Proteomic analysis of giant panda testicular tissue of different age groups

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ABSTRACT

Background. The reproductive ability of male giant pandas has been a major complicating factor in the *ex-situ* conservation of the species. While it is well known that the testis produces sperm and secretes androgens, a process that requires precise regulation of various proteins, at present, there has been no systematic study on the composition of proteins in the testis of the giant pandas. Therefore, this study aims to apply proteomics to explore the regulation of proteins in the testes of giant pandas.
Methods. Samples from the testes of three giant pandas (22 years, 18 years, 8 days) were studied to assess the protein's function. A label-free quantitative method was used to isolate testicular proteins from each male, 139,039 peptides and 11,435 proteins were obtained.
Results. Gene Ontology (GO) annotates most of the proteins involved in the processes of protein phosphorylation, oxidation-reduction, proteolysis, and signal transduction. KEGG pathway indicated that most of the proteins were involved in the pathway of signal transduction, transport, and catabolism. The protein kinase and WD40 repeats were involved in protein-protein interaction, which in turn regulates gene expression in the testicular tissue of giant pandas.
Conclusions. This study is the first to conduct an in-depth proteomic analysis of testicular tissue in giant pandas. The results revealed the important role of proteins in testicular tissue on spermatogenesis, testosterone production, and testicular microenvironment, providing clues for further research on male giant panda reproduction.

Subjects Biochemistry, Bioinformatics, Conservation Biology, Veterinary Medicine, Zoology
Keywords Proteomics, Testicular tissue, Giant pandas

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INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*), known as China's national treasure, is a flagship species, a threatened species of global concern, and a worldwide symbol of conservation (*Shen et al., 2021*). Captive giant pandas have a low reproductive capacity, both male and female pandas can have reproductive problems, such as low sperm count in males and uterine issues in females (*Cao et al., 2020*; *Ran et al., 2018*). Natural mating and artificial insemination are common methods for the reproduction of giant pandas in captivity. However, in the artificial insemination of giant pandas, fresh sperm is mainly used, while

frozen-thawed sperm is rarely utilized. The lower farrowing rate also explains the poor utilization efficiency of frozen-thawed giant panda sperm. In addition, only about 25% (3 out of 11) of giant pandas at the ideal breeding age mate natural (*Ran et al., 2018*). Furthermore, giant pandas were confirmed to have embryo diapause in 2009 (*Zhang et al., 2009*); that is, during pregnancy, the embryo floats in the womb and stops developing until it attaches to the uterine wall a few months later. The delayed embryo implantation in giant pandas results in a prolonged gestation period. Despite the longer pregnancy period of giant pandas, the newborn cubs are underdeveloped, with a weight of only 90 to 130 grams (*Cao et al., 2020*). The testicle is the main reproductive organ of the male giant panda, as it produces sperm and secretes testosterone. Due to the scarcity of samples, studies on testes from giant pandas are very limited (*Ran et al., 2018*; *Zheng et al., 2022*). Previous studies have provided important information regarding testis morphology and gene transcription, however, protein abundance, post-translational protein modification, and protein interactions have not been comprehensively studied (*Lu et al., 2018*). Proteomics serves as a robust platform for investigating individual proteins as well as intricate protein mixtures. By integrating separation methods such as gel electrophoresis and chromatography with subsequent mass spectrometry (MS)-based analysis and bioinformatics tools, it facilitates the exploration of diverse inquiries spanning medicine and fundamental scientific inquiry. Notably, it allows for a more in-depth exploration of biological processes and pathways (*Rozanova et al., 2021*; *Tsai et al., 2012*). With the successful sequencing of the human genome and key mammalian genomes used in biological research, proteomics has fundamentally transformed systems biology and biomedical science (*Holland & Ohlendieck, 2015*).

In recent years, researchers have utilized various technologies to study the proteomics of testicular tissue in different mammals. *Cossio et al. (1997)* employed two-dimensional electrophoresis (2-DE) technology to isolate proteins from the rat testis (*Cossio et al., 1997*). Subsequently, *Witzmann, Fultz & Wyman (1997)* utilized the same approach to separate proteins from the testes of bulls (*Witzmann, Fultz & Wyman, 1997*). Researchers consequently integrated 2-DE with mass spectrometry (MS) to characterize testicular proteins across various species, including humans (*Li et al., 2011*), pigs (*Huang et al., 2005*), yaks (*Yang et al., 2016*), rats (*Huo, Zhang & Lin, 2012*), as well as other species (*Sun et al., 2017*). This approach led to the identification of numerous proteins associated with testicular development, serving as biomarkers for male reproductive capacity (*Lu et al., 2018*; *Ribeiro et al., 2021*). *Paz, Morín & Del Mazo (2006)* utilized MALDI-TOF mass spectrometry to comparatively analyze the proteomic characteristics of soluble proteins expressed at different stages of mouse testis development (postnatal days 8, 18, and 45). They identified 44 proteins or variant forms and characterized their developmental patterns across various stages of spermatogenesis. A high proportion of proteins with redox or antioxidant activity, as well as enzymes involved in lipid and carbohydrate metabolic pathways, were identified. The identified proteins are widely conserved across species, predominantly in mammals (*Paz, Morín & Del Mazo, 2006*). Testicular proteomics was also performed with samples collected from pigs as an animal model. *Huang et al. (2011)* conducted a comparative proteomic study on pig testicular tissue, encompassing animals ranging from 1 week old to 1 year of age (*Huang et al., 2011*), they utilized 2-DE to separate

testicular proteins and 264 spots were identified using MALDI-TOF. The results indicate that there are 108 differentially expressed protein spots between different developmental stages. These proteins are categorized as types of expression that increase with age, decrease with age, or fluctuate during developmental stages. Understanding these differentially expressed proteins is valuable for exploring developmental biology and the pathology of male reproduction. A tandem mass tag coupled with LC-MS/MS, was used by *Huang et al. (2011)*, employing high-throughput quantitative proteomics technology, the study aimed to investigate protein expression within buffalo testicular seminiferous tubules across three distinct developmental stages: prepuberty, puberty, and postpuberty (*Huang et al., 2016*). The results show that 304 differentially expressed proteins exhibited changes of at least two-fold. Bioinformatics analysis suggests that 27 of these may be related to spermatogenesis. The aforementioned proteomic studies on the testicular tissues of various mammals have laid a solid foundation for the proteomic research of giant panda testes and provided a valuable reference framework. These studies have not only revealed the commonalities and characteristics of mammalian testes at the protein level but also provided important biological information and analytical methods for an in-depth understanding of the reproductive mechanisms of the giant panda, a unique species. Through proteomic analysis of other mammals, researchers have developed a series of efficient technical methods and bioinformatics tools that can be directly applied to the study of giant panda testicular tissue. For instance, technologies such as 2-DE, MS, and MALDI-TOF have successfully identified proteins related to spermatogenesis in multiple species. The application of these technologies will help discover similar biomarkers in giant pandas and may reveal reproductively related proteins that are unique to them.

