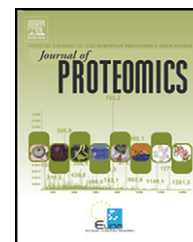


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## HnRNPL as a key factor in spermatogenesis: Lesson from functional proteomic studies of azoospermia patients with sertoli cell only syndrome<sup>☆</sup>

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### ABSTRACT

Sertoli cell only syndrome (SCOS) is one of the main causes leading to the abnormal spermatogenesis. However, the mechanisms for abnormal spermatogenesis in SCOS are still unclear. Here, we analyzed the clinical testis samples of SCOS patients by two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/TOF MS) to find the key factors contributing to SCOS. Thirteen differential proteins were identified in clinical testis samples between normal spermatogenesis group and SCOS group. Interestingly, in these differential proteins, Heterogeneous nuclear ribonucleoprotein L (HnRNPL) was suggested as a key regulator involved in apoptosis, death and growth of spermatogenic cells by String and Pubgene bioinformatic programs. Down-regulated HnRNPL in testis samples of SCOS patients was further confirmed by immunohistochemical staining and western blotting. Moreover, *in vitro* and *in vivo* experiments demonstrated that knockdown of HnRNPL led to inhibited proliferation, increased apoptosis of spermatogenic cell but decreased apoptosis of sertoli cells. Expression of carcinoembryonic antigen-related cell adhesion molecule 1 in GC-1 cells or expression of inducible nitric oxide synthases in TM4 sertoli cells, was found to be regulated by HnRNPL. Our study first shows HnRNPL as a key factor involved in the spermatogenesis by functional proteomic studies of azoospermia patients with sertoli cell only syndrome. This article is part of a Special Issue entitled: Proteomics: The clinical link.

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## 1. Introduction

Sertoli cell only syndrome (SCOS), representing a condition of the testes in which only sertoli cells line the seminiferous tubules, was first described in 1947 [1]. SCOS accounts for almost 20% azoospermia men [2]. It has been suggested that the factors associated with the primary events before or during the proliferation phase of spermatogonia are the candidate regulators of SCOS [3]. And, deletions of the azoospermia factor (AZF) subregions on the Y chromosome [4], copy number variants of sex-chromosomal [5] or single-nucleotide polymorphisms in the SEPTIN12 gene [6], are associated with the high risk of SCOS. However, the proteins responsible for abnormal spermatogenesis in SCOS remain unclear [7].

Lately, ten genes involved in human spermatogenesis were identified using microarray profile of testicular tissues from individuals with normal spermatogenesis, germinal arrest, and SCOS [8]. Similar to microarray technology, proteomics is also a kind of large-scale approach, which can evaluate complex samples of proteins on a global level rapidly and comprehensively [9]. A typical proteomic technology includes three key components: two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS) and bioinformatics. First, 2-DE enables separation of highly complex mixtures of proteins according to isoelectric point (pI), molecular mass (Mr), solubility, and relative abundance, and delivers a map of intact proteins reflecting changes in protein expression level, isoforms, or posttranslational modifications [10]. Second, these protein spots of interest can be analyzed and identified after proteolysis by liquid chromatography coupled with tandem mass spectrometry and database search [11]. Then, bioinformatics is usually used to identify proteins that are up-regulated or down-regulated in a disease-specific manner for use as diagnostic markers or therapeutic targets [12].

Proteomic methods can also be effectively used to find novel biomarkers or key proteins in reproductive biology and medicine [13,14]. Here, we used the clinical samples of testicular tissue from SCOS patients and screened the potential key proteins involved in development and progression of SCOS based on proteomic strategy. Heterogeneous nuclear ribonucleoprotein L (HnRNPL), a protein stably associated with heterogeneous nuclear RNAs (HnRNAs) complexes was first suggested as a key factor in spermatogenesis.

## 2. Material and methods

### 2.1. Specimen collection and preparation

The testis specimens were collected from 110 azoospermia patients (Nanfang hospital, Guangzhou, China) by open biopsy from 2008 to 2010. All the samples were diagnosed according to the latest diagnostic criteria for classification of testicular germ pathology by two pathologists in Nanfang hospital of Southern Medical University. Each specimen was divided into two parts: one part was fixed in Bouin's solution and stained for morphological examination; the other part was frozen in liquid nitrogen and prepared for two-dimensional electrophoresis. The specimens diagnosed as

SCOS were included for our next study. The specimens diagnosed as normal spermatogenesis were used as control. All patients gave informed, written consent to be enrolled in the study. Our study was approved by the bioethics committee of Nanfang hospital.

### 2.2. Proteomic analysis

#### 2.2.1. 2-D gel electrophoresis

Specimens from 15 SCOS patients and 15 normal spermatogenesis patients were pooled respectively for 2-D analysis. Tissue specimens were immediately frozen in liquid nitrogen, then ground to powder and homogenized in lysis buffer. The mixture was placed on a shaker at 4 °C for 1 h, and then followed by centrifugation. The supernatant was used for the first dimensional Electrophoresis after measuring the protein concentrations by modified Bradford assay. The Immobiline Dry strip (pH 3–10, length 17 cm, Bio-Rad, Hercules, CA, USA) loaded with 120 µg protein was rehydrated in 300 ml rehydration buffer for 13 h at room temperature. The first dimensional electrophoresis was performed on Protean IEF cell (Bio-Rad, Hercules, CA, USA) with a total of 60 kVh, and then followed by two-step equilibration for each step 15 min. The second dimensional electrophoresis was carried out in a homogeneous SDS-PAGE (10%) using a Protean II xi 2D cell (Bio-Rad, Hercules, CA, USA) until the bromophenol blue dye front reached the bottom of the gel. Afterwards, the gels were stained with silver. Each sample was measured in triplicates [15–18].

#### 2.2.2. Image analysis

The silver-stained 2-D gels were scanned using a Power-Look 1100 imaging scanner (Umax, Dallas, TX, USA). Differences in protein spot intensities were analyzed by PDQuest software package (version 7.1, Bio-Rad Laboratories Inc, Hercules, CA, USA). Protein spots whose intensities changed by or over 1.5-fold were marked. The spots whose intensities over-expressed by or 1.5-fold were defined as up-regulated spots, whereas, these spots under-expressed by or 1.5-fold were defined as down-regulated spots. The fold changes were represented as mean ± SD.

