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Transcriptomic Changes in Patients with Severely Impaired Spermatogenesis: A Systems Biology Study

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ABSTRACT

Background & Objective: Male infertility is a common condition, affecting approximately 7% of men worldwide. Spermatogenesis, the production of mature sperm cells, is tightly regulated by several genes and, dysregulation of these genes can impair normal spermatogenesis. This study aimed to identify key genes involved in the regulation of spermatogenesis.

Materials & Methods: We extracted transcriptomic data from testicular samples of patients with impaired spermatogenesis and normal controls (GSE145467, 10 patients and 10 control) from the GEO database. A protein–protein interaction network (PPIN) was constructed using the STRING database and visualized with Cytoscape v3.10.1. Network clusters were identified using the MCODE plugin. In addition, an lncRNA–miRNA–gene regulatory network was constructed, and key genes were identified using Cytoscape software. Finally, Gene Ontology (GO) analysis of the key genes was performed using the DAVID and TAM 2.0 servers.

Results: CDK1, KIF11, AURKA, TEKT1, TUBB4B, CDC25C, ENKUR, CALM3, CCDC39, TYMS, PRKACB, PRKACG, FAM166C, TMEM249, GTF2A2, NRAV lncRNA, miR-1, miR-9, and miR-27b were identified as novel crucial genes in the PPIN and gene regulatory network (GRN). Functional and pathway enrichment analysis revealed that these genes are involved in gap junction communication, long-term potentiation, GnRH signaling, motor protein function, basal transcription factor activity, cell cycle regulation, inflammation, apoptosis, hormone-mediated signaling, and tumor suppressor miRNA pathways.

Conclusion: This multi-layered systems biology analysis identified key genes and pathways associated with spermatogenic failure. Further experimental validation is needed to confirm these findings and evaluate their potential as therapeutic targets.

Keywords: Infertility, Spermatogenesis, Systems Biology, Transcriptome

1. Introduction

Male infertility is a complex reproductive disorder that can arise from various factors, including paracrine and autocrine signaling, hormone imbalances, gene alterations, and epigenetic modifications (1). It is clinically diagnosed when sperm parameters are abnormal or reduced. Impaired fertility is often characterized by changes in sperm quantity and/or quality, such as decreased sperm count (oligozoospermia), with or without abnormalities in sperm morphology (teratozoospermia), or motility (asthenozoospermia). In severe cases, when all three parameters are affected, the condition is referred to as oligoasthenoteratozoospermia. The absence of sperm in the ejaculate (azoospermia) may result from obstruction of the vas deferens (obstructive azoospermia, (OA) or from primary or secondary testicular failure (non-obstructive azoospermia, NOA) (2).

Spermatogenesis is a complex process of cell differentiation in which spermatogonial stem cells develop into mature spermatozoa through several stages (3). Each stage is tightly regulated by numerous genes, and studies have shown that targeted deletion of these genes in transgenic animal models can disrupt gametogenesis, fertilization, and overall fertility (4). The importance of the sperm transcriptome in fertility is well recognized, as it contributes to sperm production and function and can serve as a potential biomarker for spermatogenic defects (5). Approximately 20–25% of severe male infertility cases are attributed to known genetic factors that impair sperm production (6).

Recent advances in transcriptomics have greatly enhanced the potential to improve the diagnosis and treatment of male infertility (7). In particular, studies of the sperm transcriptome have provided valuable insights into the regulation of spermatogenesis by identifying novel biomarkers and elucidating sequential, tightly regulated gene expression patterns (8). Several studies have reported the involvement of specific genes in determining male fertility potential, and many more are likely to be identified (9, 10). Analysis of RNA sequencing and microarray data may further improve understanding of gene expression patterns, allelic expression variation and mutation detection, thereby providing insights into the molecular mechanisms underlying male infertility.

To better understand the etiology of male infertility, a thorough investigation of abnormal gene expression patterns is required. The identification of differentially expressed genes (DEGs) between healthy controls and infertile men with severely impaired spermatogenesis can provide valuable insights for diagnostics and therapeutic strategies. Few studies have simultaneously integrated protein–protein interaction networks (PPINs) and gene regulatory networks (GRNs) from testicular biopsy transcriptomes. Moreover, the marked clinical heterogeneity of non-obstructive azoospermia (NOA) and oligoasthenoteratozoospermia (OAT) has not yet been systematically elucidated at the network level.