However, proteomic studies of giant panda testes have not yet been reported. *Huo, Zhang & Lin (2012)* used the genome sequence of giant pandas to study the function of proteins, specifically those associated with specific traits (*Huo, Zhang & Lin, 2012*). established Meanwhile, a gene expression profile in giant panda testes was established by *Zheng et al. (2022)* using single-cell RNA sequencing (*Zheng et al., 2022*). Therefore, considering the importance of improving the breeding rate of the giant panda, as well as exploring the protein composition and function of the giant panda testicular tissue at different ages, this study applied a label-free quantitative mass spectrometry analysis method to the testicular tissues of three giant pandas (aged 8 days, 18 years, and 22 years) to analyze the peptides resulting from the digestion of proteins in giant panda testicular tissue. Through in-depth analysis of these samples, we were able to reveal the patterns of protein expression in the testicular tissue of giant pandas at different developmental stages and identify biomarkers related to reproductive development. The findings of this study provide new insights into the reproductive physiology of the giant panda and may help us identify potential factors affecting the success of its breeding. In addition to its significant importance for the conservation of the giant panda, it also provides valuable reference for the reproductive biology research of other mammals. Through in-depth studies of species such as the giant panda, we can better understand the reproductive mechanisms in complex biological systems and contribute to the protection of biodiversity and the maintenance of ecological balance.

MATERIALS & METHODS

Source of panda testicular tissues

The normal testicular tissue came from the frozen sample bank of Chengdu Research Base of Giant Panda Breeding (CRBGPB), located in Sichuan Province, PRC. The samples were derived from three male giant pandas: a 22-year-old male panda (H22y) who died of non-testicular causes in old age, an 18-year-old male panda (F18y) with normal tissue sections taken from removed testicles for testicular tumor, and an 8-day-old male panda (J8d), who accidentally died due to rearing issues. All samples were frozen in liquid nitrogen and then transferred to a −80 °C freezer for storage. All procedures in this study were approved by the Institutional Animal Care and Use Committee of CRBGPB (approval no. CRBGPB-2018008) and adhered to the ethical rules and regulations for animal sample use for the People's Republic of China.

Total protein extraction

The testicular tissue samples from the giant pandas were retrieved from a −80 °C freezer, then cryogenically ground in liquid nitrogen and lysed using a lysis buffer containing 100 mM NH_4HCO_3 (pH 8), 6 M urea, and 0.2% SDS. Subsequently, the lysate underwent 5 min of ultrasonication on ice. Following ultrasonication, the lysate was centrifuged at 12,000 g for 15 min at 4 °C, and the resulting supernatant was carefully transferred to a clean tube. The extracts from each sample were then treated with 10 mM DTT^{red} (Sigma, St. Louis, MO, USA) for 1 h at 56 °C to achieve reduction, followed by alkylation with an adequate amount of iodoacetamide (Sigma, St. Louis, MO, USA) for 1 h at room temperature in the dark. Following alkylation, the samples were thoroughly mixed with four times the volume of pre-cooled acetone through vortexing and incubated at −20 °C for a minimum of 2 h. Subsequently, the samples underwent centrifugation, and the resulting precipitation was collected. After two washes with cold acetone, the pellet was dissolved in a dissolution buffer containing 0.1 M triethylammonium bicarbonate (TEAB, pH 8.5) and 6 M urea.

Protein quality test

We utilized the Bradford protein quantification kit (Bio-Rad, Hercules, CA, USA) to quantify the protein. Initially, we prepared a bovine serum albumin (BSA) standard protein solution as per the manufacturer's instructions, with a concentration gradient ranging from 0 to 0.5 μg/μL. Both the BSA standard protein solutions and sample solutions, each at varying dilution multiples, were dispensed into a 96-well plate, with a final volume of 20 μL in each well. This process was repeated three times for each gradient. Subsequently, we swiftly added 180 μL of G250 dye solution to the plate and allowed the mixture to sit at room temperature for 5 min. The absorbance at 595 nm was then measured. We constructed a standard curve using the absorbance values of the standard protein solution and determined the protein concentration of the samples accordingly. For SDS-PAGE gel electrophoresis, we loaded 20 μg of the protein sample onto a 12% gel. The concentrated gel was subjected to electrophoresis at 80 V for 20 min, followed by the separation gel at 120 V for 90 min. The gel was subsequently stained with Coomassie Brilliant Blue R-250 (Amresco, Solon, OH, USA) and destained until the bands were visible.

Trypsin treatment

We took 500 μg of the protein sample and added protein dissolution solution to reach a total volume of 200 μL. Add 5 μL of 1 μg/μL trypsin and one mL of 50 mM TEAB buffer. This was mixed well and incubated at 37 °C overnight for enzymatic digestion. An equal volume of 1% formic acid was added, mixed well, and centrifuged at room temperature at 12,000 g for 5 min. The supernatant was collected and slowly passed through a C18 desalting column. Subsequently, the column was washed three times with wash solution (0.1% formic acid, 4% acetonitrile), and then eluted with an appropriate amount of elution solution (0.1% formic acid, 45% acetonitrile) twice. Finally, the eluted samples were combined and freeze-dried.

