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Transcriptome analysis of meiotic and post-meiotic spermatogenic cells reveals the potential hub genes of aging on the decline of male fertility

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ABSTRACT

Genetic and epigenetic changes in sperm caused by male aging may be essential factors affecting semen parameters, but the effects and specific molecular mechanisms of aging on male reproduction have not been fully clarified. In this study, to explore the effect of aging on male fertility and seek the potential molecular etiology, we performed high-throughput RNA-sequencing in isolated spermatogenic cells, including pachytene spermatocytes (marked by the completion of chromosome synapsis) and round spermatids (produced by the separation of sister chromatids) from the elderly and the young men. Functional enrichment analysis of differentially expressed genes (DEGs) in round spermatids between the elderly and young showed that they were significantly enriched in gamete generation, spindle assembly, and cilium movement involved in cell motility. In addition, the expression levels of DEGs in round spermatids (post-meiotic cells) were found to be more susceptible to age. Furthermore, ten genes (AURKA, CCNB1, CDC20, CCNB2, KIF2C, KIAA0101, NR5A1, PLK1, PTTG1, RAD51AP1) were identified to be the hub genes involved in the regulation of sperm quality in the elderly through Protein-Protein Interaction (PPI) network construction and measuring semantic among GO terms and gene products. Our data provide aging-related molecular alterations in meiotic and post-meiotic spermatogenic cells, and the information gained from this study may explain the abnormal aging-related male fertility decline.

1. Introduction

Couples are now more likely to delay childbearing, especially in developed countries, for economic reasons or career planning (Cedars, 2015 Jun). Although there has been much attention paid to the effects of aging on female fertility, including spontaneous abortion and chromosomal abnormalities, little attention has been paid to the impact of age on male fertility because because even 70-year-old men's sperm can still fertilize the eggs (Kühnert and Nieschlag, 2004). Nevertheless, several studies suggest that daily sperm production, sperm viability, and total sperm count are negatively correlated with age (Ng et al., 2004 Aug; Colasante et al., 2019 Jan 18; Winkle et al., 2009 Jan; Condorelli et al., 2020). Sperm genetic and epigenetic changes caused by male aging may be essential factors affecting semen parameters, but the specific molecular mechanism has not been clarified.

The median sperm concentration is $\sim 24 \times 10^6$/ml for a healthy adult (Colasante et al., 2019 Jan 18), and spermatogenesis is a highly sophisticated and coordinated process (Neto et al., 2016 Nov) that involves mitosis (amplifying to maintain the stem cell pool), meiosis (reduction division into round spermatids) and spermiogenesis

Abbreviations: DEGs, Differentially Expressed Genes; DSP, Daily Sperm Production; micro-TESE, Microscopic Testicular Sperm Extraction; DMEM, Dulbecco's Modified Eagle Medium; GO term, Gene Ontology term; PB1, First Polar Body.

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(morphological transformation of round spermatids to spermatozoa) (de Kretser et al., 1998 Apr). There are some critical molecular biological events, such as chromosome duplication, homologous chromosome synapsis, sister chromatid separation, and so on (Ohkura, 2015). Spermatocytes and round spermatids are the main cell types that make up a high proportion in the seminiferous tubules of adult testes. Pachytene spermatocytes belong to the prophase of meiosis I, which is marked by the completion of chromosome synapsis. Round spermatids with haploid genetic material are produced by the separation of sister chromatids from secondary spermatocytes (Heller and Clermont, 1963 Apr 12). Previous reports suggest that although younger and older men had similar spermatogonia production per g parenchyma (PDSP), PDSP based on late primary spermatocytes and DSP (daily sperm production) based on early spermatids were lower in older men than in younger men, suggesting that the difference in sperm production rates with age may occur in meiotic prophase and meiotic divisions (Johnson et al., 1987 Oct). Several studies have shown that the testes volume decrease, the number of germ cells decreases, and abnormal multinucleated spermatocytes and round spermatids appear as male age increase,which leads to the shrunken diameter of the seminiferous tubules and vacuolization of the seminiferous epithelium (Dong et al., 2022; Santiago et al., 2019 May 16). In addition, chromosome nondisjunction and breaks increase with aging in male germ cells, further indicating that postmeiotic cells are particularly susceptible to the effect of aging (Sloter et al., 2004 Apr; Schmid et al., 2007).

To further elucidate the transcriptome changes between the old and the young, especially highlighting the impact of aging on meiotic prophase cells and postmeiotic cells, we isolated pachytene spermatocytes (4 N) and round spermatids (N) from the elderly and young adults for high-throughput RNA-sequencing analysis. Through bioinformatics analysis of sequencing results, we found the following conclusions: 1) The foldchange level of up-/down-regulated transcripts in haploid spermatids (post-meiotic cells) identified from older men and young men is more susceptible to age than in pachytene spermatocytes; 2) Functional enrichment analysis of differentially expressed genes (DEGs) in round spermatids between the elderly and young showed that they were significantly enriched in gamete generation, spindle assembly, and cilium movement involved in cell motility; 3) PLK1 and CDC20 were identified to be the most important genes involved in the regulation of post-meiotic spermatogenic cell development and sperm quality in the elderly men. Our data uncover interesting molecular evidence regarding the aging-related effects in meiotic and post-meiotic spermatogenic cells and might provide some reference for subsequent diagnosis and treatment of aging-related male reproductive disease.

2. Materials and methods

2.1. Human testicular tissue collection

Testicular tissues of young men were obtained from the abandoned testicular biopsy tissue from obstructive azoospermia (OA) patients, and testis tissues of older men were collected from prostatic cancer patients after surgery according to protocols approved by the Medical Ethics Committee from Tongji Medical College, Huazhong University of Science and Technology. All patients signed informed consent for the collection and use of their samples for this study. Testicular samples of young men were obtained from patients (aged 22–30 years) with obstructive azoospermia who underwent micro-TESE at the Center for Reproductive Medicine, Huazhong University of Science and Technology. Testicular samples of old men were obtained from patients (aged 80–90 years) with prostatic cancer. Written informed consent was obtained from all patients. The study was approved by the ethics committees of Tongji Medical College, Huazhong University of Science and Technology.

