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Applications of single-cell RNA sequencing in spermatogenesis and molecular evolution

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ABSTRACT

Spermatogenic cell heterogeneity is determined by the complex process of spermatogenesis differentiation. However, effectively revealing the regulatory mechanisms underlying mammalian spermatogenic cell development and differentiation via traditional methods is difficult. Advances in technology have led to the emergence of many single-cell transcriptome sequencing protocols, which have partially addressed these challenges. In this review, we detail the principles of 10x Genomics technology and summarize the methods for downstream analysis of single-cell transcriptome sequencing data. Furthermore, we explore the role of single-cell transcriptome sequencing in revealing the heterogeneity of testicular ecological niche cells, delineating the establishment and disruption of testicular immune homeostasis during human spermatogenesis, investigating abnormal spermatogenesis in humans, and, ultimately, elucidating the molecular evolution of mammalian spermatogenesis.

Keywords: Single-cell RNA sequencing (scRNA-seq); Spermatogenesis; Molecular evolution; Sertoli cell

INTRODUCTION

The differentiation of spermatogonial stem cells (SSCs) into spermatocytes is a complex and continuous biological process. Mammalian spermatogenesis occurs in the seminiferous tubules of the testis, where SSCs undergo proliferation and differentiation to form spermatozoa, and involves three complex steps: mitosis, meiosis, and spermatogenesis (Nishimura & L'Hernault, 2017; Rabbani et al., 2022) (Figure 1A). (1) Mitosis: A-type spermatogonia proliferate to produce A-type and B-type spermatogonia. A-type spermatogonia do not differentiate and continue to maintain mitosis, while B-type spermatogonia differentiate into primary spermatocytes (Ehmcke et al., 2006; Oatley & Brinster, 2008; Rabbani et al., 2022; Sharma et al., 2019) (Figure 1B). (2) Meiosis: Primary spermatocytes undergo chromosomal replication, after which homologous chromosomes are linked and separated and undergo the first meiosis to form secondary spermatocytes and secondary spermatids. Secondary spermatocytes enter the second meiotic division, where sister chromatids separate to form haploid round spermatids. (3) Spermatogenesis: Round spermatids undergo complex morphological changes as well as nucleoprotein transformations and modifications, resulting in the formation of mature spermatozoa (Figure 1B).

Seminiferous tubules, where spermatogenesis occurs, provide the ecological niche required for the germination, maturation, and transportation of sperm cells. These tubules are surrounded by a specialized lamina propria, which consists of a basement membrane comprising a layer of collagen and peritubular cells (also known as myofibroblasts) (Nishimura & L'Hernault, 2017; Oatley & Brinster, 2008). Sertoli cells are located in the spermatogenic epithelium, attached to the basement membrane, and extend into the lumen of tubules, while peritubular myoid cells, located outside the seminiferous epithelium, surround the tubules without extending into the lumen. Sertoli cells are found in the embryonic testis and are closely associated with developing germ cells during embryonic development, forming spermatogenic cords called seminiferous tubules after birth. During the first round of spermatogenesis, Sertoli cells form tight junctions with each other, dividing the spermatogenic epithelium of most prepubertal male mammals into basal and adluminal (outer) chambers, ultimately forming the blood-testis barrier (Oatley & Brinster, 2012; Svingen & Koopman, 2013).

In rodents, undifferentiated spermatogonia are further divided into A-single or As (one cell), A-paired or Apr (two

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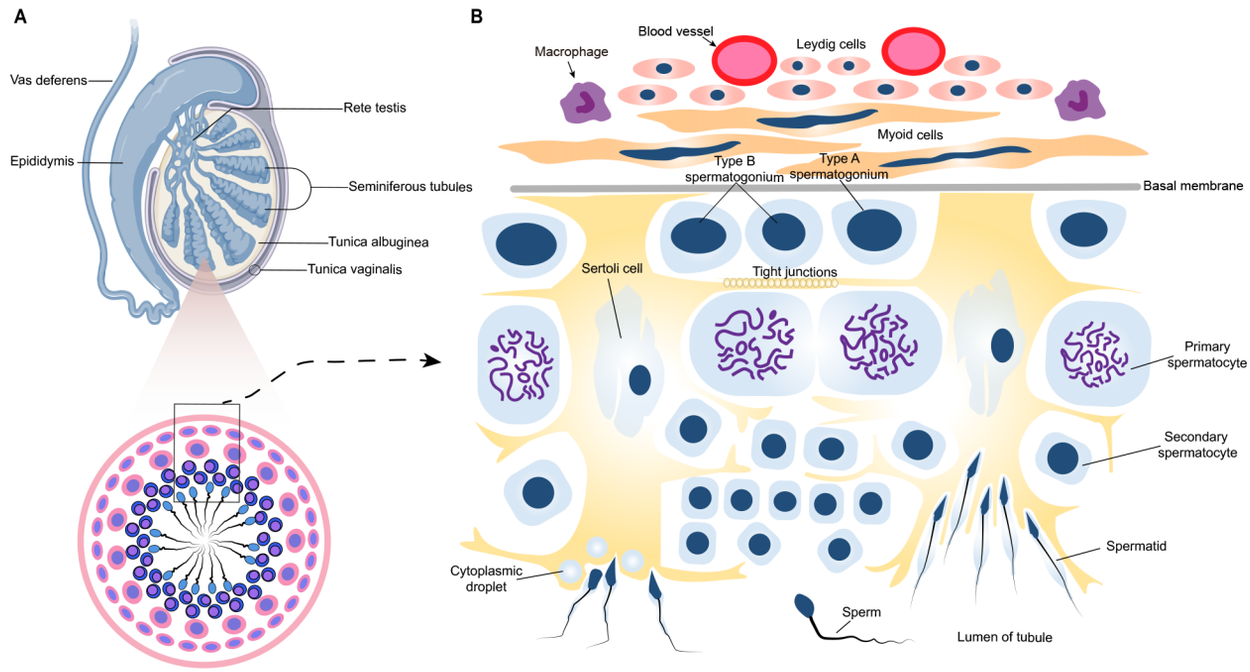

Figure 1 Model of spermatogenesis

A: Illustration of testicular anatomy, highlighting slightly flattened, oval shape of the testis with a smooth surface covered by tunica vaginalis. The tough inner albuginea, thickened at the posterior border, invaginates into the testis to form the testicular mediastinum. Connective tissue septa from the mediastinum divide the testicular parenchyma into many testicular lobules, each containing coiled seminiferous tubules for producing sperm. Spermatozoa are enriched as they progress to the testicular network, through the vas deferens, and are finally collected in the epididymis, externally connected via the vas deferens. B: Schematic of spermatogenic unit pattern within seminiferous tubules, where spermatogonial stem cells complete self-renewal and differentiation within the testis-specific niche formed by Sertoli, Leydig, and peritubular myoid cells.

cells), and A-aligned or Aal (four, eight, and 16 cells) spermatogonia. In contrast, there are two types of As spermatogonia in primates, referred to as A-dark and A-pale (Oatley & Brinster, 2008; Ramm et al., 2014). Regardless of the species, As spermatogonia are believed to make up the SSC population under homeostatic conditions, with the SSC niche anatomically localized as a result of the close association of Sertoli cells with the basement membrane of the seminiferous tubules within the septum (Ehmcke et al., 2006; Rabbani et al., 2022; Sharma et al., 2019). Sertoli cells are also acknowledged as essential contributors to the SSC niche given their close relationship with germ cells (Kanatsu-Shinohara & Shinohara, 2013; Oatley & Brinster, 2012).