#### 2.2.3. In-gel digestion and MALDI-TOF/TOF-MS identification

The significantly differentially expressed proteins spots, were cut from the stained gels with freshly prepared 15 mM potassium ferricyanide/50 mM sodium thiosulfate then destained, washed with 25 mM ammonium bicarbonate/50% acetonitrile, and dried in a SpeedVac plus SC110A vacuum concentrator (Savant, Holbrook, NY, USA). These dried gel pieces were rehydrated with 3–10 ml of 20 ng/ml trypsin solution. An in-gel digestion was performed at 37 °C for 15 h. Tryptic peptides were extracted with 5% trifluoroacetic acid (TFA) at 40 °C for 1 h and with a solution of 2.5% TFA and 50% acetonitrile at 30 °C for 1 h. The peptide mixtures were re-dissolved in 0.5% TFA, and 1 µl of the peptide solution was mixed with 1 µl of matrix (4-hydroxy- $\alpha$ -cyanocinnamic acid in 30% acetonitrile and 0.1%TFA) when analyzing by a mass spectrometer 4700 (Applied Biosystems, Foster City, CA, USA). A trypsin-fragment peak was served as internal standard for mass calibration. A list of the corrected mass peaks was the peptide mass fingerprinting (PMF) [17,18].

**Table 1 – Identification of proteins differentially expressed between the normal spermiogenesis and sertoli cell-only syndrome.**

Spot ID	Spot intensities fold changes <sup>a</sup>	Protein molecular weight (Da)	ProteinPI	Pep. count <sup>b</sup>	Accession no.	Score <sup>c</sup>	Gene name	Overall trend <sup>d</sup>
1	10.42±3.19	51,874.5	5.09	3	P14136	56	GFAP	Up
2	5.11±0.15	28,899.9	6.67	7	P18669	127	PGAM1	Up
3	10.58±3.90	28,899.9	6.67	7	P18669	127	PGAM1	Up
4	7.46±2.09	71,317.2	5.92	13	P08758	71	ANXA5	Up
5	7.37±0.78	122,946.3	5.86	1	A5A3E0	109	A26C1B	Up
6	7.84±1.19	35,387.2	5.92	3	P21108	58	PRPS1L1	Up
7	11.82±3.21	35,146.1	6.15	3	P11908	212	PRPibP	Up
8	7.73±0.93	42,331	5.91	2	Q9BYX7	181	FKSG30	Up
9	6.31±1.52	20,992.3	5.12	4	Q04760	190	GLO1	Up
10	10.53±1.29	23,569.1	5.43	8	P09211	89	GSTP1	Up
11	9.23±1.20	22,049.3	5.66	9	P32119	527	PRDX2	Up
12	10.64±1.81	71,317.2	5.92	13	P08758	290	ANXA5	Up
13	7.98±0.62	50,633.6	4.95	5	Q13748	350	TUBA3C	Up
14	7.49±0.82	15,991.1	5.52	5	P02766	333	TTR	Up
15	0.14±0.01	122,946.3	5.86	1	A5A3E0	102	A26C1B	Down
16	0.10±0.02	60,719.4	6.65	8	P14866	127	HNRNPL	Down

<sup>a</sup> Fold changes of spot intensities: normal group divided by SCOS group, represented as mean±SD.

<sup>b</sup> Calculated by amino acid count.

<sup>c</sup> Calculated from the database entry without any processing.

<sup>d</sup> Up: up-regulated in the SCOS group. Down: down-regulated in the SCOS group.

#### 2.2.4. Database searching

Protein identification was performed by the MASCOT search engine (<http://www.matrixscience.com/>, MatrixScience Ltd, London, UK) against the MSDB protein database. The search focused on the Homo sapiens subsets of the sequences in the Swiss-Prot and NCBI nonredundant protein sequence databases. The errors were in the range of 25 ppm for both the precursor mass tolerance and the fragments mass tolerance. Proteins matching more than four peptides and with a MASCOT score higher than 63 were considered statistically significant ( $P < 0.05$ ). Tolerance of one missed trypsin was set as the miss cleavages; Oxidation of methionine was selected as the differential modification and carboamidomethylation of cysteine as the static modification. The identification results

were filtered with peakErazor software (Lighthouse Data, Odense, Denmark) [18].

#### 2.3. Bioinformatic analysis

Functional network analysis of the identified proteins by proteomic methods was performed by STRING [19] (version 8.3; <http://string.embl.de/>) with the following analysis parameters: species — *Homo sapiens*, confidence level — 0.400, active prediction methods — all. Bio-association analysis was performed by Pubgene [20] (<http://www.pubgene.org/>). The bio-associations were divided in the following categories: process, function and component in Pubgene. The key bio-processes related to the thirteen proteins were retrieved for further

**Table 2 – 837 bio-process in Pubgene associated with the thirteen identified proteins (15 bio-process associated with spermatogenesis were showed).**

Description	Associated terms	Article (p-value)
Cell maturation	ANXA5, GFAP	1
Death	ANXA5, GFAP, GLO1, GSTP1, HNRNPL, PRDX2, TTR	1
Growth	ANXA5, GFAP, GLO1, GSTP1, HNRNPL, PGAM1, PRDX2, PRPS2, TTR, TUBA3C	1
Sperm motility	ANXA5	1
Cell growth	ANXA5, GFAP, GLO1, GSTP1, PRDX2, TTR	1
DNA repair	ANXA5, GFAP, GLO1, GSTP1, PRDX2, TTR	1
Cell death	ANXA5, GFAP, GLO1, GSTP1, PRDX2, TTR	1
Mitosis	ANXA5, GFAP, GSTP1, TTR	1
Regulation of growth	GFAP, TTR	1
Spermatid development	ANXA5	1
Translation	ANXA5, GFAP, GLO1, GSTP1, HNRNPL, PRDX2, PRPS1L1, PRPS2, TTR	0.994
Meiosis	ANXA5, PRDX2, TTR	0.988
Sperm capacitation	GSTP1	0.995
Cell motility	ANXA5, GFAP, PRDX2	0.974
Neuron apoptosis	ANXA5, GFAP	0.888

P = term/total (number of records containing term divided by total number of records).

analysis. Graphviz software [21] was also used to map the network of the functional and bio-process annotations of the proteins.

#### 2.4. Animals and cell cultures

Eight-week old male NIH mice were purchased from Southern Medical University Animal Centre and raised in a facility with equal periods of light and dark at  $24 \pm 3$  °C with *ad libitum* access to food and water. The protocol for animal use in the present study was approved by the university's institutional animal care and use committee.

GC-1 spg cells (a mouse spermatogonia cell line) and TM4 cells (a mouse Sertoli cell line) were kindly provided by Dr. Yaoting Gui and were cultured in Dulbecco's modification of Eagle's medium Dulbecco (Gibco, Beijing, China) plus 10% fetal bovine serum (Gibco, Montevideo, Uruguay). All cells were maintained in a humidified at 37 °C incubator with 5% CO<sub>2</sub>.