2.2. Human spermatogenic cell isolation

Human pachytene spermatocytes and round spermatids were isolated by the STA-PUT method as described previously with minor modifications (Bellvé, 1993). The STA-PUT method was used based on sedimentation velocity at unit gravity to purify the pachytene spermatocytes and round spermatids from testes. Obtained testicular samples were digested with collagenase IV (1 mg/ml) to become a loose state of a single shorter seminiferous tubule and washed with PBS and centrifuged at 500g. Then, the pellet was digested again with Trypsin containing DNase I (1 mg/ml) to prepare a single-cell suspension. Trypsinized testicular tissue suspension requires multiple pipettings and a 70 um single-cell filter to obtain a reliable and stable single-cell suspension. A cell separation apparatus was used to load the single-cell suspension followed by a 0.5–5 % BSA (bovine serum albumin) gradient. DMEM supplemented with 10 % BSA was prepared the day before to avoid bubbles in the medium. The next day, 10 % BSA should be diluted with DMEM to prepare 0.5 %~5% BSA solution with a total of 10 gradients. 0.5 %~5% BSA in DMEM were loaded into the separation apparatus chamber orderly and take care to avoid interference between different concentration layers. After ~ 3 h of sedimentation, cell fractions were harvested. Start the collection in 1 ml increments and collect a total of about 40 tubes of cell suspension. Different cell types were determined according to morphological characteristics and cell diameter under a light microscope. The purity of pachytene spermatocytes and round spermatids of each sample for RNA sequnencing reached about 90 %.

2.3. Histological analysis and immunohistochemical staining

Human testis were fixed in Bouin's solution at room temperature overnight and then embedded in paraffin. After five-micrometer sections were prepared, periodic acid Schiff (PAS) staining was performed using a standard protocal. Immunohistochemical (IHC) analysis of PLK1 (1:100; A2548; ABclonal) and CDC20 (1:100; A1231; ABclonal) expression were performed using standard protocal.

2.4. High-throughput RNA sequencing

Total RNA of pachytene spermatocytes or round spermatids from the young or the older people was isolated using TRIzol reagents (Invitrogen). A NanoDrop2000 Spectrophotometer was used to verify the RNA concentration, and 1 μg of total RNA from each sample was used to prepare the mRNA libraries according to the manufacturies' instructions (Cat.No.RS-122–2101, Illumina). All samples were sequenced using the Illumina Hiseq 4000 platform to generate 150 bp paired end reads. Fastq files were processed using Trimmomatic. Reads containing poly-N and low-quality reads were removed to obtain clean reads. The clean reads were mapped to the reference genome GRCh38 using bwa. The raw counts of each gene were calculated using bedtools. Differentially expressed genes (DEGs) were identified using the DESeq2 (1.34.0) under R environment (R-4.1.2). $|\log_2 \text{fold change}| >= 2$ and p-value < 0.05 were set as the threshold for significantly differential expression.

2.5. Single-cell sequence data analysis

The download 10x single-cell sequence data were processed with Seurat (4.0.5) (Butler et al., 2018 Jun) package, which was used for cell filtration, normalization of data, reduction of dimensionality reduction, cell cluster and differentially expressed gene identified. The U-MAP (Becht et al., 2019) and cluster analysis were performed on the combined dataset, using the top 2000 highly variable genes. Differential expressed genes of different clusters was conducted using a Wilcoxon Rank Sum test with p value < 0.05, logfc.threshold $= 0.25$. Cells types were allocated to each cluster using the known marker genes as previously reported (Nie et al., 2022). Single-cell pseudotime trajectories

were constructed with Monocle2 (2.22.0) (Qiu et al., 2017) according to the provided documention. The data reanalyzed in this paper were from published human testicular scRNA-seq datasets (Nie et al., 2022; Hermann et al., 2018). All single-cell data analyses were performed in the R environment (version: R-4.1.2).

2.6. Bioinformatics analysis

All RNA-seq data were analyzed under the R environment (version: R-4.1.2). Metascape online website (Zhou et al., 2019 Apr 3) was used for functional enrichment analysis, STRING online website (Szklarczyk et al., 2021), and Cytoscape software (Otasek et al., 2019 Sep 2) were used for protein interaction analysis and hub gene identification.

3. Results

3.1. Overview of differentially expressed genes (DEGs) between elderly and young spermatogenic cells

To investigate the effect of aging on spermatogenic cell development, we isolated the pachytene spermatocytes and round spermatids from elderly and young man testicular tissues using the STA-PUT method for high-throughput RNA-sequencing analysis and both cytological morphologies of isolated cells were provided in **Supplementary** Fig. 1**A-B**. The spermatogenesis process of the donor in this study was normal, and sperm were observed in the seminiferous tubules, as demonstrated by the PAS staining (**Supplementary** Fig. 1**.C-D**). Gene expression in spermatocytes and round spermatids from the elderly and young groups were comparable, respectively, suggesting consistency of sample expression and standardization (**Supplementary** Fig. 2**A-B**). To demonstrate cell identity in our RNA-sequencing, we examined the expression level of several representative genes in RNA-seq data: WT1 (a marker for Sertoli cells), GFRa1 (a marker for spermatogonia), SYCP3 (a marker for spermatocytes) and PRM3 (a marker for round spermatids). As expected, the results showed that WT1 and GFRa1 are poorly expressed in isolated spermatocytes and round spermatids, and SYCP3 and PRM3 are highly expressed in isolated spermatocytes and round spermatids, respectively (**Supplementary** Fig. 2**C-D**). Further principal component analysis (PCA) shows separation between the young and old groups (**Supplementary** Fig. 3**A-B**). Correlation heatmaps show good repeatability between samples within each group (**Supplementary** Fig. 3**C-D**). Clustering heat-maps were used to visualize genes that differed significantly between the elderly and the young spermatogenic cells (Fig. 1**A-B**). The results showed that, in pachytene spermatocytes, 688 genes up-regulated and 1024 down-regulated ($|\log_2$fold change$| >= 2$, p-value < 0.05) in the older group compared with the young group, as shown in the volcano map (Fig. 1**C**). Whereas in round spermatids, 1,289 genes were identified as up-regulated in the older group and 1,210

