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Decoding the Spermatogenesis Program: New Insights from Transcriptomic Analyses

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Abstract

Spermatogenesis is a complex differentiation process coordinated spatiotemporally across and along seminiferous tubules. Cellular heterogeneity has made it challenging to obtain stage-specific molecular profiles of germ and somatic cells using bulk transcriptomic analyses. This has limited our ability to understand regulation of spermatogenesis and to integrate knowledge from model organisms to humans. The recent advancement of single-cell RNA-sequencing (scRNA-seq) technologies provides insights into the cell type diversity and molecular signatures in the testis. Fine-grained cell atlases of the testis contain both known and novel cell types and define the functional states along the germ cell developmental trajectory in many species. These atlases provide a reference system for integrated interspecies comparisons to discover mechanistic parallels and to enable future studies. Despite recent advances, we currently lack high-resolution data to probe germ cell–somatic cell interactions in the tissue environment, but the use of highly multiplexed spatial analysis technologies has begun to resolve this problem. Taken together, recent single-cell studies provide an improved

understanding of gametogenesis to examine underlying causes of infertility and enable the development of new therapeutic interventions.

INTRODUCTION

The production of sperm via spermatogenesis is essential for transmitting genetic information across generations through fertilization. In the testis, germline stem cells undergo a highly ordered series of mitotic divisions, meiotic divisions, and morphological changes to give rise to sperm throughout the male reproductive life span. These differentiation processes require intricate interactions between the germ cells and the supporting somatic cell structures in the seminiferous tubules. Such elaborately choreographed events cannot be fully evaluated in vitro, but functional explorations in vivo, mainly in mouse models (detailed in comprehensive reviews 7, 80, 81, 87, 124, 136, 160, 219), have been invaluable for providing a molecular understanding of the processes governing gametogenesis.

With the advent of unbiased hybridization and sequencing technologies, it became possible to define the transcriptome (i.e., RNA expression levels of all genes) at tissue and cellular levels. Early studies relied on bulk measurements, averaging gene expression levels in an organ, tissue, or cell population, to compare developmental or pathological states of tissues within and across species. Although revolutionary at the time, these approaches can only be applied to a limited number of cell types with known genetic or cell surface markers (43, 47, 94, 151, 227). Nevertheless, by combining cell type enrichment with genomic technologies, these studies opened the door toward understanding how specific genes, proteins, and signaling pathways regulate germ cell formation and spermatogenesis (72, 73, 88, 89, 105–107, 168, 170, 171, 174, 191, 208, 217).

Because bulk methodologies relied on cell population averages, analysis of rare cell populations and population-specific and time-dependent interplays of gene expression and intercellular communication remained out of reach. In the past several years, with the arrival of single-cell genomics and spatial analysis technologies, many of these limitations have been overcome (8, 44, 71, 90, 103, 132, 158, 161, 173, 181, 212, 215). In particular, single-cell RNA sequencing (scRNA-seq) enabled us and other groups to perform unbiased characterizations of individual cells, creating a new generation of molecular atlases containing (*a*) known and newly described somatic cell types, (*b*) multiple spermatogonial stem cell populations in adults and neonates, and (*c*) a continuous trajectory of germ cell differentiation states, from meiotic prophase spermatocytes to mature sperm (1, 55, 60–63, 95, 131, 182, 187). By subclustering the recognized cell types, these studies uncovered the next level of cellular heterogeneity that exists within the major germ and somatic cell types. Through differential gene expression analysis across cell types and subtypes, it became possible to identify key regulators of gametogenesis and novel cell-specific markers, allowing in-depth analyses of both known and previously unknown cell types. Meanwhile, the new insights provided by scRNA-seq data are limited to dissociated cells, for which positional information and cell-to-cell relationships are lost. As a result, the spatiotemporal dynamics of germline–soma interactions remains missing. Such limitations are also beginning to be overcome by the recent developments in spatial transcriptomic methodologies, which enable spatially resolved examination of germ cell–soma crosstalk across stages of sperm development.

In this review, we discuss how single-cell transcriptomic analyses have improved our understanding of age-old questions regarding molecular and cellular mechanisms that govern germ cell development, as well as the transitions of this developmental program from neonate to adult. We also explore how comparisons across organs and species have brought us closer to

understanding the spermatogenesis process and its unique transcriptional properties, such as widespread transcription and the function of de novo genes. The basic understanding of the germ cell program and its evolution is relevant for understanding the causes of infertility. In particular, a deeper knowledge of the germ cell–soma interdependency in vivo is the first step toward reconstituting this process in vitro and for developing effective male contraceptives and new therapies for restoring male fertility.

HISTOLOGIC ANALYSIS REVEALED COMPLEX CELLULAR ORGANIZATION OF THE SPERMATOGENESIS PROGRAM

Spermatogonial stem cells (SSCs), the ultimate cellular reserve of male fertility, initiate the differentiation process asynchronously along the seminiferous tubule, giving rise to multiple germ cell populations within a given tubule cross section (29). These regularly repeated ensembles of spermatogonia, spermatocytes, and spermatids, mostly revealed with a modest set of protein markers, have been used to define the 12 stages of the seminiferous epithelium cycle in mice. Within a given tubule cross section, a single stage of seminiferous epithelium is detected, and the stages are arranged longitudinally along the tubule (39, 55, 70, 94, 98, 99, 147). In real time, a full cycle of spermatogenic development takes 35–36 days in mice (29).

Although the core biological programs—mitosis, meiosis, and spermiogenesis—are largely conserved, the duration of spermatogenesis varies significantly across species. Spermatogenesis occurs over 74 days in humans (75, 76) and 42 days in macaques (37, 46). Humans, great apes, and some New World monkeys also differ from most nonprimate mammals in that spermatogenic development is not as highly ordered in the former, such that a cross section of the seminiferous tubule often contains patches of cells at different stages of the seminiferous epithelial cycle, further complicating analysis of spatiotemporal patterns (129, 210).

While timing and tubule organization vary across species, the recurring patterns of cellular associations observed within a given stage are conserved across species, suggesting that the molecular events underlying the development of spermatogonia, spermatocytes, and spermatids are mostly conserved (56). However, the mechanisms regulating stage-specific transitions and the extent of evolutionary conservation remained poorly understood.

BULK RNA SEQUENCING CHARACTERIZED MOLECULAR PROFILES OF ENRICHED CELL POPULATIONS

Our molecular understanding of the intrinsic germ cell differentiation program began with the analysis of bulk sample RNA sequencing methods, for both the entire testis and cell populations enriched for some known cell types. These studies selected specific cell types by using gravity sedimentation (6, 119, 167), fluorescence-activated cell sorting (FACS) based on DNA content, transgenic reporters, cell-surface antibody labeling (65, 66, 68, 78, 149), or developmental timing based on the largely synchronous first wave of spermatogenesis in neonates (4, 114). Despite their inherent limitations, these approaches enabled transcriptional profiling of various germ cell (primordial germ cells, gonocytes, undifferentiated spermatogonia, differentiating spermatogonia, pachytene/diplotene cells, and haploid spermatids) and somatic cell (Leydig, Sertoli, and lymphatic endothelial cells) populations (1, 4, 53, 54, 58, 65, 66, 89, 112, 145, 149, 172, 188, 192), leading to identification of molecules and functional pathways central for germ cell specification, maintenance, differentiation, and meiosis regulation (1, 53, 58, 65, 66, 89, 100, 112, 145, 149, 188, 192) and transcriptomic dynamics of somatic cells across stages of the seminiferous tubule cycle (43, 52, 92, 94, 104, 138, 155, 186, 227).

SINGLE-CELL RNA SEQUENCING ENABLED THE CREATION OF A COMPREHENSIVE MOLECULAR ATLAS OF SPECIFIC CELL TYPES

While bulk sample sequencing methodologies provided valuable insights, our understanding of germ cell differentiation was based on cell populations that could be isolated using prior knowledge and known markers. Importantly, since these approaches characterize averaged profiles of cell populations, it is not possible to reveal molecularly distinct cell types in an unbiased manner. In particular, rare cell types or transient cell states are likely missed. With the arrival of scRNA-seq technologies, it became technically feasible and cost-effective to gain a snapshot of the transcriptome of thousands of individual cells (reviewed in 86).

Key Concepts: Clusters and Trajectories

scRNA-seq data from thousands of cells in a tissue sample reveal different types of cellular heterogeneity, two of which are known as clusters and trajectories. Clusters are cell populations that show distinct gene expression profiles between populations and comparatively more similar profiles within populations. These computationally observed clusters reflect major cell types or regulatory states and can be annotated by marker genes, i.e., those that are expressed higher in a cluster and carry out certain biological functions specific for that cell type. A trajectory, in contrast, is formed by a series of transcriptomic states that are not separated into clusters; rather, they form a continuum, reflecting an ordered sequence of biological changes often seen in developmental processes, such as cell differentiation. Trajectories can be either a linear cascade of functional states or more complex. For instance, one trajectory may bifurcate into two when a bipotential progenitor population differentiates into two types of descendent cells, or two parallel trajectories may coalesce when there are two routes to produce the same type of terminally committed cells. In the past several years, by applying scRNA-seq we have garnered a wealth of data on testis cell heterogeneity and germ cell differentiation. In this section, we review new insights gained regarding early transitions during embryonic development, cellular diversity of mouse and human adult testes, differences between neonate and adult germ cells, and comparisons across stages and species.