#### 2.5. Knockdown of HnRNPL in vivo and in vitro by small interfering RNA (siRNA)

Small interfering RNA (siRNA) (5'-TATGGCTTGGATCAATCTA-3', GenePharma, Shanghai, China) targeted against HnRNPL transcript were used *in vivo* and *in vitro* in our following study [22].

The seminiferous tubules were transfected with 20 nM siRNA specific for HnRNPL through efferent duct in one testis of adult mouse [23]. Another testis of the same adult mouse was injected with equivalent mock siRNA (5'-TAAGGCTATGAAGAGATAC-3', GenePharma, Shanghai, China) for control. Approximately 20 µl of siRNA was injected into the seminiferous tubules of one testis in an 8-week-old NIH mouse. Trypan blue (0.8%) was used as an indicator to ensure that the microinjection was successful. Knockdown of HnRNPL was confirmed by western blot assay and immunohistochemistry. After the adult male mice were killed by cervical dislocation, whole testes were removed, free of fat and connection organs. The part marked with trypan blue was cut off for further research.

TM4 and GC-1 spg cells were seeded at  $1 \times 10^5$  cells per well in 6-well microtiter plates. Eight hours later, the cells were transfected with 60 nM siRNA specific for HnRNPL or negative control with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two days after transfection, cells were harvested and used for protein extraction and further analysis.

#### 2.6. Western blot analysis

Western blots were performed to detect expression of HnRNPL and activated-caspase-3. Expression of HnRNPL and activated-caspase-3 proteins in the samples were probed by rabbit polyclonal primary antibody against HnRNPL (diluted 1:500; Abcam, Cambridge, United Kingdom) and monoclonal primary antibody against activated-caspase-3-p17 (diluted 1:500; Bioworld

Technology, Louis Park, MN, USA), followed by secondary horseradish peroxidase horseradish-coupled goat anti-rabbit IgG (Zhongshan Biotechnology Co. Ltd, Beijing, China). Expression of GAPDH was detected by mouse monoclonal primary antibody against human GAPDH (diluted 1:500; Zhongshan Biotechnology Co. Ltd, Beijing, China) as the loading control.

#### 2.7. Immunohistochemical analysis

Expression of HnRNPL or Ki-67 was detected by immunohistochemical staining described previously [24]. Rabbit polyclonal primary antibody against human/mice HnRNPL and rabbit monoclonal primary antibody against mouse Ki-67 (1:20; Zhongshan Biotechnology Co. Ltd, Beijing, China) was used. Polyperoxidase rabbit IgG was used as the secondary antibody (PV-9000, Lot K72703; Zhongshan Biotechnology Co. Ltd, Beijing, China). Sections were analyzed using bright field microscopy (Axioskop 2 plus; Zeiss, Germany).

#### 2.8. Immunofluorescent staining

Testicular tissue from NIH adult mice was embedded in optimal cutting temperature (OCT) compound, cryosectioned into 5-µm thick sections using a Leica Model CM1850 cryostat (Leica Microsystems, Wetzlar, Germany), mounted on slides and processed for indirect immunofluorescence as described previously [25]. Rabbit monoclonal primary antibody against activated-caspase-3-p17 (diluted 1:50; Bioworld Technology, Louis Park, MN, USA), and 4, 6-Diamidino-2-phenylindole (DAPI, aldehyde diluted 25%, Sigma Chemical Co., St. Louis, Missouri, USA) were used. The Fluorescein (FITC)-conjugated Affinipure goat anti-mouse IgG (H+L) was purchased from Zhongshan Biotechnology Co., Ltd. (Beijing).

#### 2.9. MTT assay for cell viability

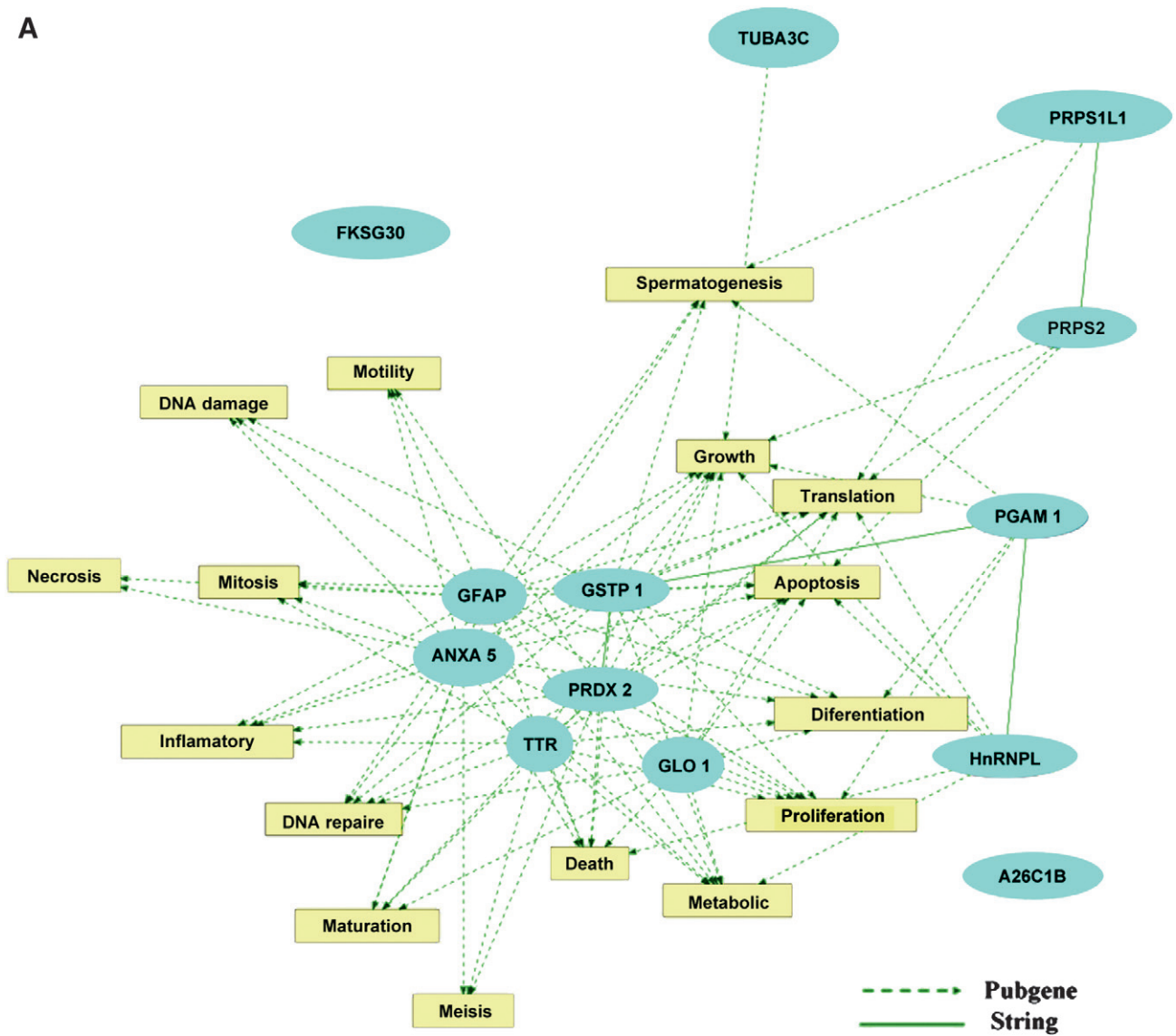
Cell viability was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, Jewelry, USA) assay every day for 4 days. GC-1 spg and TM4 siRNA-transfected cells were cultured in 96-well plates at a density of 1000 cells/well. MTT was added to each well of the titration plate and incubated for 4 h at 37 °C and then MTT-containing medium was aspirated off. One hundred microliters of DMSO was added to dissolve the crystal formed by live cells and incubated for 20 min at 37 °C. The absorbance of each well was determined in microplate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA) using an activation wave length of 570 nm.

