Original Paper

Proteomics Analysis of Testis of Rats Fed a High-Fat Diet

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Key Words

High-fat diet • Testis • Proteome • Long non-coding RNAs

Abstract

Background/Aims: The adverse effects of obesity on male fertility have been widely reported. In recent years, the relationship between the differential expression of proteins and long non-coding RNAs with male reproductive disease has been reported. However, the exact mechanism in underlying obesity-induced decreased male fertility remains unclear. **Methods:** We used isobaric tags for relative and absolute quantification to identify differential protein expression patterns in the testis of rats fed a high-fat diet and normal diet. A microarray-based gene expression analysis protocol was used to compare the differences in long non-coding RNAs in high-fat diet-fed and normal diet-fed rats. Five obviously upregulated or downregulated proteins were examined using western blot to verify the accuracy of their expression. Then, we carried out functional enrichment analysis of the differentially expressed proteins using gene ontology and pathway analysis. Finally, the metabolic Gene Ontology terms and pathways involved in the differential metabolites were analyzed using the MetaboAnalyst 2.0 software to explore the co-expression relationship between long non-coding RNAs and proteins. **Results:** We found 107 proteins and 263 long non-coding RNAs differentially expressed between rats fed a high-fat diet and normal diet. The Gene Ontology term enrichment analysis showed that the protein function most highly enriched was related to negative regulation of reproductive processes. We also found five Gene Ontology terms and two metabolic pathways upregulated or downregulated for both proteins and long non-coding RNAs. **Conclusion:** The study revealed different expression levels for both proteins and long non-coding RNAs and showed that the function and metabolic pathways of differently expressed proteins were related to reproductive processes. The Gene Ontology terms and metabolic pathways upregulated or downregulated in both proteins and long non-coding RNAs may provide new candidates to explore the mechanisms of obesity-induced male infertility for both protein and epigenetic pathways.

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Introduction

Obesity leads to various health problems such as insulin resistance, dyslipidemia, hypertension, and chronic inflammation, all of which contribute to high rates of mortality and morbidity [1]. Many of these complications are associated with an additional impact on male reproduction. Many factors are clearly associated with decreased male reproductive capacity, including environmental effects, metabolic dysfunction, and genetic polymorphisms [2-3]. In this study, we focused on the effects of obesity on male infertility.

As a result of improved living standards and dietary variation, the prevalence of obesity continues to increase [4]. Previous studies suggest that the body mass index (BMI) of obese males and semen quality determine the relationship between metabolic disorders and hormonal imbalance. Obesity reduces sperm quality and alters fertility by affecting spermatogenesis function [5, 6].

Male rats were used to simulate the habits of obese patients to study the molecular mechanism of altered protein expression in the testes of rats with high-fat diet-induced obesity (DIO). We used iTRAQ to investigate the differentially expressed proteins in the testis. Unlike previous studies on single proteins and genes, proteomics studies the disease mechanism from a holistic, multi-faceted, dynamic perspective [7, 8]. We have not only tested the protein expression in the testis tissue, but also conducted gene ontology (GO) analysis and gene and genomic (KEGG) biological pathway analysis to predict the differential expression of long non-coding RNAs (lncRNAs). If differentially expressed proteins and lncRNAs alter the same pathway, it may be important in the fertility reduction of obese men. lncRNA is a non-coding RNA that is longer than 200 nt [9]. In a recent study that explored the relationship between lncRNA and obesity, lncRNA and mRNA interactions revealed potential genes that might play important roles in the pathogenesis of obesity-related low fertility [10]. Although several lncRNAs are critical in disease development, most lncRNA functions remain unclear [11]. We examined rat testes to explore the molecular mechanisms behind the reproductive changes resulting from a high-fat diet, to reduce the adverse effects of obesity on male reproduction.