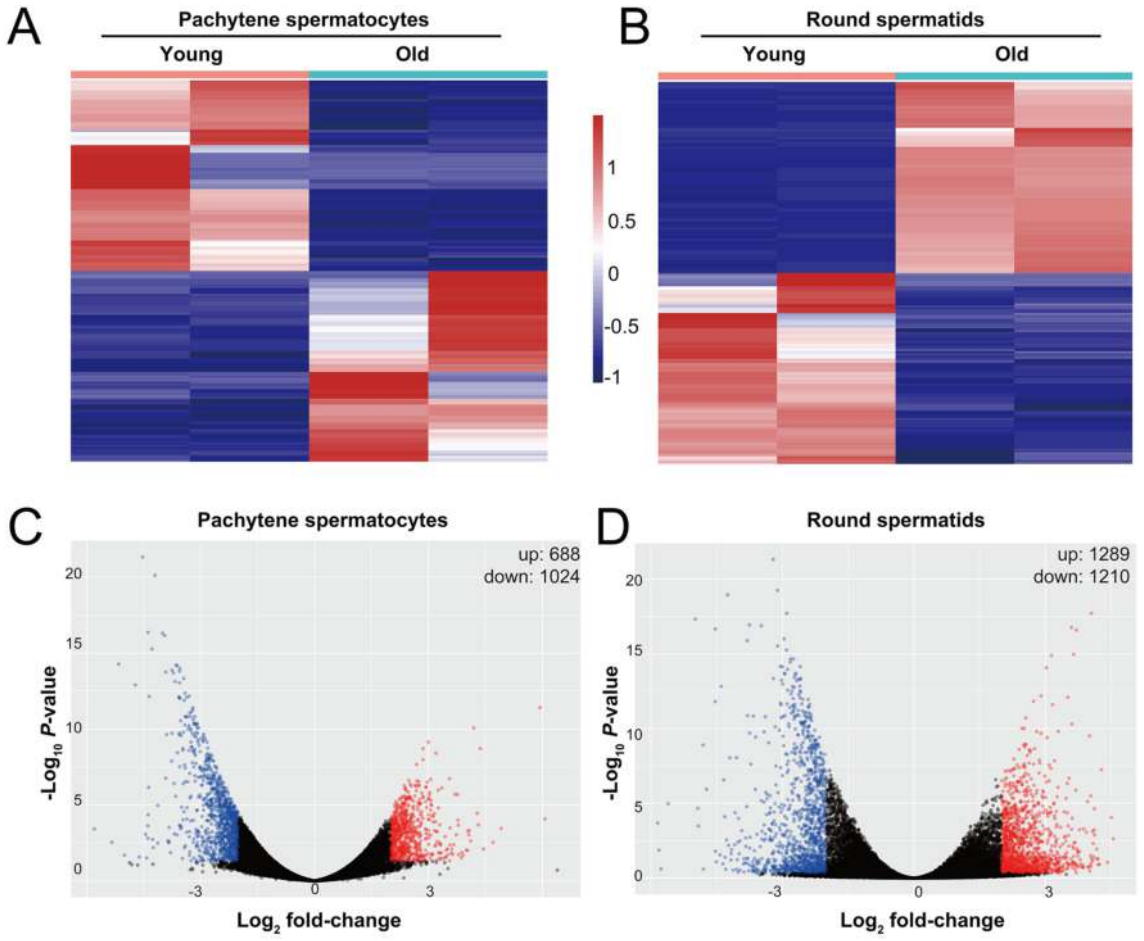

Fig. 1. Differential gene expression in pachytene spermatocytes and round spermatids from the older men was identified by RNA high throughput sequencing. (**A**) Heatmap displaying the normalized differentially expressed genes (top 500) in pachytene spermatocytes between the old and young ($|\log_2$fold-change$| >= 2$, p-value < 0.05) and hierarchical clustering of the samples. (**B**) Heatmap displaying the normalized differentially expressed genes (top 500) in round spermatids between the old and young ($|\log_2$fold change$| >= 2$, p-value < 0.05) and hierarchical clustering of the samples. (**C**) Volcano plots of genes showing differentially expressed genes of pachytene spermatocytes between the old and the young. $\log_2$fold-changes and corresponding p-values of all genes were considered to build the volcano plot. Up-regulated genes ($\log_2$fold-change $>= 2$, p-value < 0.05) are depicted in red. Down-regulated genes ($\log_2$fold-change $<= -2$, p-value < 0.05) are depicted in blue. (**D**) Volcano plots of genes showing differentially expressed genes of round spermatids between the old and the young.

down-regulated (Fig. 1**D**). Although the overall average transcriptome level was comparable in pachytene spermatocytes and round spermatids (**Supplementary** Fig. 4**A**), the foldchange of genes was significantly greater in round spermatids than that in pachytene spermatocytes (Fig. 2**A-D**). These data suggest that the level of up-/down-regulated transcripts in haploid spermatids (post-meiotic cells) is more susceptible than that in pachytene spermatocytes.

3.2. Functional analysis of DEGs in pachytene spermatocytes and round spermatids

Metascape, which is mainly based on gene ontology (including Biological Process, Cellular Components, and Molecular Functions) analysis, was used for enrichment analysis of genes. Interestingly, we utilized Metascape to analyze the DEGs in pachytene spermatocytes and round spermatids and found that in pachytene spermatocytes, the DEGs were mainly enriched in reproductive structure development (GO:0048608), positive regulation of cell motility (GO:2000147), and regulation of reproductive process (GO:2000241) (Fig. 2**E**). In round spermatids, DEGs were mainly enriched in gamete generation (GO:0007276), spindle assembly (GO:0051225), cilium movement involved in cell motility (GO:006094), fertilization (GO: 0009566), and regulation of reproductive process(GO:2000241) (Fig. 2**F**). Moreover, disease-gene correlation analyses have shown that the DEGs in round spermatids were strongly associated with male infertility, testicular regression syndrome, and chromosomal abnormalities, such as XX males and Turner syndrome (**Supplementary** Fig. 4**B**). These functional enrichment and disease correlation analyses of DEGs between the elderly and the young suggest that these differential genes may be critical genetic factors leading to lower sperm quality in older men.

