RESEARCH ARTICLE

Proteomic analysis of testis biopsies in men treated with transient scrotal hyperthermia reveals the potential targets for contraceptive development

Hui Zhu[1], Yugui Cui[1,2]*, Jin Xie[1], Ling Chen[1], Xiangxiang Chen[1], Xuejiang Guo[1], Yefei Zhu[1], Xinghai Wang[3], Jiansun Tong[3], Zuomin Zhou[1], Yue Jia[4], Yan-he Lue[4], Amiya Sinha Hikim[4], Christina Wang[4], Ronald S. Swerdloff[4] and Jiahao Sha[1]*

[1] Laboratory of Reproductive Medicine, Department of Histology and Embryology, Nanjing Medical University, Nanjing, P. R. China
[2] Center of Clinical Reproductive Medicine, The First Affiliated Hospital, Nanjing Medical University, Nanjing, P. R. China
[3] Department of Reproductive Medicine, Jiangsu Family Planning Research Institute, Nanjing, P. R. China
[4] Division of Endocrinology, Department of Medicine, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, CA, USA

Received: April 27, 2010
Revised: July 13, 2010
Accepted: July 15, 2010

Mild testicular heating safely and reversibly suppresses spermatogenesis. In this study, we attempted to clarify the underlying molecular mechanism(s) involved in heat-induced spermatogenesis suppression in human testis. We conducted global proteomic analyses of human testicular biopsies before, and at 2 and 9 wk after heat treatment. Thirty-one and Twenty-six known proteins were identified with significant differential expression at 2 and 9 wk after heat treatment, respectively. These were used to characterize the cellular and molecular events in the testes when seminiferous epithelia became damaged (2 wk) and recovered (9 wk). At 2 wk post-treatment, the changed expression of a series of proteins could promote apoptosis or suppress proliferation and cell survival. At 9 wk post-treatment, the changed expression of proteins mainly promoted cell proliferation, differentiation and survival, but resisted cell apoptosis. Among those heat-regulated proteins, HNRNPH1 was selected for the further functional study. We found that HNRNPH1 was an anti-apoptosis protein that could regulate the expression of other heat-induced proteins. In conclusion, heat-induced reversible suppression of spermatogenesis occurred by modulating the expression of proteins related to proliferation, differentiation, apoptosis and cell survival pathways. These differentially expressed proteins were found to be key molecular targets affecting spermatogenesis after heat treatment.

Keywords:
Animal proteomics / Apoptosis / HNRNPH1 protein / Human testis / Transient scrotal hyperthermia

1 Introduction

In most animals, including humans, the scrotal location of the testes keeps them cooler than the core body temperature. This lower temperature is important for germ cell viability and genetic and epigenetic modifications. Exposure of the testis to temperatures at or above body temperature results in increased germ cell death [1]. Mild testicular heating safely and reversibly suppresses spermatogenesis in several mammalian species, including mice [2, 3], rats [4, 5], monkeys [6], bulls [7], sheep [8] and humans [9, 10]. Histological changes in testes after transient exposure of the

Correspondence: Professor Jiahao Sha, Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, P. R. China
E-mail: shajh@njmu.edu.cn
Fax: +86-25-86862908

*These authors contributed equally to this work.

scrota to heat are marked by germ cell loss in rats [5], mice [2], monkeys [6] and humans [11] *via* stage- and germ cell-specific apoptotic pathways; the molecular mechanisms underlying this process are incompletely understood.

In a recent study, we examined the suppressive effect of testicular heat treatment on spermatogenesis in Chinese men [11]. We showed that testicular warming to 43°C in a water bath for 30 min each day for six consecutive days resulted in the reversible suppression of spermatogenesis. The mean sperm concentration of subjects in the heat group was 80.00 ($\pm$19.79) $\times 10^6$/mL before treatment and began to decrease at 3 wk (42.88 ($\pm$17.31) $\times 10^6$/mL) and maximally at 6 wk (20.94 ($\pm$11.39) $\times 10^6$/mL) post-heat treatment. Then the sperm concentration recovered to 34.89 ($\pm$19.02) $\times 10^6$/mL at 9 wk and to baseline levels (87.33($\pm$20.49) $\times 10^6$/mL) by wk 12 after heat treatment [11]. Although there were no discernible changes in testicular morphology at 2 wk after heat treatment, quantitative assessment of germ cell apoptosis showed that germ cell apoptosis had increased significantly at this time when compared to the pretreatment period. At 9 wk after heat treatment, the morphological appearance of the testis was improved and similar to that of the control group [11].