Materials and Methods

Animal experiments

Sprague-Dawley (SD) male rats (aged 6 weeks: Hua Fu Kang Company, Beijing, China) were housed in plastic cages and fed a normal diet in controlled conditions: room temperature of $23 \pm 2^\circ\text{C}$, relative humidity of $45 \pm 10\%$, and a 12 h light-dark cycle. After adaptation for 1 week, the rats were randomly distributed into two groups: high-fat diet and normal. Each group contained 7 rats. The rats in the normal group were fed a standard diet and the rats in the high-fat diet group were fed a high-fat diet (20% sucrose, 10% lard, 2.5% cholesterol, 0.2% sodium cholic, and 67.3% (w/w) standard chow). The two groups of animals were fed for 12 weeks (until the average body weight of high-fat diet group was $>120\%$ normal). This study was approved by the Beijing University of Chinese Medicine's Animal Ethics Committee. All animal manipulations were undertaken according to the guidelines of the Animal Care Committee and all experiments were carried out in accordance with the approved guidelines.

Semen quality analysis and testis hematoxylin-eosin staining

At the end of the 12th experimental week, the rats were killed by cervical dislocation. The deferens and epididymis were separated and spermatozoa were obtained by emptying the deferens and puncturing the epididymis in preheated human tubal cultures. Then spermatozoa were centrifuged ($1500 \times g$, 4°C , 8 min) to collect the supernatant fluid. Semen was obtained from the epididymis tail and transferred to Ham's F10 medium (Bioway Biotechnology Co., Ltd., Beijing, China) for analysis of sperm density and motility using the computer-assisted semen analyzer (CASA) (TOX IVOS, Hamilton Thorne, Beverly, MA, USA). The testicle samples collected from the rats were paraffin-embedded, sectioned (thickness, $4 \mu\text{m}$), and stained with hematoxylin-eosin (HE) for evaluation by light microscopy (Olympus, Tokyo, Japan).

Protein preparation and iTRAQ labeling

The frozen testicle tissue in the liquid nitrogen from normal group and high-fat diet group was used for iTRAQ analysis. The tissue sample of each group was from the mixture of all the 7 rats' testicle. Each sample was diluted in a lysis buffer (7 M urea, 2 M thiourea, 0.1% CHAPS, 40 mM Tris-HCL, pH 8.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, and 10 mM DTT, lysed by sonication, and then centrifuged at 25,000 × g for 20 min at 4 °C. The collected supernatant was stored at -80 °C for subsequent iTRAQ and western blot analysis. The protein concentration from each group of testicle sample was determined using the Bradford assay [12].

After the protein was quantified, 200 µg protein solution was placed in a centrifuge tube, 100 µl of iTRAQ kit Dissolution Buffer was added, and the solution was centrifuged at 12,000 rpm for 20 min. The solution at the bottom of the tube was discarded, and trypsin was added to the tube. After centrifugation at 12,000 rpm for 20 min, the pellet on the bottom of the tube contained the digested peptide.

iTRAQ labeling was done according to the kit protocol (ABI, Foster City, CA, USA). First, a reduction reagent (50 mM final concentration; 8-plex iTRAQ kit, AB SCIEX, Redwood City, CA, USA) mixed with 150 µl of isopropanol was added to the protein sample (the collected supernatant) and incubated at ambient temperature for 2 h. After rinsing a 10 kD ultrafiltration cartridge with 70% ethanol and deionized water, the protein solution was added and centrifuged at 13,400 × g for 20 min. To detect the labeling efficiency and quantitative accuracy, the protein samples (7 µl rats in the high-fat diet and normal groups, (1 µl of testis per rat) were mixed (1 µl per sample) and desalted using a Ziptip (2 µl C18 ZTC18M096, Millipore, Bedford, MA, USA) to perform MALDI-TOF-TOF (AB SCIEX 4800 Plus) for verification that the labeling reaction was complete. The reaction was terminated with deionized water and the protein samples were thoroughly mixed. After centrifugation, the dried protein sample was stored at -80°C until use. The tryptic peptides in the two protein samples from the normal and high-fat diet group were labeled with iTRAQ reagents (isobaric tags 113 and 114 for the normal and 117 and 119 for the high-fat diet group).