3.3. Protein-protein interaction network construction and identification of hub genes

Since there is a strong association between the DEGs in elderly round spermatids and male infertility diseases, we next searched for the hub genes that play a crucial role in elderly male fertility. To determine the hub genes that affect spermatogenesis by aging, we mapped the protein–protein network of DEGs enriched for male infertility-related function using the Online STRING website (**Supplementary Fig. 5**). With the help of the Cytohubba plugin in Cytoscape software, the top 10 genes involved in influencing the fertility of older men were identified, including CCNB1, AURKA, CCNB2, KIF2C, PTTG1, CDC20, KIAA0101, PLK1, RAD51AP1, and NR5A1 (Fig. 3**A**). Further functional enrichment analysis using an online string website revealed that these genes were involved in several biological processes, including condensed nuclear envelope disassembly (CCNB1, CCNB2, PLK1), histone-serine phosphorylation (CCNB1, AUKRA), positive regulation of ubiquitin-protein ligase activity (CDC20, PLK1), regulation of mitotic sister chromatid separation (CCNB1, PTTG1, CDC20, PLK1), mitotic spindle assembly checkpoint (CDC20, PLK1), mitotic metaphase plate congression (CCNB1, KIF2C), centrosome cycle (AUKRA, KIAA0101, PLK1), meiosis I (AURKA, PTTG1, PLK1). Of note, when we re-analyzed the published single-cell sequencing data from human testicular tissue (Hermann et al., 2018) and found that five (CCNB1, AURKA, CCNB2, CDC20, PLK1) of the top 10 genes showed low expression in spermatids and high expression in spermatocytes during pseudotime (Fig. 3**C**), which were abnormally upregulated in elderly round spermatids (Fig. 3**B**). In addition, three genes (KIF2C, NR5A1, RAD51AP1) showed higher expression in spermatids and lower expression in spermatocytes during pseodotime (Fig. 3**C**), which were abnormally downregulated in elderly round spermatids (Fig. 3**B**).

Furthermore, we tried to narrow down the hub genes by measuring semantics among GO terms and gene products, which provide quantitative analysis to calculate similarities between genes and gene groups. PLK1 and CDC20 were identified to be the most important genes involved in the regulation of aging on male post-meiotic cells (Fig. 3**D**). To further verify this, we downloaded the single-cell data of young and older man from the previously reported data (GSE182786) (Nie et al., 2022). Human germ cells were distributed into 6 clusters based on UMAP analysis and clusters were identified based on known cell-type specific marker genes (**Supplementary Fig. 6**). Results showed that PLK1 and CDC20 were extremely highly expressed in the Older Group1 in pachytene spermatocytes (Fig. 3**E-F**), which was consistent with the abnormal upregulation of PLK1 and CDC20 in round spermatids in our study. Although PLK1 and CDC20 seem to remain unchanged in round spermatids from the analysis (Fig. 3**G-H**), this may be caused by individual differences. Donors with histologically normal spermatogenesis were classified as Older Group1, and donors with clearly defective spermatogenesis were classified as Older Group2. Further immunohistochemistry assays showed that the expression levels of PLK1 and CDC20 in both spermatocytes and spermatids from older testicular tissues appear higher than in young testicular tissues. (Fig. 4**A-D**). These data suggest that hyperactivated activation of meiotic division-related genes in elder pachytene apermatocytes and post-meiotic spermatids may cause the decline of the ability to develop spermatids in older men.

4. Discussion

It has long been reported that older paternal age can affect sperm quality, sperm DNA integrity, chromosome structure, and epigenetic factors, but the specific molecular mechanism in aging human testis has not been well elucidated. The effects of aging on testicular molecular mechanisms have been partially elucidated in other mammals, such as mice, bovines, and dogs. Compared to the young dog, the degeneration of germ cells and atrophy of seminiferous tubules were much more severe in senile dogs (Bhanmeechao et al., 2018 Nov). Age affects gene expression and the population of cells expressing specific spermatogonial markers in the bovine testis (Giassetti et al., 2016 Jul). Microarray analysis of isolated spermatogonia stem cells/ progenitor from old (8 months) and young mice identified a number of genes that were expressed specifically in the older mice, such as *Icam1* and *Selp* (Kokkinaki et al., 2010). In addition, a large number of differentially expressed genes were identified by transcriptome and proteome analysis of spermatozoa and seminal plasma samples from infertile aging males (Bastos et al., 2017 Nov 28); however, the molecular mechanisms underlying male infertility or reduced fertility due to aging have not been well explained.

In this study, we first preliminarily revealed the potential molecular mechanisms underlying age-related male infertility phenotypes by analyzing pachytene spermatocytes and round spermatids isolated from the elderly and the young. The foldchange level of up-/down-regulated transcripts in round spermatids (post-meiotic cells) identified from older men and young men is more susceptible to age than in pachytene spermatocytes. Further protein–protein interaction network analysis identified several critical differentially expressed genes (old-vs-young) enriched in gamete generation, such as CCNB1, AURKA, CCNB2, KIF2C, PTTG1, CDC20, KIAA0101, PLK1, RAD51AP1, NR5A1. Almost all of the differentially expressed hub genes suggest to being related to DNA damage repair, chromosome stability, and cell cycle, which may correlate with the molecular mechanism of the influence of aging on the male sperm quality and chromosome stability. Of note, PLK1 and CDC20 were identified to be the most important genes involved in the regulation of aging on male post-meiotic cells. PLK1 was reported to perform several vital functions through the M phase of the cell cycle, including regulation of centrosome maturation and spindle assembly (Lane and Nigg, 1996), removal of cohesins from chromosome arms (Kang et al., 2006), inactivation of anaphase-promoting complex (APC) inhibitors (Jackman et al., 2003 Feb), and regulation of mitotic exit and cytokinesis (Roshak et al., 2000 Jun). Complete loss of PLK1 in mice is lethal (Lu et al., 2008), and the genetic variation may be responsible for recurrent miscarriages and female infertility (Maddirevula et al., 2020). PLK1

