germ cells from pluripotent cells will be covered in more detail in other articles in this issue. Here we will state briefly that several groups have reported the generation of germ cells from nonhuman primate ESCs (32, 33) and human ESCs and/or iPSCs (22–26), including evidence of rare haploid cells in some cases. These results are exciting and revolutionary but are challenged by the inability to test the function of the resulting germ cells in the human system. Therefore, safety and feasibility will need to be established in animal models. In 2011, Hayashi et al. showed that mouse ESCs and iPSCs can be induced to an epiblast-like cell that then gives rise to primordial germ cells when BMP4 was added to a culture medium (28). The resulting germ cells were transplanted into the testes of infertile mice and generated spermatogenesis with sperm that were used to fertilize oocytes using ICSI. The resulting embryos were transferred to recipient females and gave rise to viable offspring. These promising results need to be replicated in other laboratories, and more research is needed before this technique can be translated to the clinic, as some of the offspring developed malignant tumors around their neck area (28). For iPSC-based technologies, it will be important to understand the effects of reprogramming, culture, and differentiation on the genome and epigenome of in vitro–derived germ cells compared with endogenous germ cells (107).

EXPERIMENTAL METHODS TO TRACK AND QUANTIFY HUMAN GERM LINE STEM CELLS

Studies on human cells and/or tissues are a valuable stepping stone toward clinical translation. However, these studies are challenged by the limited experimental tools for quantifying human spermatogonia and testing their function. In animal studies, the most compelling experiments are those that demonstrate the ability of a test cell population to regenerate spermatogenesis and produce fertilization-competent sperm, embryos, and offspring. This is an unrealistic expectation for human studies because fertilization of human eggs and production of human embryos are not universally legal or fundable. Based on progress in animal models, human-to-human SSC transplantation is likely to be feasible in the clinical setting, but this is not an option for the routine testing of spermatogenic potential in the laboratory setting. Therefore, experimental assessment of human germ line stem cell potential must rely on descriptive endpoints and xenotransplantation for functional testing.

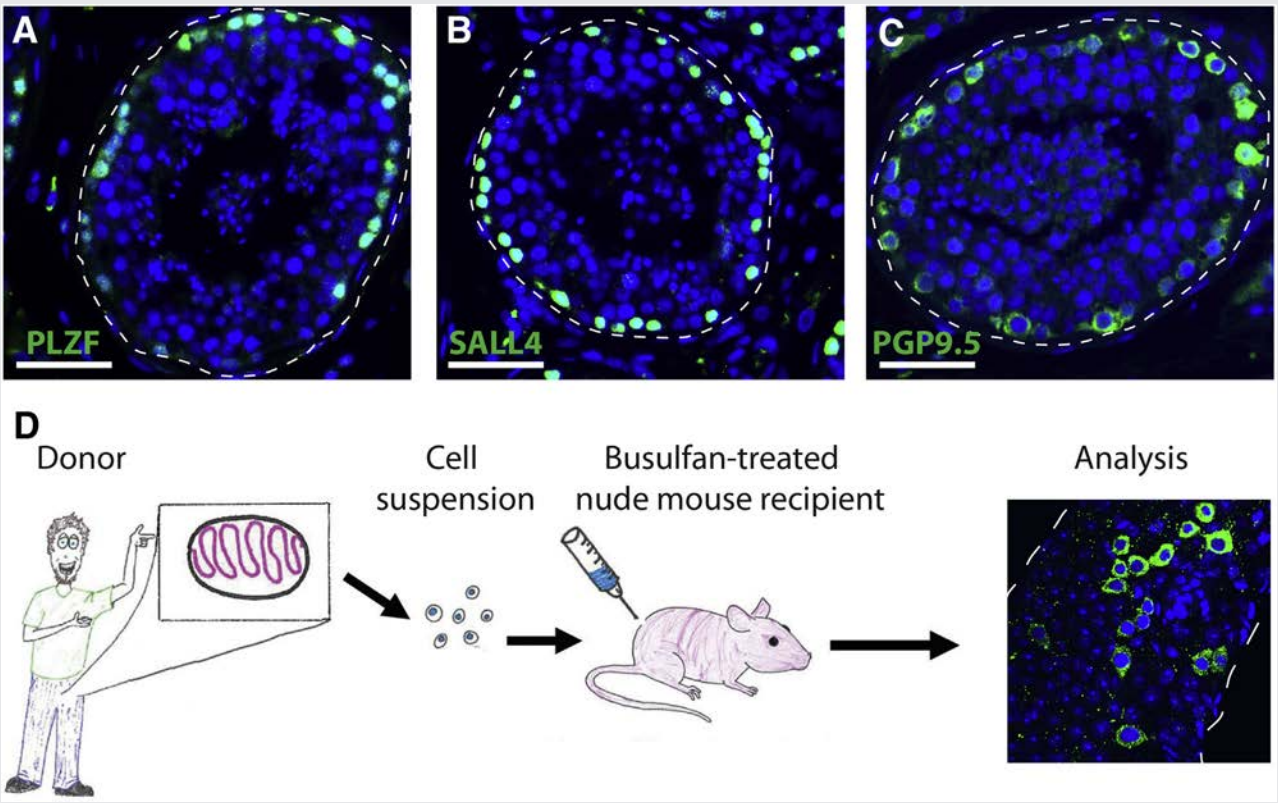
Here we propose that reliable markers of human undifferentiated spermatogonia are those with expression limited to germ cells located on the basement membrane of human seminiferous tubules and that meet one or more of the following criteria: 1) confirmed expression in Adark and/or Apale spermatogonia; 2) co-expression with other established markers of human undifferentiated spermatogonia (e.g., PLZF) or 3) that exhibit colonizing activity in the human to nude mouse xenotransplantation assay (see next paragraph and Figure 4D). Proteins that meet these criteria, based on personal experience and review of the literature, include PLZF, GFRα1, GPR125, SALL4, LIN28, PGP9.5, UTF1, FGFR3, EXOSC10, DSG2, CBL, SSEA4, CD9, OCT2, and SSX (77, 78, 108–116). Examples of PLZF, SALL4, and PGP9.5 expression in the seminiferous tubules of adult human testes are shown in Figure 4A–4C (Valli and Orwig, unpublished data).