To clarify the underlying molecular mechanism(s) involved in heat-induced increase of germ cell apoptosis and spermatogenesis suppression induced by exogenous heat administration, we investigated the differential protein expression in human testicular biopsies at different times after heat treatment. Testicular biopsies were obtained before treatment (control), at week 2 when seminiferous epithelia showed increased germ cell death and at week 9 post-treatment when recovery occurred. 2-DE and MALDI-TOF-TOF were used to assess the protein changes. Bioinformatics methods were used to construct a potential molecular network of these proteins in human testes. HNRNPH1 protein, a key protein among the network, was selected as an example for study using a mouse model, thus, clarifying its role in germ cell apoptosis and elucidating the mechanism of the heat effects on spermatogenesis.

2 Materials and methods

2.1 Subjects and testicular biopsies

This proteomic study was a subproject of a previously reported clinical trial by our group, and the detailed description of the study subjects and the experimental design has been reported [11]. Briefly, 18 subjects participated in the heat group returned daily to the clinic on six consecutive days for testicular warming to 43°C in a water bath for 30 min *per* day. Twelve subjects were recruited to participate in the testicular biopsy sub-study; refusal to participate in the testicular biopsy study did not exclude them from the main study [11]. We obtained testicular biopsies from four different men at each time point: (i) before treatment serving as control; (ii) 2 wk after heat treatment; and (iii) 9 wk after heat treatment. None of the participants had more than one testicular biopsy. The ethics committees at the First Affiliated Hospital of Nanjing Medical University, Jiangsu Family Planning Research Institute, and the Institutional Review Board of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center approved our study protocols. All subjects signed a written informed consent.

Unilateral testicular biopsies, under local anesthesia, were performed by experienced surgeons at the First Affiliated Hospital of Nanjing Medical University, China. Each biopsy specimen was divided into three portions: one was fixed in Bouin's solution, which had been used for morphological examination [11], another stored in RNA for later preparation for future gene expression studies and the other was frozen in liquid nitrogen and prepared for 2-DE and Western blot analyses, in the present study.

2.2 Heat treatment in mice

Nine young adult (7-wk-old) male ICR mice from the animal center of Nanjing Medical University (Nanjing, China) were used and maintained under a controlled environment, as previously described [12]. Three of the ICR mice were used as control. We immersed the tails and the scrotums containing the testes of the remaining animals in a thermostatically controlled water bath at 42°C for 15 min, as previously described [2]. We sacrificed these animals at 12 h and 14 days after heat exposure (three animals *per* time point). One testis of each mouse was fixed in Bouin's solution and used for morphological examination and the other testis was frozen in liquid nitrogen and prepared for the Western blot analysis.

2.3 2-DE, gel image analysis and protein identification

Proteins from the testes of each subject were extracted and separated by 2-DE as previously reported [12, 13]. Gels were silver stained, scanned, and analyzed using ImageMaster^{TM} 2D platinum software (Version 5.0, GE Healthcare, San Francisco, CA, USA). The expression level was determined by the relative volume of each spot in the gel and expressed as %Vol (%Vol = [spot volume/Σ volumes of all spots resolved in the gel]). We averaged the values from the four independent experiments of heated and control groups, respectively, calculated the means and standard deviations and assessed statistical significance with Student's *t*-tests using ImageMaster^{TM} 2D platinum software. A spot was regarded significantly differentially expressed between groups if the change of average spot intensity was greater than 1.2-fold and *p*-value was also less than 0.05.

Protein spots with significant differences between the heated and control groups were excised. Gel pieces were denatured, alkylated, trypsin digested and analyzed by an Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) under the control of FlexControl™ 2.4 software (Bruker Daltonics GmbH). MALDI-TOF spectra were recorded in the positive ion reflector mode in a mass range from 700 to 4000 Da and the ion acceleration voltage was 25 kV. Acquired mass spectra were processed using the software FlexAnalysis™ 2.4 (Bruker Daltonics, Bremen, Germany): Peak detection algorithm: SNAP (Sort Neaten Assign and Place); S/N threshold: 3; Quality Factor Threshold: 50. The trypsic autodigestion ion picks (trypsin_[108–115], MH^+842.509, trypsin_[58–77], MH^+2211.104) were used as internal standards. Matrix and/or auto-proteolytic trypsin fragments, or known contaminant ions (keratins) were excluded. The resulting peptide mass lists were used to search the IPI human database 3.30 (67 922 sequences, 28 879 402 residues) with Mascot (v2.1.03) in automated mode. The following search parameters criteria were used: significant protein MOWSE score at $p<0.05$, minimum mass accuracy 100 ppm, trypsin as enzyme, one missed cleavage site allowed, and alkylation of cysteine by carbamidomethylation as fixed modification and oxidation of methionine as variable modification. Additionally, the Mascot Score and expectation of the first non-homologous protein to the highest ranked hit was checked. Protein identification was confirmed by sequence information obtained from MS/MS analysis in ''LIFT'' (laser-induced forward transfer) mode. Acquired MS/MS spectra were also processed using the software FlexAnalysis™ 2.4 using a SNAP method set at a S/N threshold of 3.0. For MS/MS spectra searching, the spectra were used to search IPI human database 3.30 (67 922 sequences, 28 879 402 residues) automated using Mascot (v2.1.03). Search parameters for MS/MS data: 100 ppm for the precursor ion and 0.3 Da for the fragment ions. Cleavage specificity and covalent modifications were considered, as described above. The score was higher than the minimal significant ($p<0.05$) individual ion score. All significant MS/MS identifications by Mascot were manually verified for spectral quality and matching y and b ion series.