HPLC

For LC-MS/MS analysis, the peptide mixture was loaded onto a reverse-phase trap column (Thermo Scientific EASY-nLC1000 System) connected to the C18 reversed-phase analytical column (Thermo Scientific Easy Column, 10-cm-long, 75-µm inner diameter, 3-µm resin) in buffer A (0.1% formic acid). It was separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 350 nl/min (0–35% buffer B for 50 min, 35–90% buffer B for 5 min, 90% buffer B for 5 min).

RNA isolation

Twelve weeks later, spermatozoa were harvested from the rats in each group. According to the manufacturer's instructions, the total RNA was isolated using TRIzol reagent (Life Technologies) and purified using the RNeasy Protect Mini Kit (QIAGEN). The total RNA from each sample was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific), and the RNA integrity was measured by standard denaturing agarose gel electrophoresis.

LncRNA microarray experiment

RNA quantity and quality were measured using the NanoDrop ND-1000. RNA integrity was assessed using standard denaturing agarose gel electrophoresis. Arraystar Mouse LncRNA Microarray V3.0 was used for detection of lncRNA and mRNA expression. Arraystar Mouse circRNA Array and Agilent miRNA microarray were used for profiling the circRNAs and miRNAs expression. All microarray analysis was performed by KangChen Bio-tech (Shanghai, China). The concentration and specific activity of the labeled circRNAs (pmol Cy3/µg circRNA) were measured using the NanoDrop ND-1000. Each labeled circRNA (1 µg) was fragmented by adding 5 µl 10× Blocking Agent and 1 µl 25× Fragmentation Buffer, heated at 60 °C for 30 min, and diluted with 25 µl 2× GE Hybridization buffer. Hybridization solution (50 µl) was dispensed into the gasket slide and added to the lncRNA expression microarray slide. The slides were incubated for 17 h at 65 °C in an Agilent Hybridization Oven. Agilent Feature Extraction software (version 10.7.3.1) was used to analyze acquired array images. Raw signal intensities were normalized using the quantile method. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies).

Bioinformatics analysis and protein network analysis

We conducted a gene ontology (GO) analysis (<http://www.geneontology.org>) to construct meaningful annotations of genes and gene products. Ontology covers areas such as biological processes, cellular components, and molecular functions. We also conducted a KEGG pathway analysis to collect clusters of molecular interactions and response networks. $-\log_{10}$ (p-value) represents the concentration fraction, showing the significance of the pathway and GO term enrichment.

Western blotting

The extract (the supernatant collected in section 2.3) was centrifuged at 10,000 × g for 15 min at 4 °C, the supernatant was transferred to a clean tube and the protein samples were separated via SDS-PAGE. After electrophoresis, the resolved proteins were transferred to PVDF membrane. The membrane was then blocked for 1 h with TBST (50 mM Tris-HCl, pH 7.6 and 150 mM NaCl) containing 5% (w/v) nonfat milk, and incubated overnight with the primary antibodies: anti-Crisp1, anti-Epha5, anti-Mapk1, anti-Smad2 and anti-GAPDH (Proteintech, 1:10000). The membrane was washed three times with TBST and incubated with secondary antibody Peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG (Proteintech, 1:10000) for 1 h. The membrane was again washed three times and subsequently developed. The bands were visualized using SuperSignal West Micro Chemiluminescent Substrate (Pierce, part of Life Technologies) and recorded on x-ray film (Fuji Medical, Tokyo, Japan). Finally, the visualization strip was quantified using the Quantity One software on the GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

Quantitative data analysis

Scaffold Q+ (version Scaffold_4.4.8, Proteome Software Inc., Portland, OR) was used to quantitate Label Based Quantitation (iTRAQ, TMT, SILAC, etc.) peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 91.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run. Intensities for each peptide identification were normalized within the assigned protein. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations were performed using medians to multiplicatively normalize data.