In rodents, SSC transplantation is the gold standard that allows investigators to quantify germ-line stem cells by observing their biological potential to produce and maintain spermatogenesis in infertile recipient animals. At present, human–to–nude mouse xenotransplantation is the best functional assay to test the SSC-like potential of a test cell population (21, 38, 78, 89, 109, 115, 117). This method does not recapitulate complete spermatogenesis from transplanted cells like mouse–to–mouse SSC transplantation, probably owing to the evolutionary distance between humans and mice. However, human–to–nude mouse xenotransplantation does assay the ability of transplanted cells to migrate to the basement membrane of seminiferous tubules, proliferate to produce characteristic colonies of spermatogonia, and persist long term as shown in Figure 4D (78, 89, 109, 115, 117).

CONCLUSION

The assisted reproduction field is on the verge of a renaissance, which is fueled in part by exciting developments that merge new stem cell technologies with fertile outcomes. Thus, it is reasonable to expect in the next decade that the options to preserve and restore male fertility will expand from sperm freezing followed by IVF/ICSI to also include stem cell transplantation, tissue grafting, and/or culture to produce fertilization-competent gametes (Fig. 1 and Table 1). As with any rapidly developing field that has the potential to impact the clinic, it is essential to establish strict criteria to monitor progress and avoid sensationalism. Responsible technology

FIGURE 4



Experimental techniques to assay human spermatogonia. (A, B, and C) Expression of spermatogonia markers PLZF (A), SALL4 (B), and PGP9.5 (C) is limited to germ cells located on the basement membrane of human seminiferous tubules. Thus, they are reliable markers to screen test cell populations for human spermatogonia. DAPI (*blue*) stains all cell nuclei. Scale bar = 50 μm. (D) Human–to–nude mouse xenotransplantation assay. Human testicular tissue is made into a cell suspension and then transplanted into the testis of busulfan-treated infertile nude mice. Two months after the transplantation, the testes are recovered, the tunica is removed, and the seminiferous tubules are gently dispersed to make a whole mount. The tubules are then stained with antiprimate antibody (122) to recognize the colonies of human spermatogonia (*green*).

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development should ideally include [1] studies in lower mammals (e.g., rodents and other domestic species) where it is possible to examine functional readouts such as regeneration of spermatogenesis, fertilization, embryo development, and generation of healthy offspring. [2] As technologies mature, preclinical studies in nonhuman primates that have anatomy and reproductive physiology similar to humans will demonstrate conservation of biological concepts and facilitate optimization of technical/surgical approaches (e.g., biopsy, cell processing, freezing, and transplantation). Nonhuman primate studies are also amenable to functional assessments, although it is recognized that such studies will be limited to institutions with space, knowledge, and technical expertise for research in primates and specifically primate assisted reproductive technology. [3] Direct investigations of human cells or tissues are particularly valuable on the road to clinical translation but are challenged by the limited availability of human samples, study-to-study variation in sample quantity and quality, and the limited functional assays to assess experimental outcomes. Despite these challenges, human tissue studies to optimize tissue and cell-processing procedures, cryopreservation methods, and cell/tissue culture conditions will be the most relevant to the clinics that are already preserving testicular tissue for patients. Although it is not popular in the current era, descriptive studies of human germ lineage development in situ are essential to guide experimental design and interpretation of results of human stem cell studies.

Clinics worldwide are preserving testicular tissue for patients who do not have sperm in anticipation that new male fertility technologies will be available in the future. Each technology described in this review has merits and limitations that are detailed in the sections above. Owing to uncertainty about which methods will ultimately be translated to the clinic, it seems reasonable to preserve intact testicular tissue fragments in a way that maximizes viability after freezing and thawing (an active focus of research in many laboratories) and will be amenable to tissue- or cell-based approaches in the future. Variability in patient circumstances and reproductive goals dictate the parallel development of multiple technologies for preserving and restoring male fertility.

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Minireview

Emerging Methods to Generate Artificial Germ Cells from Stem Cells[1]

Fanhui Zeng,[4,6] Fajun Huang,[7] Jingjing Guo,[8] Xingchang Hu,[8] Changbai Liu,[3,9] and Hu Wang[2,5]

[5]*Medical School, China Three Gorges University, Yichang, China*
[6]*The Central Hospital of Enshi Tujia and Miao Autonomous Prefecture, Enshi, China*
[7]*School of Medical Science, Hubei University for Nationalities, Enshi, China*
[8]*College of Life and Environmental Sciences, Shanghai Normal University, Shanghai, China*
[9]*The Institute of Molecular Biology, China Three Gorges University, Yichang, China*