2.4 Bioinformatics analysis

The PathwayStudio software (version 5.0; Ariadne Genomics, MD, USA) – a specialized graph visualization engine – was used to identify relevant molecular functions of the proteins that were significantly regulated following heat treatment. The gene list comprising the genes encoding these proteins was imported into the software as *per* the manufacturer's instructions and used to develop the common targets pathway for the identification of cell processes influenced by these proteins. PathwayStudio includes an automated text-mining tool that enables the software to generate pathways from the entire PubMed database and other public sources. Thus, each identified cellular process was confirmed through the PubMed/Medline hyperlink embedded in each node.

2.5 Western blotting

The protein levels of TCP1, RBP1, HNRNPH1, Ppia, Lamin A/C, ERP57, hspa4l and β-tubulin in the human testicular specimens of the control and the heat-treated subjects, Hnrnph1 and γ-tubulin in the testes of the control and the heat-treated mice, or Hnrnph1 and γ-tubulin in the control and heat-treated GC2-spd cells (immortalized mouse spermatocyte cell line, purchased from ATCC, Manassas, VA, USA) were analyzed by Western blotting using previously described methods [13]. Antibody against hspa4l was commercially obtained from Santa Cruz Biotechnology (1:200; Santa Cruz, CA, USA), antibody against ERP57 was obtained from Sigma-Aldrich (1:1000; St. Louis, MO, USA), and antibody against Lamin A/C was obtained from Cell Signaling Technology (1:250; Danvers, MA, USA). The other antibodies against TCP1 (1:500), RBP1 (1:200), HNRNPH1 (Hnrnph1) (1:200), Ppia (1:200), β-tubulin (1:2000) and γ-tubulin (1:500) were obtained from Abcam (Cambridge, UK). β-tubulin and γ-tubulin were used as the positive control.

2.6 Immunohistochemical analysis

Bouin's solution-fixed paraffin-embedded sections from the mouse testis tissue at the six developmental stages (wks 0, 1, 2, 3, 4 and 12), and sections from human adult testes were immunostained as described previously [13, 14]. In brief, after quenching the endogenous peroxidase activity, the sections were blocked using a blocking serum and then incubated overnight at 4°C with primary antibodies to HNRNPH1 (Hnrnph1) (1:100). The sections were then incubated with HRP-conjugated secondary antibody (Beijing ZhongShan Biotechnology). Immunoreactive sites were visualized brown with diaminobenzidine and mounted for bright-field microscopy (Axioskop 2 plus; Zeiss, Germany). To confirm the specificity of these antibodies, negative controls were processed in an identical manner, except that the primary antibody was replaced by normal IgG.

2.7 Indirect immunofluorescence

Smears of testicular cell suspension (tetraploid germ cells) from adult mice, or the slices with GC2-spd cells were freshly prepared for immunofluorescence analysis. These samples were fixed with 4% paraformaldehyde in PBS for 40 min and permeabilized with 0.2% Triton X-100 in PBS for 20 min at 37°C. After blocking in PBS containing calf

serum for 2 h, samples were incubated with a 1:200 dilution of Hnrnph1 antibody at 4°C overnight. Samples were then incubated again with the secondary antigoat IgG labeled with tetraethyl rhodamine isothiocyanate (Beijing Zhong-Shan Biotechnology) at a 1:100 dilution for 1 h at room temperature. Slides were viewed with an Axioskop2 plus microscope (Zeiss, Germany) and images were captured with a CCD camera (Zeiss) driven by the software Axio-Version 4.5 (Zeiss).

2.8 Stable transfection of the GC2-spd cells with Hnrnph1-targeted siRNA

Mouse Hnrnph1-targeted siRNA plasmid expression vector (pRS-shHNRNPH1 vector, Cat. TI349493) and control eGFP-target siRNA plasmid expression vector (pRS-shGFP vector, Cat. TR30003) were purchased from OriGene Technologies (Rockville, MD, USA).

Twenty-four hours before transfection, GC2-spd cells were incubated at 37°C with fresh DMEM medium without antibiotics. The subconfluent (80–90%) GC2-spd cells were then transfected with vectors using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the instruction manual of Lipofectamine™ 2000. 2.5 μg of vectors were used in all experiments with eGFP-target siRNA vector used as the control. After antibiotic selection (3 μg/mL puromycin; Sigma-Aldrich), transfectants were pooled to avoid the effects of clonal selection and were expanded in 1.5 μg/mL puromycin. Western blot analysis was then performed to detect the expression of Hnrnph1 protein.