MS and Mascot

Tandem mass spectra were extracted using ProteoWizard version 3.0.8789. Charge state deconvolution and deisotoping were not performed. The SwissProt Rattus database searched was Mascot version 2.5.1 (Matrix Science, London, UK; version 2.5.1).

Statistical analysis

The statistical significance of microarray data was analyzed in terms of fold change using the Student's t-test (SPSS, 13.0) to correct the p-value. $p < 0.05$ was considered statistically significant.

Results

Body weight and glycolipid metabolism of experimental animals

After 12 weeks, we compared the fasting levels of blood glucose (GLU), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides (TG), as well as the body weight of DIO and normal rats. The results showed that the levels of HDL, LDL, and TG in the DIO group were not significantly higher than levels in the control group. However, the fasting blood glucose level and body weight of the DIO rats were significantly higher than in the control group (Fig. 1).

Semen analysis

CASA analysis of spermatozoa extracted from the epididymides and spermat ducts revealed that both the sperm concentration and motility percentage significantly decreased

in the DIO group compared with the control group ($p < 0.05$, $n = 7$) (Fig. 2).

Testicular morphological structure

Under light microscopy, abundant seminiferous tubules with large diameters and intact basement membranes were observed in the testicular structure of the control group, and the cell structure was clear at all stages. The mesenchyme was composed of loose connective tissue with large and round cells. Compared with the control group, obvious pathological changes were observed in the testicular tissue of the DIO group (Fig. 3). The sperm cells fell in clusters in the lumen. Many atrophic and distorted seminiferous tubules were destroyed. In each field of measurement, the number of cells was significantly reduced (Fig. 3A-3B).

Differentially expressed proteins in testes of obese rats and normal rats

In the present study, 6,795 proteins were identified in the testicular samples. We compared the expression levels of proteins in the testes of the DIO and control groups. Results showed 107 differentially expressed proteins between the DIO and control groups. Of these proteins, 67 were downregulated and 40 were up-regulated, and we

Fig. 1. Effect of high-fat diet-induced obesity (DIO) on GLU, HDL, LDL and TG. Data are expressed as mean $\pm$ SEM. * $p < 0.01$, $n = 7$, compared with the control group.

Fig. 2. Sperm concentration and motility percentage in DIO and control groups. The results are presented as the means $\pm$ SEM. A significant difference is expressed as * when ($P < 0.05$, $n = 7$), compared with the control rats.

Fig. 3. (A) Morphological changes in the testes of male rats. Magnification $\times 20$, scale bar 100 μm ; magnification $\times 40$, scale bar 50 μm . Testicular section from a normal rat showing clear cell structure, loose connective tissue and large spermatogenic cells. Testicular section from a DIO group rat, presented in the Figs as DIO (diet-induced obesity) showing atrophic and distorted seminiferous tubules and fewer spermatogenic cells. (B) Quantitative analysis of spermatogenic cells in testis. The number of seminiferous tubules containing five or more apoptotic cells was calculated using TUNEL stain. The apoptosis percentage was calculated as the ratio of apoptosis-positive seminiferous tubules to the total number of seminiferous tubules. Data ($n = 7$) are represented as mean $\pm$ SEM. * $p < 0.05$.

listed the top 20 up-regulated and 20 down-regulated proteins, a fold change cut-off ratio of >1.3 (up-regulated) or <0.77 (down-regulated) was selected to designate differentially expressed proteins ($p < 0.05$) for future study. (Table 1 and 2, respectively)

Differentially expressed lncRNAs between and normal rats

We found 263 lncRNAs differentially expressed, with fold change ≥ 2.0 and $p < 0.05$, in the samples from the high-fat diet rats compared with the normal rats. Among them, 115 lncRNAs were upregulated and 148 lncRNAs were downregulated. The top 20 upregulated and downregulated lncRNAs are listed in Table 3.

