Comparison of urinary proteome in the first two days after mating in male rats

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Abstract

Objective: To compare urine proteome changes in the first two days after mating behavior in male rats.

Methods: The urine samples of Sprague-Dawley rats on the day of mating and the day after mating were collected and analyzed by non-label quantitative proteomics by high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS), and the differential proteins in the urine proteome were screened protein function and biological pathways were analyzed.

Results: 43 differential proteins were identified by comparing the urine proteome of rats on the next day of mating with that on the day of mating, and nearly two-thirds of the differential proteins were related to spermatogenesis.

Conclusions: There were changes in the urinary proteome on the next day of mating compared with the day of mating, and some of the proteins whose functions were known to be related to spermatogenesis. Urine proteome has the potential to reflect spermatogenesis.

Keywords: urine proteome; mating; spermatogenesis

1 Introduction

Spermatogenesis is the process by which spermatogonial cells undergo proliferation and differentiation to form mature sperm, which involves chromosome fold reduction and cell deformation. There are three stages of mammalian spermatogenesis. First, the spermatogonium undergoes a series of mitosis to form a primary spermatocyte. Secondly, the primary spermatocytes produce haploid round sperm cells through meiosis. Finally, the round sperm cells are deformed into sperm with flagella. Paracrine, autocrine, and endocrine pathways all contribute to the regulation of this process, and the large number of structural elements and chemical factors involved in regulation make the network connecting various cellular activities during spermatogenesis unimaginably complex[1]. Urine is produced by the filtration of blood through the kidneys to eliminate metabolic waste, which is not controlled by the homeostatic regulation mechanism of the internal environment, and can be more sensitive to the various small changes produced by the body[2]. It has been shown that the urine metabolome can be used to distinguish between normal sperm infertile men and fertile men[3]. However, no studies have been conducted to monitor spermatogenesis by urine proteome. After a mating of male rats, mature sperm is consumed to stimulate spermatogenesis in the testis. In this study, by collecting urine samples from rats on the day of mating and the next day of mating, we compared the urinary proteome and tried to explore whether spermatogenesis could be reflected in the urinary proteome.

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2.1 Experimental material

2.1.1 Experimental animal

Five 10-week-old male Sprague-Dawley rats and five 10-week-old female Sprague-Dawley rats were purchased from Beijing Weitonglihua Experimental Animal Biotechnology Company Limited. All rats were raised in a standard environment (room temperature 22±1 °C, humidity 65%-70%). All the rats were fed in the new environment for three days before the experiment began. All experimental operations were reviewed and approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University with the approval number CLS-AWEC-B-2022-003.

2.2 Experimental method

2.2.1 Rats mating

The male rats cohabited with the female ones in a 1:1 ratio at 16:00. At 7:00 the next day, female Rats were checked for vaginal plugs, and those who detected vaginal plugs were considered to be mating between male and female.

2.2.2 Urine sample collection

Urine was collected from 20:00 to 8:00 on the mating day and temporarily stored in the -80°C refrigerator as urine samples on the mating day. After urine collection, male rats were fed separately, and urine collection continued from 20:00 to 8:00 the next day, temporarily stored in the -80°C refrigerator, and the urine samples were collected the next day after mating.

2.2.3 Urine sample processing

Urine protein extraction: Rat urine samples were removed from the refrigerator at -80 °C and thawed at 4°C. Centrifuge at 4°C, 12000×g for 30 min, take 2 mL of supernatant, add triploid volume of pre-cooled anhydrous ethanol every 500 μL of supernatant into 2 mL centrifugal tube, gently mix upside down, and precipitate overnight protein at -20°C. The mixture precipitated overnight was centrifuged at 4°C, 12000×g for 30 min, the supernatant was discarded, and the ethanol was volatilized and dried. The protein precipitate was suspended in the lysate (containing 8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris). Centrifuge at 4°C at 12000×g for 30 min, supernatant was taken into a new 1.5mL centrifuge tube to obtain urine protein. The protein concentration was determined by Bradford method.