2.9 Apoptosis detection by flow cytometry

Untreated cells (GC2-spd cells, Hnrnph1-targeted siRNA cells or eGFP-target siRNA cells) and cells at different times after incubation at 42°C were collected for apoptosis detection.

The detection of apoptosis by flow cytometry was performed according to the instruction manual of the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA). Briefly, 5×10^5 cells were collected and washed twice with cold PBS. Cells were then resuspended in 500 μL Annexin V binding buffer and 5 μL of Annexin V-FITC and 5 μL of PI were added. The cells were gently vortexed and incubated for 10 min at room temperature in the dark. Within 1 h, the cells were analyzed by flow cytometry.

2.10 Real-time RT-PCR

Total RNA was extracted from the Hnrnph1-targeted siRNA cells and eGFP-target siRNA cells using the Trizol reagent (Gibco BRL, Grand Island, NY, USA) and reverse-transcribed into cDNA with AMV reverse transcriptase (Promega, Madison, WI, USA). PCR was then performed in reactions containing a cDNA template prepared from approximately 1 μg RNA, 1 × SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 250 nM primers. Primer sequences and annealing temperatures are presented in Supporting Information Table 1. Real-time PCR was performed using the ABI PRISM 7300 SEQUENCE DETECTION SYSTEM (Applied Biosystems) and the following thermal cycling conditions: 10 min at 95°C; followed by 40 cycles of 15 s at 95°C, 1 min at 56°C∼60°C. β-actin was amplified in parallel as a loading control. The mRNA level of each gene was normalized to β-actin mRNA and presented as a percentage of control [15, 16]. Afterwards, the mRNA level of each gene in Hnrnph1-targeted siRNA cells and control cells were compared using the *t*-test. If the *p*-value was <0.05, the difference was considered statistically significant.

2.11 Statistics analysis

Data were analyzed by *t*-test for significant differences between the two groups. All the ratios were arcsine square root transformed before *t*-test analysis and the least significant difference *post hoc* test was used to examine any significant difference between groups. The results were considered statistically significant when $p<0.05$.

3 Results

3.1 Identification of altered proteins in human testes after heat treatment

Quadruple 2-DE maps of the testicular proteins from the control and 2 wk after heat-treatment groups were constructed in Supporting Information Fig. 1. By examining 2-DE gels using the ImageMaster™ 2D Platinum Software, we found 45 protein spots in the 2 wk after heat-treatment group that were significantly different when compared with those in the control group. Among the 45 protein spots, 27 spots decreased and 18 spots increased after heat treatment. Total 34 of these spots were identified successfully, corresponding to 31 known proteins. A description of these 31 proteins is presented in Supporting Information Table 2. The identified protein spots were present in the 2-DE maps of the testicular proteins from the untreated control group and the treated groups at 2 wk after treatment (Fig. 1A, Table 1).

Quadruple 2-DE maps of the testicular proteins from the control and 9 wk after heat treatment groups were constructed in Supporting Information Fig. 1. We found 36 protein spots in the 9 wk after heat-treated group that were significantly different when compared with those in the control group. Among the 36 protein spots, 9 spots

decreased and 27 spots increased after heat treatment. Twenty-six of these spots were identified successfully. A description of these 26 proteins is presented in Supporting Information Table 3. The identified protein spots were present in the 2-DE maps of the testicular proteins from the untreated control group and the treated group at 9 wk after treatment (Fig. 1B, Table 1).

3.2 Validation of the 2-DE results by Western blotting analysis

We selected five proteins, HNRNPH1, Ppia, Lamin A/C, ERP57 and hspa4l, in the network of differential proteins 2 wk after heat treatment, and four proteins (TCP1, HNRNPH1, hspa4l and RBP1) in the network of differential proteins 9 wk after heat treatment for Western blotting analysis. As shown in Fig. 2A, levels of Ppia and Lamin A/C increased and that of HNRNPH1, ERP57 and hspa4l decreased at 2 wk after heat treatment, respectively (Fig. 2A). As shown in Fig. 2B, the levels of TCP1, HNRNPH1 and RBP1 increased and that of hspa4l decreased at 9 wk after heat treatment (Fig. 2B). These results are consistent with those determined using the ImageMaster^{TM} 2D Platinum Software.