Gene ontology analysis

We used all 107 differentially expressed proteins (both upregulated and downregulated) for GO analysis in the testis samples extracted from rats. GO analysis explores proteins involved in biological process, cellular component, and molecular function. The top 10 generally changed GO terms classified by biological process, cellular component, molecular function, and ranked by fold enrichment are listed (Fig.4). The most enriched and meaningful cellular component terms were related to the extracellular region such as “extracellular vesicle,” “extracellular matrix,” “extracellular organelle,” and “extracellular membrane-bounded organelle.” The most enriched biological process terms were related to extra cellular metabolic processes such as “cellular component disassembly,” “extracellular matrix organization,” and “extracellular structure organization.” We found the most enriched molecular function terms were related to molecule structure. Representative terms were “structural molecule activity,” “extracellular matrix structural,” and “growth factor binding.”

Pathway analysis

Pathway enrichment analysis indicated that 50 pathways were significantly enriched ($p < 0.05$) among the differentially expressed proteins, including cytoskeleton remodeling,

Table 1. Differentially expressed proteins in mouse testis between DIO and control groups (up-regulated)

Accession Number	Full Name	Gene Symbol	Ratio(D/N)
NQO2_RAT	Ribosyldihydrocortinamide dehydrogenasequinone	Nqo2	2.25
EPHA5_RAT	Ephrin type-A receptor 5	Epha5	2.17
A0A0G2JSR8_RAT	Cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	1.91
B2RYK7_RAT	LOC502201 protein	Lrrc29	1.69
SMAD2_RAT	Mothers against decapentaplegic homolog 2	Smad2	1.59
A0A0H2UHF6_RAT	PRA1 family protein 3	Arl6ip5	1.58
D4A644_RAT	Protein Map7d1	Map7d1	1.53
D3ZHR8_RAT	Protein Tyw3	Tyw3	1.53
CARM1_RAT	Histone-arginine methyltransferase CARM1	Carm1	1.52
D4A6W6_RAT	Protein RGD1561333	RGD1561333	1.48
D3ZJM7_RAT	Protein Mcph1	Mcph1	1.48
ATP5E_RAT	ATP synthase subunit epsilon, mitochondrial	Atp5e	1.47
A0A0G2K523_RAT	Protein O-mannosyl-transferase 1	Pomt1	1.46
EGLN1_RAT	Egl nine homolog 1	Egl9	1.45
RS9_RAT	40S ribosomal protein S9	Rps9	1.44
A0A0G2JWB6_RAT	Protein Pxdn	Pxdn	1.42
A0A0G2K743_RAT	Uncharacterized protein		1.42
G3V950_RAT	LYR motif-containing protein 1	Lyrm1	1.42
D4A727_RAT	Protein Magea8	Magea8	1.42
ISK2_RAT	Serine protease inhibitor Kazal-type 2	Spink2	1.39

Table 2. Differentially expressed proteins in mouse testis between the DIO and control groups (down-regulated)

Accession Number	Full Name	Gene Symbol	Ratio(D/N)
CAH3_RAT	Carbonic anhydrase	Ca3	0.50
A0A0G2JTX7_RAT	Protein Col6a5	Col6a5	0.51
A0A0G2JWX4_RAT	Keratin, type II cytoskeletal 2 epidermal	Krt2	0.52
A0A0G2JTP6_RAT	Perilipin	Plin1 Plin rCG_24621	0.56
A0A0G2K0T6_RAT	Gamma-synuclein	Snca rCG_42336	0.56
A0A0H2UHF8_RAT	Alpha-1-acid glycoprotein	Orm1	0.57
A0A0G2K586_RAT	Fatty acid-binding protein, adipocyte	Fabp4	0.57
D3ZJ08_RAT	Histone H3	rCG_51970	0.57
B2RZ77_RAT	Dermatopontin	rCG_46203	0.58
PGS2_RAT	Decorin	Dcn	0.59
Q5XIH1_RAT	Asporin	rCG_43984	0.60
F7EUK4_RAT	Protein Kng2	Kng1 Kng1 Kng2	0.60
D3ZVB7_RAT	Osteoglycin	rCG_44112	0.62
A0A0G2JST3_RAT	Keratin, type II cytoskeletal 1	Krt1	0.62
LUM_RAT	Lumican	LumLcnLdc	0.62
AL1A7_RAT	Aldehyde dehydrogenase, cytosolic 1	Aldh1a7	0.63
F1LTN6_RAT	Protein Igkc	Igkc	0.64
K1C10_RAT	Keratin, type I cytoskeletal 10	Krt10 Ka10	0.64
D3ZJF8_RAT	Protein Fcgbp	Fcgbp	0.64
PRELP_RAT	Prolargin	Prelp	0.65