Enzyme digestion of urine protein: 100 μg urine protein sample was taken into 1.5 mL centrifuge tube, and 25 mmol/L NH_4HCO_3 solution was added to make the total volume 200 μL. Add 20 mM Dithiothreitol solution (DTT, Sigma), swirl and mix well, heat in a metal bath at 97°C for 10 min, and cool to room temperature. Add 50 mM Iodoacetamide (IAA, Sigma), swirl and mix, and react at room temperature for 40 min away from light. Take a 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA) and add 200 μL UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) to the filter membrane to wash the filter membrane at 18°C. Centrifuge at 14000×g for 5 min, discard the lower filtrate, and repeat once; Urine protein samples after treatment were added to the filter membrane, centrifuged at 18°C, 14000×g for 30 min, and the lower filtrate was

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discarded, leaving urine protein on the filter membrane. Urine protein was washed with 200 μL UA solution into the filter, centrifuged at 18°C, 14000×g for 30 min, and repeated twice. Urine protein was washed with 25 mmol/L NH_4HCO_3 solution into the filter membrane, centrifuged at 18°C, 14000×g for 30 min, and repeated twice. Trypsin Gold (Promega, Fitchburg, WI, USA) was added at the ratio of 1:50 for enzyme digestion, and the water bath at 37°C for 15 h. After digestion, the filtrate was collected by centrifugation at 4°C at 13000×g for 30 min. The filtrate was a polypeptide mixture. The polypeptide mixture was desalted by HLB solid phase extraction column (Waters, Milford, MA), lyophilized with a vacuum dryer, and stored at -20°C.

2.2.4 LC-MS/MS tandem mass spectrometry analysis

The polypeptide mixture of 0.1% formic acid was lyophilized and quantified with BCA kit. The peptide concentration was diluted to 0.5 μg/μL. Each sample was mixed with 6 μL and separated by Thermo Fisher Scientific (high pH reversed phase peptide separation kit). Centrifuge and collect 10 Fractions, freeze dried by vacuum dryer and redissolve in 0.1% formic acid. An iRT reagent (Biognosys, Switzerland) was added to 10 effluents and all individual samples with a sample: iRT volume ratio of 10:1 to calibrate the retention time of the extracted peptide peaks.

Ten effluents were separated using the EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA). The isolated peptides were analyzed by Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) in Data Dependent Acquisition(DDA) mode. Generate 10 raw files and import them into Proteome Discoverer software for database construction analysis using Swiss-iRT and Uniprot-Rat databases (version 2.0, Thermo Scientific). The DIA method is established by setting 39 variable Windows of the Data Independent Acquisition(DIA) model of a single sample according to the results of the database construction. A single sample of 1 μg peptide was isolated using EASY-nLC1200 chromatography (Thermo Fisher Scientific, USA). The separated peptides were analyzed by Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) in DIA mode. The newly established DIA method was used to collect DIA data and generate raw files.

2.2.5 Quantitative analysis of Label-free DIA

The raw file of a single sample collected in DIA mode was imported into Spectronaut Pulsar(Biognosys AG, Switzerland) for analysis. The abundances of peptide segments were calculated by summing the peak areas of each fragment ion in MS2. Protein abundance is calculated by adding the abundances of the respective peptide segments.

2.2.6 Data analysis

The technique was repeated 3 times for each sample, and the average value was taken for statistical analysis. The experiment was conducted before and after comparison, the day of mating and the day after mating were compared, and the differential proteins were screened. The screening conditions for differential proteins were: Fold change (FC) ≥ 1.5 or ≤ 0.67, and $P < 0.05$ for double-tailed unpaired T-test analysis. The differential proteins were analyzed by Uniprot website (https://www.uniprot.org/) and the relevant literature was searched in Pubmed database

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(https://pubmed.ncbi.nlm.nih.gov) to analyze the function of the differential proteins.