Fractionation by chromatography

First, we prepared mobile phase A (2% acetonitrile, 98% water, adjusted to pH =10 with ammonia) and mobile phase B (98% acetonitrile, 2% water, adjusted to pH =10 with ammonia). Then the lyophilized powder in mobile phase A, followed by centrifugation at 12,000 g for 10 min at room temperature. An L-3000 HPLC system with a Waters BEH C_{18} column (4.6 × 250 mm, 5 μm) was utilized at a column temperature of 50 °C. Next, we collected 1 tube per minute, combined them into 10 fractions, and lyophilized them. Finally, we reconstituted each fraction with a solution containing 0.1% formic acid.

Liquid chromatography-mass spectrometry analysis

First, we prepared a mobile phase A (100% water, 0.1% formic acid) and mobile phase B (80% acetonitrile, 0.1% formic acid). Then the lyophilized powder in 10 μL of mobile phase A, followed by centrifugation at 15,000 rpm for 20 min at 4 °C. Next, we took 1 μg of the supernatant for injection in the LC-MS system. The EASY-nLCTM 1200 Nano UHPLC system was utilized with a custom-made 2 cm × 75 μm, 3 μm pre-column, and a custom-made 15 cm × 150 μm, 1.9 μm analytical column. The Q Exactive™ HF-X mass spectrometer was employed with a Nanospray Flex™ (ESI) ion source. The ion spray voltage was set to 2.3 kV, and the ion transfer tube temperature to 320 °C. For mass spectrometry, a data-dependent acquisition mode was used with a full scan range of m/z 350–1,500. The first-stage mass resolution was set to 60,000 (at 200 m/z), the C-trap maximum capacity to 3 × 10⁶, and the maximum injection time to 20 ms. The top 40 ions were selected from the full scan based on ion intensity for higher-energy collision dissociation (HCD) fragmentation in the second stage. The second-stage mass resolution was 15,000 (at 200 m/z), C-trap maximum capacity at 5 × 10⁴, maximum injection time at 45 ms, peptide fragmentation collision energy at 27%, threshold intensity at 2.2 × 10⁴, and dynamic exclusion set to 20s. Finally, the raw mass spectrometry data file (.raw) was generated.

Database retrieval and bioinformatics analysis

Mass spectrometry data analysis was performed using Proteome Discoverer 2.2 software. The utilized database was X101SC19103253-Z01_ailuropoda-melanoleuca-uniprot.fasta (35,787 sequences). The search parameters were as follows: type of quantification was Precursor Quantification, the instrument was QE HF-X, with Precursor Mass Tolerance of ±10 ppm and Fragment Mass Tolerance of ±0.02 Da. Carbamidomethyl of cysteine

was set as a static modification, while oxidation of methionine was considered a dynamic modification. Acetylation was designated as an N-terminal modification, and trypsin was chosen as the enzyme for digestion, allowing for a maximum of 2 missed cleavage sites. Peptide Spectrum Matches (PSMs) with a confidence level above 99% were designated as reliable PSMs. Proteins containing at least one unique peptide segment were considered credible proteins. Only the trustworthy peptide spectra and proteins were retained, and false discovery rate (FDR) validation was performed, eliminating peptides and proteins with an FDR exceeding 1%. KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg), COG (Cluster of Orthologous Groups of proteins), and databases such as Gene Ontology (GO, http://www.geneontology.org) and Domain (IPR) using Interproscan software for functional annotation.

RESULTS

Proteomic profiling of testis of three pandas

In this study, proteomic evaluation was used to study the changes in protein abundance of different aged giant pandas, to determine the markers of male reproductive performance for future studies.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) *via* the iProX partner repository with the dataset identifier PXD051003. The data access link on iProX is: https://www.iprox.cn/page/project.html?id=IPX0008444000. Figure 1A illustrates the experimental workflow for proteomic analysis. As a result, we identified a total of 139,039 peptides and 11,435 proteins (Table S1), the total protein numbers of the three giant panda testicular tissue samples were 10,090, 10,178, and 10,176, respectively (Fig. 1B). In addition to that, the Venn diagram (Fig. 1C) shows that 9,268 proteins were identified in common. Furthermore, J8d, F18y, and H22y each uniquely identified 298, 116, and 126 proteins, respectively.

The raw data obtained from the mass spectrometry needed to be controlled by a series of quality controls after the database was searched, including peptide length distribution, precursor ion mass tolerance distribution, unique peptide number distribution, protein coverage distribution, and protein molecular weight distribution. Firstly, each mass spectrometer has its measurement range, limiting the detectable peptide length. Peptides that are too long or too short cannot be detected by the mass spectrometer. The distribution of peptide lengths mainly falls between 7 and 25 residues (Fig. 2A), indicating the appropriate selection of protease enzymes. Secondly, there is a difference between the measured and theoretical molecular weights of peptides, which is important for evaluating mass spectrometer performance and result quality. The peak concentration around 0 indicates minimal mass deviation (Fig. 2B). Furthermore, after comparing identified peptide sequences with a protein database, proteins with identical peptide segments were grouped. Unique peptides within each group contribute to the protein's specificity and reliability. A shallower curve indicates a higher number of unique peptides, signifying a more reliable identification of proteins (Fig. 2C). For a given identified protein, a higher