ABSTRACT

Germ cells are responsible for the transmission of genetic and epigenetic information across generations. At present, the number of infertile couples is increasing worldwide; these infertility problems can be traced to environmental pollutions, infectious diseases, cancer, psychological or work-related stress, and other factors, such as lifestyle and genetics. Notably, lack of germ cells and germ cell loss present real obstacles in infertility treatment. Recent research aimed at producing gametes through artificial germ cell generation from stem cells may offer great hope for affected couples to treat infertility in the future. Therefore, this rapidly emerging area of artificial germ cell generation from nongermline cells has gained considerable attention from basic and clinical research in the fields of stem cell biology, developmental biology, and reproductive biology. Here, we review the state of the art in artificial germ cell generation.

differentiation, gamete biology, gametogenesis, germ cells, germline, infertility, stem cells

INTRODUCTION

Successful sexual reproduction will be essential for the continuity of the human race. However, infertility affects 15%–25% of couples of reproductive age [1, 2]. Evidence is increasing that the quality and quantity of germ cells produced by both men and women have declined over the past two decades due to myriad genetic [3, 4] and environmental causes [5, 6]. The advent of in vitro fertilization and other assisted reproductive techniques has helped infertile couples to parent their own children [7]; however, nearly 10% of couples are still unable to have children due to a complete lack of germ cells [8]. Although infertility by itself may not threaten affected couples' physical health, it may lead to serious declines in social well-being [9].

Stem cells are a class of undifferentiated cells that have the unique ability to self-renew and differentiate into specialized cell types. The potential for using stem cells to produce new tissues or repair damaged tissues has largely spurred the currently recognized field of regenerative medicine. Stem cells are categorized by their differentiation potential, with the full classification including totipotent, pluripotent, multipotent, oligopotent, and unipotent [10]. Generally, embryonic stem cells (ESCs) and the newly found induced pluripotent stem cells (iPSCs) [11, 12] are considered to be pluripotent because they have the ability to differentiate into most known embryonic and adult cell lineages. Stem cell niches provide microenvironments that maintain stem cells in an undifferentiated state, which supports their self-renewal by blocking differentiation signals. Moreover, tissue-specific signals emanating from the niche not only support self-renewal of stem cells occupying a given niche but also redirect the fate of early differentiating progenitor cells back into a stem cell state [13, 14].

Mammalian germ cell lineages begin with primordial germ cells (PGCs), which generate spermatozoa and oocytes in suitable microenvironments provided by the male and female gonads, respectively. Based on the potential of pluripotent stem cells to generate embryonic lineages, including to the germline, many researchers have recently developed strategies to generate germ cells from pluripotent stem cells in vitro (Fig. 1). Subsequently, artificial germ cells generated from non-pluripotent stem cells, such as somatic stem cells, have been reported. These approaches may offer us clues to understand the basic biology of gametogenesis, which holds the potential to open new avenues for treating or curing human infertility. In this review, we discuss historical studies to the most recent advancements in the generation of germ cells using embryonic and nonembryonic stem cells.

GERM CELL DERIVATION STRATEGIES AND PLATFORMS

To date, several different strategies are available for deriving gamete-like cells from stem cells in culture: 1) spontaneous differentiation, 2) differentiation in conditioned medium from somatic cells and/or coculture with somatic cells in medium supplemented with defined growth factors, 3) genetic manipulation, and 4) niche reprogramming (Fig. 2A).

Based on factors known to regulate sex determination, germ cell development, and pluripotent stem cell differentiation, systems for monitoring artificial germ cell development from

[1]This work is supported by Science Foundation of CTGU (KJ2014B066).
[2]Correspondence: E-mail: biomed_wang@yahoo.com
[3]Correspondence: E-mail: changbai_liu@yahoo.com
[4]Correspondence: E-mail: fanhui_zeng75@126.com