The development of single-cell transcriptome sequencing technology has enhanced our understanding of the complex process of spermatogenesis. In this review, we describe the molecular characteristics of germ and somatic cells involved in human spermatogenesis and discuss changes in the testicular environment observed in patients with Sertoli cell-only syndromes and cryptospermia. We also cover the molecular evolution of mammalian spermatogenesis, principles of single-cell transcriptome sequencing, and tools used for routine data analysis.

SINGLE-CELL RNA SEQUENCING (scRNA-seq) PRINCIPLES

Since Tang et al. (2009) first proposed a high-throughput sequencing strategy for single cells, single-cell sequencing technology has developed rapidly, with the emergence of the STRT-seq, CEL-seq, Smart-seq, Smart-seq2, Drop-seq, InDrop, and 10x Genomics sequencing strategies (Haque et al., 2017; Macosko et al., 2015; Svensson et al., 2017; Zhang et al., 2019; Zheng et al., 2017; Ziegenhain et al., 2017). However, all scRNA-seq strategies share a common initial step in which cellular mRNA is converted to cDNA, which is subsequently amplified via molecular biology methods such as polymerase chain reaction (PCR) and *in vitro* transcription (IVT) (Macosko et al., 2015; Svensson et al., 2018; Zhang et al., 2019). Single-cell sequencing libraries are then generated to quantify the expression levels of gene products. In the final step, cellular clusters are annotated and different expression patterns among cellular clusters are analyzed through a programmed language approach (Zhang et al., 2019) (Figure 2A).

The introduction of droplet microfluidic technology has facilitated the execution of single-cell lysis reactions in nanoliter scales, enabling the separation and encapsulation of hundreds of thousands of droplets per second. This approach, characterized by a relatively straightforward arrangement of microchannels for sample introduction and collection, uses a water-in-oil emulsion to create tiny droplets significantly improving reaction throughput (Macosko et al., 2015; Zhang et al., 2019; Zheng et al., 2017). The use of 10x Genomics has substantially lowered reaction costs and enabled the sequencing of millions of cells using droplet microfluidics, with its single-cell sequencing technology targeting mRNA molecules through primers that contain poly-T sequences. To differentiate the origin and number of mRNA molecules, unique molecular identifiers (UMIs) and barcodes are added to the 5' ends of the primers for mRNA quantification and cell identification, respectively (Zheng et al., 2017) (Figure 2B). This technology also improves cell and mRNA capture efficiency by modifying magnetic bead materials to hydrogels and by extending the barcode length from 8 bp to 16 bp,

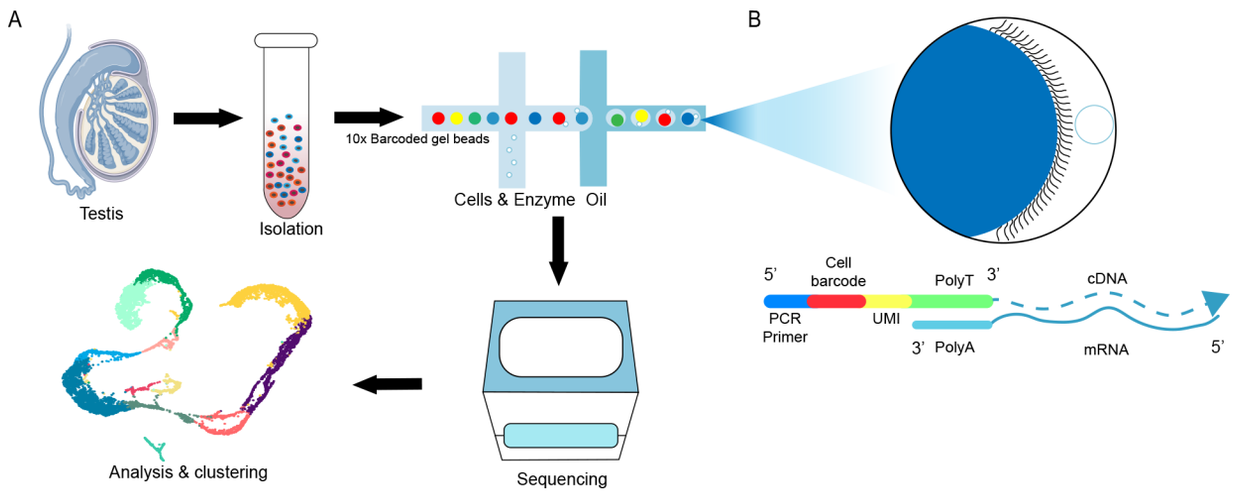

Figure 2 Model view of scRNA-seq workflow analysis and principles

A: ScRNA-seq workflow on 10x Genomics platform. Testicular tissues were prepared into single-cell suspensions, with the 10x Genomics library platform then used. Cells were separated using water-in-oil emulsions, with each droplet containing gel beads for reverse transcription, followed by cDNA isolation and sequencing, and finally, cell clustering using the algorithm. B: Microstructure of gel beads. Each gel bead carried pre-coded primers, with different barcodes within each gel bead to distinguish transcripts from different cells. UMI sequence was used for mRNA quantification, in addition to the poly-T sequence, responsible for capturing mature mRNA.

enabling the potential capture of up to approximately 4.29 billion cells (Zhang et al., 2019; Zheng et al., 2017). However, the underlying design principles of 10x Genomics impose certain limitations, such as the focus on capturing and sequencing only the 3' ends of mRNA molecules and the inability to capture nonadenylated RNA molecules (Cole et al., 2018) or full-length transcripts.