3 Experimental results and analysis

3.1 Comparison of urine proteome between the day after mating and the day after mating

3.2.1 Differential protein

The urine protein of the next day of mating was compared with that of mating day. The screening conditions for differential protein were FC≥1.5 or ≤0.67, and the two-tailed unpaired T-test was P<0.05. The results showed that 43 differential proteins could be identified on the day after mating compared with the day of mating. First, the identified differential proteins were analyzed by using STRING database for protein interaction, and the results were shown in Figure 1. Then the differential proteins were arranged in the order of FC from largest to smallest and retrieved by Uniprot. The results are shown in Table 1.

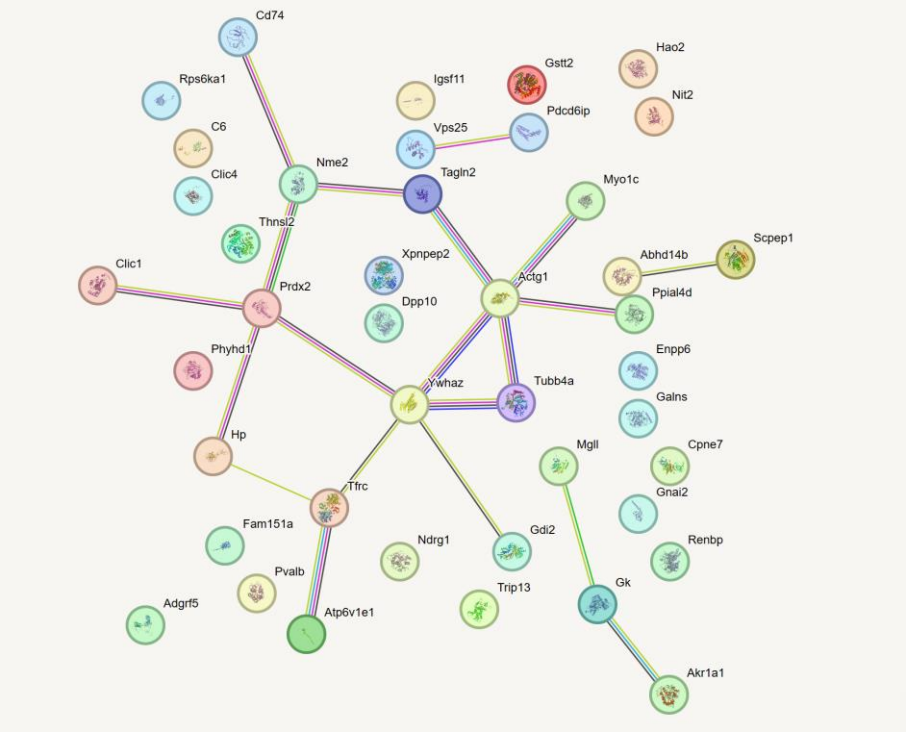

Figure 1. Interaction of urine differential proteins between the next day of mating and the day after mating

Table 1. Urine differential proteins between the next day of mating and the day after mating

Uniprot ID	Protein names	Fold change	Trend	P value	Related to implantation
Q8R431	Monoglyceride lipase	13.75	↑	5.71E-03	4
P0C0A1	Vacuolar protein-sorting-associated protein 25	8.48	↑	5.31E-03	5
Q63531	Ribosomal protein S6 kinase alpha-1	7.80	↑	3.12E-02	6 7
P30713	Glutathione S-transferase theta-2	5.02	↑	2.05E-02	8 9