The Transition from Prospermatogonia to Spermatogonial Stem Cells

One of the advances gained from scRNA-seq was to refine our knowledge about molecular subtypes of the earliest stages of germ cell specification and differentiation. The founding germ cells in the mammalian gonad are called primordial germ cells, which give rise sequentially to primary transitional (T1)–prospermatogonia (ProSG), secondary transitional (T2)-ProSG, and SSCs (137, 196) (**Figure 1**). The two stages of prospermatogonia, T1-ProSG and T2-ProSG, were defined histologically, based on size and location within the tubule, but they lacked markers that specifically distinguish them (137). T1-ProSG are mitotically quiescent cells that undergo genome-wide chromatin and epigenetic changes (96, 216). At postnatal day 1 (P1), T1-ProSG begin converting into T2-ProSG, which subsequently initiate proliferation and are considered immediate precursor cells of SSCs (137). Although the T2-ProSG to SSC transition was poorly understood, it was believed that the ProSG population is molecularly heterogeneous, consisting of (*a*) neurogenin 3 (*Ngn3*)-expressing progenitors in mice that transition into the founding SSC population between P3–6 and (*b*) progenitors lacking *Ngn3* expression, which directly differentiate into A2 spermatogonia, initiating the first wave of spermatogenesis at approximately P3 (96, 216, 221, 222) (**Figure 1**).

An unbiased, pooled analysis of embryonic day (E)18.5, P2, and P7 mouse testes expanded the number of distinct germ cell states in the late fetal and early neonatal stages to six, including three prospermatogonia [T1-ProSG, intermediate prospermatogonia (I-ProSG), T2-ProSG] and three

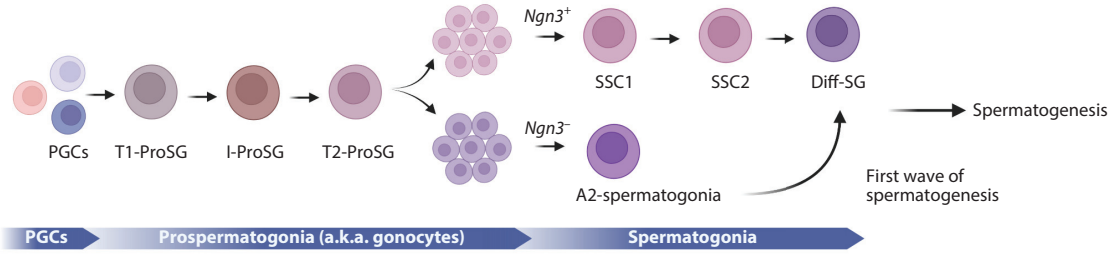

Figure 1

Transitions from PGCs to SSCs in the fetal-to-neonatal testis. PGCs are the founder germ cells that give rise to transitional ProSG populations in the gonad. T1-ProSG give rise to I-ProSG and T2-ProSG sequentially. Descendant cells of T2-ProSG can be broadly classified into two subpopulations based on *Ngn3* expression. *Ngn3*$^{+}$ cells give rise to SSCs, which are the foundational unit of fertility and continuously balance self-renewal with differentiation to sustain continued sperm production throughout a male's adult life (i.e., steady-state spermatogenesis), while *Ngn3*$^{-}$ cells transition directly to A2-spermatogonia to initiate the first wave of spermatogenesis. Figure adapted from images created with BioRender.com. Abbreviations: Diff-SG, differentiating spermatogonia; I-ProSG, intermediate ProSG; PGC, primordial germ cell; ProSG, prospermatogonia; SSC, spermatogonial stem cell; T1-ProSG, primary transitional–ProSG; T2-ProSG, secondary transitional–ProSG.

SSC states [SSC-1, SSC-2, and differentiating (Diff)-SG] (137, 196). Here scRNA-seq allowed the identification of cell type– and state-specific molecular markers. For example, T1-ProSG express *Utf1*, *Dnmt3l*, and *Piwil4*. The latter two genes encode proteins important for reestablishing DNA methylation marks that were erased during primordial germ cell development in order to silence transposons (2, 3, 5, 9, 108, 156, 203), re-establish parental genomic imprinting in germ cells (10) and prevent meiotic catastrophe (9, 15). I-ProSG, a previously unknown transitory population identified in the P2 testis, continues to express *Utf1* but loses *Dnmt3l* and *Piwil4* expression. Instead, I-ProSG expresses genes related to migration and actin organization, including *Elmo1* and *Palld*. As expected, T2-ProSG appear at P3 and are actively proliferating, consistent with their *Mki67* and *Cdk1* expression. These cells subsequently transition into SSC-1, SSC-2, and Diff-SG, which express *Dazl* and *Cdkn2a*, and are further distinguished by the expression of *Lhx1*, *Cd82*, *Etv4*, *Rarg*, and *Stra8*. Based on computational ordering, these cells appear to follow a linear developmental trajectory, but whether the differentiation in vivo follows the computationally predicted ordering needs to be validated.

Similarly, by marking the germline of fetal and neonatal mouse testis using a combination of lineage reporters (Blimp1-Cre, Tdtom, and Id4^{EGFP}) it has become feasible to enrich and functionally characterize germ cell subtypes (117). The Blimp1-Cre is expressed in primordial cell germ cells. When combined with a conditional red fluorescent allele (TdTomato or Tdtom), the Cre-positive cells will irreversibly label primordial germ cells and all descendent cells with the Tdtom reporter (150). Among the Tdtom^{+} cells, a subset of germ cells will express a developmental transcription factor known as inhibitor of differentiation 4 (identified as Id4^{EGFP}) (16). Clustering of all cells with both reporters and comparing across developmental time points led to the identification of 10 germ cell clusters, 4 of which are enriched in E16.5 gonads, 2 are in the P0 testis, and the remaining 4 are from germ cells from P3–6. Furthermore, Law et al. (117) elegantly show that two fetal cell subpopulations with varying levels of ID4 expression (i.e., Id4^{EGFP} bright versus dim cells) have distinct abilities to reconstitute the germline of a germline-ablated recipient: The Id4^{EGFP} bright cells establish donor-derived spermatogenesis, whereas the dim cells do not, suggesting that SSC potential is restricted to a subset of prospermatogonia, and cells that acquire expression of the right markers gain a competitive advantage for colonizing the testis niche.

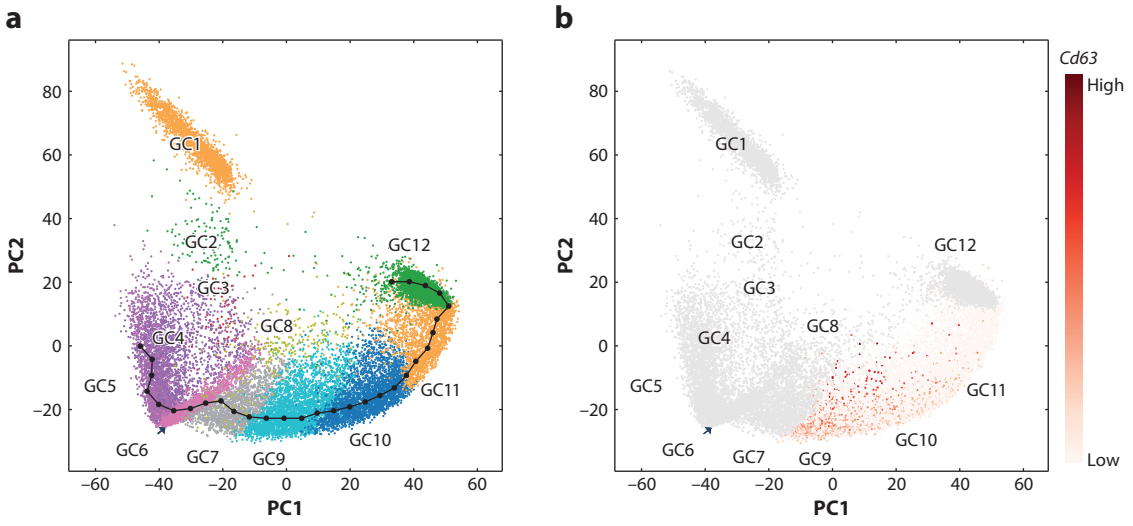

Figure 2

The continuous differentiation trajectory of germ cells in the adult mouse testis, as defined by scRNA-seq data (figure adapted from Reference 55). (*a*) Principal component analysis (PCA) plot of ~20,000 mouse germ cells, shown in PC1-PC2. Each point is a cell, colored and labeled by 12 germ cell (GC) states. Cells in GC1 are SPG cells, which form a discrete cluster. GC2 is a transitional state initiating meiosis, and GC3–GC12 describe a linear trajectory from spermatocytes to spermatids. The solid black line links pseudotime points that trace the sequentially advancing differentiation states, although these points do not have a simple, linear correspondence to real time. (*b*) A recolored version of panel *a*, where round spermatids, GC9–GC11, are colored by *Cd63* expression level, while other GCs are in the background color (*light gray*).