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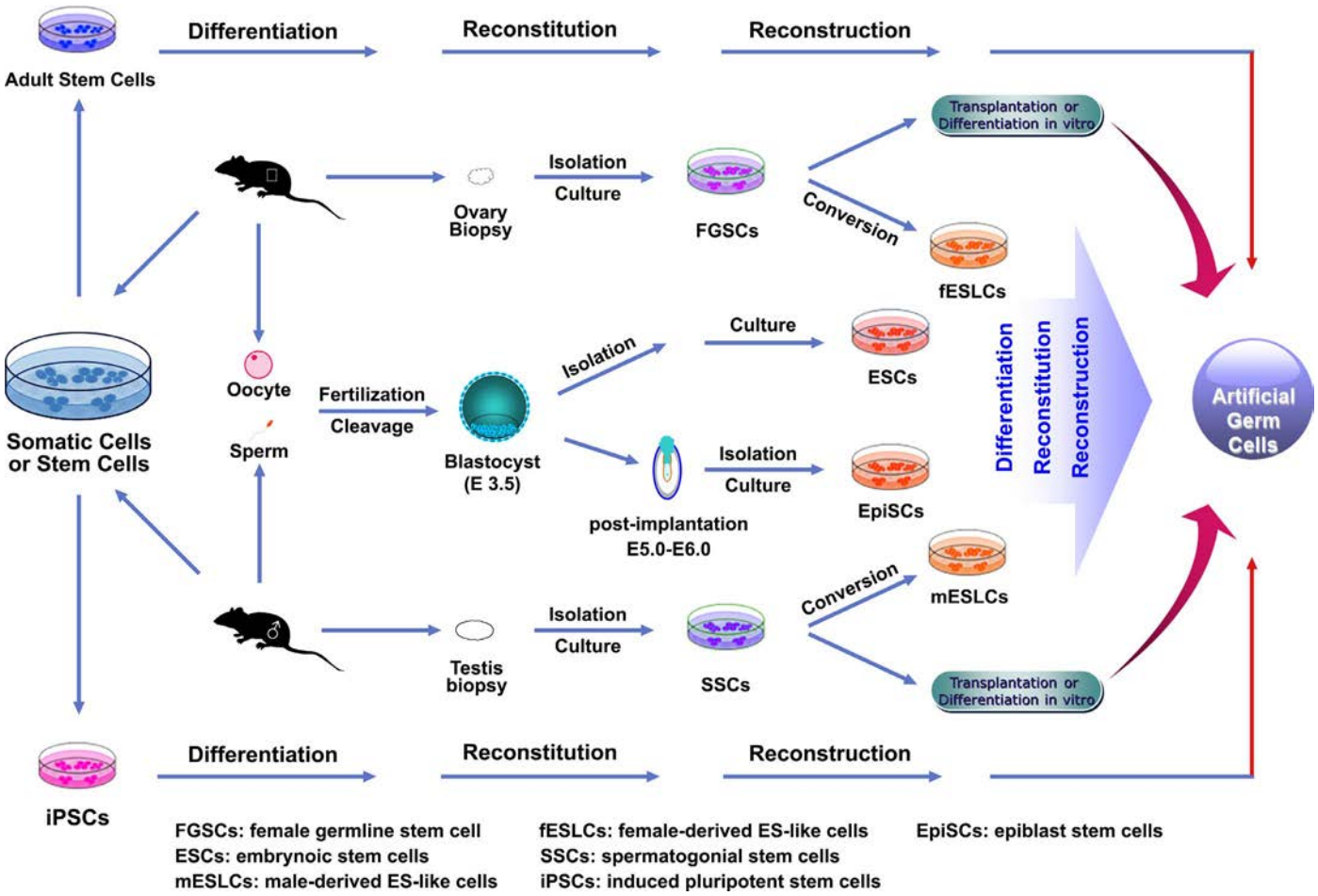

FIG. 1. Schematic representation of artificial germ cell generation. Under appropriate differentiation procedure, ESCs (originated from the inner cell mass of blastocysts), iPSCs (derived from somatic cell reprogramming), EpiSCs ([epiblast stem cells] derived from postimplantation late epiblasts), SSCs (subpopulation of type A spermatogonia), and FGSCs (GSCs for oocyte production) can be used for artificial germ cell generation. Germline-derived ES-like cells could be converted from GSCs (SSC and FGSCs) in vitro; then, the artificial germ cells would be derived following the pluripotent stem cell differentiation protocol. In vivo microenvironments provide structural cues that are crucial for tissue maintenance and function. Thus, newly found techniques, including gonad architecture reconstruction from differentiated progenitors of germ-like cells and in vitro reconstitution of the multistepped germ cell lineage specification pathway, will be helpful for functional artificial germ cell generation in vitro.

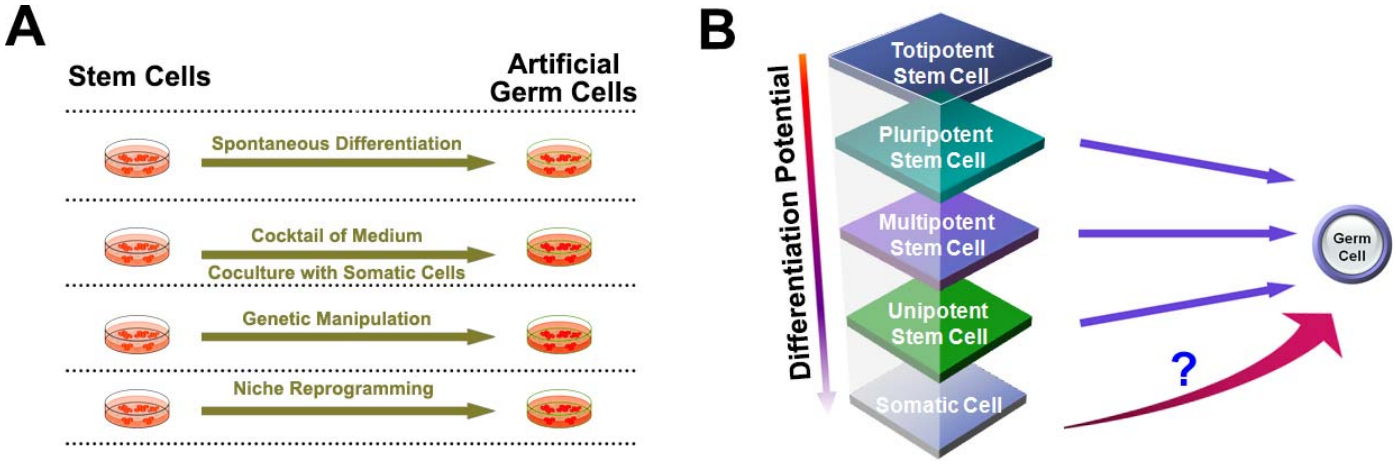

FIG. 2. Different strategies for stem cell differentiation into germ cells. **A**) Methods of artificial germ cell generation currently under development include spontaneous differentiation, cocktail of differentiation induction factors and/or coculture enhancement, genetic manipulation, and niche reprogramming. **B**) Mammalian ESCs are considered to be totipotent and thus able to differentiate into almost all cell types, including germ cells. Artificial germ cells could be derived from different types of cells (pluripotent stem cells, multipotent stem cells, and unipotent stem cells); however, whether the in vitro-derived germ cells could be generated by somatic cell direct transdifferentiation with specific transcription factors similar to those of other cell lineage transdifferentiation processes is still unknown.