In this study, we aimed to identify key DEGs in testicular tissue from patients with severely impaired spermatogenesis. We hypothesized that integrating DEGs with curated protein–protein interactions and experimentally supported non-coding RNA (ncRNA) interactions would reveal coherent cell cycle and axonemal modules associated with severely impaired spermatogenesis.

2. Materials and Methods

This study was conducted in five steps:

a) Dataset selection: Identification of a suitable microarray dataset of testicular samples from individuals with normal and impaired spermatogenesis.

b) Data processing: Normalization of the dataset and identification of differentially expressed genes (DEGs).

c) Networks construction: Development of PPINs and GRNs based on DEGs.

d) Network analysis: Identification of clusters and hub or bottleneck nodes (critical genes) within the PPIN and GRN.

e) Functional enrichment: Pathway analysis of critical genes to explore their biological functions and potential relevance to spermatogenesis

2.1 Data Collection and Processing

The dataset of testicular samples with normal and impaired spermatogenesis (GSE145467; 10 testicular biopsies from patients with impaired spermatogenesis and 10 testicular biopsies from healthy controls) (7) was retrieved from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The characteristics of the samples are provided in Supplementary Table S1. DEGs were identified using the GEOquery and limma packages in R through the GEO2R interface. DEGs were selected based on an adjusted p-value (Adj.P) < 0.0001 and log fold change |logFC| > 2. These DEGs were subsequently used for downstream network and functional enrichment analysis.

2.2 PPIN and Topological Analysis

The DEGs were submitted to the STRING v12.0 web server (https://string-db.org/) to construct the PINN. Nodes with a confidence score ≥ 0.7 were visualized using Cytoscape v3.10.1. Hubs and bottlenecks (critical genes), along with other topological network properties, were identified using the NetworkAnalyzer plugin in Cytoscape. Hubs are defined as proteins with a high number of interactions, whereas bottlenecks are defined as nodes with high betweenness centrality. The top 10% of nodes with the highest degree and betweenness

centrality were identified using a Venn diagram tool (https://bioinformatics.psb.ugent.be/webtools/Venn/).

2.3 Cluster Detection

Highly interconnected sub-networks within the PPIN were identified using the MCODE app in Cytoscape with the following parameters: Degree Cutoff = 2, Node Score Cutoff = 0.2, K-Core = 2 and Max-Depth = 100. Clusters with a score > 3 and containing more than five nodes were selected as final sub-networks for further analysis.

2.4 GNR Construction

Identification of LncRNA-miRNA-Gene Regulatory Networks

Experimentally validated miRNA target genes regulating the DEGs were retrieved from miRTarBase (https://bio.tools/mirtarbas). miRTarBase is a well-curated repository providing experimentally confirmed miRNA- gene interactions identified using methods such as reporter assays, microarrays, next-generation sequencing and western blotting.

LncRNAs Repressing DEGs and microRNAs

LncRNA-regulated DEGs were retrieved from the LncRNA2Target v2.0 database (http://123.59.132.21/lncrna2target), a comprehensive resource of experimentally validated lncRNA-gene interactions identified using RNA-seq. LncRNA-miRNA interactions were obtained from the LncBase v2 database (www.microrna.gr/LncBase), which provides in silico predicted miRNA targets on lncRNAs identified using the DIANA-microT algorithm. This database includes millions of potential miRNA binding sites enriched with detailed metadata and MRE scores. Moreover, LncBase v2 offers insights into cell type–specific miRNA-lncRNA regulation across 66 distinct cell types from 36 human and mouse tissues, supported by precise lncRNA expression profiles derived from the analysis of more than 6 billion RNA-seq reads.

MicroRNAs Suppressing LncRNAs

MicroRNAs repressing LncRNAs were extracted from miRTarBase.

Construction of GRN and Topological Analysis

The relationships among miRNAs, genes, and lncRNAs (miRNA-gene, miRNA-lncRNA, lncRNA-gene and lncRNA-miRNA) were integrated to construct the regulatory lncRNA-miRNA-gene network using Cytoscape v3.10.1. Duplicate interactions were automatically removed by Cytoscape to generate the final network. Topological properties and hub genes were identified using the NetworkAnalyzer plugin. For further analysis, the final network was defined as the intersection of the top 10% of the genes ranked by both degree and betweenness centrality.

2.5 Functional Pathways Analysis

Biochemical pathway enrichment analyses were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) for the top 10% of shared genes with the highest degree and betweenness centrality from both the PPIN and GRN. This analysis facilitated the identification and clustering of relevant proteins. To assess functional pathways regulated by miRNAs within the GRN, the TAM 2.0 server (http://www.lirmed.com/tam2/) was used. Pathways with p-values < 0.05 were considered statistically significant.