Taken together, these studies advanced our molecular understanding of the ProSG-to-SSC developmental transition. However, the methodological differences and the lack of direct comparisons of the two data sets make it difficult to reach a consensus about the number of intermediate states in these early developmental transitions. Resolving the fate and contributions of prospermatogonia subtypes will be achievable in the future using next-generation sequencing-based lineage tracing methods (e.g., 183), where cells sharing the same DNA barcode originate from the same founder cell and are identified as part of the same lineage.

Insights from scRNA-seq Analysis of Adult Mouse Spermatogenesis

Analysis of adult mouse spermatogenesis was carried out by several groups, including ours (20, 42, 55, 60–63, 77, 95, 115, 131, 146, 187). By scRNA-seq analysis of adult mouse testis, our group identified 12 germ cell (GC) states, referred to as GC1–GC12, consisting of spermatogonia, spermatocytes, and round and elongating spermatids (**Figures 2*a*** and **3*a***). They apparently follow a continuous germ cell trajectory without distinct clusters, with the exception of the transition from spermatogonia into meiosis, which is a discrete programmatic change in cell transcriptome (55) (**Figure 2*a***). Below we describe more details.

Spermatogonia. While our initial clustering of all germ cells grouped all spermatogonia (SPG) cells into one cluster, a focused reclustering of ~1,200 SPG cells identified four molecular subtypes, consistent with undifferentiated spermatogonia, type A, type B, and preleptotene spermatocytes reported in related work (42, 95, 187).

Of the multiple spermatogonia subtypes, stem cell activity resides in an undifferentiated population of isolated spermatogonia known as A_{single} (A_s) (36, 85, 148), which either self-renew or

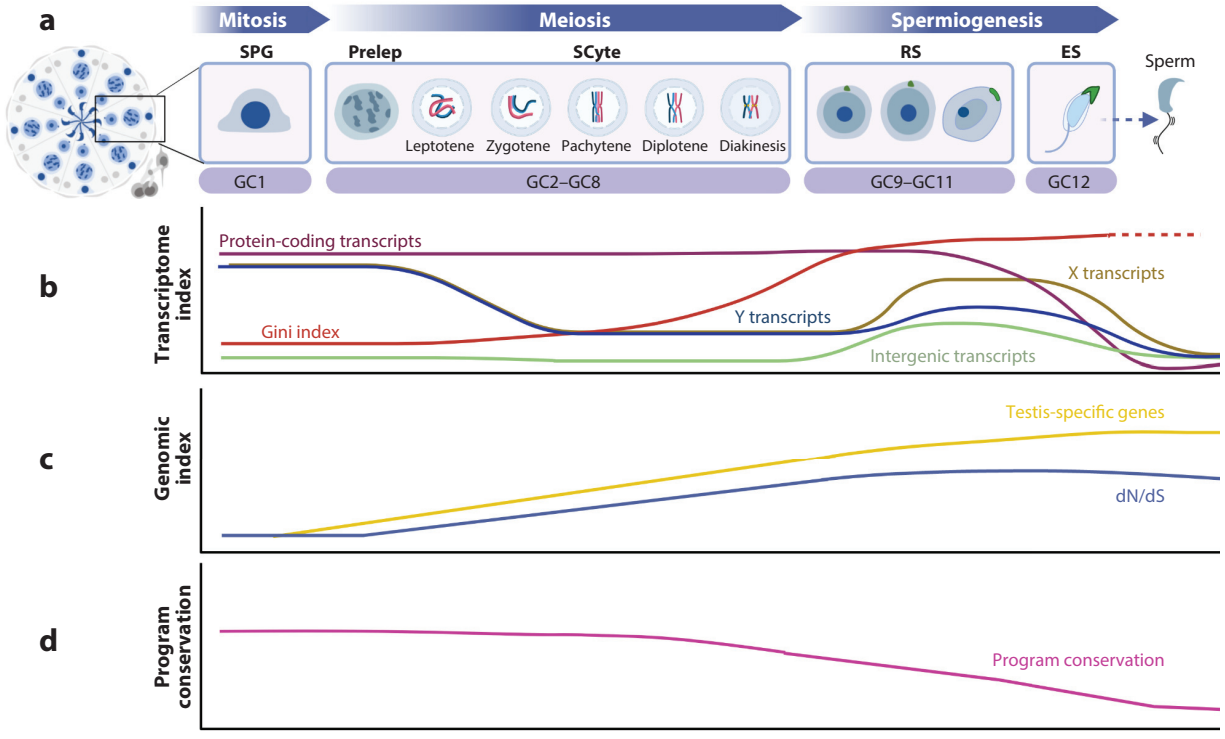

Figure 3

Progression of cellular states during spermatogenesis and dynamic patterns of key molecular attributes. (*a*) Depiction of 12 germ cell (GC) states identified by scRNA-seq analyses of adult mouse testis (55), corresponding to previously known germ cell types: GC1, spermatogonia (SPG); GC2–GC8, spermatocytes (SCyte) progressing through stages of meiotic prophase; GC9–GC11, round spermatids (RS); and GC12, elongating spermatids (ES). (*b*) Temporal expression pattern of several classes of genes and Gini, a measure of single-cell transcriptomic complexity. (*c*) Increased expression for testis-specific genes in later stages, in parallel with dN/dS (ratio of nonsynonymous versus synonymous nucleotide substitution rate measured across species), a measure of positive selection. (*d*) Decrease of transcriptomic conservation between species during germ cell development. Figure adapted from images created with BioRender.com. Abbreviations: Prelep, preleptotene; scRNA-seq, single-cell RNA-sequencing.

divide to generate A_{pair} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia (also known as the transient amplifying progenitor; **Figure 4**) (36, 83, 148). Collectively, A_s, A_{pr}, and A_{al} make up the population of undifferentiated spermatogonia and comprise ~0.3% of the rodent testis cells (45, 84, 147, 148, 157). Interestingly, scRNA-seq and subclustering of the undifferentiated spermatogonia population in our data did not uncover distinct functional subtypes, suggesting that undifferentiated SPG neither exist in stable states nor form a hierarchy (55, 68). However, it remains possible that a developmental hierarchy exists but has very subtle transcriptomic differences, which would require much deeper sequencing per cell and/or a larger number of cells to validate experimentally. Alternatively, this hierarchy may be maintained at the level of protein content, or when cell–cell interactions are intact.

A more recent analysis of neonate and adult SSCs revealed that undifferentiated SSCs toggle between a quiescent and a mitotically active state, which then transition to early and late progenitor cells coincident with upregulation of MAPK/AKT and mTORC1 (193). Inhibition of the mTORC1 pathway using rapamycin increased the fraction of activated SSCs and decreased the