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Q63060	Glycerol kinase	4.87	↑	6.89E-03	10
P63259	Actin, cytoplasmic 2	4.50	↑	3.11E-02	11 12 13
Q6Q629	Inactive dipeptidyl peptidase 10	4.02	↑	2.29E-02	14 15
Q63355	Unconventional myosin-Ic	3.91	↑	2.17E-02	16
P63095	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	3.75	↑	2.59E-02	17 18
Q5BJP9	Phytanoyl-CoA dioxygenase domain-containing protein 1	3.72	↑	3.92E-02	
P04897	Guanine nucleotide-binding protein G(i) subunit alpha-2	3.60	↑	1.70E-02	19 20
Q6PCU2	V-type proton ATPase subunit E 1	3.07	↑	4.20E-04	21 22
Q9Z0W7	Chloride intracellular channel protein 4	2.92	↑	1.93E-02	23
Q6JE36	Protein NDRG1	2.76	↑	2.77E-02	24
P06866	Haptoglobin	2.73	↑	1.72E-02	25 26
Q99376	Transferrin receptor protein 1 (Fragment)	2.71	↑	1.29E-02	27 28
Q6MG61	Chloride intracellular channel protein 1	2.34	↑	2.26E-02	23
Q6P9T8	Tubulin beta-4B chain	2.33	↑	2.62E-02	29 30
Q99MA2	Xaa-Pro aminopeptidase 2	2.31	↑	3.91E-02	
Q497B0	Omega-amidase NIT2	2.24	↑	3.23E-02	
Q9QZA2	Programmed cell death 6-interacting protein	2.15	↑	3.33E-02	
H1UBN0	Copine-7	2.15	↑	4.97E-02	
Q811M5	Complement component C6	2.04	↑	3.51E-02	
P50399	Rab GDP dissociation inhibitor beta	2.03	↑	2.16E-02	31
Q6DGG1	Protein ABHD14B	1.94	↑	4.52E-02	
P51635	Aldo-keto reductase family 1 member A1	1.94	↑	1.40E-02	32 33
Q07523	Hydroxyacid oxidase 2	1.93	↑	4.56E-02	
Q5XHZ9	Pachytene checkpoint protein 2 homolog	1.90	↑	3.95E-02	34 35
Q5XFX0	Transgelin-2	1.86	↑	2.75E-02	36 37
Q32KJ6	N-acetylgalactosamine-6-sulfatase	1.85	↑	2.57E-02	
P10247	H-2 class II histocompatibility antigen gamma chain	1.85	↑	4.86E-02	
P51607	N-acylglucosamine 2-epimerase	1.84	↑	1.47E-02	
Q920A6	Retinoid-inducible serine carboxypeptidase	1.74	↑	2.10E-02	
Q9WVT0	Adhesion G protein-coupled receptor F5	1.69	↑	3.91E-02	38
Q5M7T9	Threonine synthase-like 2	1.64	↑	3.75E-03	
P63102	14-3-3 protein zeta/delta	1.60	↑	3.57E-02	39 40
P35704	Peroxiredoxin-2	1.58	↑	4.64E-02	41 42
B0BND0	Ectonucleotide pyrophosphatase/phosphodiesterase family member 6	1.57	↑	9.37E-03	

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Q5U2P2	Immunoglobulin superfamily member 11	1.57	↑	2.19E-02	
P10111	Peptidyl-prolyl cis-trans isomerase A	1.56	↑	3.49E-02	43
P19804	Nucleoside diphosphate kinase B	1.54	↑	1.12E-02	44 45
Q642A7	Protein FAM151A	1.51	↑	3.94E-02	
P02625	Parvalbumin alpha	0.40	↓	1.07E-02	46

3.2.2 Differential protein function analysis

The 43 identified differential proteins were searched through PubMed database, and 26 of them or other members of their family were reported to be associated with spermatogenesis.

Monoglyceride lipase is highly expressed in the testes and is directly involved in regulating human testicular physiology as part of the endocannabinoid system, including spermatogenesis and the function of stromal cells [4].

Mutations in Vacuolar protein-sorting associated protein 33 B from the same family as Vacuolar protein-sorting associated protein 25 cause sterility in C. elegans, Spermatocyte with division arrest indicates that this protein is involved in the formation of sperm-specific organelles [5].

Ribosomal protein S6 kinase alpha-1 substrate Ribosomal protein S6 is critical for spermatogenesis, and knockdown of this protein in Chinese bream resulted in spermatogenesis defects, including germ cell loss, mature sperm retention, and cavity formation [6]. The protein can also regulate the blood-testicular barrier of supporting cells through Akt1/2, and then regulate the organization of F-actin, regulate the adhesion function of the intercellular interface, and promote the transport of blood-testicular barrier of spermatocytes in the pre-leptotene stage during rat spermatogenesis [7].