3. Result

3.1 Analysis of Raw Data

A total of 659 differentially expressed genes (DEGs) were identified from the GSE145476 dataset, comprising 33 upregulated and 629 downregulated genes. Selection criteria included an adjusted p-value (Adj. P) < 0.0001 and |logFC|>2. The full list of DEGs is provided in Supplementary Table S2.

3.2 Construction of a PPI Network and Topological Analysis

The PPI network for 659 DEGs was constructed using the STRING v12.0 database and visualized with Cytoscape v3.10.1. The resulting network consisted of 564 nodes and 1391 edges, with a network density of 0.009, a diameter of 15, 156,404 shortest paths, and a clustering coefficient of 0.317. Topological properties were analyzed using the NetworkAnalyzer plugin to identify nodes with the highest degree and betweenness centrality. The intersection of the top 10% ranked by degree and the top 10% ranked by betweenness centrality was determined by a Venn diagram and further visualized in Cytoscape. The main results are presented in Figure 1 and Table 1.

3.3 Cluster Detection

Clusters represent highly interconnected regions within the PPIN. Using the MCODE plugin in Cytoscape, four significant clusters were identified, each with a MCODE score greater than three and containing more than five nodes. The seed proteins of these clusters were FAM166C, CCDC39, TMEM249 and GTF2A2. The identified clusters and their characteristics are presented in Figure 2.

3.4 Construction and Topological Analysis of the miRNA-lncRNA-Gene Regulatory Network

In this study, miRNAs targeting both mRNAs and lncRNAs were identified using the miRTarBase database. A total of 766 genes were regulated by 1,677 miRNAs, resulting in 18,578 interactions. Additionally, 629 miRNAs were predicted to regulate 82 lncRNAs through 914 interactions. According to the LncRNA2Target v2.0 database, 54 lncRNAs were found to regulate 2,666 genes via 4,613 interactions. Furthermore, 6,180 lncRNAs were identified as targets of 681 miRNAs, accounting for 53,073 interactions.

3.5 Integrated lncRNA–miRNA– Gene Regulatory Network

The miRNA–gene, miRNA–lncRNA, lncRNA–gene and lncRNA–miRNA interactions were imported into Cytoscape v3.10.1 and merged to construct an integrated lncRNA–miRNA–gene regulatory network. Topological analysis was performed using the NetworkAnalyzer plugin. Nodes ranking in the top 10% for both degree and betweenness centrality were identified, and the common nodes were visualized using a Venn diagram. The key nodes are illustrated in Figure 3. Notably, the NRAV lncRNA emerged as a critical regulatory element in the GRN and was downregulated in patients with impaired spermatogenesis.

3.6 Functional pathway Analysis

Functional pathway analysis using the DAVID server revealed that the critical genes identified within the PPIN are involved in key biological processes, including gap junctions, long-term potentiation and GnRH signaling. In addition, motor proteins, basal transcription factors, and the cell cycle were identified as major pathways regulated by cluster-specific critical genes. Analysis of miRNAs in the GRN using the TAM 2.0 server indicated that these miRNAs are associated with the regulation of inflammation, apoptosis, hormone-mediated signaling pathways, and tumor suppressor activity and, the cell cycle. A summary of these findings is provided in Table 2.

Table 1. List of common hubs and bottlenecks (critical) genes identified as 10% nodes by a degree and betweenness centrality in the PPIN.

Name of genes	Degree	Betweenness centrality
CDK1	48	0.17867783
KIF11	31	0.0940799
AURKA	27	0.04712771
TEKT1	24	0.06532077
TUBB4B	23	0.13708926
CDC25C	22	0.08528449
ENKUR	21	0.10474559
CALM3	18	0.20928081
CCDC39	16	0.0765165
TYMS	16	0.05971629
PRKACB	15	0.098499
PRKACG	15	0.098499

Table 2. Top functional pathways regulated by critical genes in the PPIN and the cluster-specific nodes, identified using DAVID and the TAM 2.0 server. (P-value < 0.05)