Table 3. Differentially expressed lncRNAs

Probe name	Fold change	Regulation	Probe name	Fold change	Regulation
CUST_3277_P428311958	9.57	up	CUST_458_P428311958	19.62	down
CUST_2772_P428311958	9.08	up	CUST_5519_P428311958	18.85	down
CUST_8089_P428311958	8.65	up	CUST_2111_P428311958	17.15	down
CUST_9208_P428311958	8.54	up	CUST_9135_P428311958	15.42	down
CUST_2117_P428311958	8.12	up	CUST_8347_P428311958	14.33	down
CUST_1197_P428311958	8.02	up	CUST_2931_P428311958	13.49	down
CUST_384_P428311958	7.94	up	CUST_6638_P428311958	12.14	down
CUST_7285_P428311958	7.76	up	CUST_4899_P428311958	12.02	down
CUST_545_P428311958	7.31	up	CUST_396_P428311958	11.75	down
CUST_4267_P428311958	7.12	up	CUST_8936_P428311958	11.45	down
CUST_4145_P428311958	6.88	up	CUST_6993_P428311958	11.18	down
CUST_8075_P428311958	6.80	up	CUST_8371_P428311958	10.60	down
CUST_7733_P428311958	6.65	up	CUST_8040_P428311958	9.37	down
CUST_6167_P428311958	6.53	up	CUST_887_P428311958	9.36	down
CUST_4331_P428311958	6.41	up	CUST_4772_P428311958	8.47	down
CUST_3632_P428311958	6.37	up	CUST_2963_P428311958	8.16	down
CUST_9613_P428311958	6.13	up	CUST_2786_P428311958	7.87	down
CUST_8256_P428311958	6.05	up	CUST_8955_P428311958	7.68	down
CUST_8886_P428311958	5.70	up	CUST_7631_P428311958	7.57	down
CUST_3683_P428311958	5.44	up	CUST_7878_P428311958	7.22	down

keratinocyte differentiation, immune response, cell cycle, platelet activation, and beta-alanine metabolism (Fig. 5).

Interactions between Differentially Expressed Proteins

We established a protein-protein interaction (PPI) network based on 13 possible target-related proteins of reproduction and metabolism. Ingenuity pathway analysis (IPA) (<http://www.ingenuity.com>) identifies protein networks from known interactions (either direct or indirect) between proteins. In addition, IPA defines common functional and canonical pathways, thereby offering additional information about the complex interactive lines between these proteins. The network comprises 35 proteins (Lcn5, PLCL2, SELENOS, PER3, LHCGR, CYP17A1, PLIN1, PLIN4, PTGR2, Ncoa6, FABP, MEDAG, PPARG, FABP4, RETSAT, ESR2, EPHA5, Spink2, S100A8, BRD8, BCL2, BANF1, HIST1H1D, MANBA, IL4, SMARCE1, MYC, CSTF1, APOA4, HIST1H1T, CARM1, SERINC3,

Fig. 4. Gene ontology enrichment for protein targets from testis samples. Gene ontology analysis of protein-target genes according to cellular component, biological process, and molecular function.

Crisp1/Crisp3, CPOX, and FARP1), among which 13 proteins were quantified by iTRAQ. The remaining 22 proteins interact with the quantified proteins according to IPA (Fig. 6).