3.3 PathwayStudio analysis of the altered proteins following heat treatment

The potential involvement of the differentially expressed proteins, listed above, in a relevant molecular network was analyzed by PathwayStudio software (Fig. 3). Among the 31 differential proteins of testis 2 wk after heat treatment, 25 proteins were observed to participate in the complex functional network and were involved in the events of prolif-

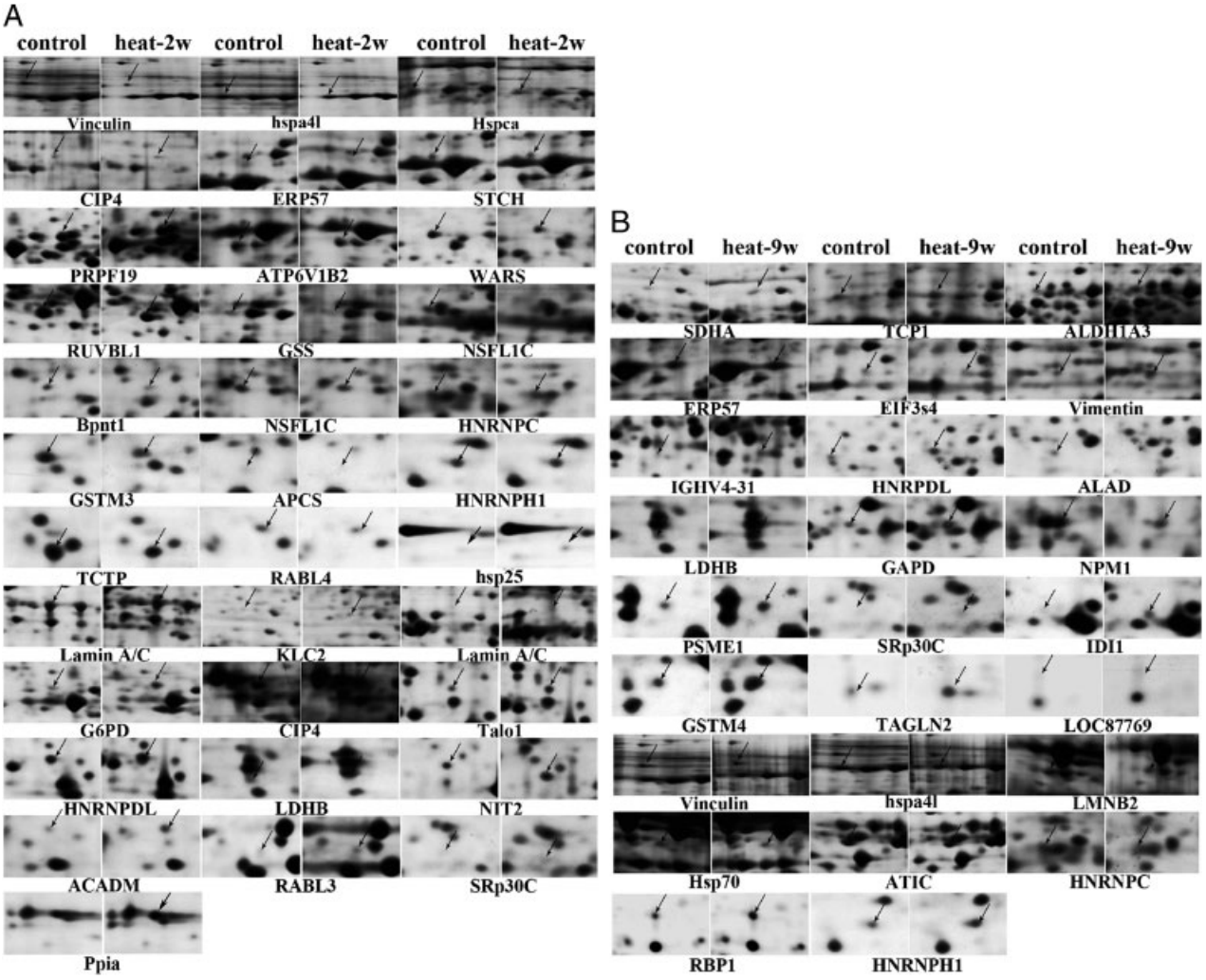

Figure 1. Partial 2-DE gel images showing differential expression of the identified protein spots in the untreated human testes and in the subjects 2 wk after heat treatment (A), and those in the untreated human testes and in the subjects 9 wk after heat treatment (B).