#### 2.10. Flow cytometry analysis

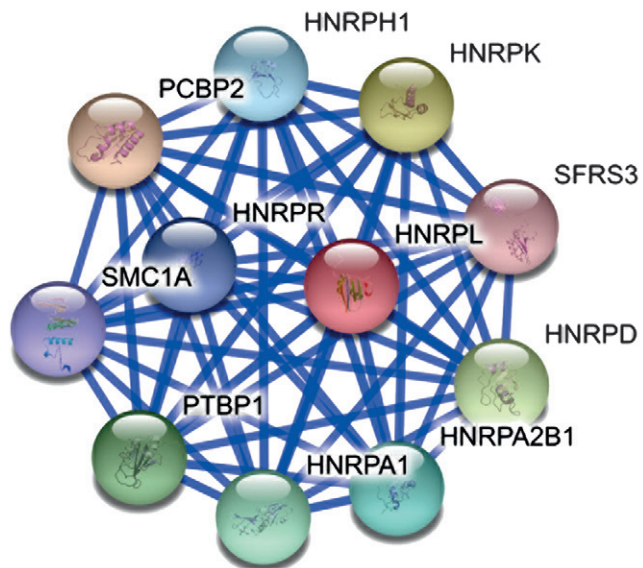
Apoptosis of TM4 or GC-1 spg cells were measured by Hoechst 33342/PI Double Stain Kit (keygenbio, Nanjing, China) according to the manufacturer's instructions. After staining, cells were analyzed on a Becton flow cytometer (FACSCalibur™,

**Fig. 1 – Analysis of bio-association network by String and Pubgene programs. (A) Bioinformatic analysis of bio-association network by String and Pubgene programs. Bio-process associated with the thirteen proteins was analyzed by Pubgene (dotted lines). Function association among the thirteen proteins was performed by String (solid line). The bio-association network was visualized in Graphviz software (B) The single protein function partner network of HnRNPL in String analysis.**

A



B



Becton Dickinson, San Jose, CA, USA) with flowjo software (Treestar, Inc., San Carlos, USA).

### 2.11. Real-time quantitative PCR

RNA samples from GC-1 spg and TM4 cells were extracted by RNAisoPlus (Takara, Dalian, China) following manufacturer's instructions. Primescript RT master mix (Takara, Dalian, China) was used to construct the template cDNA for real-time PCR (ABI Prism 7500, Perkin Elmer, Foster City, Calif, CA, USA) using SYBR Premix Ex Taq (Takara, Dalian, China). Gene specific primers are retrieved from Primer Bank (<http://pga.mgh.harvard.edu/primerbank/>) or RTPrimerDB (<http://medgen.ugent.be/rtpriimerdb/index.php>) as follows: GAPDH: 5'-ACCCAGAAGACTGTGGATGG-3' (sense) and 5'-ACACATTGGGGGTAGGAACA-3' (antisense); HnRNPL: 5'-TTGTGGCCCTGTCCAGAGAATT-3' (sense) and 5'-GTTCGTGTAGTCCCAAGTATCTTG-3' (antisense); Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1): 5'-ATTT-CACGGGGCAAGCATACA-3' (sense) and 5'-AAATCGCACAGTCCGCTGAGTACG-3' (antisense); (B-cell lymphoma 2) Bcl-2: 5'-GTCGCTACCGTCTGACTTC-3' (sense) and 5'-CAGACATGCACCTACCAGC-3' (antisense); Inducible nitric oxide synthases (iNOS): 5'-GGCAGCC TGTGAGACCTTTG-3' (sense) and 5'-GCATTGGAAGTAAGCGTTTC-3' (antisense). The initial denaturation phase was 30 s at 95 °C followed by an amplification phase of denaturation at 95 °C for 5 s, and annealing at 60 °C for 34 s for 40 cycles. Relative quantification of PCR products was calculated after normalization to GAPDH.

### 2.12. Statistical analysis

Student's t test was used for analyses of RT-PCR and 2-DE results.  $P < 0.05$  was regarded as statistically significant. All calculations were performed with the use of SPSS 13.0 (SPSS Inc, Chicago, Illinois, USA).

## 3. Results

### 3.1. Thirteen proteins are identified as potential regulators involved in SCOS by proteomic approach

The proteins were isolated by 2-DE, quantified and normalized using PDQuest 7.1 software package between normal spermatogenesis group and SCOS group (Supplementary Fig. 2). Sixteen differential spots whose intensities changed by or over 1.5-fold and had statistically significant difference between two groups were selected for subsequent identification by MALDI-TOF/TOF MS. Excluded the three overlapped proteins, thirteen proteins were identified. Eleven proteins were significantly up-regulated and two were down-regulated in SCOS. These up-regulated proteins included Glial fibrillary acidic protein (GFAP), Phosphoglycerate mutase 1 (PGAM1),

Annexin A5 (ANXA5), Ribose-phosphate pyrophosphokinase 3 (PRPS1L1), Ribose-phosphate pyrophosphokinase 2 (PRPS2), Kappa-actin (FKSG30), Lactoylglutathionelyase (GLO1), Glutathione S-transferase P (GSTP1), Peroxiredoxin-2 (PRDX2), Tubulin alpha-3C/D chain (PRDX2) and Transthyretin (TTR). The down-regulated two proteins were ANKRD26-like family C member 1B and Heterogeneous nuclear ribonucleoprotein L (HnRNPL). Information about these protein spots such as fold changes of intensities, molecular weight, PI, gene name, access number, and overall trends was described in Table 1.