	Terms	P. value	Genes
PPI	Gap junction	5.47E-05	PRKACG, CDK1, TUBB4B, PRKACB
	Long-term potentiation	0.001602	PRKACG, CALM3, PRKACB
	GnRH signaling pathway	0.003062	PRKACG, CALM3, PRKACB
MCODE	Motor proteins	2.62E-10	DNAI2, DNAH2, DNAH17, DNAH7, KIF11, TUBB4B, DNAI1, DNAL1, DNALI1, KIF15
	Basal transcription factors	1.53E-04	GTF2A2, TAF10, TAF9, TAF5
	Cell cycle	0.006113	CCNB2, PTTG1, CDK1, TTK
GRN	Inflammation	1.00E-300	miR-24-1, miR-9-1, miR-320a, miR-124-2, miR-181a-1, …
	Apoptosis	3.50E-29	miR-24-1, miR-9-1, miR-181a-1, miR-16-2, miR-101-1, miR-7-2, miR-9-2, …

Terms	P. value	Genes
Hormone-mediated Signaling Pathway	7.34E-27	miR-24-1, miR-34a, miR-9-1, miR-221, miR-126, miR-7-3, miR-124-2, miR-101-2, …
Tumor Suppressor MiRNAs	1.71E-26	miR-125a, Let-7b, miR-34a, miR-7-1, miR-126, miR-7-3, miR-124-2, miR-26b, …
Cell Cycle	2.58E-25	Let-7b, miR-24-1, miR-34a, miR-185, miR-9-1, miR-221, miR-320a, …

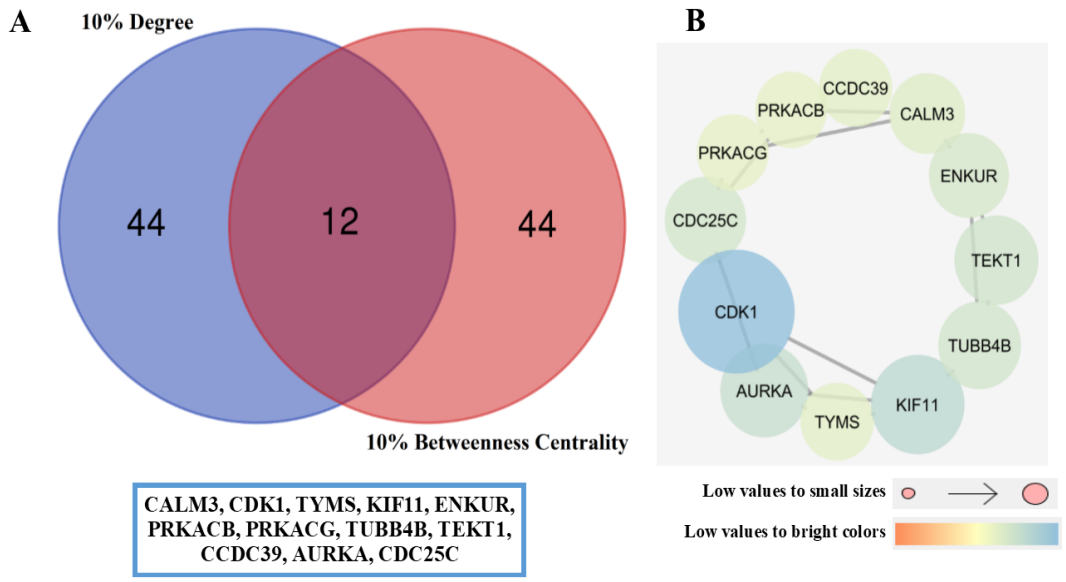

Figure 1. Common nodes with the highest degree/betweenness centrality and PPIN. A) Venn diagram showing the intersection of the top10% nodes by degree and top 10% nodes by betweenness centrality. B) The network constructed from the intersecting nodes using Cytoscape. Nodes with large size and dark color indicate higher degree (**Prepared by Authors, 2025**).