Glutathione S-transferase plays an important role in spermatogenesis and normal sperm function [8], The ineffective genotype of Glutathione S-transferase theta-1, the same family as Glutathione S-transferase theta-2, has been associated with dysspermatogenesis and may contribute to susceptibility to dysspermatogenesis and male infertility in the Chinese population [9].

Glycerol kinase 2, which has a high degree of homology with Glycerol kinase kinase, is crucial for the correct arrangement of crescent mitochondria to form mitochondrial sheath during mouse spermatogenesis. Knockout of this gene will lead to the disorder of mitochondrial sheath of sperm flagella [10].

Actin is involved in various aspects of spermatogenesis, and with spermatogenesis, the Actin cytoskeleton exhibits active remodeling during this process, participating in the shaping and differentiation of sperm cells [11 12]. However, the molecular mechanisms by which Actin cytoskeletal tissue responds to spermatogenesis in spermatogenic epithelial cells remain largely unexplored [13].

The prostate secretes enzyme-containing vesicles called prost glands into human semen. At neutral or slightly acidic pH, the prost gland fuses with the sperm, thereby transferring certain molecules into the sperm. Dipeptidyl peptidase IV activity, which was not present in sperm, was transferred from the prost gland to sperm, allowing sperm to acquire a new membrane binding enzyme and change the catalytic activity of its surface [14]. Guinea pig sperm contains Dipeptidyl peptidase II, and the protein is confined to a single compartment in the acrosomal sperm [15].

During spermatogenesis, spermatogonial cells undergo mitosis and meiosis to form bundles of

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sperm cells that remain connected to each other via cytoplasmic Bridges, which are individualized to form individual sperm cells. The process of individuation involves the formation of cytoskeletal proteins and membrane complexes around the sperm nucleus, the individualised complex, which breaks down the shared membrane into a single membrane surrounding each sperm cell. Unconventional myosin 95F myosin, which belongs to the Unconventional myosin family as well as Unconventional Myosin-IC, is a component of the individualization complex and is involved in membrane recombination during individualization. Its function is critical to the individualization process, and mutations in partial loss of function of this protein can lead to male infertility[16].

Guanine nucleotide-binding protein G(s) subunit alpha is expressed in a tissue-specific and age-dependent manner in the reproductive organs of the ram. The protein is expressed at high levels in the epididymis, suggesting that it may influence the composition of the epididymal lumen fluid, thus, it affects the microenvironment of sperm maturation and may play an important role in spermatogenesis and the development of the testis and epididymis in the ram reproductive system [17]. Guanine nucleotide-binding protein G(o) subunit alpha, from the same family as Guanine nucleotide-binding protein G(s) subunit alpha, The high level of expression in rat spermatocytes during pachytene stage suggests that it may play a role in this stage of spermatogenesis [18].

Guanine nucleotide-binding protein G(i) subunit alpha-1、Guanine nucleotide-binding protein G(i) subunit alpha-2、Guanine nucleotide-binding protein G(i) subunit alpha-3 and Guanine nucleotide-binding protein G(o) subunit alpha were detected in mouse spermatocytes and sperm cells. When spermatocytes develop into sperm cells, Guanine nucleotide-binding protein G(o) subunit alpha levels are reduced [19]. Guanine nucleotide-binding protein G(i) subunit is associated with the developing acrosome, which may play a role in acrosome biogenesis. The Guanine nucleotidebinding protein G(i) subunit is found in the acrosomal region of mammalian sperm and is part of a complex required for signal transduction to induce acrosomal exocytosis [19 20].

V-type proton ATPases play an important role in the capacitation process of rabbit sperm [21]. Rat round sperm cells regulate intracellular pH through V-type proton ATPases, HCO3- entry pathways, Na+/HCO3- dependent transport systems, and postulated proton conduction pathways. These pH adjustments appear to be specifically designed to withstand the acid challenge [22].

There are Chloride intracellular channel protein in bovine epididymis sperm。Chloride intracellular channel protein 1, Chloride intracellular channel protein 4 and Chloride intracellular channel protein 5, are present in the sperm, and occupy different positions in the cell. Both are able to bind to PP1γ2 in sperm, and since PP1γ2 is a key enzyme regulating sperm motility, the PP1γ2-binding protein Chloride intracellular channel protein may play an important role in sperm function[23].