The introduction of Smart-seq has addressed many of the challenges associated with sequencing full-length transcripts. Hagemann-Jensen et al. (2022) enhanced the sensitivity of transcript capture from individual cells by replacing potassium monovalent cations with sodium in the reverse transcription buffer, doubling the detection sensitivity of Smart-seq2 compared to Smart-seq. However, while full-length transcriptome sequencing technologies offer detailed splicing analyses of polyadenylated RNA molecules at single-cell resolution, the lack of UMI information has hampered the reliable quantification of splicing events. Furthermore, these technologies typically only capture a few cells at a time and have not been applied to droplet-based high-throughput platforms. To overcome these limitations, Salmen et al. (2022) developed the VASA-seq (vast transcriptome analysis of single cells by dA-tailing) technique, which captures nonpolyadenylated and polyadenylated transcripts in both plate and droplet microfluidic formats. This technology involves the end repair and tailing of RNA molecules (poly-A tailing), followed by reverse transcription and cDNA synthesis, achieving full coverage of the gene body and high-throughput sequencing. Combining the technical advantages of 10x Genomics and Smart-seq, VASA-seq is expected to become a dominant technology in sequencing. Given the critical role of variable splicing events in embryonic development and spermatogenesis, VASA-seq provides a promising tool for exploring underlying mechanisms in developmental biology.

Although scRNA-seq technology has been pivotal in revealing cellular heterogeneity and complex biological processes, it is not without some limitations. Notably, dropout events in scRNA-seq can occur, where mRNA from certain cells is not efficiently detected during sequencing (Haque et al., 2017), potentially impacting estimations of gene expression levels in cells and affecting the clarification of cell types, states, and functions. Such dropout events primarily stem from factors such as randomness, technical constraints (including cell lysis, reverse transcription, and PCR amplification efficiency), and cellular heterogeneity. These issues may lead to the underestimation of genes with low abundance, misclassification of cell types, and misinterpretation of biological processes. In response, researchers have increased sequencing depth, employed UMIs to differentiate between biological and technical variations, developed statistical models to correct for dropout events, and integrated scRNA-seq with single-cell proteomics for a more comprehensive analysis of cellular states (Labib & Kelley, 2020). Although dropout events remain a challenge, these approaches are progressing refining the precision of single-cell biological information.

ANALYSIS OF HIGH-DIMENSIONAL SINGLE-CELL DATA

Quantifying gene transcript expression levels serves as a precursor to the critical task of clustering and annotating cellular groups within samples (Luecken & Theis, 2019). The Seurat package (https://satijalab.org/seurat/), developed by Butler et al. (2018), integrates single-cell sequencing data using canonical correlation analysis (CCA). This package was later updated to incorporate multiomics datasets through the "bridge integration" method (Hao et al., 2024), enhancing its utility in eliminating batch effects, preserving actual biological effects, and completing migration of multimodal information for discrete and continuous variables (Butler et al., 2018; Stuart et al., 2019). With continuous developments and updates (Butler et al., 2018; Hao et al., 2021, 2024; Satija et al., 2015; Stuart et al., 2019), the current version of Seurat (v5) includes multiomics data integration (Hao et al., 2024), establishing it as an indispensable tool for single-cell data analysis. Concurrently, Wolf et al. (2018) developed Scanpy, a Python-based toolkit for single-cell data analysis that includes deep learning potential, offering a versatile ecosystem that complements Seurat and supports advanced analytical tools, such as pySCENIC, CellPhoneDB, Cell2location, scVelo, and

CellRank, further expanding its analytical scope (Virshup et al., 2023). A summary of common analysis methods for single-cell data is provided in Table 1, outlining the specific applications of each method. However, in actual applications, these methods may need to be adjusted according to data characteristics and research objectives, and other related techniques may exist beyond those listed in the table.

SCRNA-SEQ REVEALS HUMAN SPERMATOGENESIS

In mammals, SSCs produce millions of spermatozoa daily. For spermatogenesis to proceed normally, SSC self-renewal and differentiation must be regulated precisely by the stem cell niche, a process governed by soluble factors and adhesion molecules from the surrounding microenvironment (Kubota et al., 2004). Although many studies have explored the roles of various factors in spermatogenesis, a comprehensive understanding is lacking. The introduction of single-cell transcriptome sequencing technology offers new avenues to explore and address this issue.

Fate determination of human SSCs (hSSCs)

How SSCs balance self-renewal and differentiation remains incompletely understood. Using single-cell transcriptome sequencing to resolve the states of SSCs (SSEA4+ and c-KIT+) during spermatogenesis in adult males, Guo et al. (2017) identified the existence of four SSC states. In State 1, self-renewing hSSCs express significant quantities of stem cell-related transcription factors, such as ID4, ETV5, TCF3, FGFR3, and TSPYL5. Notably, TXNIP inhibits glucose absorption by hSSCs in this state (Guo et al., 2017), a mechanism that may explain why the SSC pool in the testis undergoes differentiation when nutrients such as glucose are taken up. In State 2, the transitional initial state, hSSCs up-regulate genes related to the cell cycle and DNA replication but down-regulate the expression of pluripotency genes and deregulate the limiting effect on glucose uptake (Guo et al., 2017). In State 3, the transitional end-state hSSCs, stem cell signaling is attenuated, RNA splicing is up-regulated, and several critical mitochondrial activities (utilizing glucose) are up-regulated to support adenosine triphosphate (ATP) and lipid production for growth and differentiation. In State 4, differentiated hSSCs promote spermatogonial differentiation by up-regulating the expression levels of differentiation-related transcription factors and signaling pathways (Guo et al., 2017) (Figure 3A). Notably, this study revealed the different states and corresponding molecular mechanisms of SSCs during self-renewal and differentiation, with insights from State 1 offering novel perspectives on how SSCs maintain stemness via TXNIP-mediated glucose uptake inhibition.

Subsequently, while parsing the transcriptional states of various cellular clusters in the adult human testis, Guo et al. (2018) identified an additional state of SSCs, termed State 0, representing the most naïve and undifferentiated phase of these cells. Analysis of testes from infants aged 12–13 months identified State 0 as the dominant state in infant SSCs, indicating it as the most primitive in adults, characterized by markers such as PIWIL4, TSPAN33, MSL3, and EGR4 (Guo et al., 2018). Interestingly, most State 0 SSCs exhibited low expression of ST3GAL2, an enzyme that catalyzes SSEA4 formation (Guo et al., 2018; Saito et al., 2003), suggesting that State 0 SSCs do not express SSEA4, an SSC surface marker. These findings clarify the absence of State 0 SSCs in earlier studies capturing SSEA4+ and c-KIT+ SSCs. Furthermore, although State 0 differs from the State 1, SSCs in different states may represent substable (heterogeneous) cellular phenotypes that allow SSCs to adapt to dynamic ecological niche regulation and maintain homeostatic regulation within the testis (Figure 3A).