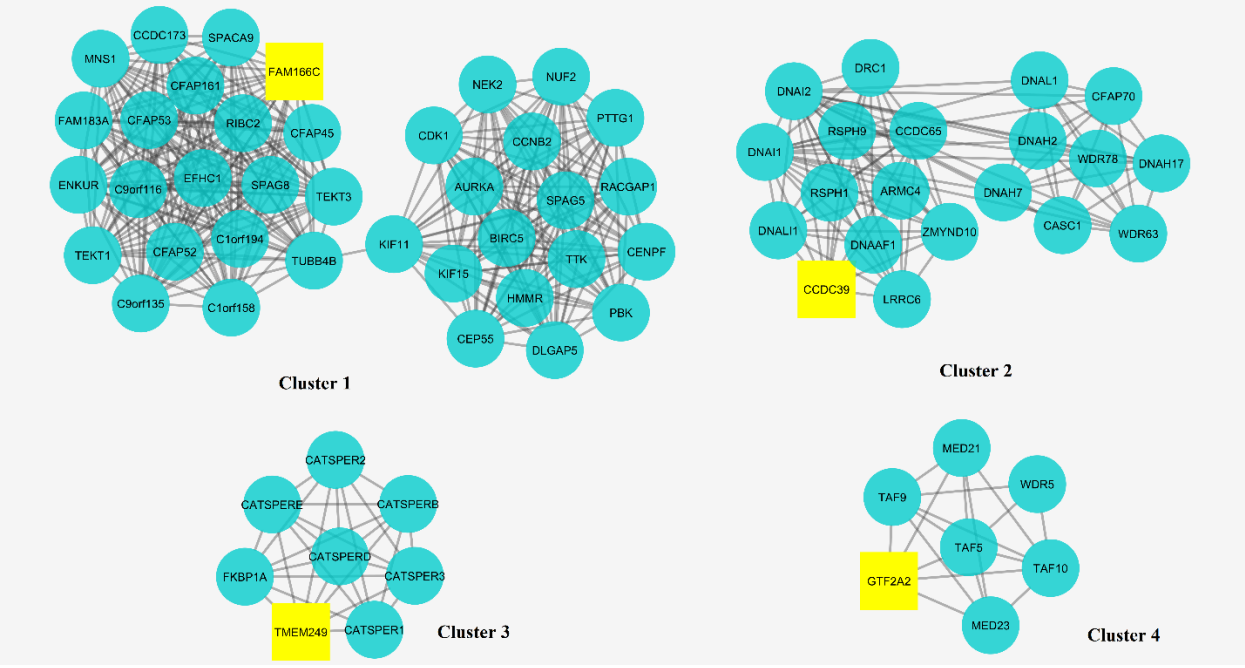

Figure 2. Clusters identified in the PPIN using MCODE. Seed nodes are hiloghted with a yellow rectangles. Clusters have an MCODE score > 3 and contain more than five nodes (**Prepared by Authors, 2025**).

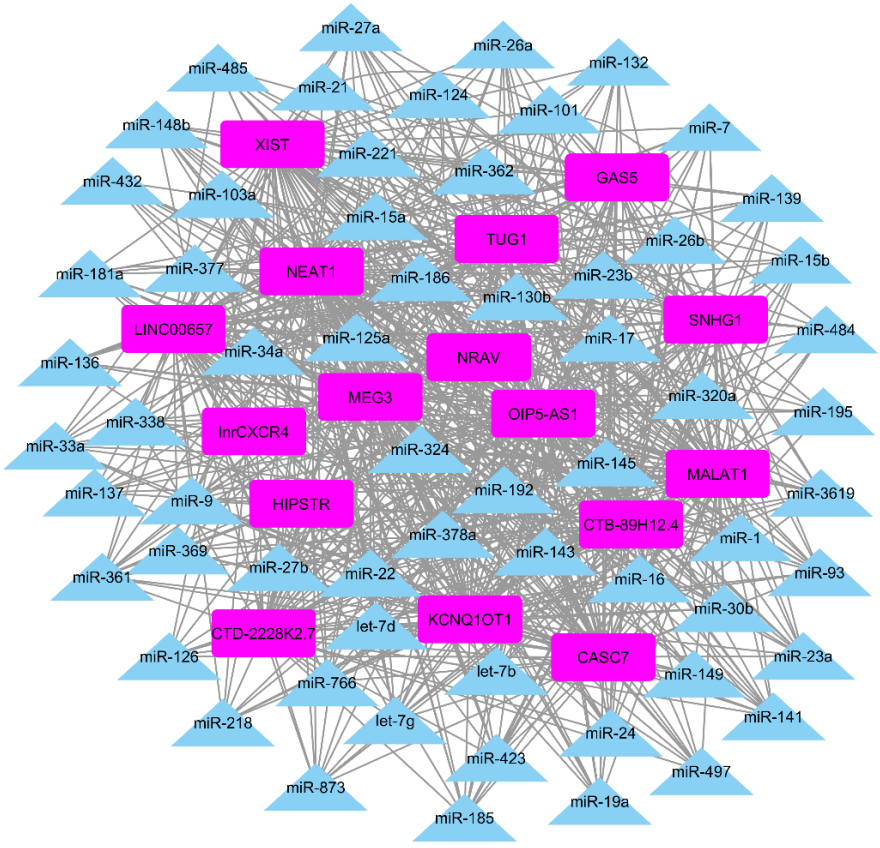

Figure 3. GRN showing the common nodes between the top 10% nodes by degree and betweenness centrality. miRNAs are represented by blue triangles and genes/lncRNAs are represented by purple rectangles (**Prepared by Authors, 2025**).