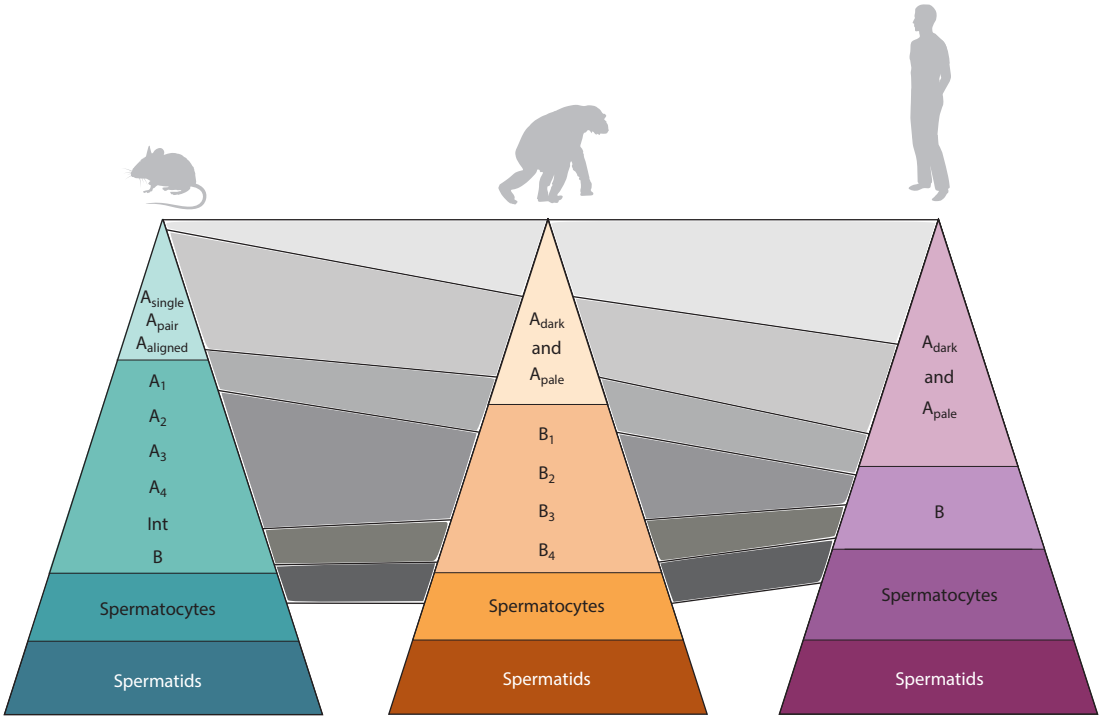

Figure 4

Alignment of functionally equivalent spermatogenesis cell types across three mammalian species: mouse, monkey, and human (figure adapted from Reference 182). From top to bottom are undifferentiated spermatogonia states (such as A_{single} and A_{pair} in mouse and A_{dark} to A_{pale} in primates) and successive stages of differentiating spermatogonia. The height of different layers depicts relative population size. In mice, for example, the undifferentiated population, A_{single}, accounts for only ~0.3% of the germ cells in the testis. In human and monkey, undifferentiated spermatogonia, A_{dark} to A_{pale}, are transcriptionally concurrent rather than sequential. While different nomenclatures were adopted for cell types in different species, scRNA-seq data have defined molecularly similar states across species, indicated by the line segments between species.

fraction of early progenitors but did not affect the proportion of late progenitors, demonstrating that mTORC1 is involved in the transition from activated SSC to early progenitors (193). How these molecular states align with A_s, A_{pr}, and A_{al} or with our SPG1–SPG4 subtypes (and those identified by others) is not clear (20, 42, 55, 60–63, 77, 95, 115, 131, 146, 187).

Meiotic and postmeiotic cells. The spermatocyte populations, defined in our analysis as GC2–GC8 (expressing *Hormad1*, *Spag6*, *Sycp3*, *H2afx*, *Piwil1*, *Spo11*, and *Tdrd5*), progress through successive stages of meiotic prophase corresponding to leptotene, zygotene, pachytene (early, mid, and late), and diplotene (**Figure 3*a***). Our unbiased scRNA-seq analysis provided one of the first comprehensive molecular descriptions of these cell states. Although the classification of meiotic prophase I has relied on enrichment of known protein markers with stage-defined roles, it is important to note the RNAs were often expressed more broadly, providing lower distinguishing power (55). To better predict cell states, other groups coupled scRNA-seq with the analysis of germ cells collected during the first wave of spermatogenesis, which helped to validate gene signatures assigned to cell states in adult experiments corresponding to steady-state spermatogenesis (42, 57, 77). Ideally, by combining state-specific molecular markers identified by scRNA-seq with

high-throughput, multiplexed single-molecule fluorescent in situ hybridization, decades of historically described cell states can be linked with their scRNA-seq-defined molecular states.

Importantly, in addition to the definition of molecular states and associated markers for meiotic prophase, scRNA-seq captures other properties of meiotic prophase cells, such as the silencing of the X and Y chromosomes in pachytene cells (**Figure 3*b***). This is due to meiotic sex chromosome inactivation (MSCI), which is part of a broader mechanism of silencing of unsynapsed chromatin (200). Although bulk RNA-seq of pachytene cells suggested that some genes escape MSCI (34, 188), single-cell data in mouse failed to detect such genes (20, 55, 95, 131), suggesting that previously identified escapees may be an artifact of using a heterogeneous pool of cells, which included earlier or later MSCI stage cells. Curiously, in the postmeiotic stages, a subset of X and Y chromosome genes are reexpressed, but the Y chromosome genes tend to be expressed at significantly lower levels than X chromosome genes despite sharing a similar developmental environment and history (e.g., both being sequestered for silencing). This phenomenon suggests that the regulation of X and Y genes differs in haploid spermatids, possibly due to differences in the epigenetic landscape previously reported, with higher levels of H3K9me3 methylation on the Y chromosome than on autosomes or the X chromosome (142).

Finally, we and others have identified multiple subtypes of spermatids, corresponding to clusters 9–12 in our data set, consistent with early, late, round, and elongating spermatids (GC9–GC12; with markers *Acrv1*, *Tssk1*, *Prm1*, *Tnp1*, and *Hspa1L*) (**Figure 3*a***). Many of the marker genes encode proteins required for the histone-to-protamine exchange or involved in cytoskeletal remodeling, flagella formation, and motility, consistent with the process of spermiogenesis to produce spermatozoa (20, 55, 95). It is important to note that while the expression of spermiogenesis-associated genes peak in round spermatids, many of these genes begin to be expressed from pachynema and onward (55).

Other than reconstructing developmental maps and gene expression networks, scRNA-seq analysis validated a number of known regulators (*Egr1/Egr4*, *Bcl6b*, *Nrf1*, *E2f4*, *Nfyb*, *Ctcfl*, and *Rfx2*) (35, 49, 185, 198, 205) and discovered previously undescribed regulators (*Zcwpw1*, *Sox30*, *Zbtb33*, *Zbtb7a*, *Rfx3/Rfx4*, and *Runx3*) (20, 55, 95), some of which have been functionally investigated (20, 82, 122, 134). Taken together, scRNA-seq studies have deepened our understanding of the gametogenesis process and identified transcriptional regulators at a level of granularity far exceeding those in earlier data sets for sorted bulk populations (61, 65, 118, 188). This rich resource can now be leveraged for (*a*) identifying or predicting novel functions for hundreds of uncharacterized genes during spermatogenesis and (*b*) defining stages of germ cell arrest underlying different forms of infertility (95, 115).

Specifically, knowledge about germ cell subtypes (e.g., early versus late round spermatids) can be applied to enrich and compare these haploid cells, especially for functions such as developmental competency. For example, injections of $CD63^{high}$ (corresponding to our GC9 population) or $CD63^{low}$ round spermatids (corresponding to GC10 and GC11) into oocytes yielded striking differences in the formation rate of two-cell-stage embryos and blastocysts. CD63 is a cell surface marker that distinguishes early from late round spermatids (**Figure 2*b***). Curiously, the early spermatids ($CD63^{high}$) showed a significantly lower blastocyst formation efficiency, suggesting that the completion of the reductive division of meiosis by itself is not sufficient for embryonic competency (20), and other factors are required to enable haploid cells to acquire competency. But what the factors are, or which maturation steps are needed, remains to be determined. Nevertheless, the identification of a developmentally competent spermatid subpopulation has important clinical relevance, as these can be isolated from testicular biopsies in human for round spermatid injection (ROSI) into oocytes, which may improve reproductive outcomes for patients with late spermatogenic arrest.

In the coming years, layering additional multiomic and spatial technologies will help us decipher cellular heterogeneity due to underlying chromatin and epigenetic stochasticity and/or changes in microenvironment.

Transcriptional Differences Are Observed Between the Neonate and Adult Germ Cell States

Numerous studies have analyzed germ cells purified across multiple time points during the first week of postnatal development to understand how the earliest pool of SSCs is established and how neonatal SSCs compare to adult SSCs (42, 57, 117, 123, 178, 196). Comparisons of neonatal and adult spermatogonial cell population centroids reveal that many genes are differentially expressed, suggesting possible programmatic differences between early and adult SSCs (57, 77). However, it remains possible that the reported transcriptomic differences may be due to shifts in SSC composition, which indirectly affect observed expression profiles within a spermatogonia pool (57). Curiously, differences between neonate and adult spermatogenesis are not restricted to SSCs but are also noted in prophase I of meiosis, with hundreds of differentially expressed genes, including those implicated in cell cycle, DNA replication, crossover formation, and double-strand break (DSB) repair: *Brip1* (190), *Brca1* and *Brca2* (12), *Rad51* (64), *H2afx* (197), and *Atm* (57, 159). These differences in gene transcription during meiotic prophase may explain the fundamental differences in DSB number and repair between the first wave of spermatocyte development and subsequent waves of spermatogenesis.

Insights from scRNA-seq Analysis of Human Spermatogenesis

The rapid adoption of scRNA-seq has led to the analysis of multiple human testis tissues, including the developing gonad, perinatal gonad, and adult testis of fertile and infertile men (50, 59, 60, 63, 77, 121, 187, 206). Further, several groups also performed direct comparisons of cell types and states across species.