Co-expression analysis between lncRNAs and proteins

The metabolic GO and pathways involved in the differential metabolites were analyzed using the Metabo Analyst 2.0 software (<http://www.metaboanalyst.ca>). As a result, we identified seven GO terms and two metabolic pathways found in both lncRNA and protein results associated with high-fat diet-induced male metabolic changes. These were characterized as single-organism catabolic process, protein complex subunit organization, cellular component organization, growth factor binding, oxidoreductase activity, extracellular matrix component, extracellular space, β -alanine metabolism, and retinol metabolism (Tables 4 and 5).

Western blotting results

We used western blot techniques to validate five differentially expressed proteins (Crisp1, Mapk1, Smad2, Epha5 and GAPDH). Antibodies against Smad2, Crisp1, Mapk1 and Epha5 were used to validate the differentially expressed proteins in the testis of male rat between the DIO and control groups. The iTRAQ analysis of protein expression for Smad2, Epha5, and Mapk1 was upregulated 1.59-, 2.17-, and 1.33-fold, respectively, whereas Crisp1 was downregulated 0.76-fold compared with the normal group. Western blotting results showed that the expression level of Crisp1 considerably decreased, but the expression levels of Mapk1, Smad2, and Epha5 increased in the high-fat diet group compared with those in normal group (Figs 7A-7B). This result was consistent with the protein expression levels obtained using the iTRAQ approach.

Discussion

Obesity is a high risk factor for insulin resistance, type 2 diabetes, high-fat diet vascular diseases, endocrine disorders, and decreased fertility [11]. Studies show that lncRNA is

Fig. 5. The top 10 pathways related to metabolism and reproduction of all differentially expressed proteins.

Fig. 6. Network analysis of the differentially expressed proteins using the IPA software. Proteins are represented as nodes. Nodes in red represent upregulated proteins, whereas nodes in green represent downregulated ones. Proteins represented by white nodes were not observed. Solid lines indicate direct interactions or regulation, whereas dashed lines indicate indirect effects mediated by additional proteins.

Table 4. Overlap between protein and lncRNA GO analysis

Name	p-value (protein)	Count (protein)	p-value (lncRNA)	Count (lncRNA)
Single-organism catabolic process	3.729E-12	26	5.05E-06	148
Protein complex subunit organization	2.315E-10	31	4.36E-09	305
Cellular component organization	1.377E-07	53	5.36E-06	213
Growth factor binding	3.165E-02	5	1.35E-07	33
Oxidoreductase activity	7.071E-02	11	1.44E-05	133
Extracellular matrix component	7.675E-11	11	3.68E-07	34
Extracellular space	6.356E-12	29	8.16E-20	247

Table 5. Overlap between protein and lncRNA pathway analysis

Name	p-value (protein)	Count (protein)	p-value (lncRNA)	Count (lncRNA)
Retinol metabolism	2.521E-02	72	0.4685499	10
Beta-alanine metabolism/ Rodent version	5.005E-03	31	0.0826913	7

associated with obese male fertility [10]. The incidence of infertility in obese males is significantly higher than that in normal males [13]. Obesity and male infertility are closely related. Increased BMI has a negative impact on the levels of luteinizing hormone, testosterone, gonadotropin, sex hormone binding protein, and estradiol in men [14, 15].

In our study, 107 differentially expressed proteins were identified in the high-fat diet group. Among them, we focused on those proteins that have anti-oxidative functions and play a role in spermatogenesis. We also confirmed that lncRNAs were differentially expressed in testis samples from DIO and normal rats.

Among the 13 proteins in the protein network we established and quantified by iTRAQ, we identified Spink2 (serine protease inhibitor Kazal-type 2) gene, which belongs to the Spink family of proteins characterized by the presence of a Kazal-type serine protease inhibitor-pancreatic secretory trypsin inhibitor domain. Previous studies indicate that Spink family members are expressed in diverse tissues. However, only Spink2 is expressed in testes. Thus, it is considered essential for spermatogenesis [16, 17]. Moreover, Spink2 is necessary to inactivate acrosin during its transit through the endoplasmic reticulum and the Golgi apparatus. In the absence of Spink2, the Golgi apparatus is disorganized, preventing the production of the acrosome and inducing a block at the round spermatid stage [18]. Interestingly, we found the Spink2 gene was significantly upregulated in the rat testis with DIO, in contrast to previous reports. Because the sperm concentration and motility percentage in the high-fat diet group decreased, we speculate that Spink2 acts as a regulator to maintain the quantity of spermatozoa and normal spermatogenesis.