Table 1. Identification of altered proteins in human testes after heat treatment

	2 w/control		9 w/control	
	Upregulated	Downregulated	Upregulated	Downregulated
Number of differentially expressed protein spots	18	27	27	9
Number of identified protein spots	14	20	19	7
Number of identified proteins	13	19	19	7

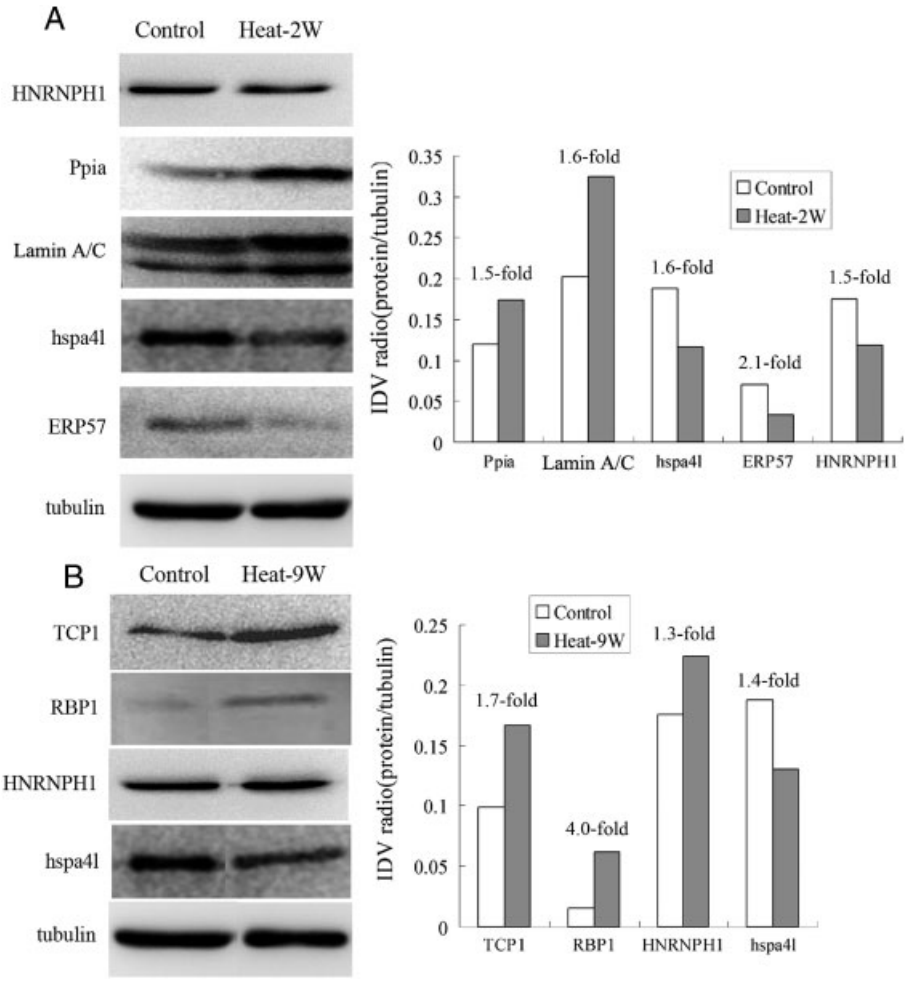

Figure 2. Western blot analysis was performed using anti-HNRNPH1, Ppia, Lamin A/C, ERP57, hspa4l and β-tubulin polyclonal antibodies for aliquots of total protein extracts prepared from the untreated human testes and from those 2 wk after heat treatment (A). Western blot analysis was performed using anti-TCP1, HNRNPH1, RBP1, hspa4l and β-tubulin polyclonal antibodies for aliquots of total protein extracts prepared from the untreated human testes and from those 9 wk after heat treatment (B).

eration, differentiation, cell survival, apoptosis, DNA fragmentation, motility, mitosis, RNA splicing, *etc*. The most prevalent events involving these 25 proteins were ''proliferation,'' ''differentiation,'' ''cell survival'' and ''apoptosis.'' A total of 15 proteins (occupied 60% proteins in the network) participated in the event of proliferation, 14 proteins (occupied 56% proteins in the network) participated in the event of differentiation and 17 proteins (occupied 68% proteins in the network) participated in the events of cell survival and apoptosis. Many proteins in the functional network were involved in more than one event (Fig. 3A).

In testis from the 9 wk after heat-treatment group, we identified 17 proteins in the complex functional network which participated in the events of proliferation, differentiation, cell survival, apoptosis, regulation of signal transduction, mitosis, RNA splicing, spermatogenesis, *etc*. The most prevalent events involving these 17 proteins were also ''proliferation,'' ''differentiation,'' ''cell survival'' and ''apoptosis.'' Twelve proteins (occupied 71% proteins in the network) participated in the events of proliferation and apoptosis. Eleven proteins (occupied 65% proteins in the network) participated in the event of cell survival and differentiation (Fig. 3B).