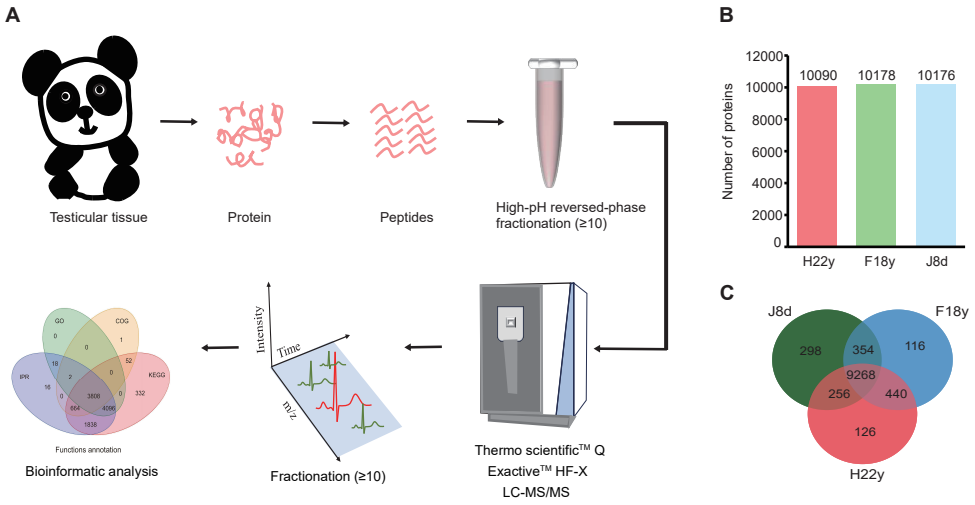

Figure 1 The workflow of panda testis proteomic analysis. (A) Experimental flow chart of deep proteomic analysis of testicular tissue of giant panda. (The panda element is original. Proteins, peptides, test tubes, and other elements are all derived from images in the analysis report of Novogene Corporation, and the copyright of the images belongs to the company. Authorization for their use has been obtained.) (B) Number of proteins identified in testicular tissue samples from three giant pandas. (C) The Venn diagram illustrates the amount of protein in the testicular tissues (J8d, F18y, and H22y) of giant pandas at different ages.

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count of supporting peptides enhances its credibility. Hence, the coverage of identified proteins indirectly reflects the overall accuracy of the results. The chart demonstrates consistent coverage across intervals, indicating high identification accuracy (Fig. 2D). Additionally, the (0, 0.1) interval has the highest count of peptides, suggesting the highest credibility within this range. Finally, the distribution of protein molecular weights is a crucial indicator of identified protein sizes. The chart displays a distribution of proteins across a wide range of molecular weights from 0 to above 100 kDa (Fig. 2E), signifying a broad spectrum of identified proteins.

Functional analysis of identified testis proteins

Currently, commonly used functional databases for annotation include Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Cluster of Orthologous Groups of Proteins (COG). These databases were utilized to functionally annotate proteins identified in the giant panda testicular tissue, aiming to understand the functional characteristics of different proteins and facilitate insights into the functionality of giant panda testicular tissue. As depicted in the Venn diagram (Fig. 3A), the total number of proteins annotated by GO, KEGG, COG, and IPR are 7,924, 10,790, 4,527, and 10,442, respectively. Among them, 3,808 proteins were commonly annotated. Additionally, KEGG, COG, and IPR individually annotated 332, 1, and 16 proteins, respectively. GO analysis was performed on the proteins identified from the testicular tissues of giant pandas to extract functional information about the proteins. Analysis results of the top 10-degree values in each category were selected and presented in histograms (Fig. 3B). Among