First-generation scRNA-seq studies. Smart-Seq technology, which was relatively costly and low-throughput, enabled the first exploration into human SSC (hSSC) heterogeneity, hierarchy, and differentiation (61). A study analyzed 92 single cells (60 SSEA4$^+$ and 32 c-KIT$^+$) that passed quality control and revealed four distinct cell clusters that delineate the transition from undifferentiated hSSCs (SSEA4$^+$) to differentiated c-KIT$^+$ hSSCs (61). Cells in cluster 1 express a subset of markers previously associated with mouse SSCs, such as *GFRA1*, *BCL6*, *ID4*, and *ETV5*, while markers such as *KIT*, *SOHLH2*, *SYCE3*, *SSX3*, *SYCP3*, and *NR6A1* were more highly expressed in clusters 3 and 4, consistent with differentiated cells. Key distinctions between undifferentiated and differentiated cells are proliferation status and metabolic requirements: Cells in cluster 1 are more quiescent and have low expression of genes involved in glucose uptake, whereas cells in clusters 3 and 4 have higher activity for genes involved in cell cycle and mitochondrial function, including adenosine triphosphate (ATP) production needed for growth and differentiation. Interestingly, despite the dynamic transcriptome changes, epigenomic profiling using the assay for transposase-accessible chromatin with sequencing (ATAC-seq) and DNA methylation data revealed no significant differences between undifferentiated and differentiated spermatogonia (61). Consistent with work in mouse, these data suggest that the chromatin conformation in spermatogonial cells maintains high plasticity, which somewhat contradicts the traditional model of hierarchical SSC subtypes. As in mouse, the epigenetic landscape in human germ cells is established early and maintained throughout gametogenesis (65, 128).

Second-generation scRNA-seq studies. Shortly after the study based on Smart-Seq, Drop-Seq technologies produced larger data sets that captured the sequential development of multiple

spermatogonia, spermatocyte, and spermatid subtypes. With such data, an unexpected undifferentiated spermatogonial cell population was identified, which expresses *TSPAN33*, *PIWIL4*, *UTF1*, and *PLPPR3* (77, 182, 187, 206). Using cell surface markers, we and others sorted these cells from human testis and performed xenotransplantation into the testis of a germline-ablated, immune-deficient mouse to test whether this population has SSC activity (79, 144, 202). Although human SSCs do not undergo complete spermatogenesis in the mouse environment, transplanted cells migrate from the lumen to the basement membrane, where they produce chains of spermatogonia that may survive for months. Therefore, the relative ability of colony formation in vivo can be used as a proxy for stem cell activity. Importantly, costaining of a testis single-cell suspension with either TSPAN33 or PLPPR3 in combination with c-KIT identifies very few TSPAN33$^+$ c-KIT$^+$ or PLPPR3$^+$ c-KIT$^+$ double-positive cells, confirming that TSPAN33$^+$ and PLPPR3$^+$ cells are both undifferentiated spermatogonia cell types (60, 182, 195). Upon transplantation, both the TSPAN33$^+$ c-KIT$^-$ and the PLPPR3$^+$ c-KIT$^-$ populations generated higher numbers of colonies in vivo than the negative fraction or the cells expressing the generic SSC marker ITGA6 (along with c-KIT$^-$), suggesting that both the TSPAN33$^+$ and PLPPR3$^+$ SSCs do indeed maintain stem cell activity. However, whether TSPAN33$^+$ and PLPPR3$^+$ are completely distinct, partially overlapping, or the same cell populations remains to be determined. Combinatorial marker selection followed by SSC transplantation will help resolve these confusions.

A current challenge to studying human SSC biology and differentiation hierarchy is our inability to maintain and expand these cells in vitro. Unlike rodent spermatogonia, which can be maintained and expanded in culture and can even complete spermatogenesis and restore fertility when transplanted into infertile recipients (67, 97, 110), protocols to culture human spermatogonia have yet to be developed. Current protocols rely on knowledge learned from mice and have not been reliably replicated in different research groups. They are also limited in survival rates and duration (reviewed in 51, 201). These technical difficulties likely came from a lack of known key signals or growth factors. In mice, Gdnf and Fgf2 are required components of the cocktail used for long-term culture of SSCs (111); however, our scRNA-seq data and that of others suggest that additional signaling pathways, such as AKT, TGFB, BMP, and MAPK, may need to be present to efficiently maintain human SSCs in vitro. In this regard, a careful analysis of expression patterns of ligand–receptor pairs in spermatogonia and supporting somatic cells may help develop a robust spermatogonia culture and differentiation regimen.

Linking hSSC molecular identity with histological descriptors. The identification of two transcriptionally distinct undifferentiated populations in the human gonad suggests that they may represent the A_{dark} and A_{pale} spermatogonia populations (60, 182), which in the past were defined histologically by nuclear staining intensity (27, 30, 31) and were considered the quiescent and active stem cells, respectively (28). To examine whether cells in either of the two undifferentiated hSSC populations are enriched for A_{dark} or A_{pale}, we combined periodic acid–Schiff (PAS) and hematoxylin staining with markers for populations 1 and 2 for immunohistochemistry in human testis cross sections. Interestingly, markers for either population 1 or 2, PIWIL4 and MORC1, respectively, were detected in A_{dark} and A_{pale} spermatogonia, suggesting that the original histological terms based on nuclear appearance do not necessarily reflect transcriptional differences (182). And this was consistent with bulk RNA-seq analysis of enriched A_{dark} and A_{pale} populations using laser capture microdissection methods (91).

Comparison Across Spermatogenesis Stages and Species

As spermatogenesis has been studied using multiple model organisms in different laboratories, adopting somewhat different nomenclatures, it has been difficult to translate knowledge from mice

to higher primates and vice versa. For instance, in the classical terminology spermatogonia cells in rodents are classified as A_s, A_{pr}, A_{al}, A1–A4, Int, and B (36, 83, 148); in monkeys they are classified as A_{dark}, A_{pale}, B1–B4; and in humans they are A_{dark}, A_{pale}, and B (26–29). Additional interspecies differences are found in histological organizations of the testis, the duration of the seminiferous epithelium cycle, the dynamics of stem cell renewal and differentiation, and the quantity and morphology of sperm produced (reviewed in 45, 162), thus raising questions about how various steps in germ cell differentiation are related across species. To translate knowledge acquired from mice to the study of spermatogenesis and fertility in higher primates, and to make better use of the diverse genetic and physiological tools in nonhuman models, we need a comprehensive and unbiased analysis of the gametogenesis process that identifies equivalent cell types and states.

To compare the developmental trajectories of germ cells across multiple species requires a close examination of the concept of pseudotime. When a series of connected transcriptomic states are ordered, and their direction of flow annotated by marker genes for the beginning state (e.g., stem cells or progenitor cells) and the end state (e.g., fully differentiated cells), one can quantify the duration and intervals along the trajectory, as when we divide an hour into 60 minutes. For instance, the full journey from one end to the other can be divided into 100 pseudotime units, with the earliest stem cell state at time-0 and the latest differentiated state at time-100. However, it is important to note that the unit of duration is computationally constructed, using a distance metric between two transcriptomic states, and depends strongly on the genes selected (e.g., highly expressed and/or highly variable genes in this organ system). As such, an interval in pseudotime does not need to have a linear relationship with real time. For instance, cells covering the first 10% of the pseudotime may span a much longer interval in real time than the last 10% of the pseudotime. Further, the density of occupancy does not need to be uniform: There can be many more cells residing in the first 10% than in the last 10% of the trajectory. Differential occupancy along the pseudotime reflects uneven speed of progression from one biological state to the next, such that a fast-moving portion of the differentiation process may contain fewer cells than a slow portion, where many more cells are found, as if caught in a traffic jam. In other words, while each scRNA-seq data set captures a one-moment snapshot of the cell community, the density of occupancy along the trajectory reflects the speed of progression if we were to follow a cohort of cells in time. Finally, the testis contains cells from all stages of the seminiferous tubule cycle, and the overall pattern observed for the entire tissue may smooth out differential occupancy in each tubule. To fully characterize the variable speed of progression requires synchronization among the tubules, such as in a pause-and-release experiment by chemical perturbation.

We recently reported a comparative study of the transcriptomes of human, rhesus macaque, and mouse along the spermatogenesis trajectory (182). For somatic cells, while the transcriptomes of most cell types align well across species, there are cases of misalignment, for example, when the muscular pericytes in human and monkey are best matched to myoid cells in mouse, and the immature Leydig cells in human and monkey resemble the interstitial progenitor cells in mouse. Among the germ cells, between-species transcriptomic similarity is the highest in spermatogonia, becomes lower for cells entering into meiosis, and reaches the lowest point at the end of spermatogenesis (**Figure 3*d***). This suggests that the developmental program is more conserved in spermatogonia and becomes more divergent in postmeiotic stages (182). This trend holds true when the analysis is focused only on the transcription factors, indicating more divergent transcription regulation in later stages. This decrease in transcriptomic conservation during the course of spermatogenesis and increased rate of gene evolution of the spermiogenesis genes (**Figure 3*c***) were also observed in another study that assayed a wider range of species (143). Therefore, these changes in gene regulation during spermatogenesis can reflect natural selection of reproductive traits.