4. Discussions

Spermatogenesis is a complex process of cell differentiation in which spermatogonial stem cells develop through several stages into mature spermatozoa. A crucial aspect of assessing male infertility is the precise categorization of impaired spermatogenesis. Each stage of this process is regulated by numerous genes, and defects in these genes can affect mature sperm production and fertilization **(4)**. Investigation of these genes may lead to the development of effective therapeutic strategies. Therefore, in this study, we analyzed transcriptomic data from testicular samples of patients with impaired spermatogenesis to identify key regulatory genes.

In this study, analysis of the PPIN, MCODE clusters, and GRN from testicular samples of patients with impaired spermatogenesis revealed that *CDK1, KIF11, AURKA, TEKT1, TUBB4B, CDC25C, ENKUR, CALM3, CCDC39, TYMS, PRKACB, PRKACG, FAM166C, TMEM249, GTF2A2* and *NRAV lncRNA* are key genes involved in impaired spermatogenesis. Among these, *CDK1, KIF11, AURKA, TEKT1, TUBB4B, CDC25C, ENKUR, CALM3, CCDC39, TYMS, PRKACG, FAM166C, TMEM249, GTF2A2* and *NRAV lncRNA* are downregulated in patients with impaired spermatogenesis, while *PRKACB* is upregulated. miR-1, miR-9, and miR-27b show the highest expression levels in the GRN. MicroRNAs (miRNAs) play a crucial role in regulating spermatogenesis, and their dysregulation can impair spermatogenesis and cause male infertility. Specifically, miRNAs are involved in the proper development and function of sperm by regulating processes such as cell proliferation, differentiation and apoptosis. Aberrant miRNA expression has been associated with various forms of impaired spermatogenesis, including azoospermia (absence of sperm) and oligospermia (low sperm concentration) **(11)**. Notably, miR-27b expression is increased in ejaculated sperm from azoospermic patients, and its upregulation correlates with reduced progressive sperm motility while being inversely associated with CRISP2 protein levels **(12)**. Comprehensive studies on highly expressed miRNAs and their association with impaired spermatogenesis are limited and need further exploration.

CDK1 and *KIF11* were identified as critical genes in our study. Clement et al **(13)** reported that conditional knockout (cKO) of the *Cdk1* gene in mouse spermatocytes resulted in irregular cell morphology, unevenly sized nuclei, and prominent nucleoli. Their findings demonstrated that *CDK1* is essential for meiotic metaphase progression in mouse spermatocytes **(13)**. KIF11, a motor protein belonging to the kinesin-like family, plays a crucial role in spindle dynamics, chromosome positioning, centrosome separation, and bipolar spindle formation during mitosis **(14)**. Hara-Yokoyama et al **(15)** further showed that *KIF11* is

expressed in spermatogonia and spermatocytes and is required for spindle formation during both mitosis and meiosis (15). Deletion of *Aurka* in spermatogonia results in the loss of all developing germ cells in the testis, whereas deletion in spermatocytes leads to reduced testis size, lower sperm count, and decreased fertility, indicating disruption of meiosis or defects in spermatogenesis (16). *TEKT1* and *TUBB4B* are also critical genes. Larsson et al (17) demonstrated that *TEKT1* plays an essential role in the nucleation of the flagellar axoneme of mature spermatozoa and in the assembly of the basal body (17). Feng et al (18) reported that *TUBB4B* is involved in the spermatogonial division through interactions with various cell cycle regulatory proteins and plays a specific role in the regulation of spermatogonial proliferation and the cell cycle (18). *CDC25A,* a member of the *CDC25* gene family, is critical for spermatogenesis, as it activates cyclin-dependent kinases (CDKs) required for cell cycle progression in male germ cells. Reduced *CDC25A* expression is associated with spermatogenic failure and infertility, particularly in cases of azoospermia and meiotic arrest. Its presence in spermatocytes and round spermatids underscores its role in both cell cycle regulation and sperm physiology. Overall, *CDC25A* is essential for normal sperm development, and its dysregulation can contribute to male infertility (19). Enkurin (ENKUR) is an essential protein involved in spermatogenesis, particularly in sperm motility and function. Research suggests that *ENKUR* is essential for proper bending and swimming patterns of sperm flagella, which are required for navigation through the female reproductive tract and successful fertilization. Jungnickel et al (20) reported that *ENKUR*-deficient mice exhibit impaired sperm motility and subfertility, as their sperm have difficulty reaching the oviduct and effectively fertilizing oocytes. The involvement of *ENKUR* in the regulation of sperm calcium signaling also indicates that it may also affect additional aspects of sperm function, underscoring its crucial role in male fertility and the complex processes of spermatogenesis (20). These findings suggest that *ENKUR* and *TEKT1*, both of which contribute to sperm motility via flagellar mechanisms, may cooperate during the later stages of spermatogenesis. Therefore, these results may serve as confirmation or reprioritization of known biomarkers involved in this process.