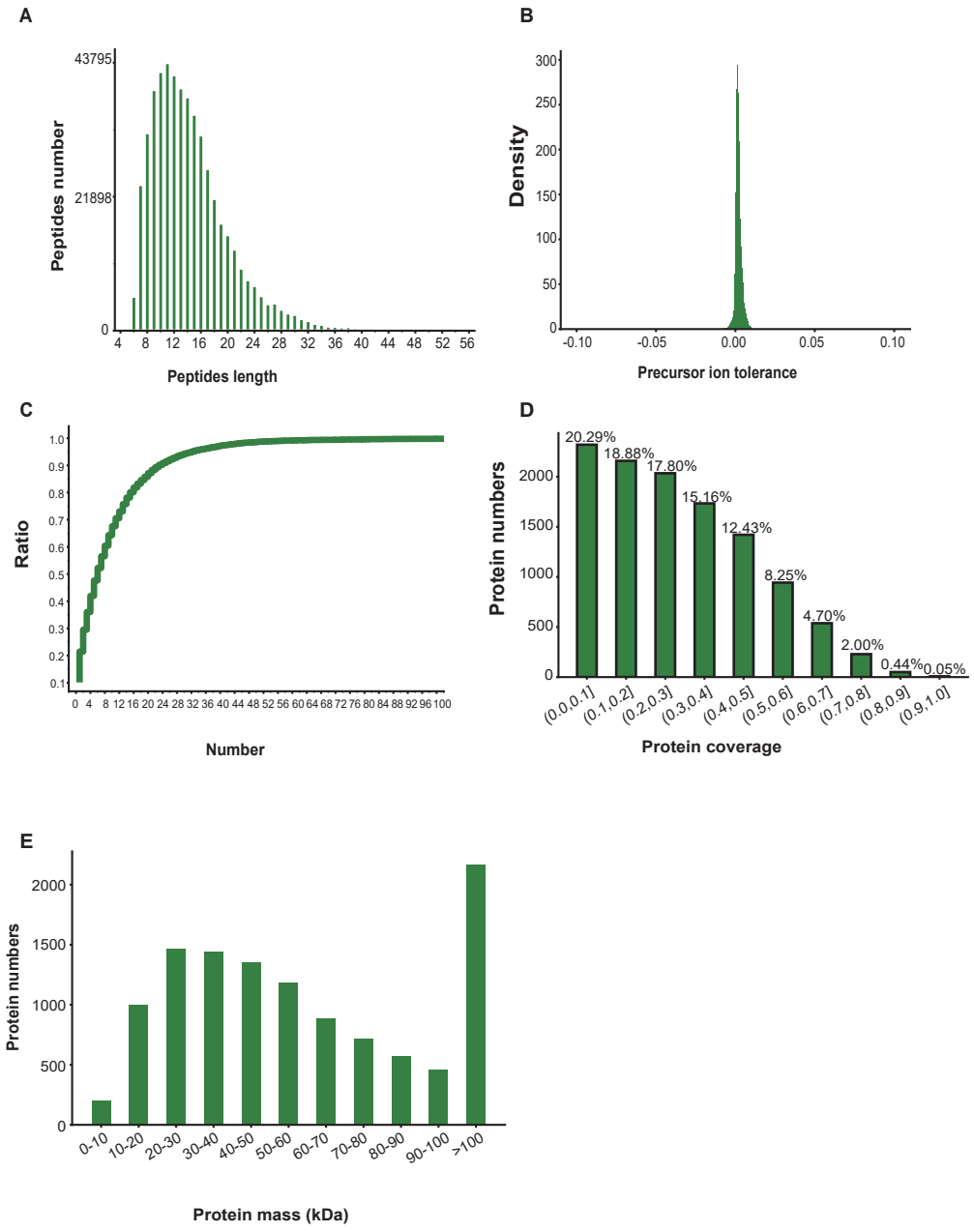

Figure 2 **Data quality control content.** (A) Peptide length range distribution. The *x*-axis represents the number of amino acid residues in peptide segments, and the *y*-axis represents the count of peptides at each length. (B) Precursor ion mass tolerance distribution. The *x*-axis represents mass deviation, and the *y*-axis shows the density distribution of precursor ion errors. (C) Map of the number of unique peptides in the identified protein. The *x*-axis represents the number of unique peptides, while the *y*-axis depicts the cumulative percentage of proteins containing these unique peptides as their count increases. (D) Protein coverage distribution. The *x*-axis represents intervals of protein coverage (the proportion of the protein's length covered by detected peptides relative to its total length), and the *y*-axis shows the number of proteins within each interval. (E) Protein molecular weight distribution. The *x*-axis represents the identified protein molecular weight (kDa), while the *y*-axis shows the count of identified proteins.

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them, the top five enrichment biological processes were: protein phosphorylation, redox processes, proteolysis, signal transduction, and metabolic processes. The function of the enriched molecule is mainly the binding reaction, such as protein binding, ATP binding, *etc*. Metabolic processes are key processes that maintain the activity of testicular tissues. Metabolites are not only essential for cell signaling and energy transfer but are also directly influenced by the physiological and pathological changes in tissues and accurately reflect the physiological changes.

The cluster of COG is the database for the orthologous classification of proteins and the proteins that make up each COG are assumed to be derived from a common protein ancestor (*Li et al., 2016a*). The COG classification of proteins is of great significance for functional annotation and evolutionary research (*Li et al., 2016b*). The identified proteins were divided into 26 COG categories, with the top group being General function prediction only (group R) (Fig. 3C). Translation, ribosomal structure and biogenesis (group J), and signal transduction mechanisms (group T) tied for second place. Posttranslational modification, protein turnover, and chaperones (group O) were third. There were 4,527 proteins annotated based on the COG database. However, about 60.41% of the identified proteins were not assigned as functional annotations.

KEGG is a pathway-based categorization of orthologous genes that provides biochemical metabolic pathway and signal transduction pathway analyses for a gene or protein dataset (*Zhai et al., 2020*). In this study, 33 pathways were mapped, mainly related to cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems (Fig. 3D). The most annotated pathway was signal transduction (980 proteins). Signal transduction proteins play a crucial role in the testes, where they mediate the actions of hormones and other signaling molecules, which are essential for testicular function, including spermatogenesis (the production of sperm), Leydig cell function (testosterone production), and the overall regulation of the testicular microenvironment (*Luo et al., 2022*; *Smith & Walker, 2014*). Some of the other major pathways include transport and catabolism, cell growth and death, associated with lipid/carbohydrate/amino acid metabolism, translation, folding, sorting, and degradation pathways.

IPR and subcellular localization analysis

Protein domains are reusable segments of proteins that play an important role in protein evolution. As shown in Fig. 3E, this study divided the identified proteins into 21 domains, of which the top three domains were: the protein kinase domain (319 proteins), WD40 repeat (237), and WD40-repeat-containing domain (215). The cell of an organism is a highly ordered structure, and the intracellular layer can be divided into different organelles or cellular regions according to different spatial distributions and functions. There were 15 types of subcellular localization of the identified proteins (Fig. 3F), the most common of which was nucleus protein, accounting for 32.89%. The second and third largest were cytoplasm protein (17.98%) and plasma membrane protein (10.48%), respectively.