The N-myc downstream regulatory gene (NDRG) family consists of NDRG-1, NDRG-2, NDRG-3 and NDRG-4 members. It has been reported that Ndrg3 is a key gene that causes homologous lethal in early embryonic development, regulating male meiosis in mice. NDRG3 expression was specifically enhanced in germ cells and reached a peak level in spermatocytes of pachytene stage.In NdrG3-deficient germ cells, ERK activation is attenuated and double-strand break repair and synapsis complex formation in meiosis are impaired [24].

Haptoglobin, an iron transporter expressed in Sertoli, Leyden, and germ cells of the rat testis

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but not in the epididymis, may play an important role in iron metabolism in the testis. Testis Haptoglobin mRNA levels steadily increase during maturation after birth, indicating its involvement in spermatogenesis [25]. Sertoli cells play a key role in spermatogenesis and express receptors for follicle-stimulating hormone (FSH) and testosterone (T), the major hormonal regulators of spermatogenesis.FSH stimulation of Sertoli cells in pigs resulted in an increase in inhibin-α, inhibin-β, plaquoglobin, haptoglobin, D-3-phosphoglycerate dehydrogenase, and sodium/potassium transport atpase in Sertoli extracellular vesicles [26].

The Transferrin receptor protein was identified in the proteome of newborn mouse testis, suggesting that it is involved in meiosis. Transferrin receptor proteins are essential for the progression of spermatocyte meiosis, particularly for DNA double-strand break repair and synapsis [27]. The Transferrin receptor protein is only found in human spermatocytes and early spermatocytes.In patients with sperm genetic disorders, Transferrin is always present in Sertoli cells, and Transferrin receptor protein is only found when spermatocytes are present. In human seminiferous tubules, Sertoli cells are dedicated to Transferrin production, storage, and storage. Spermatocytes and early sperm cells use Transferrin[28].

Many processes in spermatogenesis depend on the dynamic changes of the cytoskeleton, the movement of organelles, and especially the regulation of microtubules. Data from transgenic mouse models suggest that the coordination of microtubule dynamics is critical for male fertility [29]。 During spermatogenesis, a structure called "nuage" appears and disappears as spermatogenic cells differentiate. nuage can be divided into four types: Irregularly Shaped Perinuclear Granule (ISPG), Intermitintermital Cement (IMC), and Satellite Body (SB), Chromatoid Body (CB). ISPG, IMC and SB were observed in pachytene spermatocytes, while CB was observed in round sperms. In rat round sperm cells, Tubulin beta is translated from mRNA stored in CB, and assembled with Tubulin alpha outside CB to form a structural unit of microtubules: αβ-heterodimer, to construct microtubules in the sperm flella[30].

Rab GDP dissociation inhibitor beta and family member Rab GDP dissociation inhibitor alpha are involved in the organization of actin cytoskeleton and also regulate cell morphology and cell motility. The expression of this protein is decreased in patients with asthenospermia [31].

Increased ability to metabolize specific steroids during testicular development, such as increased expression of Aldo-keto reductase family 1 member C3, the same family member as Aldo-keto reductase family 1 member A1, during testicular development in domestic cats [32]. The level of Aldo-Keto Reductase mRNA in silkworm testis is higher than that in other tissues, which plays an important role in the spermatogenesis of silkworm [33].

Pachytene checkpoint protein 2 homolog was searched by Uniprot database. The results showed that this protein is expressed in the male reproductive nucleus, and is involved in biological processes such as sperm development, sperm formation, male meiosis, synaptic complex, meiosis recombination, double-strand break repair, and meiosis recombination checkpoint signaling pathway. This protein plays a key role in chromosome recombination and chromosome structure development during meiosis.In the early stage of meiosis recombination, it mediates the non-crossed pathway and also influences the crossed and non-crossed pathways to effectively complete homologous chromosome association, which is necessary for the effective sex chromosome