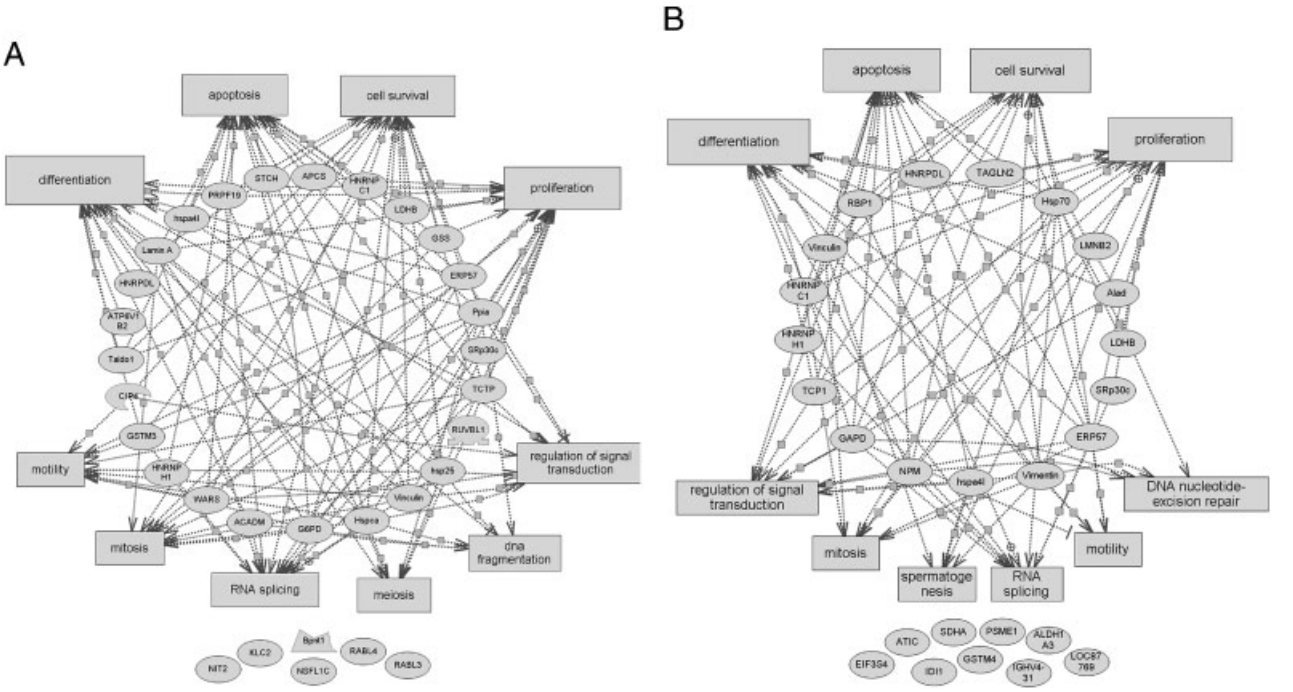

Figure 3. Key components of the molecular pathways involved in spermatogenesis suppression at 2 wk after heat treatment (A) and spermatogenesis recovery at 9 wk after heat treatment (B). Oval-shaped signs represent proteins and cube-shaped signs represent cellular processes. Regulatory events are represented by arrows, and the effects are represented as ''+'' (activation) or ''−'' (downregulation).

3.4 Expression of the HNRNPH1(Hnrnph1) protein in the human and mouse testes

The sequence analysis using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov) indicated that human HNRNPH1 protein showed a 99% identity to the mouse Hnrnph1 protein (Supporting Information Fig. 2A). Conserved domain analysis using Simple Modular Architecture Research Tool (http://smart.embl-heideberg.de) showed that both the mouse Hnrnph1 and human HNRNPH1 proteins had three RRM, RNA recognition motif, domains (Supporting Information Fig. 2B). These results indicated that the mouse Hnrnph1 protein was highly homologous to the human HNRNPH1 protein.

By immunohistochemical analysis, we detected the localization of the Hnrnph1 protein in the mouse testes at different developmental stages (Fig. 4). The results showed that in testes of newborn (day 0) and 1-wk-old mice, the Hnrnph1 protein was found to be expressed only in the somatic cells, including the nucleus of Sertoli cell and cytoplasm of Leydig cells. No immunostaining was detected in the spermatogonia (Fig. 4A and B). At the time when spermatocytes appeared in testes of 2-wk-old mice, the Hnrnph1 protein was found to be expressed in the nucleus of spermatocytes in additional to somatic cells (Fig. 4C). In testes of 3-, 4- and 12-wk-old mice, the Hnrnph1 protein remained expressed in the spermatocyte, Sertoli cells and Leydig cells, and was weakly positive in spermatids (Fig. 4D–F). In adult human testes, HNRNPH1 was mainly localized in the nucleus of spermatocytes (Fig. 4G), which was similar to that observed in adult mouse testis.