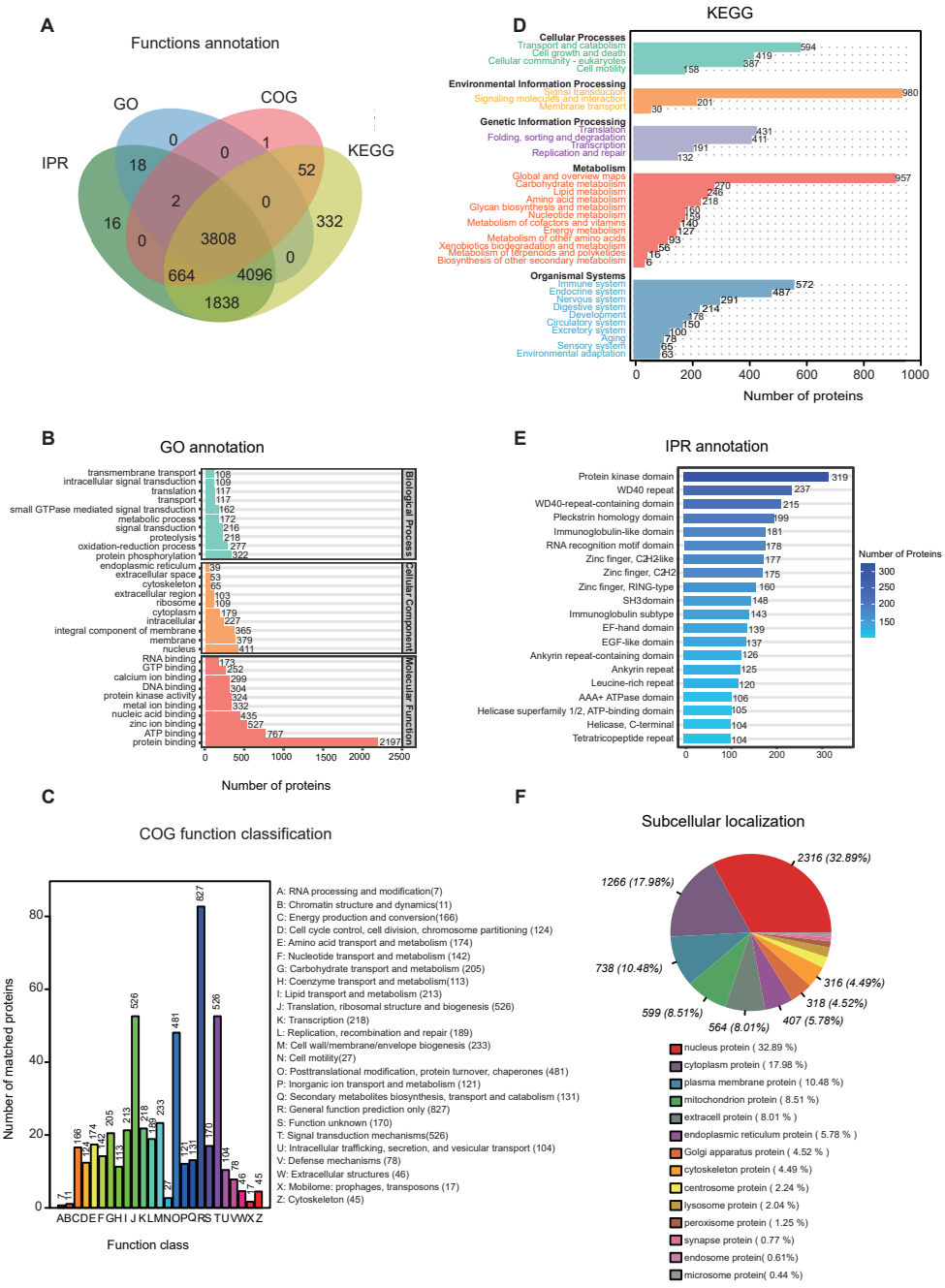

Figure 3 **Annotation of protein function identified in testicular tissue of giant pandas.** (A) A Venn diagram illustrates the number of identified proteins by using the GO, KEGG, IPR, and COG databases. (B) GO analysis of identified proteins includes biological processes, molecular functions, and cellular components. (C) Proteomic COG analysis allows for inferring the function of the identified protein by comparing the protein sequences of the NCBI database. (D) The identified proteomic KEGG pathway analysis reveals the biological functions of different proteins. (E) Domain-annotated proteins with patterns, structures, or features using Interproscan software (F) Subcellular localization information of the identified protein.

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GO analysis of age-specific unique proteins in three different age groups

Based on our previous research, we have identified the presence of certain unique proteins in the testicular tissues of giant pandas at three distinct age stages (specifically 8 days, 18 years, and 22 years old). To gain a deeper understanding of the biological significance of these proteins and their roles in the reproductive system, we conducted a functional enrichment analysis on these unique proteins. The results of this analysis will help us to elucidate their involvement in biological processes, their molecular functions, and their cellular locations. This comprehensive approach aims to provide insights into the specific functions of these proteins and their potential implications for the reproductive health and fertility of giant pandas. Figure 4A represents the GO analysis of unique proteins from the F8d sample, which has enriched a total of 20 biological processes. The top five enriched biological processes are Cell morphogenesis, Growth, Heart development, Glycoprotein metabolic process, and Cell junction organization. Figure 4B illustrates the GO analysis for unique proteins in the F18y sample, revealing enrichment in seven biological processes: Progesterone-mediated oocyte maturation, Estrogen signaling pathway, Response to alcohol, Oxidative phosphorylation, Membrane lipid biosynthetic process, Regulation of G protein-coupled receptor signaling pathway, and Modulation of chemical synaptic transmission. Finally, Fig. 4C represents the GO analysis of unique proteins in the H22y sample, which has enriched a total of three biological processes: Response to bacterium, Positive regulation of calcium ion transport, and Metabolism of water-soluble vitamins and cofactors.

Protein quantification and differential analysis

Proteome Discoverer 2.2 first obtained the relative quantification value of each PSM in each sample based on the peak area of the original lower spectrum and then corrected the relative quantification value of the unique peptide according to the quantification information of all PSMs contained in the identified unique peptide. Then it corrected the relative quantification value of each protein according to the quantitative information of all unique peptides contained in each protein. The results showed that the top five proteins with the highest abundance among the proteins identified by H22y were D2HU49, G1LEJ5, P18970, D2H496, and D2HVQ7. The five most abundant proteins identified by F18y were G1LEJ5, D2H496, D2HU49, G1LX08, and G1MDU7. In addition, the top five proteins identified from J8d were G1LEJ5, D2HU49, P18970, G1LVM5, and G1LP52 (Table S2). Ultimately, nine unique proteins were highly abundant in the three pandas, and we analyzed their abundance patterns from young to old times and found that these proteins were more abundant in the young and old periods, except for G1LEJ5, which had the opposite characteristics (Table S2). Among them (Table 1), G1LEJ5, D2HU49, and D2H496 are all uncharacterized proteins, which have potential research prospects and have a certain significance for the study of testicular tissue in giant pandas.