Investigating the transition from primordial germ cells (PGCs) to primitive SSCs, Guo et al. (2021) analyzed approximately 32 500 unsorted testicular cells from human embryos (W6–8), fetuses (W12, W15–16), and postnatal

Table 1 Overview of methods for single-cell RNA sequencing analysis

Method	Description	Software/Tool	Application
Library Size Normalization	Adjusts for differences in sequencing depth	Seurat (Hao et al., 2024), Scanpy (Wolf et al., 2018)	Essential for downstream analysis
Total Count Normalization	Normalizes total counts per cell to compare gene expression	Seurat, Scanpy	Accounts for cell size and content variability
Log-Transformation	Transforms data to stabilize variance and improve linearity	Seurat, Scanpy	Commonly used before clustering
Principal Component Analysis (PCA)	Reduces dimensionality for visualization and clustering	Seurat, Scanpy, PCAtools	Identifies major sources of variation in data
t-Distributed Stochastic Neighbor Embedding (t-SNE)	Visualizes high-dimensional data in two or three dimensions	Seurat, Scanpy, t-SNE R package (Linderman et al., 2019)	Exploratory data analysis
Uniform Manifold Approximation and Projection (UMAP)	Reduces dimensionality for visualization	Seurat, Scanpy	Alternative to t-SNE
k-Nearest Neighbors (kNN) Graph	Constructs graphs for cell-cell interactions	Seurat, Scanpy	Defines cell neighborhoods for clustering
Louvain or Leiden Clustering	Community detection algorithms for cell clustering	Seurat, Scanpy,	Identifies distinct cell populations
Differential Gene Expression (DGE) Analysis	Identifies differentially expressed genes between groups	DESeq2 (Love et al., 2014), MAST (Finak et al., 2015)	Finds markers for specific cell types or conditions
Pseudotime Analysis	Orders cells along a development or differentiation trajectory	Monocle (Cao et al., 2019)	Infers developmental processes
Cell Chat Analysis	Identifies and quantifies cell-cell communication	CellPhoneDB (Garcia-Alonso et al., 2021), CellChat (Jin et al., 2021)	Understands signaling interactions between different cell types
Gene Regulatory Network Analysis	Infers regulatory relationships between genes and transcription factors	pySCENIC (Aibar et al., 2017; Van de Sande et al., 2020)	Elucidates complex regulatory mechanisms governing gene expression
RNA Velocity Analysis	Predicts future state of cells based on current gene expression	scVelo (Gayoso et al., 2024)	Studies cell differentiation and development trajectories

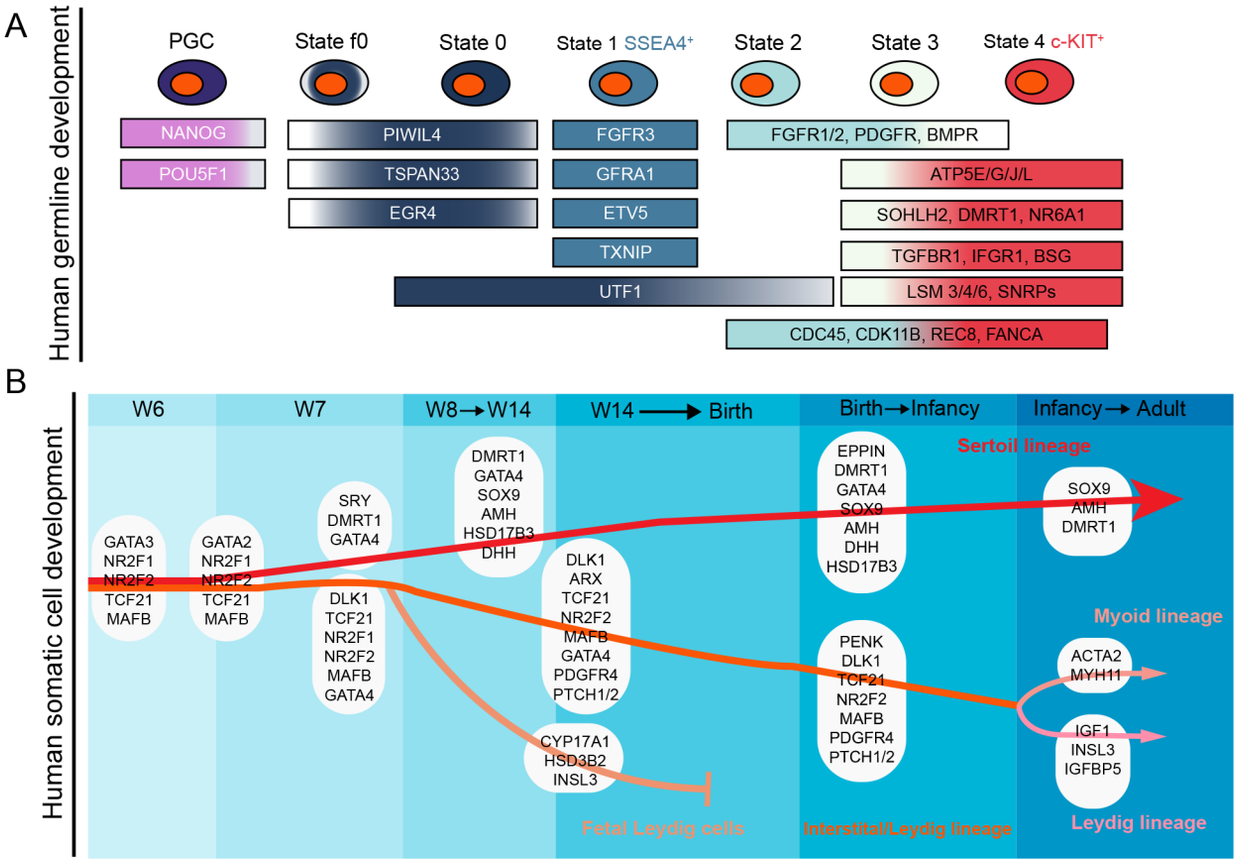

Figure 3 Developmental models of human germ cell lines and testis somatic cell lines

A: Expression programs of certain genes during transition from human PGC to SSC. B: Patterns of human testis somatic cell development from embryo to adult.

individuals (M5). Using UMAP cluster analysis, they observed tight clustering of cells from weeks W6 and W7, as well as among those from W8, W12, W15, and W16. By comparing these germ cells with previously published data, they determined that the first batch of germ cells consisted predominantly of W6–12 cells and some W15 cells, exhibiting a transcriptional profile similar to that of PGCs, marked by TFAP2C, KIT, NANOG, POUF51, and SOX17 (Guo et al., 2021; Wen & Tang, 2019). Germ cells in the subsequent batch primarily consisted of W15, W16, and M5 cells, which expressed State 0 markers rather than PGC markers, including PIWIL4, EGR4, MSL3, and TSPAN33 (Guo et al., 2018, 2021), with progressively stronger expression over time, in contrast to the expression patterns of new genes. As these cells transitioned from the pluripotent state of PGCs during the fetal to infant stages, they were designated as interval State f0 cells. Subsequent cell taxa corresponded to adult states 0–4 (Figure 3A), consistent with previous research (Sohni et al., 2019).