### 3.2. HnRNPL is predicted as a key regulator involved in apoptosis, death and growth of spermatogenic cells

Pubgene was used to analyze the bio-process of identified proteins. Eight hundred and thirty seven bio-processes were predicted to be associated with these identified thirteen proteins by Pubgene (15 bio-processes associated with spermatogenesis were shown in Table 2). Most of these proteins were involved in apoptosis or cell death (ANXA5; GFAP; GLO1; GSTP1; HNRNPL; PRDX2; PRPS2; TTR), and cell growth (ANXA5; GFAP; GLO1; GSTP1; HNRNPL; PGAM1; PRDX2; PRPS2; TTR; TUBA3C).

Interestingly, HnRNPL, PGAM1, GSTP1 and PRDX2 were predicted as functional partners by String 8.3 program. Moreover, single protein analysis indicated that HnRNPL was a key function partner with many hnRNPs family members, such as hnRNPK or hnRNP A2/B1, which are involved in spermiogenesis (Fig. 1B). HnRNPL was involved in bio-process of apoptosis, death or growth and played as a function partner with many HnRNPs family members, suggesting a potential key role in spermiogenesis (Fig. 1A).

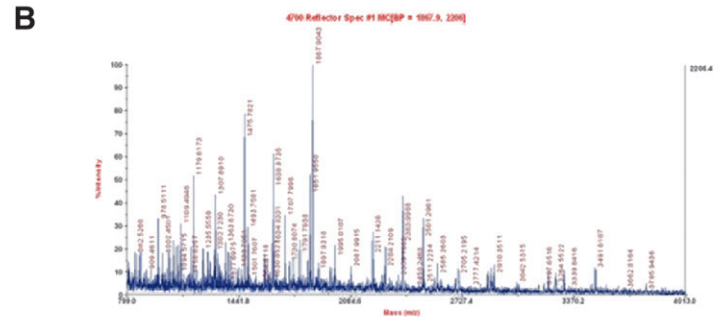
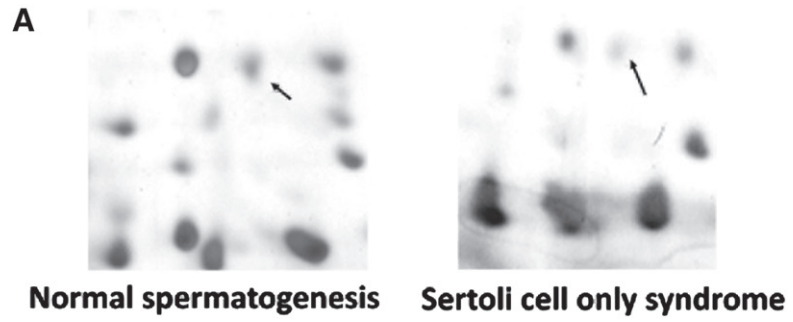
### 3.3. Low expression of HnRNPL in testis of SCOS patients

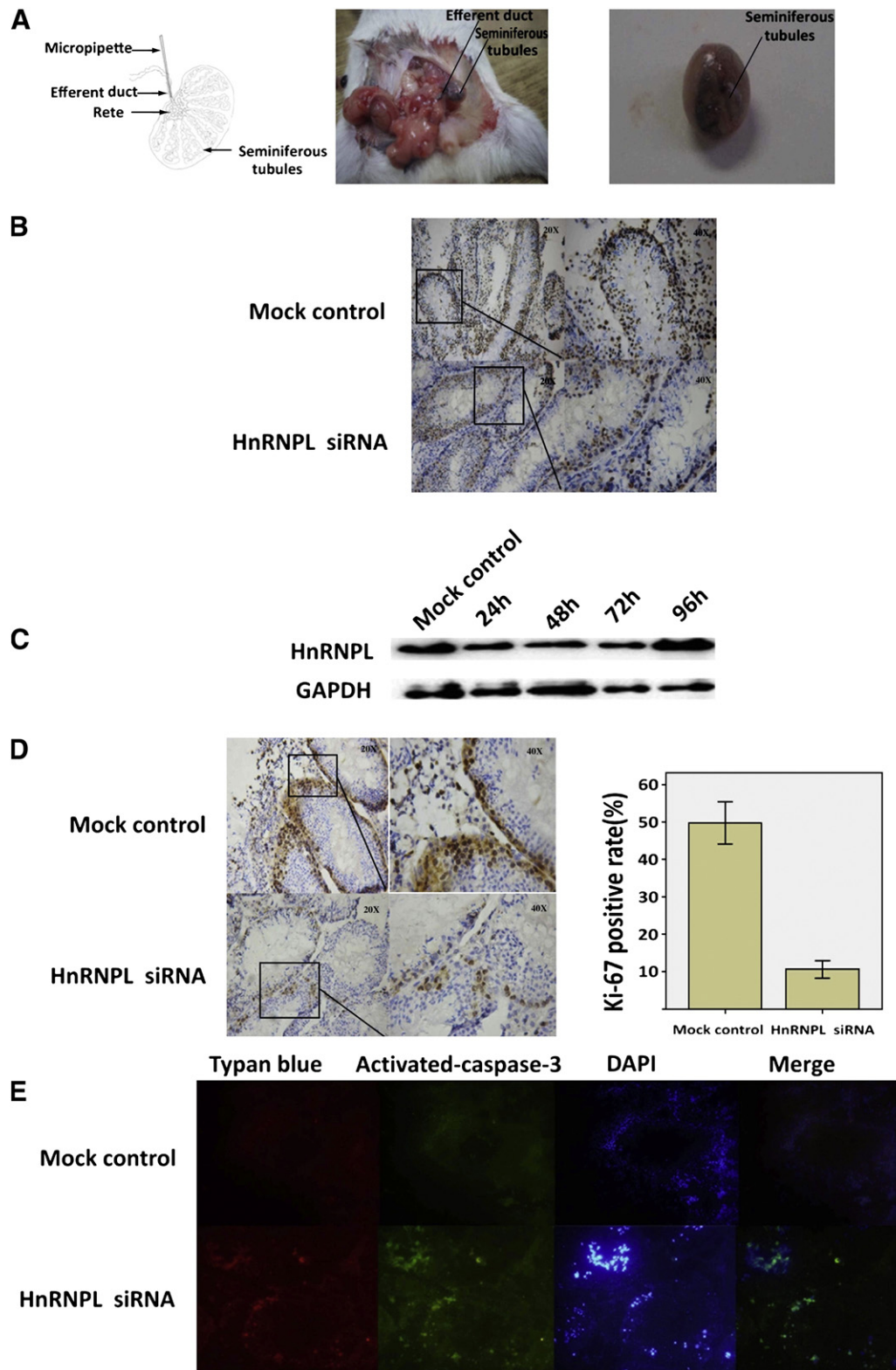
In order to further know the clinical significance of HnRNPL in testis of SCOS patients, we used western blot and immunohistochemical analysis to study expression of HnRNPL protein in SCOS. Our results indicated that HnRNPL was downregulated in testis of SCOS patients, consistent with the results identified by proteomic methods (Fig. 2).