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synapsis. Mouse Pachytene checkpoint protein 2 is required for recombination and normal higher-order chromosome structure during meiosis. Pachytene checkpoint protein 2 plays a potential role in the non-crossed repair of double-strand breaks in meiosis. The spermatocytes of male mice with a complete mutation in this gene can fully associate with their chromosomes. However, in the process of chromosome recombination, the loss of this leads to the failure of repair of double-strand breaks, cell death at the pachytene stage, and the lack of post-meiotic cells in the testicular tissue [34] [35]. In males, Pachytene checkpoint protein 2 is required for sex chromosome syndesmosis and the formation of sex bodies (transcription-silencing subnuclear domains formed by the X and Y chromosomes) [35].

Acute heat stress impels translation, protein folding and protein degradation processes in chicken testis, leading to apoptosis and interfering with spermatogenesis. After acute heat stress, Transgelin is upregulated in the testis to resist heat-induced damage [36]. The expression level of Transgelin gene was low in testicular tissue of aged animals [37].

Adhesion G protein-coupled receptor A3, which belongs to the same family as Adhesion G protein-coupled receptor F5, is a known marker of spermatogonial stem cells, and 55% of mice that have this gene deleted, even with normal spermatogenesis and epididymal sperm count, they are infertile from puberty [38].

The 14-3-3 protein plays a key regulatory role in both mitosis and meiosis. In mice, 14-3-3 protein epsilon is essential for normal sperm function and male fertility [39]. Mature spermatids need to be released from their attached sertoli cells. Identified proteins involved in the adhesion between sertoli cells and mature spermatids include 14-3-3 protein zeta/delta, which was only found in the tubular segment lysate during sperm release. But what exactly is its role in spermatogenesis, and how does it interact or influence other signal transduction pathways in the testes remain unknown [40].

Peroxiredoxin-2 has some antioxidant properties and may be involved in maintaining oxidative balance in the spermatogenic environment of mice [41]. Peroxiredoxin-2 also maintained the normal development of reproductive cells in newborn rats [42].

Peptidyl-prolyl cis-trans isomerase A is upregulated in the testes of mice treated with environmental estrogen, which reduces male sperm count and leads to male infertility. However, the molecular mechanism of its effect on male infertility remains unclear [43].

Nucleoside diphosphate kinase B is distributed in the manchette microtubule structure of sperm (a transient microtubule structure in elongated sperm that plays an important role in nuclear enrichment and sperm tail formation). Nucleoside diphosphate kinase A is distributed instantaneously in the round sperm nucleus and asymmetrically in the cytoplasm of the elongated sperm nucleus base. Nucleoside diphosphate kinase subtypes may have specific functions in phosphate transfer networks involved in human spermatogenesis and flagellar motility [44]. Nucleoside diphosphate kinase plays a key role in spermatogenesis by increasing the level of glutathione peroxidase 5 in mouse cells to eliminate reactive oxygen species [45].

The combination of injection of testosterone undecanoate and oral levonorgestrel enhanced the expression of Parvalbumin alpha and inhibited spermatogenesis. Parvalbumin alpha protects testicular cells from apoptosis and promotes cell survival, and may be an early molecular target for

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hormone-induced spermatogenesis inhibition [46].

4 Discussion

Rats were self-controlled in the experiment, and the interval between two urine collection was only one day, so as far as possible, the interference of individual differences and their own growth and development could be excluded. Therefore, even with a small sample size, the results of this study could preliminarily show that there were significant differences in the urine proteome of rats on the second day after mating and on the mating day, and most of the differences were related to spermatogenesis. Although the correlation between the remaining differential proteins and spermatogenesis has not been found in the database, the results of this study suggest that these proteins may still be related to spermatogenesis and can be further studied as target proteins of spermatogenesis. This study demonstrates the potential of urine proteome in studying spermatogenesis, and provides a method of urine proteomics for exploring the pathogenesis of abnormal spermatogenesis in males, discovering targets and new diagnostic methods. Further experiments may consider expanding the sample size of experimental animals or collecting clinical samples for research. At the same time, it also reflects the sensitivity of urine proteome and opens up a new field of urine exploration.

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