While single-cell transcriptome sequencing technology has revealed that SSCs are more diverse than can be categorized using karyotypes (A-pale, A-dark, B-type), a complete transcriptional profile associated with karyotypic categorization had not yet been established. The existence of different SSC states suggests that spermatogonial differentiation is not binary but rather a more gradual process, possibly linked to their functional roles. Recognizing this dynamic process is vital for understanding the mechanisms that regulate stem cell proliferation and differentiation, as well as SSC behaviors related to reproductive fitness, senescence, and germline mutations.

ScRNA-seq mapping reveals human testicular somatic cytogenesis

SSCs are pivotal not just for maintaining testicular homeostasis but also for their essential role in establishing and maintaining ecological niches along the tubular wall, facilitating orderly SSC self-renewal and differentiation. Consequently, the study of somatic cell development is crucial. Researchers have identified markers of Sertoli cells (SOX9, AMH, and INHA) (Baert et al., 2015; Hemendinger et al., 2002; Sohni et al., 2019), testicular Leydig stromal cells (IGF1, DLK1, and INSL3) (Baert et al., 2015; Guo et al., 2020, 2021), and testicular peritubular myofibroblasts (ACTA2 and MYH11), utilizing these markers to classify specific cell subpopulations from high-dimensional single-cell data, laying the foundation for further analysis. Based on testicular single-cell data from embryos, fetuses, infants, and adolescents and observations of immunological staining for specific cellular markers, Guo et al. (2020, 2021) reported that human Sertoli and Leydig cells share common progenitors. Furthermore, they classified the embryo-fetal testicular somatic cell population into seven developmental stages (A–G): A: common progenitor cells; B: embryonic Leydig progenitor cells; C&D: fetal Leydig progenitor cells; E: fetal Leydig cells; F: embryonic Sertoli progenitor cells; and G: fetal Sertoli cells. Notably, in contrast to mice, human fetuses lack myeloid cells (Wen et al., 2016). Heterogeneous progenitors of Leydig and Sertoli cells are present within the embryo during the early developmental stages at W6–7, with differentiation into Sertoli

and Leydig cell lineages at W7–8. Interestingly, in the Leydig cell lineage, the expression levels of genes related to DNA replication, proliferation, and the cell cycle are up-regulated, indicating an expansion phase aligning with the post-W8 increase in Leydig cell numbers. In contrast, genes expressed in the Sertoli cell lineage are related to chromatin assembly (Guo et al., 2021). The Leydig cell lineage also shows an increase in the expression of Gene Ontology (GO) terms related to the extracellular matrix, cell adhesion, and glycoproteins and an up-regulation in the expression of genes related to the Notch and Hedgehog signaling pathways, consistent with the known role of fetal Leydig cells in the production of androgens in mouse embryos (Guo et al., 2021; Shima et al., 2013, 2015).

To further characterize the developmental timing of fetal Leydig cells, immunofluorescence staining for CYP17A1 (a gene related to steroid synthesis) revealed no fluorescent signal in the germinal ridge epithelium at W5.5 weeks, whereas a strong fluorescent signal was observed in the Leydig cell (non-cordate) zone after W7, suggesting that Leydig cells first appear after approximately seven weeks of embryonic development, consistent with the single-cell data (Guo et al., 2021). In addition, not all Leydig cells in testicular tissue sections from eight-week-old mice exhibited positivity for CYP17A1, and no CYP17A1 signal was found in postnatal fetal samples. These observations led to the hypothesis that during Leydig progenitor cell development, some cells first differentiate into fetal Leydig cells, providing signaling molecules needed for testicular development, while other cells (CYP17A1-) remain capable of self-renewal, providing a differentiation pool for Leydig and peritubular myoblast-like cells after birth. Furthermore, monitoring CYP17A1 signaling revealed that fetal Leydig cells differentiate or vanish after birth, consistent with mouse observations (Svingen & Koopman, 2013). Guo et al. (2020) demonstrated that, in the human testis during adolescence, Leydig and myoid cells share similar progenitors during the prepubertal phase. To further determine the differentiation fate of CYP17A1− LCs, fetal Leydig progenitors (C, D, E), neonatal Leydig cells, and postnatal and adult Leydig/muscle-like cells were extracted and combined. Pseudotemporal analyses using Monocle demonstrated that fetal Leydig progenitors give rise to postnatal and prepubertal Leydig/muscle-like progenitors (Guo et al., 2021).

Cell cluster analysis at different developmental stages revealed that somatic cells from W8–16 exhibit distinct cellular markers specific to the Leydig or Sertoli lineages, which are absent in the W6 to W7 period (Guo et al., 2021). Further investigations revealed that the embryonic mesenchymal lineage expresses DLK1 and TCF21, while the embryonic support lineage expresses SOX9, AMH, DMRT1, and SRY (Guo et al., 2021). To identify key transcription factors, Guo et al. (2021) identified a group of GATA family transcription factors with sequential and nonoverlapping expression profiles: notably, GATA3 was broadly expressed throughout the germinal epithelium during W5 and in the mesenchymal cell lineage in W6–7, before gradually disappearing in W8. While GATA2 expression was not observed by immunofluorescence, GTAT2 was found to be expressed during human gonadal development, predominantly in extragonadal tissues and mesonephric sites, based on single-cell spatial transcriptome and smFISH techniques (Garcia-Alonso et al., 2022). GATA4 is highly expressed in the gonads, while GATA4+ somatic epithelial cells differentiate into the first wave of Sertoli cells *in vivo*, with ultimate stable expression in the Sertoli cell lineage (Garcia-Alonso et al., 2022; Guo et al., 2021). Other transcription factors, such as NR2F1, NR2F2, MAFB, and TCF21, are expressed relatively early (similar to GATA2). However, the expression of TCF21 and NR2F2 is sustained in the Leydig lineage and absent in the Sertoli lineage (Guo et al., 2021) (Figure 3B).