### 3.4. Knockdown of HnRNPL in vivo leads to impaired growth and apoptosis of spermatogenic cells in testes of NIH mice

To investigate the putative role of HnRNPL on spermatogenesis, expression of HnRNPL in the testis of mice was knocked down in vivo by efferent duct administration of siRNA into seminiferous tubules (Fig. 3A). The HnRNPL protein was suppressed significantly in the testis of mice as early as 24 h, and consistently suppressed at 48 h and 72 h, recovered at 96 h after injection (Fig. 3C). Immunohistochemical staining also indicated the expression of HnRNPL was significantly

**Fig. 2 – Expression of HnRNPL protein in testis of SCOS patients. (A)** A section of the gel of the testis specimens was enlarged to show the expression of HnRNPL in patients with normal spermiogenesis or SCOS. **(B)** MS of in-gel trypsin digests of the protein and analysis of the depicted peptide spectrum resulted in the identification of HnRNPL. **(C)** HnRNPL expression in testis of SCOS patients by western blotting. **(D)** HnRNPL expression in testis of SCOS patients by immunohistochemical staining.

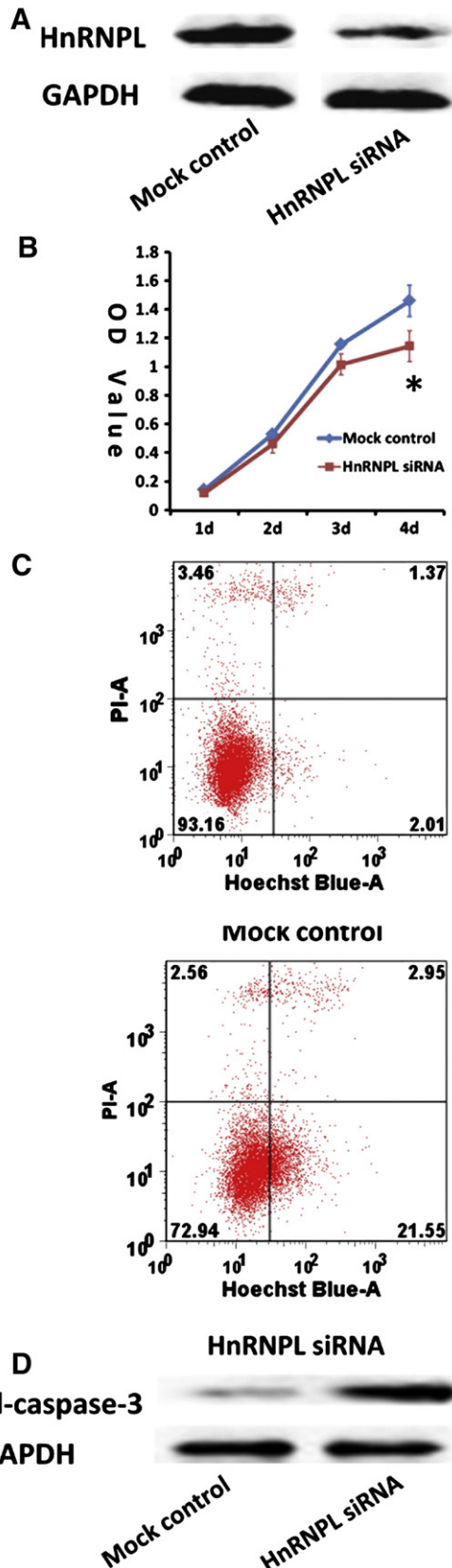




**Fig. 3 – Knockdown of HnRNPL caused growth inhibition and apoptosis of spermatogenic cells in vivo. (A)** Microinjection pipette was inserted into the seminiferous tubules for introducing HnRNPL siRNA with trypan blue. **(B)** Expression of HnRNPL protein in the testis from 24 to 72 h after administration of siRNA against HnRNPL. **(C)** Expression of HnRNPL in spermatogenic cells after administration of siRNA against HnRNPL by immunohistochemical staining. **(D)** The mean ratio of spermatogonia to sertoli cells in the HnRNPL siRNA-treated testis and mock control testes. **(E)** The number of activated caspase-3 positive cells after HnRNPL siRNA was injected into seminiferous tubules in NIH mice.



reduced in testis after HnRNPL siRNA injection (Fig. 3B). Because expression of HnRNPL at 72 h after *in vivo* siRNA administration was lowest, we observed the subsequent effects at 72 h after HnRNPL siRNA injection.



The Ki-67 index is one of the most commonly used markers of proliferating cells [26]. Immunohistochemical results showed that Ki-67 positive cells were spermatogoniums and spermatocytes. The Ki-67 negative cells were later spermatids, sperms, and sertoli cells [27]. Compared with that in paired testes in the same mouse, the number of Ki-67 positive cells in different stage of spermatids decreased significantly after HnRNPL siRNAs were injected with in seminiferous tubules *in vivo* (Fig. 3D). Ten round tubules were randomly selected, and the numbers of sertoli cells, Ki-67-positive spermatogonias were counted for quantitative evaluation of spermatogenesis [28]. The mean ratio of spermatogonia to sertoli cells in the HnRNPL siRNA-treated testis (10.57%) was significant lower than that in mock control testes (49.79%) (Fig. 3D).

The caspase family of cysteine proteases plays a key role in apoptosis. Caspase 3, a key executioner in the apoptotic pathway, has been extensively studied in caspase family members [29]. The caspase-3 positive cells (green) were mainly localized in the bottom and the center of lumen which germ cells were localized (Fig. 3E). Interestingly, compared with that in paired testes in the same mouse, the number of caspase-3 positive cells increased significantly after HnRNPL siRNAs injected into seminiferous tubules in NIH mice.

### 3.5. Knockdown of HnRNPL leads to impaired growth and apoptosis of GC-1 spg cells but inhibits apoptosis of TM4 cells *in vitro*

In order to know the potential regulatory effects of HnRNPL on spermatogonium and sertoli cells, a mouse spermatogonia cell line GC-1 spg cells and a mouse sertoli cell line TM4 cells were used in our study. We knocked down HnRNPL expressions of GC1 spg and TM4 cell lines respectively and determined the effects of HnRNPL on cell proliferation and apoptosis by MTT assays and flow cytometry analysis.

MTT assay showed that cell proliferation of GC1 spg cells decreased significantly after HnRNPL was knocked down (Fig. 4B). Differently, down-regulation of HnRNPL had no significant effects on cell proliferation of TM4 cells (Fig. 5B).