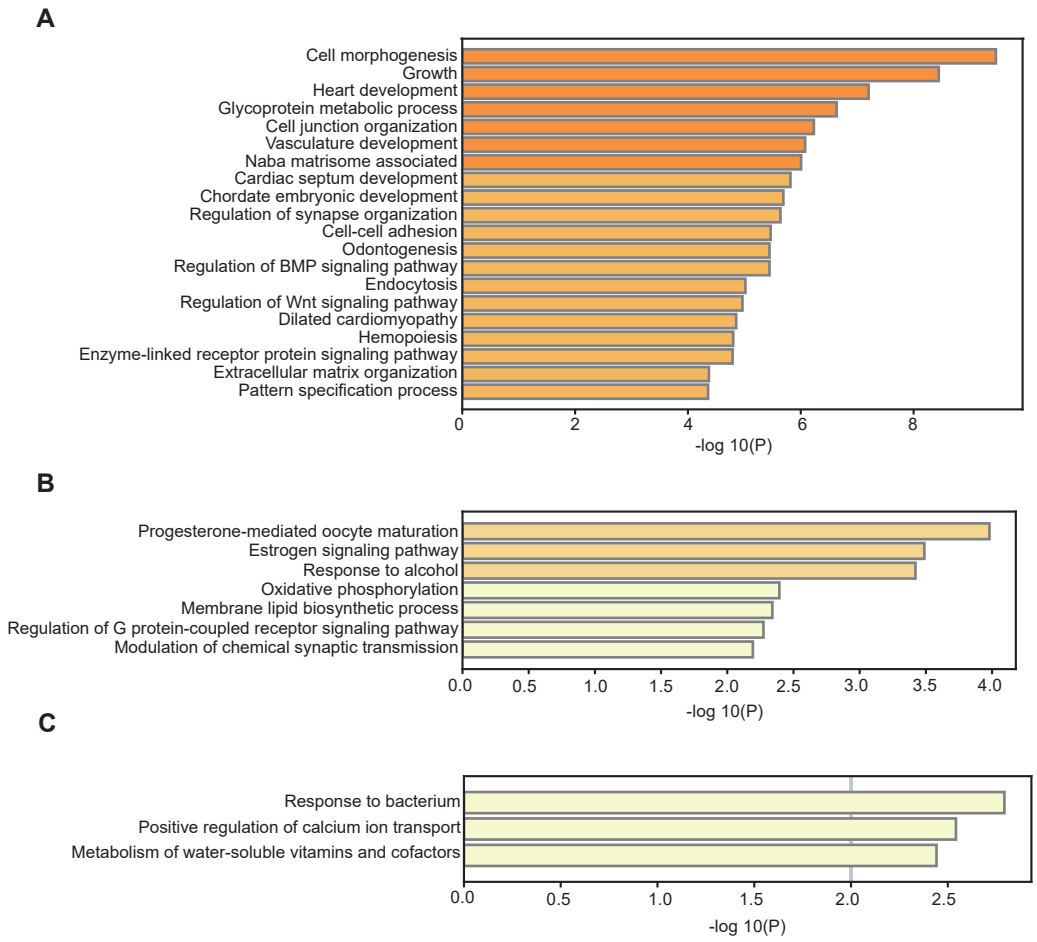

Figure 4 Gene Ontology (GO) analysis of unique proteins in the testicular tissues of giant pandas at three distinct ages (specifically 8 days, 18 years, and 22 years old). (A) The GO analysis of unique proteins of sample F8d. (B) The GO analysis of unique proteins of sample F18y. (C) The GO analysis of unique proteins of sample H22y.

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DISCUSSION

In 2016, the giant panda's conservation status was downgraded from "endangered" to "threatened" by the IUCN (*Ma et al., 2018*). This was due in part to the substantial growth of the captive population, however, even though the captive population has grown, the natural mating ability of male giant pandas is still poor, with the majority of births in captivity due to artificial insemination (*Zhu et al., 2022*). The male reproductive system and testicular development are still poorly understood. To address this lack of knowledge, we employed high-depth quantitative proteomics based on non-labeled methods to identify and analyze proteins in giant panda testicular tissue (*Zheng et al., 2022*). A total of 139,039 peptides and 11,435 proteins were identified in the testicular tissues of giant pandas. This effort aimed to enhance our understanding of the mechanisms underlying testicular tissue development and provide new perspectives for the captive breeding of giant pandas.

Table 1 Genes and descriptions of the top-ten proteins of the three giant pandas.

Protein	Description	Gene	Protein functional description
D2HU49	Uncharacterized protein	*PANDA_015769*	Molecular carrier activity
G1LEJ5	Uncharacterized protein	*ALB*	DNA binding; fatty acid binding
P18970	Hemoglobin subunit alpha	*HBA*	Involved in oxygen transport from the lung to the various peripheral tissues.
D2H496	Uncharacterized protein (Fragment)	*ACTB*	Unknown
D2HVQ7	Heat shock protein 90 alpha family class A member 1 (Fragment)	*HSP90AA1*	ATP hydrolysis activity; ATP-dependent protein folding chaperone;
G1LX08	Tubulin alpha chain	*LOC100470688*	Hydrolase activity
G1MDU7	Heat shock protein family A (Hsp70) member 5	*HSPA5*	ATP hydrolysis activity; ATP-dependent protein folding chaperone
G1LVM5	Vimentin	*VIM*	Unknown
G1LP52	Collagen type VI alpha 3 chain	*COL6A3*	Serine-type endopeptidase inhibitor activity

In mammals, the testis is the organ with the highest transcriptional activity. After gene transcription, translation, and post-translational protein modification, the transcriptional results are finally presented at the metabolic level (*Chalmel & Rolland, 2015*; *Li et al., 2021*; *Wang et al., 2023*). Studies have shown that most of the proteins are involved in a series of biological roles in the GO enrichment analysis of testicular tissue from giant pandas, among which the most critical to maintaining testicular tissue activity is metabolic processes. Metabolic processes play a crucial role in the testicular tissue of giant pandas, as they provide the necessary energy and building blocks for cellular activities. Under normal physiological conditions, the metabolites in the testes are always in a state of dynamic or homeostatic equilibrium, and eventually, ATP is produced through oxidative catabolism, which provides energy for various metabolic reactions and physiological activities (*Cui et al., 2019*). Protein phosphorylation is a crucial regulator of protein and cellular function (*Bilbrough, Piemontese & Seitz, 2022*). Phosphorylation refers to the attachment of phosphate groups to proteins, mainly on serine, threonine, and tyrosine, catalyzed by kinases (*Wang & Wang, 2019*). Each phosphoform of a protein, the protein state defined by the specific combination of phosphorylated residues, is chemically and biologically distinct and results in unique outcomes (*Zhang et al., 2023*). In the study of giant panda testicular tissue, protein phosphorylation may be involved in regulating the process of spermatogenesis, as phosphorylation-dependent ubiquitination and proteasome degradation may be involved in the regulation of spermatogenesis (*Zhang, Situ & Guo, 2022*).