pluripotent cell lines have been explored using vital markers expressed under control of germline-restricted reporter genes (i.e., green fluorescent protein [GFP] under the control of germ cell specific promoters). The Mvh-Venus/GFP [15, 16], Stella-GFP [17], and Acr/Gsg2-GFP [18] reporters have been applied in such studies to visualize and sort out in vitro-derived germ cells. Moreover, signaling molecules, such as bone morphogenetic protein (BMP) 4 [19], WNT3A [20], and retinoic acid [21] have been identified as key factors responsible for deriving the induction of pluripotent stem cells into germ cell precursors.

Previous studies have already demonstrated that neonatal mouse testicular architecture can be reconstructed in the subcutis of nude mice [22–25], and development of functional germ cells has been reported. Importantly, using the gas-liquid interphase organ culture method, it has been convincingly demonstrated that functional sperm can be generated in vitro [26–28] and within spontaneously reconstructed epithelium from immature testicular cells [29]. Therefore, organ culture is becoming a useful platform for research associated with gametogenesis and could potentially be refined for artificial germ cell generation from accessible cell sources.

GERM CELL DIFFERENTIATION FROM SOMATIC STEM CELLS

Immunologic and genetic issues are primary challenges of stem cell-based therapeutic approaches and are important to consider in artificial germ cell generation. Fortunately, derivation of autologous stem cells will open new avenues for generating immunocompatible artificial germ cells (Fig. 1). Some evidence has shown that germ-like cells can be generated from somatic stem cells derived from mouse [30, 31], sheep [32, 33], or human [34–36] mesenchymal stem cells; fetal mouse skin [37, 38] as well as porcine skin [39–42] or muscle [42, 43] stem cells; and human amniotic fluid stem cells [44, 45]. Combined, these studies provide examples in which somatic stem cells differentiated into male or female germ cells in culture. Although these presumptive germ cells expressed germ cell-related markers, they did not progress beyond the early stages of germ cell development, even after transplantation into the testis or ovaries. Thus, research on artificial germ cell generation directly from adult somatic stem cells is far from being optimized.

GERM CELL DIFFERENTIATION FROM GERMLINE STEM CELLS

In mammals, the germline is unique because it is the only cell lineage that undergoes cell division of two types, mitosis and meiosis, as a requisite for forming haploid gametes. The general developmental steps in gametogenesis for production of sperm or eggs are largely elucidated. Moreover, germline stem cell (GSC) lines may become more suitable for artificial germ cell generation under appropriate culture conditions because sperm and eggs can develop from GSC lines (Fig. 1). Recently, new evidence demonstrated that female GSCs (FGSCs) isolated from mouse [46–49], rat [50], human [48, 49], and pig [51] could generate viable offspring or differentiate into oocytes in vivo. Additionally, mouse FGSCs cultured in vitro can differentiate into oocyte-like cells [52] mediated by BMP4 signaling [53]. Although these newly found GSCs hold great potential for artificial oocyte production in vitro, support for the traditional view (i.e., no postnatal follicular renewal occurs in mammals) still exists. Lei and Spradling [54] concluded that adult mouse ovaries lack stem cells after they failed to detect ''germline cysts'' using a lineage tracing approach, and Zhang et al. [55] failed to get mitotically active female germline progenitors from postnatal mouse ovaries. However, as Bhartiya et al. [56] suggested, absence of evidence is not necessarily the evidence of absence. No matter what the various opinions on FGSC, extensive collaborations from different labs supporting the evidence for and against FGSC in mammals should be carried out to address these issues, and many other problems also need to be solved. These include more specific FGSC marker analysis, more efficient isolation and purification protocols, culture condition optimization, stronger lineage tracing studies, and elucidation of the signaling pathways driving FGSC differentiation in vivo. More importantly, it remains to be determined if oocyte-like cells differentiated from FGSCs in vitro maintain the capacity for fertilization with sperm. Likewise, roles for FGSCs in oogenesis, menopause, and even ovarian-related disease remain to be determined.

As the other gender of GSCs, spermatogonial stem cells (SSCs) in testes have been widely studied. Still, in vitro spermatogenesis in mammals has only been reported by one group [57]. It should be noted that the presumptive SSCs from that study were ''experimentally immortalized,'' and similar studies have yet to be performed by other laboratories. Thus, unmodified SSCs are still difficult to differentiate into haploid gametes in vitro. Although some studies have shown that SSCs could support spermatogenesis and then differentiate into functional sperm using seminiferous tubule transplantation and organ culture (standard gas-liquid interphase method) in vitro [18, 58–60], such protocols are still complex and difficult to perform. Interestingly, other three-dimensional (3D) in vitro culture systems using two different matrices (soft agar and methylcellulose) reveal the potential for artificial germ cell generation [61, 62]. A new report has shown that mouse male SSCs can be converted into oocyte-like cells in culture [63], but whether the FGSCs can be converted into spermatogenic cells is still unknown.

It should be stressed that almost all of the artificial or presumptive germ-like cells reported in this review have not resulted in robust, reproducible protocols for deriving gametes, much less approached the gold standard of demonstrating the capacity to produce healthy offspring by fertilization [2]. A major problem in the field is that conventional techniques used for presumptive germ cell identification often lead investigators down incorrect paths due to false positives generated by 1) PCR marker analysis (caused by cell preparation and/or genotyping sample contamination or by misleading pseudogene or retrogene detection), 2) antibody labeling artifacts/nonspecific signals, and 3) subjective gating/thresholding to analyze DNA content.