Organ development during embryogenesis relies on the establishment of a vascular network, which provides oxygen and nutrients and plays a guiding role in the pattern of organ development. Vascularization is necessary to induce the formation of primitive cord-like structures in adult testicular seminiferous tubules (DeFalco et al., 2014). Fetal macrophages, particularly those derived from the yolk sac and serving as the primary myeloid cells during testicular morphogenesis, are essential for vascular remodeling and compartmentalization of organ spaces, facilitating interactions with various cell types to promote testicular development (DeFalco et al., 2014). In the mature testis, macrophages are generally classified into mesenchymal types, originating from the yolk sac during fetal development and later being replaced by bone marrow-derived ones, and peritubular types, which emerge in the postnatal testis as progeny of bone marrow-derived monocytes (Lokka et al., 2020; Wang et al., 2021). Mesenchymal macrophages are reportedly derived primarily from the fetal liver, whereas peritubular macrophages are generated from embryonic precursors at birth (Lokka et al., 2020). In recent research, using scRNA-seq and 10x Visium, two different types of rare tissue-resident macrophage clusters were identified in the early gonads of human fetuses: (1) SIGLEC15+ fetal testicular macrophages (ftMs), with osteoblastic osteoblast-like features (SIGLEC15, ACP5, ATP6V0D2, and MMP9); and (2) TREM2+ ftMs, with microglia-like features (TREM2, P2RY12, and SALL1) (Garcia-Alonso et al., 2022). Compared with tissue repair macrophages (92.2%) in developing tissues, SIGLEC15+ and TREM2+ ftMs accounted for 2.8% and 5%, respectively. SIGLEC15+ ftM cells, present in the peritubular space around the testicular cords, interact with endothelial and mesenchymal cells via extracellular matrix proteins (Garcia-Alonso et al., 2022) (Figure 4A). The number of SIGLEC15+ ftM cells peaks at approximately 8–14 gestational weeks, coinciding with the period of mesonephric endothelial cell migration, during which endothelial cells from the mesonephric kidney migrate to the developing testis and assemble in the coelomic cavity arteries, necessary processes for testicular cords, thus mirroring findings that osteoblasts promote bone angiogenesis through metalloproteinase 9 (MMP9) (Garcia-Alonso et al., 2022). This evidence suggests that SIGLEC15+ macrophages are important for the formation of somatic arteries in the fetal testis. TREM2+ ftMs are the first immune population predominantly located within the developing testicular cords (Figure 4A). TREM2+ macrophages with microglial cell-like features are also present in fetal skin. Although microglia-like cells are associated with peripheral nerves in other tissues (skin, intestines, sciatic nerve), they do not express core microglial genes, including SALL1. Testis TREM2+ ftMs, including SALL1 (Garcia-Alonso et al., 2022), share the same transcriptomic profile as brain microglia, potentially reflecting a common origin and function. As TREM2+ ftMs interact with Sertoli cells and spermatogonia through binding within seminiferous tubules, it is suggested

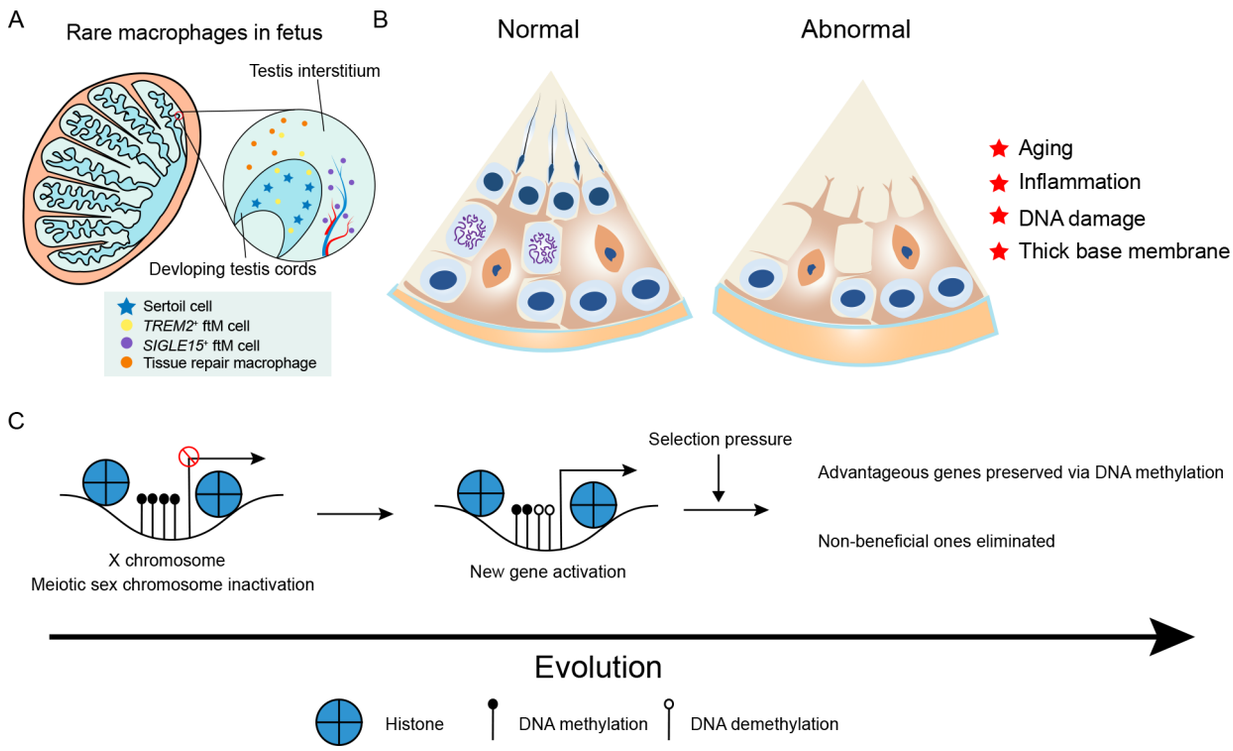

Figure 4 Rare macrophage localization, abnormal spermatogenesis, and MSCI evolution

A: Schematic showing spatial location of rare macrophages in fetuses. B: Schematic showing differences between normal and abnormal spermatogenesis. C: Schematic showing importance of MSCI during evolution and activation and reservation of new genes depending on MSCI mechanism.

that these cells may play a role in removing apoptotic germ cells.

The above studies suggest that the regulation and maintenance of the ecological niche of testicular stem cells play essential roles in organ development, vascular network formation, undifferentiated precursor cell pooling, and testis regenerative potential. During fetal life, a portion of Leydig progenitor cells differentiate into fetal Leydig cells, which provide essential signaling molecules and androgens for testicular development. Another portion continues to maintain self-renewal to ensure the stability of the stem cell pool, providing additional reserve cells for subsequent testicular development. During gonadal development, several rare macrophages play essential roles in testicular cord formation and vascular network establishment. Although single-cell sequencing technology has been used to analyze rare cell populations and their transcriptional profiles during testicular development, epigenetic regulation is still unclear. Furthermore, the mechanisms underlying the regulation and transformation of several pluripotency marker genes and transcription factors have not been fully elucidated. As such, additional research on the process of transcription splicing, working mode of the spliceosome, and diversity of transcripts at any given time is needed.