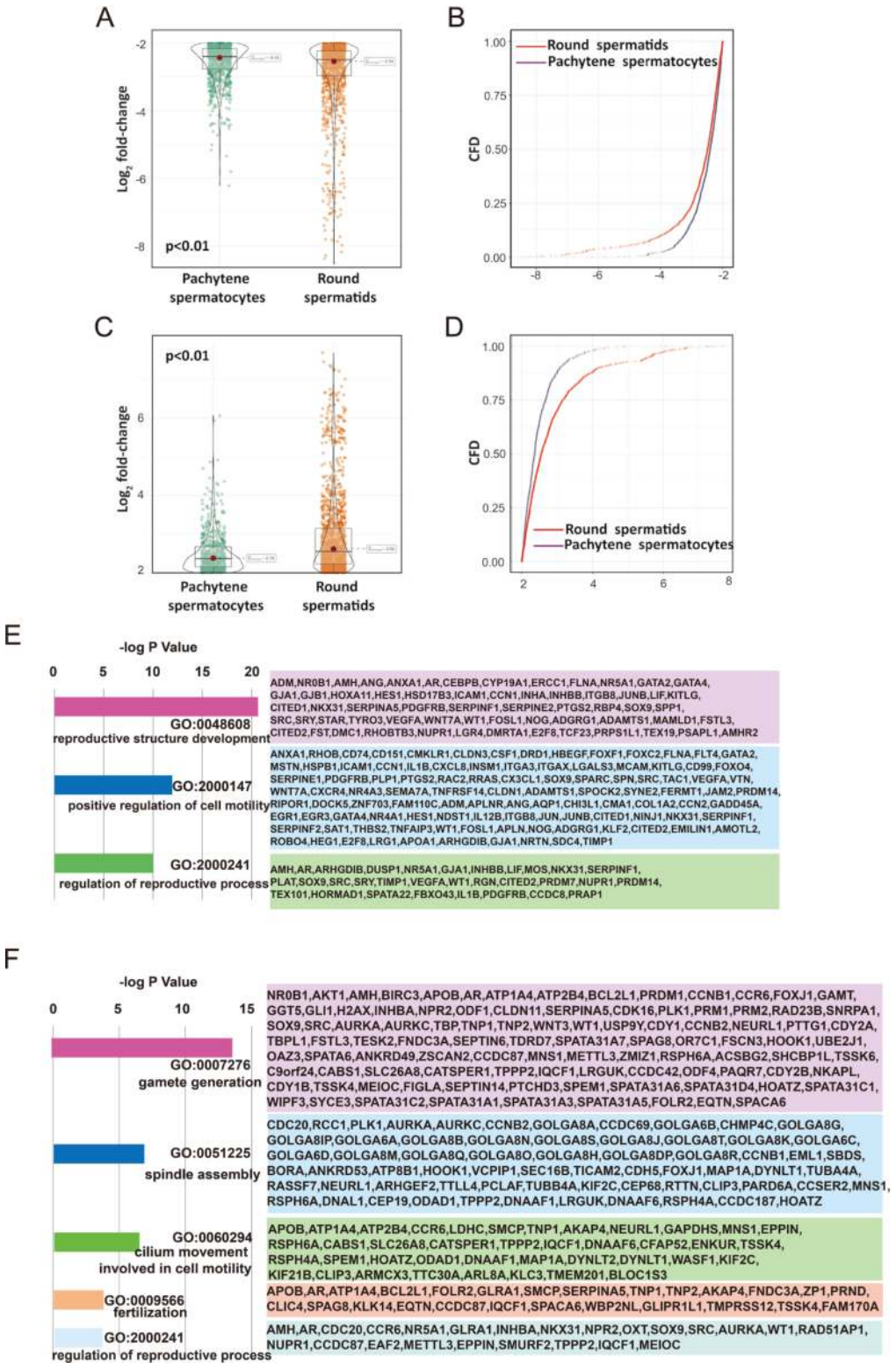

Fig. 2. Transcriptome analysis of differentially expressed genes (old-vs-young) in spermatogenic cells. (**A**) Differential analysis of down-regulated genes between pachytene spermatocytes and round spermatids in the elderly. (**B**) Cumulative frequency distribution (CFD) plot depicting the fold-changes of the down-regulated genes in the elderly between pachytene spermatocytes and round spermatids. (**C**) Differential analysis of up-regulated genes between pachytene spermatocytes and round spermatids in the elderly. (**D**) Cumulative frequency distribution (CFD) plot depicting the fold changes of the up-regulated genes in the elderly between pachytene spermatocytes and round spermatids. (**E**) The GO enrichment result (old-vs-young) of DEGs in pachytene spermatocytes. The horizontal axis represents the *p*-value in each enriched GO term. On the right are the DEGs in pachytene spermatocytes enriched in the corresponding functional GO term. (**F**) The GO enrichment result (old-vs-young) of DEGs in round spermatids. DEGs were submitted for GO enrichment analysis using the metascape online analysis. On the right are the DEGs in round spermatids enriched in the corresponding functional GO term.

expression is higher in aged female oocytes and may be associated with increased oocyte aneuploidy and altered embryo development (D'Aurora et al., 2019). Interestingly, evidence shows that older men with prostate cancer have a more significant tumor mutational burden (TMB) and lower survival rates, and PLK1 has been identified as a key gene associated with disease-free survival in men with prostate cancer (Wang et al., 2021 Feb 25). Similarly, CDC20 was required for full ubiquitin ligase-activity of the anaphase promoting complex (Fang et al., 1998 Aug). During the transition from metaphase to anaphase, normal assembly and activation of APC promote normal separation of sister