The expression levels of *CALM1* and *CALM3* increase in early meiotic cells (leptotene-zygotene spermatocytes) and remain constant throughout meiosis (21). The protein encoded by *CCDC39* is essential for the assembly of the regulatory dynein arm and inner dynein arm complexes that control ciliary beating. Mutations in this gene are associated with primary ciliary dyskinesia type 1 (22). The *TYMS* gene, which encodes thymidylate synthase, plays a crucial role in regulating DNA synthesis and cell division during germ cell development. Targeted inactivation of *TYMS* in mice results in embryonic development after implantation, highlighting its essential role in cell proliferation and differentiation (23). Therefore, these findings may serve as confirmation or reprioritization of the known roles of *TYMS, CDK1*, and *CDC25C*, which are involved in DNA replication and division, ultimately contributing to reduced sperm production.

PRKACG is a catalytic subunit of protein kinase A (PKA) involved in energy metabolism, hyper-activation and sperm capacitation (24). Dysregulation of this protein has also been associated with infertility. *FAM166C* is specifically expressed in early and late spermatids**,** suggesting a role in the final stages of spermatogenesis. High levels of RNA and protein expression are detected in round spermatids, elongating spermatids, pachytene spermatocytes, and spermatogonia in human testes. *FAM166C* is classified as a microtubule inner protein (MIP) located in the axoneme of cilia and flagella, indicating its importance in maintaining of proper microtubule organization. The protein is also localized in regions where the acrosome develops in spermatids, suggesting a possible role in acrosome biogenesis (25). In *Tbc1d21*-null mice, which exhibit severe sperm tail defects with a disrupted axoneme, proteomic analysis revealed an upregulation of *TEKT1*. This abnormal accumulation of *TEKT1* in the midpiece region suggests that its mis-regulation contributes to structural defects of the sperm tail (26). Another critical gene is *TMEM249*. Huang et al (27) demonstrated that the transmembrane protein 249 (TMEM249) plays an essential role in the assembly of the CatSper channel during sperm tail formation (27). This network of genes represents a calcium-dependent motility and maturation module. Studies have also shown that *GTF2A2* is expressed at various stages of spermatogenesis. In mice, *Gtf2a2* expression is upregulated during spermatogenesis, particularly in pachytene spermatocytes and haploid spermatids, suggesting that *GTF2A2* may be involved in the transcriptional regulation required for the progression of spermatogenesis (28). Li et al (29) reported that *Rnf216*-deficient mice (Rnf216$^{-/-}$) are infertile. In these mice, the expression of *PRKACB* is upregulated. *RNF216* interacts with *PRKACB* and promotes its degradation via the ubiquitin-lysosome pathway. Their results indicate that *RNF216* plays a key role in the regulation of meiosis and PKA stability in the testis (29). Therefore, *PRKACB* could be a potential therapeutic target for patients with impaired spermatogenesis.

The *NRAV* (Negative Regulator of the Antiviral Response) gene, a long non-coding RNA, is not currently known to be directly associated with spermatogenesis. However, existing studies indicate that other non-coding RNAs, such as miRNAs, piRNAs and lncRNAs, play a significant role in male fertility. While *NRAV* has consistently been identified as a negative modulator of the antiviral response (30), its recurrent suppression in infertile patients suggests a potential role as an epigenetic regulator that warrants further investigation.

Functional pathway analysis demonstrated that critical genes identified in the PPIN and MCODE clusters are involved in several key processes, including gap junction communication, long-term potentiation, GnRH signaling,

motor protein function, basal transcription factor activity and cell cycle regulation. Furthermore, in the GRN, the top pathways regulated by critical miRNAs are associated with inflammation, apoptosis, hormone-mediated signaling, tumor suppressor miRNAs and cell cycle regulation.

Gap junctions play a vital role in the function of various cells within the male reproductive system. In this study, we demonstrated that this signaling pathway is regulated by *PRKACG*, *CDK1*, *TUBB4B* and *PRKACB*. These genes contribute to key processes, including proliferation, meiosis and differentiation of male germ cells in the testis, as well as the final maturation of sperm in the epididymis (31).