ABNORMAL SPERMATOGENESIS

According to the World Health Organization, the global prevalence of infertility in childbearing-age couples is 12.5%–17.5% (Njagi et al., 2023). To date, however, no single causative factor has been identified. In men, infertility is most commonly due to low or absent sperm levels or abnormal sperm morphology and motility. One of the most severe manifestations is Sertoli cell-only syndrome, which, as its nomenclature suggests, presents as a disorder of spermatogenesis, with tissue sections showing the disappearance of spermatogenic cells in the curved seminiferous tubules of the testis, leaving only testicular support cells. This syndrome is clinically defined as nonobstructive azoospermia (NOA). Although single-cell and polygenic mutations are associated with the pathogenesis of NOA, its etiology cannot be determined in 80% of patients, in whom the condition is considered idiopathic germ cell aplasia (iGCA) (Alfano et al., 2021). Male infertility is also associated with an increased risk of cancer, heart metabolic disease, and premature death (Kasman et al., 2020).

Based on single-cell transcriptome sequencing of testes from individuals with iGCA, Alfano et al. (2021) revealed the presence of senescence, inflammation, and DNA damage within the somatic microenvironment. Notably, compared to normal spermatogenic testes, immune cell populations in iGCA samples exhibited up-regulation in several immune-related GO pathways, including “MHC class II antigen presentation”, and “γ-interferon signaling pathway”. Furthermore, T cells were only present in iGCA samples, dominated by CD8+ tissue-resident memory (TRM) T cells, CD8+ CD69+ T cells expressing granzymes K and M, proinflammatory factors CCL4 and CCL5, and cytokine IL-32, possibly due to previous inflammation or autoimmune-induced localized chronic inflammation (Alfano et al., 2021). Further resolution of the somatic cells revealed that Leydig cells in the iGCA testicular samples possessed similar transcriptional profiles to those of neonatal Leydig cells (Alfano et al., 2021; Guo et al., 2020).

Collagens I and IV are the main forms of collagen in the basement membrane, with collagen I also forming a thin layer around the germinal tubules in normal, mature males. However, in iGCA patients, there is a notable thickening of

collagen I within the germinal tubules (Figure 4B) (Alfano et al., 2021). This aberrant collagen accumulation in the basal layer signifies tissue aging. In addition, HMGB1, a prototypical nuclear danger-associated molecular pattern (DAMP) protein, is translocated to the cytoplasm and secreted into the extracellular milieu as a signal following inflammatory injury. HMGB1 belongs to a large class of senescence-activated secreted phenotypic proteins (SASPs), whose secretion is associated with senescence. SASPs are related to chronic DNA damage, and the presence of phosphorylated H2AX (γH2AX) in the nucleus is correlated with the presentation of DNA damage in iGCA testes (Alfano et al., 2021).

Based on the above findings, senescence, inflammation, and DNA damage clearly occur within the somatic cell microenvironment of patients with iGCA (Figure 4B). Structurally aberrant nuclei may directly contribute to the dysfunctional transcriptional landscape in iGCA, and vice versa; a harsh microenvironment may lead to structural damage to otherwise weak nuclei, followed by substantial transcriptional changes. In addition, prolonged inflammatory stress may lead to DNA damage overload, resulting in genomic instability and cancer induction. Di Persio et al. (2021) compared the spermatogenesis of healthy subjects to cryptozoospermic men using scRNA-seq. A significant decrease in the percentage of cells from the pachytene spermatogonia to spermatozoa stage in the testes of cryptozoospermic men was detected following pathological characterization, and significant increases in the numbers of peritubular myoid cells and macrophages were observed at the somatic cell level. The percentages also increased significantly, with the most pronounced change occurring in peritubular myeloid cells. In addition, PIWIL4+ SSCs increased but A-dark reserve SSCs decreased (Di Persio et al., 2021).

Interestingly, extended expression of the transcription factor EGR4 in SSCs resulted in decreased expression of UTF1 at the transcriptional and protein levels, altering the chromatin structure of spermatogonial cells, potentially associated with a decrease in the A-dark reserve of SSCs. In cryptozoospermia, disruption of the testicular microenvironment caused transcriptional changes in spermatogonia, ultimately affecting spermatogenesis (Di Persio et al., 2021). Studies have shown that male reproductive health declines with age, and aging is often associated with elevated body mass index (BMI) (>30), as evidenced by collagen deposition in the seminiferous tubule walls, spermatogenesis disruption, and testicular inflammation (Nie et al., 2022). Previous research has suggested that obesity-related declines in male fertility may be due to hormonal imbalances, inflammation, and elevated testicular temperature (Craig et al., 2017). Given the extensive age-related somatic cell modifications in the testes of aging men, it is highly likely that these somatic cells undergo age-related alterations, impairing their ability to support spermatogonial cell self-renewal and differentiation.

However, the explanatory power of scRNA-seq is limited, with a combination of multiomics techniques (e.g., 10x Visium; scRNA-seq; scATAC-seq; CUT&Tag; Proteomics; Metabolomics) needed to further investigate the causes of abnormal spermatogenesis. Large-scale cohort studies are also necessary to determine the factors that contribute to the development of this disease. The identification of proinflammatory factors in human testes with abnormal spermatogenesis is also needed to establish anti-inflammatory treatments to ameliorate decreased fertility.

MOLECULAR EVOLUTION OF MAMMALIAN SPERMATOGENESIS

The rapid evolution of mammals is significantly influenced by competitive selection of spermatozoa. To achieve reproductive success, males are subjected to various evolutionary pressures. Variations in reproductive traits, such as testis size, sperm production rate, sperm morphology, and characteristics of other testicular somatic cells, are fundamentally regulated at the molecular level within the testis (Ramm et al., 2014). Studies have indicated that testes exhibit the most pronounced changes in gene expression across mammalian organs, highlighting their evolutionary significance (Brawand et al., 2011; Cardoso-Moreira et al., 2019). Testis-specific genes often show signs of positive selection in their coding sequences (Murat et al., 2023). In addition, many genes that emerged through evolutionary processes are predominantly expressed in the testis, suggesting a role in the rapid evolution of testicular phenotypes. Spermatogenic cell chromatin undergoes extensive remodeling during spermatogenesis, culminating in the tight assembly of DNA in the sperm head. This remodeling leads to the transcriptional leakage of a small number of genes in the genome, leading to the frequent emergence of new testis-expressed genes throughout evolution (Figure 4C) (Murat et al., 2023). The differentiation of sex chromosomes from ancestral autosomes also triggered meiotic sex chromosome inactivation (MSCI) in eutherians and marsupials (therians), promoting the establishment of backup gene copies on the X chromosome to replace parental genes during meiosis (Figure 4C) (Larson et al., 2018; Murat et al., 2023; Necsulea & Kaessmann, 2014). Despite MSCI, the X chromosome is enriched in testis-expressed genes, potentially facilitating the preservation of male-beneficial mutations on this chromosome (Murat et al., 2023).