Interestingly, genes expressed in later stages of germ cell development tend to have higher dN/dS scores (ratio of nonsynonymous versus synonymous nucleotide substitution rate measured across species) (**Figure 3*c***), a measure of positive selection, as well as lower pLI scores, a measure of a gene's tolerance to its loss of function, indicating less functional constraints for genes expressed in later stages (143). It should be noted that different assessments of natural selection capture selection on different evolutionary timescales and may not have a mechanistic relationship with expression levels for specific genes. For instance, between-species comparisons (using indices such as dN/dS) highlight genes involved in selection forces acting in different species in the distant past (i.e., tens of millions of years ago), while scores based on human variation data (such as the pLI) reflect fitness effects in recent human populations (the past hundreds to thousands of years). While gene expression in different germ cells may directly affect the fitness of these cells in one testis, competition among germ cells is a biological phenomenon vastly different from the competition among individuals in a population or across species.

TRANSCRIPTIONAL SURPRISES IN THE TESTIS

The testis has long been recognized as the fastest-evolving organ at the phenotypic and molecular levels (11, 14, 69, 162, 188, 218). Comparative analyses across multiple organs and species revealed some unexpected molecular features of the testis, including pervasive transcription, higher complexity of germ cell states, and birth of new genes.

Pervasive Transcription

Pervasive transcription refers to the observation that, according to RNA-seq data, vast regions of the genome, including the intergenic regions, can be transcribed (25, 40). The percentage of the genome reported as transcribed increases with sequencing depths and can reach 70–80%, far beyond the ~2% covered by the protein-coding genes. Even after considering the many types of noncoding RNAs, there still seems to be a baseline level of transcription—sometimes called leaky transcription—at regions of the genome not coding for known proteins or known noncoding RNAs. The transcriptional activity at these unexpected regions usually occurs at a much lower level than that of protein-coding genes or the known noncoding RNAs. Importantly, different tissues exhibit different activity patterns across the known genes (both coding and noncoding) and different baseline patterns along the rest of the genome. For example, a comparative analysis of six organ sources (brain, cerebellum, heart, kidney, liver, and testis) and five species (human, rhesus macaque, mouse, opossum, and chicken) using the same sequencing depth (8 million randomly selected reads per sample), revealed that more autosomal protein-coding genes are detected in the testis than other organs, and this is true in all five species (188). The higher detection rate in the testis also extends to long noncoding RNAs and intergenic elements that include pseudogenes, transposable elements, and unannotated intergenic sequences.

The concept of pervasive transcription is related to the concept of transcriptome complexity, usually measured by how many genes are expressed in a given organ or a cell type (at a certain level of detection sensitivity). Here, being expressed can be defined by a present/absent threshold in microarray data or by whether the gene has at least one read in RNA-seq data. The latter depends strongly on the samples' read depth and the specific technology (e.g., full-length RNA-seq or 3′-biased). In general, most studies in this area have shown that the testes have one of the most complex transcriptomes of any organ in the body, and this holds true across multiple vertebrate species, including placental mammals, marsupials, monotremes, and birds (139, 176, 188). For example, in a set of similarly collected bulk tissue RNA-seq data, most tissues express 60% to 70% of the protein-coding genes, while testis tissue expresses 84% (163).

Since the earliest RNA-seq studies used samples representing the entire testis, it is not clear if the higher level of pervasive transcription and transcriptomic complexity is driven by the diversity of different cells in the organ or by an innate property of somatic cells or germ cells in the testis. Since germ cells far outnumber somatic cells in the testis, it is suspected that the germ cells could be the source of high transcriptomic complexity. Several groups performed RNA-seq analyses on enriched cell populations corresponding to somatic cells (such as Sertoli cells) and several broadly defined stages of developing germ cells isolated from the mouse and human testis (65, 66, 188). Such studies relied on known cell surface markers to sort and collect cell populations that roughly correspond to major cell types and major stages of germ cells, then performed RNA-seq analysis on the pseudobulk samples. The results confirmed that the germ cell populations have more genes detected than other tissues for a given read depth, and that later-stage germ cells tend to have more complexity than those at earlier stages.

Subsequently, several groups extended the comparison of transcriptome complexity by using scRNA-seq (60, 213). Contrary to the RNA-seq findings based on bulk tissue or enriched cell population, scRNA-seq showed that the number of genes detected in later-stage cells, such as the elongated spermatids, is smaller than in earlier stage cells, including spermatogonia and spermatocytes (**Figure 3*b***). In other words, while bulk tissue analyses show that the testis has a higher complexity than other organs, and enriched cell populations show that later-stage cells are more complex, single-cell data show the contrary: that the later-stage cells are progressively less complex. For example, we reported that at the same read depth (also known as the library size for the cell), the number of genes detected in a cell decreases from spermatogonia to spermatocytes to round and elongated spermatids (55) (**Figure 3*b***). Further, the transcriptome inequality for each cell can be measured by its Gini index, where a cell with a larger number of genes expressing a comparable number of transcripts would be more equal than a cell with fewer genes accounting for a disproportionately high fraction of the observed transcripts in the cell. By this measure, we found that germ cells in later stages tend to show higher inequality, as if concentrating the expression on fewer genes so as to serve a narrower range of biological function.

This seeming contradiction—increasing transcriptome complexity during germ cell differentiation based on analyzing mixtures of cells, yet decreasing complexity when assessed in individual cells—can be resolved if the population-level transcriptome covers a wider range of cell states in the testis than in other organs and covers more diverse cell states in the late-stage populations than in early-stage ones. In this scenario, the apparently higher level of pervasive transcription and transcriptome complexity in the testis, and in late-stage germ cells, is not the innate property of individual cells; rather, it is the emergent property of different cell ensembles, where the spermatids collectively covered a larger state space of the transcriptome, and apparently expressed more genes when measured as the whole mixture. The other side of this narrative, however, is that while an individual germ cell occupies a single point along the path of differentiation, and may be transcriptionally narrow in its current profile, this cell may, in time, travel through many other points along the developmental trajectory and will eventually cover a wide range of functional states along its full journey. The time-averaged profile of one such cell, or the ensemble average of many cells taken in a "snapshot," would collectively exhibit a high level of transcriptomic complexity even though the individual cells, or a single frame of a cell's "movie," may be less complex.

A related observation is that later-stage germ cells tend to have a larger library size: a greater number of transcripts being sequenced than early-stage germ cells. As a result, if we do not compare cells at the same sequencing depth, the absolute number of genes detected per cell can be higher in later cells, or at least higher than when we impose the condition of equal depth. This caveat raises the question of absolute versus relative complexity when comparing across cells. The

observed library size for a cell is influenced by the true, original number of transcripts in the cell, the rate of conversion from messenger RNA to complementary DNA, and the sequencing depth. For a given experiment, all the captured cells in the sample experienced the same biochemical reactions and are sequenced without bias; thus, the observed library size may indeed reflect the real transcriptome size of the cell. Whether the larger number of transcripts in later stages (and the resulting larger number of genes detected) is due to higher RNA production or decreased turnover is not answerable by scRNA-seq data per se. Similarly, whether more RNA would lead to more protein being translated can only be addressed by proteomic measurements (13). In fact, preliminary proteomic data suggest that later germ cell stages are less translationally efficient, possibly contributing to an increase in residual RNAs (101, 175, 207). Therefore, most of the current single-cell RNA-seq data only provide information about the level of mature transcripts, while the speed of active transcription can only be learned by alternative approaches, such as the computational comparison of the spliced and unspliced portions of the transcriptome (113) or the direct measurement of nascent transcripts in situ (180).

Evolutionary Relevance of Transcriptomic Complexity of the Germ Cells

Many groups have attempted to understand the developmental and evolutionary impact of transcriptional regulation in spermatogenesis. For example, we found that, of the known male infertility genes in human and mouse, many have the highest expression in spermatogonia, while the rest have stage-specific patterns spanning the rest of the germ cell differentiation trajectory (55). Such data on transcriptional dynamics provide specific hypotheses regarding the likely stage-of-action of individual genes known to be implicated in infertility. The next question is, Can such stage-specific expression patterns also lead to new hypotheses about the genetic basis of reproductive success, and further, about the evolution of spermatogenesis across species?

One of the ideas being explored is that pervasive transcription may be beneficial during evolution due to transcription-coupled repair (TCR) (179), which removes mutations from the transcribed, noncoding strand and thus could reduce germline mutation rate for genes highly expressed in germ cells. If this were the case, one would also expect that pervasive transcription in germ cells could bring an evolutionary benefit of correcting newly emerged mutations for a wide fraction of the genome, thus contributing to the fidelity of inheritance and providing a stabilizing influence on genome evolution. A related process is transcription-associated mutagenesis (TAM) (93), which accrues damage such as oxidative lesions in the coding, nontranscribed strand, thus becoming a source of mutagenesis in germ cell DNA. In a recent study (213), the authors reported that genes with higher expression levels during spermatogenesis tend to have lower mutation rates, and the reduction is more evident on the transcribed strand. This led to the hypothesis that transcriptional scanning by TCR acts more strongly than TAM and could reduce germline mutation rates. Further, pervasive transcription in germ cells could be evolutionarily advantageous by repairing more genes than in cells from other organs.