As seen in Fig. 4C, knockdown of HnRNPL could lead to the apoptosis of GC-1 spg cells. However, knockdown of HnRNPL could not result in increased apoptosis of TM4 cells (Fig. 5C), consistent with regulatory effects of HnRNPL on cell proliferation of TM4 cells.

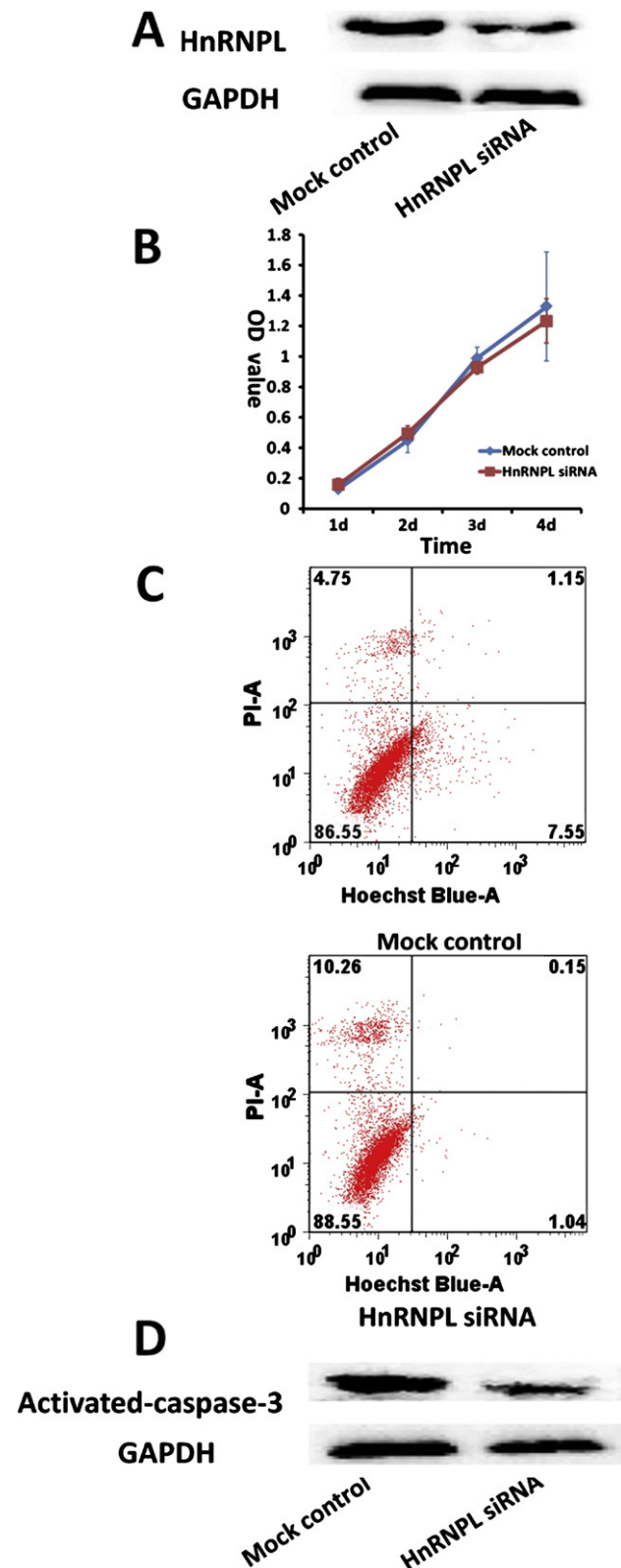
Expression of caspase-3 increased significantly in GC-1 spg cells but decreased in TM4 cell after HnRNPL was knocked down (Fig. 4D; Fig. 5D), suggesting the role of activated caspase-3 in HnRNPL related apoptotic pathway.

**Fig. 4 – Down-regulation of HnRNPL inhibited growth and promoted apoptosis of GC-1 spg cells *in vitro*.** (A) Expression of HnRNPL in GC-1 spg cells after transfection of siRNA specific for HnRNPL. (B) Proliferation of GC-1 spg cells after transfection of siRNA specific for HnRNPL. (C) Rate of apoptosis of GC-1 spg cells after transfection of siRNA specific for HnRNPL by flow cytometry and Hoechst/PI staining. (D) Expression of activated-caspase-3 of GC-1 spg cells after knockdown of HnRNPL by western blot analysis.

### 3.6. Knockdown of HnRNPL leads to reduced expression of CEACAM1 in GC-1 cells and decreased expression of iNOS in TM4 cells

HnRNPL is a global regulator of alternative splicing, binding to diverse C/A-rich elements [30]. CEACAM1, bcl-2 and iNOS

have been reported to be regulated by HnRNPL [31–33] and be closely related to spermatogenesis [34–36]. Therefore, we studied the regulatory effects of HnRNPL on expressions of CEACAM1, bcl-2 and iNOS (Fig. 6). Knockdown of HnRNPL led to the reduced expression of CEACAM1 but no change in expression of the iNOS in GC-1 cells. Interestingly, expression of iNOS decreased significantly and expression of the CEACAM1 did not change after HnRNPL was knocked down in TM4 cells. Expression of BCL2 had no significant change in both GC-1 spg and TM4 cells.