Recent research using single-nucleus RNA-seq (snRNA-seq) to analyze the testes of 11 species, encompassing three major mammalian lineages (eutherian, marsupial, and egg-laying monotremes) and birds (evolutionary outgroups), found that rapid testicular evolution was driven by late spermatogenic gene expression changes, amino acid substitutions, and novel genes, possibly due to reduced pleiotropic constraints, haploid selection, and chromatin structure conducive to transcription (Murat et al., 2023). The study also identified conserved gene expression patterns across species, with genes predominantly expressed in spermatogonia and testis-supporting cells clustering on the X chromosome throughout evolution, potentially offering a selective advantage for males (Figure 4C) (Murat et al., 2023). Further research revealed transcriptional differences between X and Y spermatozoa, with MSCI also present in monotremes, suggesting it may be a universal feature of the mammalian sex chromosome system (Murat et al., 2023). Previous analysis of the chromatin profiles associated with X chromosome reactivation in mice showed that nascent escape genes in spermatocytes carry high levels of repressive H3K9me3 before reactivation (Ernst et al., 2019). Comparative scRNA-seq-based studies in humans, macaques, and mice have also demonstrated that interspecies sex chromosomes all exhibit features of MSCI (Lau et al., 2020; Shami et al., 2020), although a small fraction of genes show transcriptional leakage, with the DNA damage response (DDR) pathway playing a dual role in maintaining genome integrity and

influencing MSCI (Lau et al., 2020).

While many single-cell transcriptomic datasets are available, comprehensive data on single-cell full-length transcriptomes remain scarce. Single-cell full-length transcriptomes offer the unique advantage of identifying different transcriptional variants, vital for elucidating specific molecular mechanisms. To understand the effects of post-transcriptional changes on spermatogenesis, data focusing solely on single-cell translation are necessary, presenting a significant challenge. Nonetheless, a method for detecting ribosome-binding sequences, known as ribosome-sequencing (Ribo-seq), has been established, with further refinement enabling Ribo-seq to be performed at the single-cell level (scRibo-seq) (Vaninsberghe et al., 2021). Notably, scRibo-seq marks an important advance in identifying significant cell-to-cell diversity in translation across individual cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

This paper provides a comprehensive review of single-cell sequencing technology and downstream analysis tools, as well as the molecular dynamics of germ and somatic cells during human spermatogenesis, disruption of homeostasis within the testes of patients with abnormal spermatogenesis, and molecular evolution of spermatogenesis in mammals. The application of scRNA-seq technology has significantly advanced our understanding of spermatogenesis and molecular evolution, overcoming various limitations inherent in traditional bulk sequencing approaches. This advancement has been instrumental in identifying critical cell types and molecular pathways, enhancing our understanding of reproductive strategies and their evolutionary development. The ability to detect rare cells and low-abundance transcripts has been particularly valuable, offering new perspectives on male infertility and reproductive medicine.

While single-cell sequencing technology has shed light on the heterogeneity of spermatogenesis, it falls short in precisely mapping annotated SSCs in scRNA-seq datasets to species A-dark and A-pale states due to the absence of a universal standard for defining these cells, which currently relies solely on molecular markers. Future efforts need to focus on the construction of a more extensive testicular scRNA-seq database that categorizes cells in each developmental state, not only by molecular markers but also by cell-to-cell mapping relationships, potentially using tools like Cell BLAST (Cao et al., 2020). Moreover, while scRNA-seq has played a crucial role in revealing the causes of abnormal spermatogenesis, aging, and reduced fertility due to disease, a more comprehensive understanding of disease development and gene expression regulation will require joint multi-omics analyses and more extensive cohort studies.

The emergence of single-cell spatial transcriptomics in recent years has distinguished itself from traditional scRNA-seq by enabling library construction and sequencing directly on tissue sections (including formalin-fixed paraffin-embedded (FFPE) samples), thereby preserving the original spatial information of cells. For spermatogenesis, this technology has aided in elucidating how cellular interactions and the local microenvironment influence the progression of SSCs through various developmental stages, including self-renewal, differentiation, and eventual maturation into spermatozoa. Notably, spatial transcriptomics and single-molecule FISH (smFISH) techniques have facilitated the identification of two rare macrophage populations within the testis during human gonadal development (Garcia-Alonso et al., 2022). The integration of spatial transcriptomics into spermatogenesis research has not only enhanced our understanding of the complex cellular dynamics within the testis but also provided a platform for uncovering the molecular mechanisms that underpin SSC maintenance and differentiation. Mapping gene expression patterns in relation to cellular location has enabled researchers to identify key regulatory factors and signaling pathways that are active in specific regions of the testis, such as the spermatogonial niche and blood-testis barrier (Chen et al., 2021). This spatial resolution is crucial for understanding the microenvironmental cues that guide SSC behavior and for identifying potential therapeutic targets for treating male infertility and related disorders.

Over the course of evolution, animals have developed sexual reproduction, a process that significantly enhances the exchange of genes between populations and has enabled adaptation to selection pressures (Pincheira-Donoso and Hunt, 2017; Rosenthal and Ryan, 2022). Male reproduction, in particular, is subject to intense selection pressure, leading to the emergence of mechanisms such as MSCI in mammals, which helps protect against the continuation of certain mutations (Abe et al., 2022; Larson et al., 2018). Despite this, certain genes remain unaffected by MSCI, allowing for a reduction in the expression of genes that favor the emergence of new traits. Nevertheless, the impact of new genetic information and genes on offspring development remains uncertain, presenting a challenge in the transmission of genetic traits. The evolutionary process is slow, with MSCI and leakage mechanisms evolving as strategies for organisms to adapt to selective pressures.

Research on spermatogenesis in certain livestock species, such as cattle, goats, sheep, and pigs, remains underdeveloped and only a limited number of datasets are available (Huang et al., 2023; Yang et al., 2021; Yu et al., 2021; Zhang et al., 2021), hindering in-depth study of spermatogenesis and reproductive disorders in domestic animals. This gap is exacerbated by the incomplete annotation of the GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases for livestock and the lack of livestock-specific CellPhoneDB databases, slowing progress of related field research. Addressing these deficiencies requires the accumulation of additional histological data to establish livestock-specific databases.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.B.C. wrote and edited the manuscript. M.F.Z. and F.Y. contributed perspectives and conducted proofreading. J.L.H. conceived and performed the final editing of the manuscript. All authors read and approved the final version of the manuscript.

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