Long-term potentiation (LTP) is another signaling pathway regulated by critical genes in the PPIN. Samavat et al (32) investigated the role of LTP in enhancing synaptic information storage in the hippocampus of adult rats. Although LTP is primarily associated with brain function, it shares fundamental mechanisms with spermatogenesis, including calcium signaling and the regulation of synaptic strength. These shared mechanisms suggest that similar processes may influence sperm regulation and maturation, although this connection remains theoretical and indirect (32). Gonadotropin-releasing hormone (GnRH) signaling plays a pivotal role in regulating spermatogenesis through its effects on the hypothalamic-pituitary-gonadal (HPG) axis. GnRH is synthesized and released by hypothalamic neurons and acts on the anterior pituitary to stimulate the secretion of two key gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins are essential for initiating and maintaining spermatogenesis (33). The critical genes *PRKACG* and *PRKACB* regulate these pathways in spermatogenesis.

Motor proteins, which are essential for intracellular transport, and basal transcription factors, which are critical for gene expression, are regulated by miRNAs within the GRN. This intricate regulatory network maintains cellular functionality, adaptability and homeostasis, with *KIF11* serving as a central regulator (34). Similarly, the signaling pathways that regulate the cell cycle, inflammation and apoptosis are essential for maintaining cellular integrity and responding appropriately to stimuli. The key gene *CDK1* coordinates these processes by controlling cell cycle progression, immune response and programmed cell death, supporting previous finding on the delicate balance of these mechanisms in health and disease (35). Hormone-mediated signaling and tumor suppressor miRNAs play critical roles in endocrine regulation and cancer biology. Our findings suggest that miRNAs exert a significant influence on these pathways, highlighting their therapeutic potential for treating endocrine disorders and cancer (36).

While several transcriptomic studies have previously identified gene expression profiles in infertile men, this study advances the field by incorporating both PPINs and gene regulatory networks (GRNs) within a systems biology framework. Notably, the inclusion of lncRNA–miRNA–mRNA interactions revealed NRAV lncRNA, miR-1, miR-9, and miR-27b as novel regulatory nodes in impaired spermatogenesis. Furthermore, the co-downregulation of *CDK1*, *KIF11*, *AURKA*, *TEKT1* and *TUBB4B* suggests a coordinated disruption of meiotic progression and structural integrity, a phenomenon that has not been widely reported. Collectively, this multilayered network analysis highlights new candidate targets for future experimental validation.

5. Conclusion

In summary, this study provides a comprehensive network-based analysis (PPIN and GRN) to identify critical genes and functional pathways involved in the regulation of spermatogenesis. Our findings highlight *CDK1*, *KIF11*, *AURKA*, *TEKT1*, *TUBB4B*, *CDC25C*, *ENKUR*, *CALM3*, *CCDC39*, *TYMS*, *PRKACB*, *PRKACG*, *FAM166C*, *TMEM249* and *GTF2A2*, as well as NRAV lncRNA, miR-1, miR-9 and miR-27b, as potential key regulators of impaired spermatogenesis. These critical genes are linked to signaling pathways essential for reproduction and cellular regulation, including gap junctions, GnRH signaling, motor protein function, basal transcription factors, the cell cycle, inflammation, apoptosis, hormone-mediated signaling pathways, and tumor suppressor miRNAs. We also propose that long-term potentiation may play a role in spermatogenesis, although this requires further investigation. These findings should be interpreted with caution due to limitations such as small sample size, potential cellular heterogeneity in biopsy material, and reliance on invalidated in silico analyses. Nevertheless, this multilayered systems biology approach provides novel insights and generates testable hypotheses for future experimental validation.

6. Declarations

6.1 Acknowledgments

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6.2 Ethical Considerations

The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study. (Ethical code: IR.SBMU.RETECH.REC.1402.566)

6.3 Authors' Contributions

F. Saberi, Z. Dehghan, and H. Zali contributed to the conception and design of the study, Z. Dehghan, N.

gholijani, Sh. Mehdinejadiani, Z. Taheri, J. Moayedi, and S. Mortezaali were involved in the collection, analysis and interpretation of the data. All authors contributed to the preparation of the manuscript, and author A revised it critically. All authors have read and approved the final version of the manuscript.

6.4 Conflict of Interest

The authors have no conflict of interest.

6.5 Fund or Financial Support

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6.6 Using Artificial Intelligence Tools (AI Tools)

The authors declare that no artificial intelligence (AI) tools were used in the writing, analysis, or preparation of this manuscript.

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