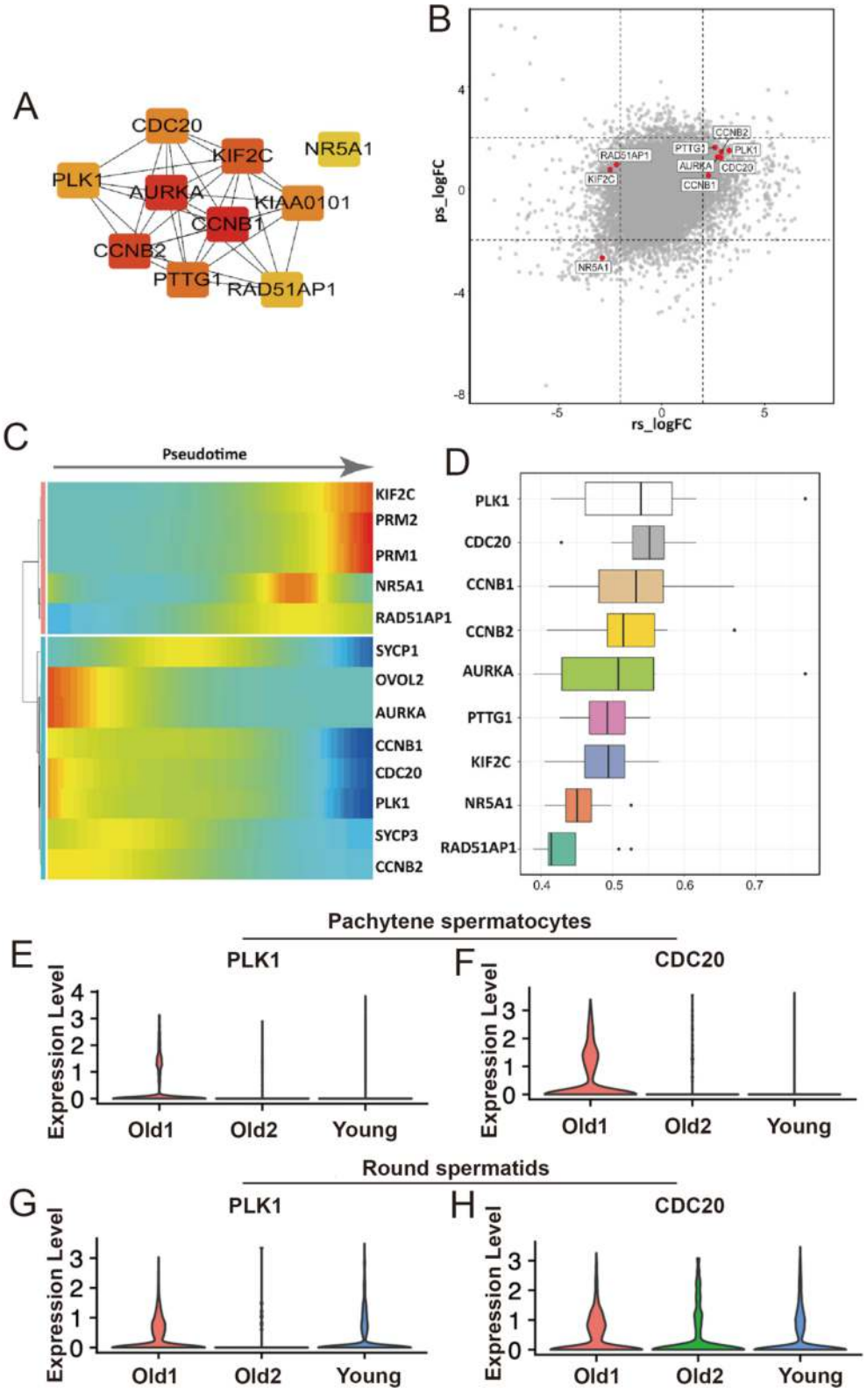

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Fig. 3. Analyses of the Protein-Protein interaction network (PPI) and hub genes identification. (**A**) The top 10 hub genes identified in the PPI network of DEGs (old-vs-young) enriched in spermatogenesis of the round spermatids. Cytohubba plugin of the Cytoscape software were used. (**B**) The dot plot shows the log fold-change (old-vs-young) of all genes in pachytene spermatocytes and round spermatids. ps, pachytente spermatocytes; rs, round spermatids. (**C**) Heatmaps show the hierarchical relationship of the hub genes (old-vs-young) in round spermatids across the pseudotime of human spermatogenesis. Expression of the genes was assessed by reanalyzing single-cell RNA-seq data (GSE106487) of the adult human testis. Pseudotime (left to right) corresponds to the development trajectory of spermatogenesis (pachytene spermatocytes to round spermatids). (**D**) Hub genes were further narrowed down through semantic analysis among GO terms and gene products. The X-axis represents the correlation coefficient, and the higher the value, the stronger the correlation. On the Y-axis are the names of the different genes that were analyzed. (**E**) Expression level of PLK1 in pachytene spermatocytes of the young and older group.(Older Group1: donors with histologically normal spermatogenesis; Older Group2: donors with clearly defective spermatogenesis). (**F**) Expression level of CDC20 in pachytene spermatocytes of the young and older group. (**G**) Expression level of PLK1 in round spermatids of the young and older group. (H) Expression level of CDC20 in round spermatids of the young and older group.