While this hypothesis is interesting, previous studies found that TAM has a stronger impact than TCR (19, 154), with the net result of highly expressed genes containing more mutations, not fewer. A recent large-scale study of somatic and germline mutation patterns (141) could not observe a reduction of mutation rate with increasing gene expression in germ cells. It also found that the mutational strand asymmetry increased with expression level in somatic tissues but not in the germline. Another group identified several theoretical and empirical problems with the hypothesis (125). One of them is that natural selection acts directly on organismal fitness, not gene-specific mutation rate, which could only be involved in a second-order selection. Another problem is that the Xia et al. analysis (213) did not control for confounders such as DNA replication timing, GC

content, and nucleosome occupancy, and it excluded genes without de novo mutations when comparing expression levels with the genes' de novo mutation rates. On the whole, the transcriptional scanning hypothesis is at present not well supported.

In this line of integrated analysis, it is important to appreciate the vast differences in functional units and timescales being considered. A molecular lesion may occur in the DNA at a specific nucleotide position in a specific cell. Most of such lesions are corrected by proofreading activities of DNA polymerase during DNA replication, and a small minority may persist as a base pair mismatch in the double-stranded DNA, forming a heteroduplex. While many such heteroduplexes are reverted to the original nucleotide pair, a fraction of them may be converted to the wrong nucleotide pair, locking in a somatic mutation as a homoduplex, such that at that location the DNA in the cell is different from that of the parental cell. Such somatic mutations occur constantly in every cell lineage. When they arise in germ cells, a tiny minority may affect the fitness of the germ cell, in the specific context of life-and-death dynamics among the germ cells in the testis. Those that confer a higher fitness to descendant germ cells may increase their frequency in the testis of older males (133), but only a small fraction of these will be transmitted to the next generation by the sperm that fertilized the egg. In fact, out of hundreds of millions of sperm being produced, one will fertilize the egg, representing one of the strongest cases of evolutionary bottleneck, where the impact of drift far exceeds the impact of positive selection in the germ cell population. By large-scale whole-genome sequencing of parent–child trios, we can discover ~70 such de novo mutations in the entire human genome for each trio, but whether one such mutation can increase the organismal fitness of the child may have little to do with the cellular fitness of the sperm. The effective population size of the germ cells in a testis has little to do with the effective population size of the organism at the population and species scales. Most of the de novo mutations in humans and in laboratory animals do not rise to become common variants, and therefore would not be accurately captured by databases for common variants, such as the Single Nucleotide Polymorphism Database (dbSNP). The metrics of natural selection also covers disparate systems and distinct timescales. For example, allele frequency spectrum in human population genomic data may reflect the selective pressure experienced by some populations in the recent past, and this selection could have little to do with the between-species DNA substitution patterns shaped by each species' life history over millions of years. On some special occasions, natural selection at a locus may act across scales, such that the genomic signature of selection at the locus can be seen in both within-species common/rare variant patterns and between-species substituting patterns (for a review, see 204). But those are exceptions rather than rules, and selection must be sufficiently strong to rise above the impact of random genetic drift. In short, apparent correlations between gene expression dynamics in germ cells and signatures of natural selection can come from many sources of confounding effects and can lead to overinterpretation.

Birth of New Genes

During evolution, new genes are generated through three main mechanisms: DNA-based gene duplication, retrotransposition, and conversion from noncoding DNA (126). These new genes are usually identified by comparing the genome and transcriptome data of closely related species and can be defined as genes present in a newer species/lineage while absent in the older ones. The evolutionary history of the gene can be inferred from sequence-specific patterns; for example, true de novo genes will have no discernible orthologs in related species, while retrotransposed genes will have an ancestral gene that contains introns (126, 223). De novo genes are well characterized in flies (18, 109, 224), while for mammals there is a curated list of mammalian-specific testis-enriched genes (130, 169, 223), which tend to be highly or uniquely expressed in the testis when compared to older genes that are more broadly expressed across tissues. This pattern of

high expression in the testis for de novo genes or newly derived mammalian-specific genes in the testis led to the out-of-testis hypothesis, which posits that new genes arose by being first expressed in the testis and then acquiring a broader expression pattern across other tissues. The testis may be the birth ground for new genes due to the permissive open-chromatin environment, coupled with pervasive transcription of postmeiotic germ cells. The strong selective pressure on reproductive function tends to quickly fix a gene if it is favorable or remove it if it has negative consequences.

The identification of new genes in flies raised a question: Are these new genes critical for viability and/or fertility? To address this question, two studies assessed these genes in flies via two different approaches and reached contradictory results. The first study examined the functional significance of 192 de novo genes using RNA interference (RNAi) (18) and concluded that over a third of these protein-coding genes (likely originating 3–35 million years ago) are essential for viability (18), suggesting that new genes evolve essential functions very quickly and participate in developmental processes outside of the testes. However, the second study revisited the role of these same genes using gene knockouts, rather than RNAi, and failed to replicate the results of all but two genes, suggesting that the majority of the lethality observed in RNAi knockdowns could be a result of off-target effects of RNAi, and only a small fraction of them (2 out of 192) acquired an essential role in development (109).

In mice, two functional analyses of conserved mammalian and testis-specific genes also led to different conclusions. In the first study, 17 out of the 19 testis-specific genes seemed to be required for normal male fertility (177). The second analysis of another 54 conserved genes with testis-specific expression patterns, not overlapping with the 19 examined in Reference 177, failed to demonstrate any biological effect on spermatogenesis upon single-gene knockout. These differences in reported effects on fitness may be due to different genes being selected for targeting. Taken together, there remain contradictory observations between genes that are enriched in the testis and their functional relevance in fertility (140).

SOMATIC CELLS: COMPOSITION, MOLECULAR ATTRIBUTES, COMMUNICATION, AND EVOLUTION

The somatic compartment of the testis is critical for germ cell development, but remains poorly understood due to the relative rarity of such cells and the relative lack of cell surface markers to clearly identify and effectively purify them. As a result, somatic cell types were for many years only identified histologically (**Figure 5*a***), and the importance of their functional roles, including relevant signaling molecules, were mainly explored by using genetic strategies (24, 38, 100, 115, 165, 184, 194, 199, 220). With single cell RNA-seq, we and others identified five major somatic cell types: Sertoli, myoid, Leydig, endothelial, and macrophage (55, 77, 95, 187). A deeper examination of the data led to the identification of a resident adult mesenchymal population, referred to as Tcf21$^+$ (or telocyte), and an innate lymphoid II (ILCII) cell population (55, 95) (**Figure 5*b***). A comparison of top markers for the Tcf21$^+$ population and the telocytes reported by other groups (55, 95) indicates that these cells are likely to be the same cell type. Our functional analysis of Tcf21$^+$ cells shows that they are a reserve somatic progenitor population that maintains somatic cell homeostasis during normal tissue maintenance, aging, and regeneration after damage (184). The functional role of the ILCII population in the testis is less well known, but they resemble T_H2 cells in terms of cytokine production. In other tissue, they contribute to immune responses by secreting effector cytokines and regulating the function of other innate and adaptive immune cells (153). In an in vitro system, we find that the ILCII population secretes high levels of interleukin 13 (IL-13) but not IL-4, consistent with earlier work detecting the presence of IL-13 in the testis (135). Importantly, IL-13 and its receptor are necessary for maintaining an M2

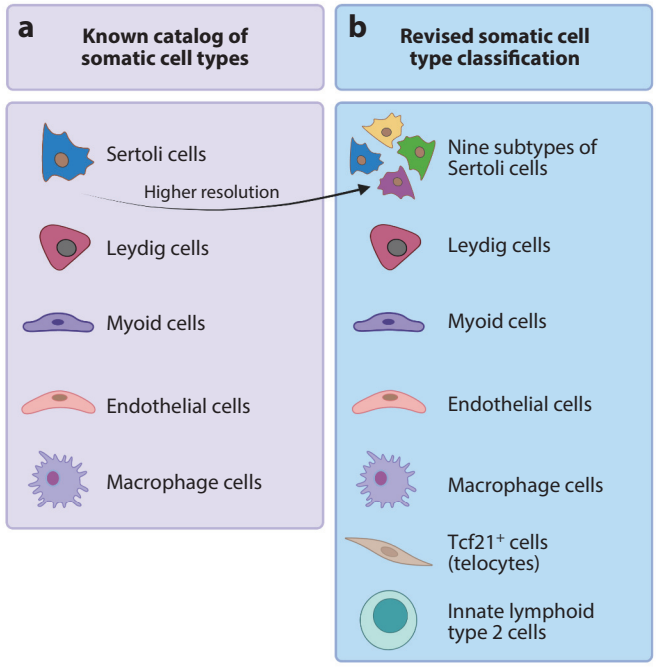

Figure 5

Current view of somatic cell diversity in the testis. (*a*) Previously known somatic cell populations and (*b*) newly characterized populations from studies using single-cell RNA sequencing. Figure adapted from images created with BioRender.com.

macrophage population (135), indicating that ILCII cells may function in immune surveillance and tissue homeostasis.