In the proteome KEGG analysis of the testicular samples, the most important pathways were signal transduction, global and overview maps, and transport and catabolism. In motif analysis, the protein kinase, WD40 repeat, and WD40-repeat-containing domains exist in large numbers in various regulatory situations. Protein kinases (PKs) are metabolic switches that regulate cellular signaling. They catalyze phosphoryl transfer from ATP to amino acid side chains of their protein substrates (*Gógl et al., 2019*). The catalytic activity of protein kinases is regulated, and they can be thought of as molecular switches that are controlled

through protein-protein interactions and post-translational modifications (*Arter et al., 2022*). Protein phosphorylation, catalyzed by kinases, and its reversal by phosphatases represents a cardinal intracellular control mechanism. This process is vital for modulating the activity of proteins involved in a spectrum of critical cellular activities, including cell cycle progression, cell expansion, cellular differentiation, migration, metabolic pathways, and programmed cell death (apoptosis) (*Ardito et al., 2017*; *Deribe, Pawson & Dikic, 2010*). The WD40 repeats are among the top ten most abundant domains in eukaryotes. Proteins containing WD40 repeat proteins play a key role in many cellular functions including signal transduction, protein degradation, and apoptosis. Owing to the numerous roles of WD-repeat proteins in cellular processes, any malfunctions in these proteins might lead to diseases (*Hu et al., 2023*; *Jain & Pandey, 2018*). Based on the above analysis, protein kinases and WD40 repeat proteins play a broad role in cellular processes within the cells of the giant panda's testicular tissue, including signal transduction, cell cycle regulation, apoptosis, and gene regulation. In addition to these roles, they also have a significant function in protein-protein interactions, thereby regulating gene expression in the testicular tissue of the giant panda. In the proteome of testicular tissue of giant pandas at different ages, we have found many quantitative proteins that play an important role. Among them, the proteins with higher abundance and characterized are P18970, G1LVM5, G1LP52, G1LX08, G1MDU7, and D2HVQ7. The gene description of the P18970 protein is a hemoglobin subunit alpha. Hemoglobin (Hb) is a major protein involved in the transport of oxygen. It consists of Hb- α and Hb- β subunits, which are normally expressed by cells of erythroid lineage (*Barbarani, Łabedz & Ronchi, 2020*; *Saha et al., 2017*). Each constituent monomer of Hb contains one heme molecule in its hydrophobic pocket (*Dasauni et al., 2021*). O_2 is essential for living organisms. Molecular O_2 binds to Hb and is delivered to every organ in the body (*Sato & Takeda, 2023*). Therefore, P18970 may affect the metabolism and signal transduction of testicular tissue in giant pandas (*Bustamante-Marin et al., 2015*). The gene for the G1LVM5 protein is Vimentin. Vimentin is the main component of the intermediate filament family. It is widely expressed in a variety of cells and is believed to maintain cell integrity and resist pressure, and is involved in cell adhesion, migration, and signal transduction (*Battaglia et al., 2018*; *Costigliola et al., 2017*; *Dave & Bayless, 2014*). Alterations in the arrangement of vimentin filaments correlate with heightened apoptosis in germ cells, highlighting the crucial role of vimentin filaments in preserving the structural integrity of the vas deferens epithelium (*Wang et al., 2002*). The gene for the G1LP52 protein is COL6A3, and its function is to bind to proteins as well as to bind to and inhibit, prevent, or reduce the activity of serine-type endopeptidases. The gene description of the G1LX08 protein is tubulin alpha-3 chain. Tubulin alpha-3 chain is an essential component of the cytoskeleton. Together with beta-tubulin, it forms a tubulin heterodimer, which then assembles into microtubules. Microtubules are highly dynamic cellular structures that not only provide structural support and maintain cell shape but also participate in a variety of cellular processes, including intracellular material transport, cell division, and neural development (*Islam & Iskander, 2004*). Both D2HVQ7 and G1MDU7 are members of the heat shock protein family A. Heat shock proteins (Hsps) are integral to a variety of cellular processes, where they monitor and support the correct folding

of proteins, guard against the formation of protein aggregates, assist in the reactivation of misfolded proteins, orchestrate the assembly of multi-protein complexes, aid in the transmembrane movement and relocation of proteins, and are involved in the mechanisms of protein degradation (*Janowska et al., 2019*). The aforementioned proteins may play a crucial role in the testicular tissue of giant pandas, as evidenced by their abundance variations across different age groups. However, to precisely understand the specific functions of these proteins and how they impact the reproductive health of giant pandas, a series of comprehensive experimental studies are required. These experiments may include quantitative analysis of protein expression patterns, their specific localization within cells, and research into their mechanisms of action in the development and function of germ cells. Furthermore, researchers may also need to explore the interactions of these proteins with other cellular components, as well as their regulatory mechanisms in response to environmental stress or physiological changes. Through in-depth study of these proteins, it is hoped that a better understanding of the complexity of the giant panda's reproductive system can be achieved, providing a scientific basis for the protection of this endangered species.

CONCLUSIONS

We performed proteomic analysis of giant panda testicular tissue for the first time using label-free quantification. Our findings demonstrate that various proteins play important roles in the testicular tissue of the giant panda, for example, signal transduction, metabolism, *etc*. Through some bioinformatics analysis of the data, our findings demonstrate that the identified proteins play an important role in spermatogenesis and intratesticular environment. This study lays a foundation for the further study of the regulatory mechanism of key proteins in the testicular tissue of giant pandas, which will help improve our knowledge of male reproductive development in this threatened species and help with its continued *ex-situ* conservation. While this study has made initial progress in proteomic analysis of giant panda testicular tissue, limitations exist, primarily due to the small sample size ($n = 3$) resulting from the rarity of the species. The limited sample size may not fully capture the proteomic profile of giant panda testes, accurately reflect age-related protein changes, or distinguish the specific impacts of factors like age, health, and environment on protein expression. Additionally, the complexity of protein interactions and regulatory networks within cells may not be adequately revealed with such a small sample size, constraining our exploration of biological processes. Future research should aim to increase sample size and consider supplementary methods like transcriptomics and metabolomics for a more comprehensive understanding. Interdisciplinary collaboration and technological advancements in sample collection and preservation could also help overcome current limitations and advance the study of giant panda reproductive biology, providing stronger scientific support for the conservation and breeding of this endangered species.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Jing Peng analyzed the data, prepared figures and/or tables, and approved the final draft.
- Tao Wang analyzed the data, prepared figures and/or tables, and approved the final draft.
- Feiping Li performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Shenfei Wang performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Mengshi Zhang performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- James Ayala performed the experiments, prepared figures and/or tables, and approved the final draft.
- Yuliang Liu performed the experiments, prepared figures and/or tables, and approved the final draft.
- Rong Hou performed the experiments, prepared figures and/or tables, and approved the final draft.
- Kailai Cai conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.

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