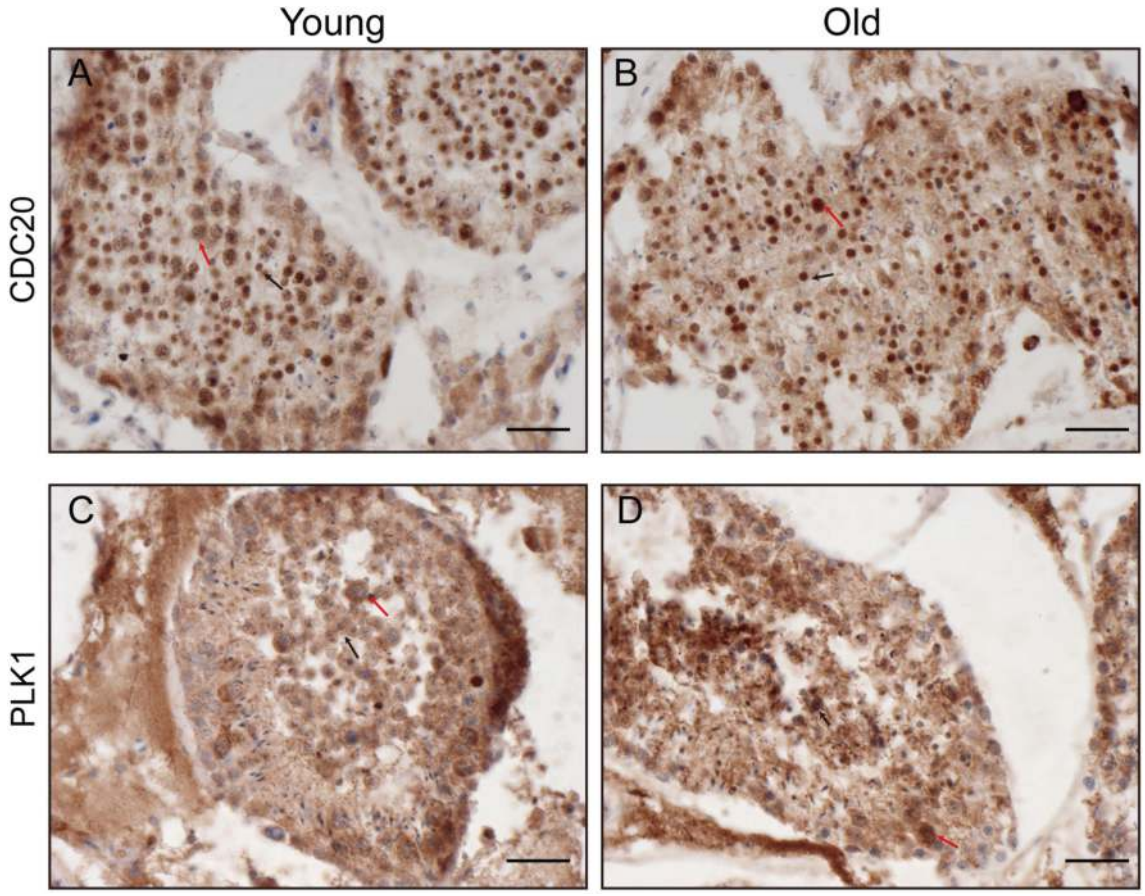

Fig. 4. Immunohistochemical (IHC) staining of CDC20 and PLK1 in testicular tissue in young and older men. (A) IHC analysis of CDC20 in testis from young men. **(B)** IHC analysis of CDC20 in testis from older men. **(C)** IHC analysis of PLK1 in testis from young men. **(D)** IHC analysis of PLK1 in testis from older men. The red arrows represent spermatocytes, and the black arrows represent round spermatids. Scale bars = 50 μm.

chromatids. The transcription level of CDC20 was significantly reduced in the first polar body (PB1) of the aged MII oocytes compared with that of the young MII oocytes, which may provide insight into age-related aneuploidy in the oocyte (Jiao et al., 2014 Jun). Although PLK1 and CDC20 are not differentially regulated in elder pachytene spermatocytes, their high expression in elder round spermatids suggests that abnormal activation of PLK1 and CDC20 may be a potentially important cause of chromosome nondisjunction and breaks increasing with aging in male germ cells.

It is important to note that aging-related spermatogenic alterations into the testis, particularly in spermatocytes and spermatids, have not been well studied. The information gained from this study may explain the abnormal changes observed in spermatozoa and provide some references for subsequent diagnosis of aging-related reproductive decline in the clinic. To explore the effect of aging on male fertility, we need to consider multiple aspects, such as the somatic cells in the testis other than spermatogenic cells, especially the Sertoli cells that maintain the testicular microenvironment and the Leydig cells that perform hormone synthesis. In addition, we should consider aging as a single variable in the study as far as possible, excluding diseases, infections, environmental factors, and so on, in the hope that more comprehensive and authoritative studies on aging causing infertility will promote the development of new therapies to overcome fertility decline.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2023.147883.

References

Bastos, P., Freitas, M.J., Gomes, A., Vitorino, R., Fardilha, M., 2017 Nov 28. Unravelling the Power of Omics for the Infertile Aging Male. Curr. Pharm. Des. 23 (30), 4451–4469.

Becht, E., McInnes, L., Healy, J., Dutertre, C.-A., Kwok, I.W.H., Ng, L.G., Ginhoux, F., Newell, E.W., 2019. Dimensionality reduction for visualizing single-cell data using UMAP. Nat. Biotechnol. 37 (1), 38–44.

Bellvé, A.R., 1993. Purification, culture, and fractionation of spermatogenic cells. Methods Enzymol. 225, 84–113.

Bhanmeechao, C., Srisuwatanasagul, S., Ponglowhapan, S., 2018 Nov. Age-related changes in interstitial fibrosis and germ cell degeneration of the canine testis. Reprod. Domest. Anim. 53 (Suppl 3), 37–43.

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., Satija, R., 2018 Jun. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat. Biotechnol. 36 (5), 411–420.

Cedars, M.I., 2015 Jun. Introduction: Childhood implications of parental aging. Fertil. Steril. 103 (6), 1379–1380.

Colasante, A., Minasi, M.G., Scarselli, F., Casciani, V., Zazzaro, V., Ruberti, A., Greco, P., Varricchio, M.T., Greco, E., 2019 Jan 18. The aging male: Relationship between male age, sperm quality and sperm DNA damage in an unselected population of 3124 men attending the fertility centre for the first time. Arch. Ital. Urol. Androl. 90 (4), 254–259.

Condorelli, R.A., La Vignera, S., Barbagallo, F., Alamo, A., Mongioì, L.M., Cannarella, R., Aversa, A., Calogero, A.E., 2020. Bio-Functional Sperm Parameters: Does Age Matter? Front Endocrinol (lausanne). 11, 558374.

D'Aurora, M., Budani, M.C., Franchi, S., Sarra, A., Stuppia, L., Tiboni, G.M., Gatta, V., 2019. Dynactin pathway-related gene expression is altered by aging, but not by vitrification. Reprod. Toxicol. 88, 48–55.

de Kretser, D.M., Loveland, K.L., Meinhardt, A., Simorangkir, D., Wreford, N., 1998 Apr. Spermatogenesis. Hum. Reprod. 13 (Suppl 1), 1–8.

Dong, S., Chen, C., Zhang, J., Gao, Y., Zeng, X., Zhang, X., 2022. Testicular aging, male fertility and beyond. Front Endocrinol (lausanne). 13, 1012119.

Fang, G., Yu, H., Kirschner, M.W., 1998 Aug. Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol. Cell 2 (2), 163–171.

Giassetti, M.I., Goissis, M.D., Moreira, P.V., de Barros, F.R.O., Assumpção, M.E.O.D., Visintin, J.A., 2016 Jul. Effect of age on expression of spermatogonial markers in bovine testis and isolated cells. Anim. Reprod. Sci. 170, 68–74.

Heller, C.G., Clermont, Y., 1963 Apr 12. Spermatogenesis in man: an estimate of its duration. Science 140 (3563), 184–186.