GERM CELL GENERATION FROM PLURIPOTENT STEM CELLS

Steady progress has been made in the generation of germ cells from pluripotent ESCs or iPSCs (Fig. 1).

Germ Cells Differentiated from ESCs

In mice, the first evidence of germ cell generation from ESCs was provided by Hubner et al. [64]. They found that mouse ESCs (mESCs; both XX and XY chromosomes) tagged by Oct4-GFP in the absence of feeder cells and added growth factors condition spontaneously and differentiate into presumptive oocyte-like cells, which not only coexpress oocyte-specific markers (SCP3, ZP2, and ZP3) but also form follicle-like structures. Interestingly, these putative oocytes could even develop into blastocyst-like structures via subsequent parthe-

nogenic activation. Toyooka et al. [19] found that mESCs could be induced into Mvh-GFP-positive and/or Mvh-lacZ-positive cells via embryoid body (EB) differentiation or coculture with somatic cells expressing BMP4. The flow-sorted Mvh-postive cells could participate in spermatogenesis in vivo after transplantation into host testis; however, spermatozoa produced were not tested for fertility. Geijsen et al. [65] and West et al. [66] then described haploid gametes generated from ESC differentiation that were competent to form blastocysts following injection into oocytes. Another study showed that oocyte-like cells could be derived from mESCs with XY chromosomes when cultured in conditioned medium collected from testes of newborn male mice [67]. Meiotic progression of germ-like cells were reportedly derived in vitro from mouse and human pluripotent cell lines (ESCs and iPSCs) by ectopic expression of germ cell-specific or meiosis-related proteins, including DAZ [68, 69], DAZL [68–71], BOULE [68, 69], DDX4 [71], and GASZ [72].

Although these studies have suggested that mESCs can differentiate into female and male germ cells in vitro, the differentiation strategies employed were complex, inefficient, and differed substantially among the respective laboratories. It remains to be determined if germ-like cells generated from pluripotent stem cell lines by such approaches are capable of generating offspring. This breakthrough was provided by Nayernia et al. [73], who demonstrated the birth of live offspring from mESC-derived sperm-like cells. They used a dual fluorescent reporter system (Stra8-driven GFP reporter and Prm1-driven DsRed reporter) to track the progression of mESC-derived germ cells during their derivation process. Cells with sperm-like characteristics were isolated by fluorescence-activated cell sorting and then injected into mouse oocytes that developed into live offspring. There have been many attempts (Table 1) using different approaches to allow germ-like cells to progress into or complete meiosis. However, several groups [74, 75] have reported that germ-like cells derived from ESCs fail to progress through meiosis appropriately.

Germ cell derivation from cynomolgus monkey ESCs [76, 77] and human ESCs (hESCs) is also being rigorously investigated [78]. In one approach, hESCs were differentiated into germ-like cells using a 3D coculture system based on calcium alginate encapsulation of the ESCs with testicular somatic cells [79]. Although germline-specific markers were clearly expressed by primate cells derived in these systems, functional assays for testing successful generation of human artificial germ cells are largely lacking.

Germ Cells Differentiated from iPSCs

Despite ethical issues centered on germ cell derivation from pluripotent stem cell lines in culture [80], iPSC technology holds tremendous potential for generating patient-specific artificial gametes. Studies reporting that male hESCs and human iPSCs (hiPSCs) can be differentiated into haploid spermatogenic cells in culture have achieved two significant endpoints: artificial generation of adult-type spermatogonia and postmeiotic round spermatids [81]. However, it remains unknown whether these haploid cells are functional. Proof-of-concept for these studies is provided by recent breakthroughs demonstrating the germ cell differentiation potential of iPSCs by Saitou's group. First, male (XY) mESCs and iPSCs bearing Prdm1-mVenus and DPPa3-ECFP (BVSC) reporters were induced into epiblast-like cells (EpiLCs) [82, 83] and then further into PGC-like cells (PGCLCs), which differentiated into viable spermatozoa in recipient mice that were able to generate healthy offspring [84]. Next, to determine whether their reconstitution strategy could be translated to oocyte production, female ESCs and iPSCs expressing BVSC transgenes were induced into EpiLCs and then further into PGCLCs via a differentiation pathway similar to that in males, followed by aggregation with embryonic gonadal somatic cells as in vitro. The PGCLCs underwent oogenesis, including X-reactivation, imprint erasure, cyst formation, and meiosis, after the reconstituted ovarian cells were transplanted under adult mouse ovarian bursa. Functional germinal vesicle oocytes were generated from PGCLCs in up to 1 mo but displayed some differences from normal oocytes, including abnormal elliptical shape at a higher frequency, and a subset of them failed to extrude the second polar bodies. The PGCLC-derived oocytes were matured into eggs in vitro and then contributed to healthy and fertile offspring after fertilization with spermatozoa [85, 86]. These results demonstrated that mESCs and iPSCs could be used for reconstituting the entire processes of spermatogenesis and oogenesis, which might provide the long-sought-after foundation for studying mammalian gametogenesis in vitro. Recently, Nakaki et al. [87] reported a refined strategy for mouse germ cell reconstitution that did not depend on a cocktail of added cytokines; rather, expression of transcription factors (Blimp1, Prdm14, and Tfap2c) in EpiLCs was sufficient to induce PGCLCs development that could further contribute to spermatozoon production and healthy offspring.