Enrichment and analysis of more than 1,200 Sertoli cells from either $Sox9^{EGFP}$ or Amh-Cre; $Rosa^{mT/mG}$ mice led to the identification of nine molecularly recognizable Sertoli cell subtypes, all of which were observed in two transgenic lines we analyzed (55), consistent with earlier studies detecting Sertoli cells with differences in cell size, nuclear shape, and marker gene expression patterns at different stages of the seminiferous epithelial cycle (70, 94, 98, 99). Single-molecule fluorescent in situ hybridization (smFISH) for some of these markers, correlations with the nine subtypes, and the previously reported tubule stage–specific marker genes identified by microarrays (70, 211) further supported that the nine Sertoli cell subtypes may represent cells differentially distributed in the 12 spatially ordered stages of the seminiferous tubule. Within a given tubule cross section in mouse, a single stage of seminiferous epithelium is detected; therefore, we expected that the Sertoli cell subtype would correspond one-to-one with stages. Instead, we found that multiple Sertoli cell types aligned with a given stage of the seminiferous tubule cycle, suggesting that Sertoli cells within a stage may be molecularly heterogeneous. These observations raise important questions about functional properties of Sertoli cells within a segment. Currently it is believed that a single Sertoli cell type coordinates the differentiation of all germ cells in the section simultaneously, but it may be that its transcriptome cycles across states within a stage to accommodate the maturation of different germ cell populations within a phase of the cycle. As a result, we are capturing multiple Sertoli cell states that correspond to different stages of the seminiferous tubule cycle.

ScRNA-seq analysis has also enabled us to explore the molecular evolution of somatic cells. The importance of germline–soma compatibility has been elegantly demonstrated by cross-species transplantation experiments. In one study, rat SSCs are capable of completing spermatogenesis in the mouse testis (32), whereas macaque or human SSCs colonize the mouse testis but fail to initiate meiosis (144). To perform cross-species comparisons of somatic cells, we clustered more than 6,000 somatic cells from human, monkey, and mouse testes and identified myoid cells, endothelial cells, Leydig cell precursors, macrophages, and several rarer cell types not extensively studied in the testis, such as T cells and two types of pericytes (182). Unlike in mouse, we did not capture sufficient numbers of Sertoli cells, innate lymphoid cells, and mature Leydig cells in the human and macaque data sets, likely due to the mechanical or physiological stress of cryopreservation and dissociation methods (55, 60, 63, 116, 182). Despite identifying the same set of somatic populations across species, we observed notable between-species differences for many somatic cells (182). For example, mouse myoid cells appeared to be transcriptionally similar to primate pericytes with smooth muscle properties, rather than to primate myoid cells. These global differences in the transcriptome are also noted when comparing ligand–receptor pairs across soma and germ cells (182). For example, ligand–receptor pairs have changed either the source cells or the target cells, or both, generating interesting hypotheses regarding the evolution of germ cell–soma communication in mammals that need to be explored. These global changes in regulatory pathways across species may underscore the failure of cross-species transplantation experiments.

RECONSTRUCTING A SPATIALLY RESOLVED ATLAS OF SPERMATOGENESIS

A limitation of scRNA-seq is that spatial information is lost during tissue dissociation, preventing the analysis of cellular context. Additionally, in the current scRNA-seq data sets, we rely on the expression levels of ligand–receptor pairs in different cell types to infer potential cellular communication, but we do not know whether these cells are juxtaposed or within a distance in which diffusible ligands can act. In this regard, spatial data are needed to understand local differences of cell composition and the distribution of signaling molecules. A spatially resolved atlas, at subcellular to cellular resolution for both germ and somatic cells, may refine or revise the classic 12-stage model of the seminiferous epithelium. Furthermore, defining stage-specific compositional differences and intercellular signaling pathways will aid in the development of a chemically directed regimen for germ cell differentiation in vitro.

Spatial analysis can currently be performed with two main approaches (reviewed in 113, 120, 164). The first leverages spatially barcoded RNA-seq libraries corresponding to RNA captured at predefined spots, beads, or custom-selected regions of interest. It measures expressed genes in an unbiased fashion by next-generation sequencing but cannot reveal the true cellular location of individual transcripts (except that they are collected near a certain spot). With adequate sequencing depth for each library, spot capture–based data have a high level of multiplexity, but remain coarse in spatial resolution, from 50 μm in Visium (209) to 10 μm in Slide-seq2 and NanoString's GeoMx (74, 189). Newer technologies such as PIXEL-seq (48) and Seq-Scope (23) have reported submicron resolution. The second approach measures RNA molecules directly in the tissue using in situ hybridization or sequencing. Here, combinatorial DNA barcodes are added to traditional smFISH oligos (41, 214) to increase the number of molecular entities analyzed. With single-molecule data, these methods attain subcellular resolution but are limited to a panel of predefined targets. These spatial transcriptomics technologies have been applied to many different tissues, disease states, and model organisms (127, 161, 164). However, the technologies are still in their infancy, and applications to the study of spermatogenesis remain limited.

One of the first spatial analyses of the mouse and human testis used Slide-seq2 (17, 166), in which ~29,000 beads dispersed over the adult mouse testes cross section captured close to 800 transcripts per bead. In mouse, this analysis described spatially resolved expression patterns, some of which were stage specific. For example, sperm mitochondrial-associated cysteine-rich protein (*Smcp*) and a hyaluronan-binding protein 4 (*Hapb4*) transcript are enriched in the center of the tubule, while *Lyar*, which encodes a cell-growth-regulating nucleolar protein involved in preribosomal RNA processing, is enriched in spermatocytes near the basement membrane. Moreover, other spatially partitioned genes were restricted to only one of the four roughly defined seminiferous epithelium stages (Stages I–III, IV–VI, VII–VIII, and IX–XII). The identification of spatially partitioned genes was not unexpected, as germ and Sertoli cells in the testis are known to have stage-specific expression patterns, but these data extended past knowledge by showing that Leydig cells and macrophages also exhibit stage-specific expression patterns. Furthermore, in human but not mouse testes, differentiating and undifferentiated spermatogonia reside in different cellular microenvironments, suggesting spermatogonia fate is differentially influenced by microenvironment between humans and mice (21, 22, 220). Despite the exciting findings, the resolution for spot- or bead-based methods is limited due to many cells overlapping on one bead, preventing an examination of cellular communication between two cells in physical contact with one another or subcellular localization and sequestration of RNA molecules.

REMAINING GAPS AND CHALLENGES IN STUDYING GERM CELL BIOLOGY

Single-cell genomics profiling has provided insights into the cellular composition and molecular signatures in the testis, creating cell atlases that contain both known and novel cell types and a refined germ cell developmental trajectory (55, 60–63, 95, 182, 187). We and others have used the atlas to understand the evolution of germ cell differentiation in humans (60, 62, 63, 182) and additional primate species (116, 182) and to decipher mechanisms of aging and infertility in mice and man (33, 95, 115, 146, 152, 225).

Although the scRNA-seq data sets to date covered many testis developmental stages and many species, comparisons across data sets remain challenging. As a result, the classification of germ and somatic cell subtypes varies among published reports. To build a common atlas for future research it is necessary to perform comprehensive multistudy alignments of cell types and align across species. Such a reference atlas will include a consensus classification of somatic cell types and germ cell stages so that gene expression markers can be reliably compared across data sets.

The transcriptome captures a cell's past, present, and future state. However, the transcriptome only represents one aspect of cell function. In the testis, a higher divergence between the transcriptome and proteome relative to other organs is consistent with the known strong translational regulation in spermatogenesis (101, 175, 207). Generally speaking, the uncoupling of transcription and translation makes unified cell clustering difficult, but more importantly, highly orchestrated translational regulation in different germ cell stages calls for a better understanding of what drives these programs (102). By combining multiple omics technologies, such as single-cell transcriptomics, proteomics (13), genomic DNA sequencing (such as DR-seq and G&T-seq), DNA methylation (such as scMT-seq and snmCT-seq), and chromatin accessibility (such as ATAC-seq, or combined ATAC-RNA-seq) (226), we can refine our understanding of cellular heterogeneity, developmental trajectories, and regulatory networks. Such a rich resource will be further bolstered by spatial analysis within individual cells, which will offer insights into tissue architecture and local signaling events. Furthermore, defining stage-specific compositional differences and signaling pathways in the testis will aid in the development of new methods for germ cell differentiation in vitro.

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