Hermann, B.P., Cheng, K., Singh, A., Roa-De La Cruz, L., Mutoji, K.N., Chen, I.-C., Gildersleeve, H., Lehle, J.D., Mayo, M., Westernströer, B., Law, N.C., Oatley, M.J., Velte, E.K., Niedenberger, B.A., Fritze, D., Silber, S., Geyer, C.B., Oatley, J.M., McCarrey, J.R., 2018. The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. Cell Rep. 25 (6), 1650–1667.e8.

Jackman, M., Lindon, C., Nigg, E.A., Pines, J., 2003 Feb. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat. Cell Biol. 5 (2), 143–148.

Jiao, Z.X., Xu, M., Woodruff, T.K., 2014 Jun. Age-related increase in aneuploidy and alteration of gene expression in mouse first polar bodies. J. Assist. Reprod. Genet. 31 (6), 731–737.

Johnson, L., Nguyen, H.B., Petty, C.S., Neaves, W.B., 1987 Oct. Quantification of human spermatogenesis: germ cell degeneration during spermatocytogenesis and meiosis in testes from younger and older adult men. Biol. Reprod. 37 (3), 739–747.

Kang, Y.H., Park, J.-E., Yu, L.-R., Soung, N.-K., Yun, S.-M., Bang, J.K., Seong, Y.-S., Yu, H., Garfield, S., Veenstra, T.D., Lee, K.S., 2006. Self-regulated Plk1 recruitment to kinetochores by the Plk1-PBIP1 interaction is critical for proper chromosome segregation. Mol. Cell 24 (3), 409–422.

Kokkinaki, M., Lee, T.-L., He, Z., Jiang, J., Golestaneh, N., Hofmann, M.-C., Chan, W.-Y., Dym, M., 2010. Age affects gene expression in mouse spermatogonial stem/progenitor cells. Reproduction 139 (6), 1011–1020.

Kühnert, B., Nieschlag, E., 2004. Reproductive functions of the ageing male. Hum. Reprod. Update 10 (4), 327–339.

Lane, H.A., Nigg, E.A., 1996 Dec. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J Cell Biol. 135 (6 Pt 2), 1701–1713.

Lu, L.-Y., Wood, J.L., Minter-Dykhouse, K., Ye, L., Saunders, T.L., Yu, X., Chen, J., 2008. Polo-like kinase 1 is essential for early embryonic development and tumor suppression. Mol. Cell Biol. 28 (22), 6870–6876.

Maddirevula, S., Awartani, K., Coskun, S., AlNaim, L.F., Ibrahim, N., Abdulwahab, F., Hashem, M., Alhassan, S., Alkuraya, F.S., 2020. A genomics approach to females with infertility and recurrent pregnancy loss. Hum. Genet. 139 (5), 605–613.

Neto, F.T.L., Bach, P.V., Najari, B.B., Li, P.S., Goldstein, M., 2016 Nov. Spermatogenesis in humans and its affecting factors. Semin. Cell Dev. Biol. 59, 10–26.

Ng, K.K., Donat, R., Chan, L., Lalak, A., Di Pierro, I., Handelsman, D.J., 2004 Aug. Sperm output of older men. Hum. Reprod. 19 (8), 1811–1815.

Nie, X., Munyoki, S.K., Sukhwani, M., Schmid, N., Missel, A., Emery, B.R., DonorConnect, Stukenborg, J.-B., Mayerhofer, A., Orwig, K.E., Aston, K.I., Hotaling, J.M., Cairns, B. R., Guo, J., 2022. Single-cell analysis of human testis aging and correlation with elevated body mass index. Dev. Cell 57 (9), 1160–1176.e5.

Ohkura, H., 2015. Meiosis: an overview of key differences from mitosis. Cold Spring Harb. Perspect. Biol. 7 (5).

Otasek, D., Morris, J.H., Bouças, J., Pico, A.R., Demchak, B., 2019 Sep 2. Cytoscape Automation: empowering workflow-based network analysis. Genome Biol. 20 (1), 185.

Qiu, X., Mao, Q.i., Tang, Y., Wang, L.i., Chawla, R., Pliner, H.A., Trapnell, C., 2017. Reversed graph embedding resolves complex single-cell trajectories. Nat. Methods 14 (10), 979–982.

Roshak, A.K., Capper, E.A., Imburgia, C., Fornwald, J., Scott, G., Marshall, L.A., 2000 Jun. The human polo-like kinase, PLK, regulates cdc2/cyclin B through phosphorylation and activation of the cdc25C phosphatase. Cell. Signal. 12 (6), 405–411.

Santiago, J., Silva, J.V., Alves, M.G., Oliveira, P.F., Fardilha, M., 2019 May 16. Testicular Aging: An Overview of Ultrastructural, Cellular, and Molecular Alterations. J. Gerontol. A Biol. Sci. Med. Sci. 74 (6), 860–871.

Schmid, T.E., Eskenazi, B., Baumgartner, A., Marchetti, F., Young, S., Weldon, R., et al., 2007. The effects of male age on sperm DNA damage in healthy non-smokers. Hum Reprod. 22 (1), 180–187.

Sloter, E., Nath, J., Eskenazi, B., Wyrobek, A.J., 2004 Apr. Effects of male age on the frequencies of germinal and heritable chromosomal abnormalities in humans and rodents. Fertil. Steril. 81 (4), 925–943.

Szklarczyk, D., Gable, A.L., Nastou, K.C., Lyon, D., Kirsch, R., Pyysalo, S., et al., 2021. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. Nucleic Acids Res. 49 (D1), D605–D612.

Wang, L., Pan, S., Zhu, B., Yu, Z., Wang, W., 2021 Feb 25. Comprehensive analysis of tumour mutational burden and its clinical significance in prostate cancer. BMC Urol. 21 (1), 29.

Winkle, T., Rosenbusch, B., Gagsteiger, F., Paiss, T., Zoller, N., 2009 Jan. The correlation between male age, sperm quality and sperm DNA fragmentation in 320 men attending a fertility center. J. Assist. Reprod. Genet. 26 (1), 41–46.

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A.H., Tanaseichuk, O., Benner, C., Chanda, S.K., 2019 Apr 3. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat. Commun. 10 (1).