In mammals, Crisp1 is the first described member of the evolutionarily conserved Cysteine-Rich Secretory Protein (Crisp) family. It is a sperm protein expressed in the proximal regions of the epididymis, associates with sperm during maturation, and participates in

Fig. 7. (A) Protein expression levels of Crisp1, Mapk1, Smad2, and EphA5 with reference to GAPDH, measured by western blot in high-fat diet and normal groups. (B) Summary plot showing densitometric readings of the corresponding protein blots. Data are expressed as mean $\pm$ SEM. * $P < 0.05$, $n = 7$.

fertilization [19-22]. It may have an effect on male reproduction through the TGF- β -mediated signaling pathway. Our results showed that Crisp1 was significantly downregulated.

From the protein network we established, we found 13 differentially expressed proteins forming a complex network related to reproduction. It is necessary to perform further studies to reveal the underlying mechanisms. Hist1h1t forms less compacted chromatin compared to other H1 histone subtypes. Formation of more relaxed chromatin may be required to promote chromatin architecture required for proper chromosome regulation during meiosis [23]. We hypothesize the protein regulates the formation of sperm cells by influencing the synthesis of RNA. Lcn5 associates with spermatozoa in the epididymal fluid. It may act as retinoid carrier protein required for epididymal function or sperm maturation [24]. We also found that retinol metabolism components were altered at both lncRNA and protein levels. We speculate that obesity may affect the expression pattern of lncRNAs, further regulate the expression level of target proteins, and eventually lead to decreased spermatozoa during maturation.

Our pathway analysis revealed that the biological processes associated with differential expression of high-fat diet-induced obese rat testicular proteins include cytoskeleton remodeling, keratinocyte differentiation, immune response, cell cycle, platelet activation, and beta-alanine metabolism. Cytoskeleton remodeling may be associated with dysplasia whereas platelet activation may be due to decreased testicular tissue ischemia and oxygen levels [25, 26]. Obesity-related alanine metabolic disorders may have an impact on the sperm membrane structure integrity and destroy oxidation resistance, which is a major factor in the decline of sperm fertility capacity [27, 28]. β -alanine is the only limiting amino group for the synthesis of imidazole dipeptide acid and is a potential functional amino acid. β -alanine has a variety of physiological functions: regulating neurotransmitters and hormones, regulating body metabolism, serving as intermediate metabolites for variety of active substances (Coenzyme A, pantothenic acid, etc.), combating fatigue, and improving athletic ability. Due to variety of physiological functions, β -alanine has attracted a wide range of studies [29, 30]. In this study, we found the β -alanine metabolism pathway not only regulated by proteins, but also by lncRNAs. According to this phenomenon, β -alanine metabolism may play an important role in male reproduction of obese rats induced by high-fat diet. The exact mechanism remains to be further explored.

In summary, we detected the differential expression of proteins and lncRNAs in the samples from high-fat diet rats and analyzed their potential roles through bioinformatics. The GO term enrichment analysis showed that the function most highly enriched was related to negative regulation of reproductive processes. Pathway analysis showed that metabolic pathways might be related to the high-fat diet-induced decline in male fertility. We also found some GO terms and metabolic pathways upregulated or downregulated in both protein and lncRNA analyses. Our results provide a foundation for future studies to investigate the molecular role of proteins and lncRNAs in high-fat diet associated decreases in male fertility.

Abbreviations

lncRNAs (long non-coding RNAs); GO (Gene Ontology); iTRAQ (isobaric tags for relative and absolute quantification); DIO (diet-induced obesity);

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Disclosure Statement

No potential conflicts of interest relevant to this article were reported.

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