GERM CELL GENERATION FROM NICHE REPROGRAMMING

Gametogenesis is regulated by ovarian and testicular microenvironments that develop specialized cell-cell and growth factor interactions (nonstem and stem cells), or niches. Therefore, generation of germ cells from stem cells in vitro may likewise require complex niches, including appropriate hormones (endocrine and paracrine), extracellular matrix architecture, and other environmental factors.

Durruthy Durruthy et al. [88] reported that undifferentiated hiPSCs derived from mRNA-based reprogramming with OSKM (OCT3/4, SOX2, KLF4, and cMYC) or OSKMV (OSKM plus a germ cell-specific marker, VASA) factors could directly differentiate into germ cells after transplantation into the seminiferous tubules of germ cell-depleted immunodeficient mice. Significantly, the OSKMV-based iPSCs did not form tumors, whereas the OSKM-based iPSCs that remained outside the seminiferous tubule did. The same group also found that hESCs and azoospermia factor (*AZF*)-deleted hiPSCs inside murine seminiferous tubules could receive critical molecular cues directing germline differentiation; however, outside seminiferous tubules, *AZF*-deleted iPSCs maintained their undifferentiated state and formed tumors [89]. Thus, these studies suggest that niche cells, potentially Sertoli cells, are critical to suppress somatic cell differentiation and favor the germline fate in hiPSCs.

CONCLUSIONS AND PERSPECTIVES

Complete restoration of gametogenesis in culture will be important to our understanding of biological events at the cellular and molecular levels that are important for germline development. Understanding these processes will have an enormous impact on applications in the biomedical and agricultural communities. The studies mentioned above suggest that artificial or in vitro-differentiated germ cells could be readily derived from stem cells, including somatic, germline, and pluripotent stem cells.

Significantly, without any gene delivery, unipotent SSCs from mouse [90–94] and human [95, 96] can convert into an

TABLE 1. Pluripotent stem cell differentiation into germ cells.

Source[a]	Method[b]	Endpoint				Reference
		Marker expression	PGC formation[c]	Haploid cells[c]	Offspring production	
mESCs	EBs	+	+	–	–	[17]
mESCs	EBs	+	+	–	–	[104]
mESCs	EBs	+	+	–	–	[105]
mESCs	Adherent + EB	+	+	–	–	[106]
mESCs	Adherent + EB	+	+	–	–	[107]
mESCs	Adherent + BMP4/8b + SCF + LIF + EGF	+	+	–	–	[108]
mESCs (XY)	EBs + BMP4	+	+	+	–	[19]
mESCs (XY)	EBs	+	+	+	Blastocyst	[65]
mESCs (XY)	Adherent + RA	+	Unshown	+	+	[73]
mESCs (XY)	Adherent	+	Unshown	-	–	[74]
mESCs (XY)	Adherent + RA	+	Unshown	+	–	[109]
mESCs (XY)	EB + testis extraction	+	Unshown	-	–	[67]
mESCs (XY)	EB + BMP4	+	+	–	–	[110]
mESCs (XY)	EBs	+	+	+	–	[66]
mESCs (XY)	EBs + RA	+	Unshown	+	–	[111]
mESCs (XY)	EBs + granulosa cells	+	+	-–	–	[112]
mESCs (XY)	EBs + BMP4	+	+	–	–	[113]
mESCs (XY)	EB + RA + testosterone	+	Unshown	–	–	[114]
mESCs (XY)	EB + RA	+	+	+	–	[115]
mESCs (XY)	Adherent + coculture	+	+	–	–	[116]
mESCs (XY)	EBs + BMP2/4	+	+	–	–	[117]
mESCs (XY)	Adherent	+	+	–	–	[75]
mESCs (XY)	EBs	+	+	–	–	[118]
mESCs (XY)	EBs	+	+	–	–	[119]
mESCs (XY)	Adherent + RA	+	+	–	–	[120]
mESCs (XY)	EBs + RA	+	+	–	–	[121]
mESCs (XY)	Adherent	+	-	–	–	[122]
mESCs(XY)	EBs + BMP4 + RA	+	–	–	–	[123]
maGSCs (XY)	Adherent + RA	+	Unshown	+	Blastocyst	[97]
miPSCs (XY)	EBs + BMP4 + EGF + GDNF + SCF	+	+	–	–	[124]
miPSCs (XY)	EBs + RA + testosterone	+	Unshown	–	–	[125]
miPSCs (XY)	Adherent	+	+	–	–	[126]
miPSCs (XY)	EBs + RA	+	Unshown	–	–	[127]
miPSCs (XY)	EBs + RA	+	Unshown	–	–	[128]
mESCs (XX, XY)	Adherent	+	Unshown	–	–	[64]
mESCs (XX, XY)	EBs	+	+	–	–	[129]
mESCs (XX, XY)	EBs	+	+	–	–	[130]
mESCs (XX, XY)	EBs	+	Unshown	–	–	[131]
mESCs (XX, XY)	Adherent	+	Unshown	–	–	[132]
mESCs (XX, XY)	Adherent	+	Unshown	–	–	[133]
mESCs (XY), miPSCs (XY)	Adherent + BMP4/8b + SCF + LIF + EGF	+	+	Unshown	+	[84]
miPSCs	Adherent + BMP4/8b + SCF	+	+	–	–	[134]
mESCs (XY), miPSCs (XY)	EBs	+	+	–	–	[135]
mESCs (XX)	Adherent + EBs	+	Unshown	–	–	[136]
mEpiSCs (XX)	Adherent + BMP4	+	+	–	–	[137]
miPSCs	Adherent + BMP4	+	+	–	–	[138]
mESCs (XX), miPSCs (XX)	Adherent + BMP4/8b + SCF + LIF + EGF	+	+	–	+	[85]
cyESCs (XY)	EBs	+	+	–	–	[77]
cyESCs (XY)	EBs + BMP4 + RA + SCF	+	Unshown	–	–	[76]
hESCs (XX)	Adherent	+	Unshown	–	–	[139]
hESCs (XX)	EBs + BMP4/7/8b	+	+	–	–	[140]
hESCs (XX)	Adherent + BMP4/7/8b	+	+	–	–	[141]
hESCs (XX)	Adherent + BMP4	+	+	–	–	[142]
hESCs (XY)	Adherent	+	+	+	–	[143]
hESCs (XY)	Adherent + BMP4	+	+	–	–	[144]
hESCs (XY)	Adherent + bFGF	+	+	–	–	[145]
hESCs (XX, XY)	Adherent	+	+	–	–	[16]
hESCs (XX, XY)	Adherent + RA	+	Unshown	–	–	[146]
hESCs (XX, XY)	EBs + coculture	+	Unshown	–	–	[147]
hESCs (XX, XY)	EBs	+	Unshown	–	–	[78]
hESCs (XX, XY)	Adherent + BMP4/7/8b	+	+	–	–	[148]
hESCs (XX, XY)	EBs + BMP4 + WNT3A	+	+	+	–	[20]
hESCs (XX, XY)	Adherent	+	Unshown	+	–	[81]
hESCs (XX, XY)	EBs	+	+	+	–	[149]
hESCs (XX, XY)	EBs	+	+	–	–	[150]
hESCs (XX, XY), hiPSCs (XX, XY)	Adherent + BMP4/7/8b	+	Unshown	+	–	[69]
hESCs (XX, XY), hiPSCs (XY)	Adherent + coculture	+	+	–	–	[151]
hESCs (XX, XY), hiPSCs (XX, XY)	Adherent + RA	+	+	–	–	[152]
mESCs (XY)	Adherent + EBs + Dazl overexpression	+	Unshown	+	Blastocyst	[70]
mESCs	EBs + lin28 overexpression	+	+	–	–	[153]