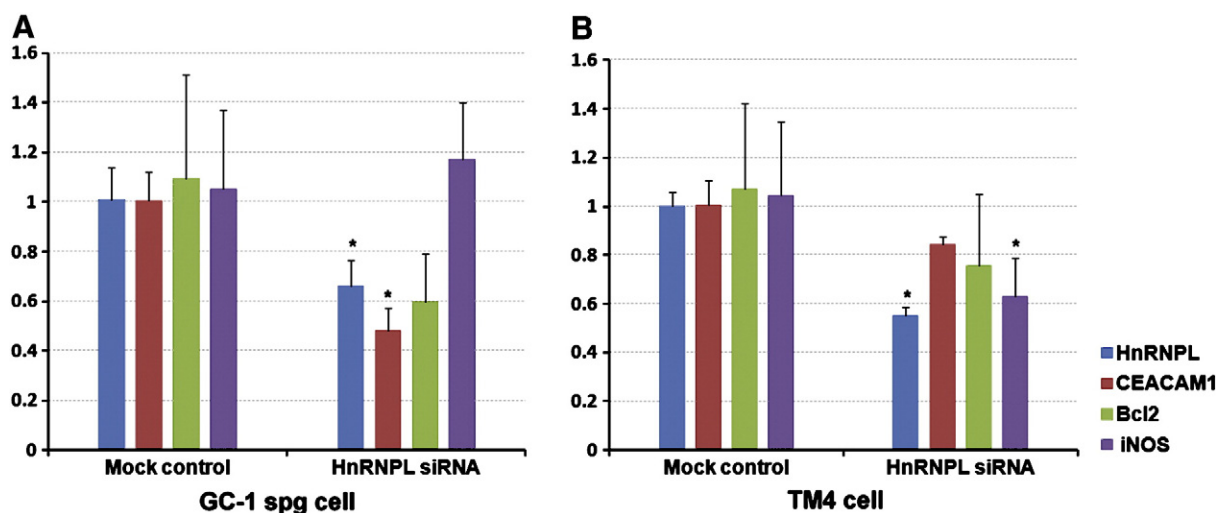


## 4. Discussion

Proteomics is an effective approach to study interactions, organization, and function of proteins at a global level. It is also a powerful approach for understanding of spermatogenesis [13]. In an abnormal spermatogenesis mouse model caused by hyperthermia, 36 differentially expressed proteins have been identified by proteomic methods. Among these proteins, Heterogeneous nuclear ribonucleoproteins (hnRNPs) have been confirmed as the factors contributing to spermatogenic disorder in mice [15]. Also in a mouse model for spermatogenesis, 28 novel proteins with unknown functions in somatic cells or germ cells were also identified by proteomic approach [37]. However, these studies only use testicular specimens from mouse models. The studies using clinical samples of abnormal spermatogenesis by functional proteomic methods are still very limited. In clinical practice of abnormal spermatogenesis, SCOS accounts for the majority of non-obstructive azoospermia [38]. Here, we studied clinical samples of SCOS patients by functional proteomic analyses to understand the mechanism for SCOS and identify the key proteins involved in spermatogenesis.

Thirteen proteins are identified as potential regulators involved in SCOS by proteomic approach. Of these proteins, some were confirmed as spermatogenesis associated proteins. ANXA5 is related to the degenerative response of germ cells [39]. PRDX2 is associated with peroxidase enzymatic activity of mammalian spermatozoa [40]. TTR interacts with retinol-binding protein, participating the progress of spermatogenesis [41]. GST plays an important part in final stages of spermatozoa maturation and provides protection against germ cells from teratogens and carcinogens [42]. GFAP mutation of parental sperm contributes to Alexander disease [43]. These results showed that proteomic approach is an effective approach to find the key proteins involved in SCOS and spermatogenesis. Besides these proteins confirmed by other groups, we also found some novel proteins, which might also be implicated in SCOS and spermatogenesis.

**Fig. 5 – Knockdown of HnRNPL had no effect on growth but inhibited apoptosis of TM4 cells *in vitro*.** (A) Expression of HnRNPL in TM4 cells after transfection of siRNA specific for HnRNPL. (B) Proliferation of TM4 cells after transfection of siRNA specific for HnRNPL. (C) Rate of apoptosis of TM4 cells after transfection of siRNA specific for HnRNPL by flow cytometry and Hoechst/PI staining. (D) Expression of activated-caspase-3 of TM4 cells after knockdown of HnRNPL by western blot analysis.



**Fig. 6** – Knockdown of HnRNPL led to reduced expression of CEACAM1 in GC-1 cells and decreased expression of iNOS in TM4 cells. The mRNA levels of HnRNPL, CEACAM1, iNOS and bcl-2 were analyzed by quantitative real-time reverse transcription-PCR 24 h following transfection. Statistical analysis of the data was done using Student's *t* test and  $P < 0.05$  was considered significant. The error bars represent the standard deviations. \*:  $P < 0.05$ , compared with mock control group.

Our study further revealed HnRNPL as a key regulator of spermatogenesis in SCOS. The HnRNPL protein, one member of an RNA binding protein HnRNP family, is a major component of the HNRNP complex [44]. HnRNPL plays an important role in the formation, packaging, processing, and function of mRNA [45]. Some members of the HnRNP family have been found to participate in progress of spermatogenesis [15,16,46]. HnRNP K is a key early molecular target for spermatogenesis suppression induced by hormone treatment [16]. HnRNP A2/B1 is also involved in spermatogenesis [46]. In our study, HnRNPL was first predicted as a key regulator in apoptosis, death and growth of spermatogenic cells by bioinformatic methods. It was also considered as a partner with many hnRNPs family, such as hnRNP K and hnRNP A2/B1. Further, *in vitro* and *in vivo* experiments demonstrated that knockdown of HnRNPL led to inhibited proliferation, increased apoptosis of spermatogenic cell but decreased apoptosis of sertoli cells, supporting a key role of HnRNPL in regulating spermatogenesis in SCOS. It has been reported that HnRNPL could regulate the expressions of CEACAM1, bcl-2 and iNOS [31–33]. CEACAM1, a member of the CEA family, has been shown to function as intercellular adhesion molecules [47]. Alternative splicing of CEACAM1 pre-mRNA generates two cytoplasmic domain splice variants characterized by the inclusion (L-isoform) or exclusion (S-isoform) of exon 7 [31]. Depletion of HnRNPL by RNAi promotes exon 7 inclusion [31], which is a substrate of caspase-3-mediated cleavage in apoptotic cells [48]. Here, we also found that apoptosis and inhibitory growth of spermatogenic cell induced by HnRNPL was associated with suppressed CEACAM1 and activated caspase-3. The elevated expression of activated caspase-3 in the GC-1 spg cells may contribute to low expression of CEACAM1. Moreover, CEACAM1 is related to adhesive interactions between sertoli cells and germ cells, which is required for proliferation and differentiation of spermatogenic cells [34]. HnRNPL may be involved in the process of spermatogenesis via influence on expression

of activated-caspase-3 and CEACAM1 in spermatogonia. Our results also suggested that Inducible NOS (iNOS) may account for apoptosis induced by HnRNPL. Inducible NOS, one of three key enzymes generating nitric oxide (NO) from the amino acid L-arginine, is constitutively expressed in Leydig cells, sertoli cells and germ cells [36]. NO plays important role in germ cell apoptosis [49], sertoli cell numbers [36], production of testosterone [50], junction dynamics [51]. Additionally, NO has been implicated in the apoptotic death of a number of cell types [50].

Briefly, low expression of HnRNPL in spermatogonium leads to increasing apoptosis of germ cells and may damage the conjunctions between spermatogenic cells and sertoli cells in the process of spermatogenesis. Interestingly, low HnRNPL expression inhibits apoptosis in sertoli cells, which can explain to some extent why the seminiferous tubules contain only Sertoli cells in the testes of SCOS patients.

## 5. Conclusions

HnRNPL was predicted as a key regulator involved in apoptosis, death and growth of spermatogenic cells. *In vivo* and *in vitro* studies indicated that HnRNPL was a key regulator of spermatogenesis in SCOS.

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