TABLE 1. *Continued.*

Source[a]	Method[b]	Endpoint				Reference
		Marker expression	PGC formation[c]	Haploid cells[c]	Offspring production	
mESCs	Adherent + Max knockdown	+	+	−	−	[154]
hESCs (XX, XY)	Adherent + RA + Stella overexpression	+	+	−	−	[155]
hESCs (XX, XY)	EBs + BMP4/7/8b + Dazl/Daz/Bouble overexpression	+	+	+	−	[68]
hESCs (XY)	EBs + BMP4 + RA	+	Unshown	+	−	[79]
hESCs (XX, XY), hiPSCs (XX, XY)	Adherent + Vasa/Dazl overexpression	+	Unshown	−	−	[71]
hESCs (XY)	Adherent + Blimp1/Prdm14/Tfap2c overexpression	+	+	+	+	[87]
hiPSCs (XY)	Transplantation	+	+	−	−	[88]
hiPSCs (XY)	Transplantation	+	+	−	−	[89]

[a] cyESCs, cynomolgus monkey embryonic stem cells; maGSCs, multipotent adult germline stem cells; mEpiSCs, mouse epiblast stem cells; miPSCs, mouse induced pluripotent stem cells.
[b] bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GDNF, glial cell-derived neurotrophic factor; LIF, leukemia inhibitory factor; RA, retinoic acid; SCF, stem cell factor.
[c] Unshown indicates data not shown.

ESC-like state in vitro, called germline-derived ES-like cells (gESLCs), which have the same potential as ESCs to differentiate into haploid male gametes [97]. Importantly, FGSCs share many similar characteristics with SSCs [98, 99], whereas a recent report shows that FGSCs can be converted into pluripotent stem cells similar to those of SSC conversion [100]. Thus, in theory, these female germline-derived pluripotent stem cells should have ability to differentiate back into germ cells. These studies suggest not only that gESLCs may be used for germ cell derivation in vitro (Fig. 1), but also that germ cell generation from the gESLC differentiation system provides a unique platform to dissect cellular and developmental mechanisms underlying gametogenesis. This platform may help us find new approaches to treat sex reversal-associated disease.

Today, evidence is accumulating that somatic cells can be transdifferentiated into other cell types without acquiring a pluripotent state [101, 102]; however, whether the somatic cells can directly transdifferentiate into spermatocytes or oocytes by ectopic expression of germline-specific transcription factors is unknown (Fig. 2B). Additionally, the combination of niche reprogramming and newly found 3D printing techniques [103] may be useful for stem cell-based artificial germ cell generation.

Several problems remain to be solved before artificial germ cells can be applied for treating infertility. These include efficient and highly reproducible protocols for in vitro derivation of genetically and epigenetically healthy, patient-specific gametes from stem cells. Health status of such artificially generated germ cells could theoretically be diagnosed with markers on genome-wide levels (i.e., epigenome, transcriptome, proteome, and metabolome). Once these standards—and any additional safety and ethical standards—are surpassed, clinical application of artificially generated human gametes may someday be warranted for the purpose of treating relevant infertility cases.

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