Imitating the human testis model of heat treatment, we constructed the mouse testis model of reversible suppression of spermatogenesis induced by heat treatment. The morphological appearance of the mouse testes showed seminiferous epithelia beginning to damage at 12 h after heat treatment (42°C, 15 min) and showed seminiferous epithelia recovery at 14 days after heat treatment (Fig. 5A). By Western blot analysis, we found the expression of the Hnrnph1 protein decreased at 12 h and increased at 14 days in the mouse testis after heat treatment (Fig. 5B), which was similar to the expression characteristic of the human HNRNPH1 protein in the testes after heat treatment (Figs. 1 and 2). The above results allowed us to study the function of human HNRNPH1 protein using the mouse model.

3.5 The expression level of Hnrnph1 protein related to GC2-spd cell apoptosis

According to localization characteristics of Hnrnph1 protein in the spermatocytes of mouse testes, we detected the expression of Hnrnph1 protein in the GC2-spd cells (an immortalized mouse spermatocyte cell line) using immunofluorescence and found it was highly expressed in the nucleus of GC2-spd cells (Fig. 6B). This expression pattern of Hnrnph1 protein in GC2-spd cells was the same as that observed in the isolated mouse tetraploid germ cells

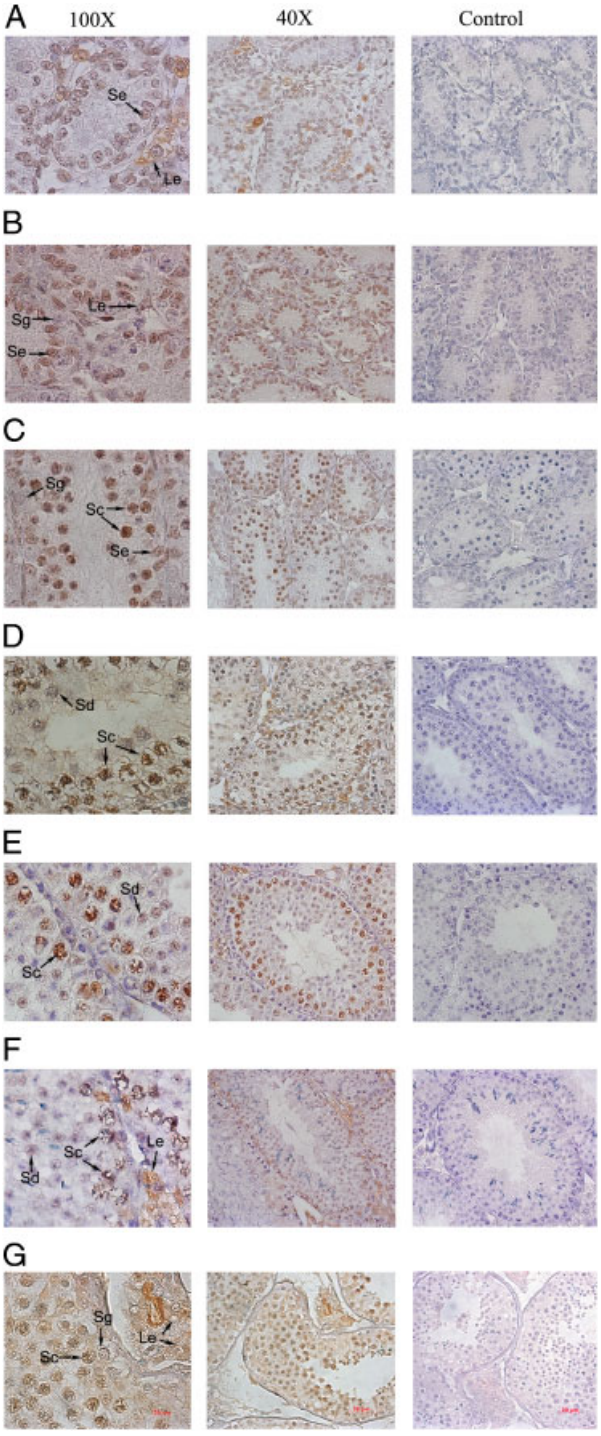

Figure 4. Localization of the HNRNPH1 (Hnrnph1) protein in the mouse and human testes analyzed by immunohistochemistry. (A–F) Localization of the Hnrnph1 protein in the mouse testes of different developmental stages (A: 0 W; B: 1 W; C: 2 W; D: 3 W; E: 4 W; F: 12 W). (G) The localization of HNRNPH1 protein in human adult testis. Se: Sertoli cell; Le: Leydig cell; Sg: spermatogonia; Sc: Spermatocyte ; Sd: spermatid. Scale bar: 20 μm.

(Fig. 6A). Thus, we selected GC2-spd cells as the model for the functional study of Hnrnph1 protein.

To prove that Hnrnph1 protein was related to the heat-induced suppression of spermatogenesis, we studied the relationship between apoptosis and the expression level of the Hnrnph1 protein. We found that 24 h after heat incubation (incubated at 42°C for 3 h and then returned to the incubation at 37°C for 24 h), the apoptotic rate of GC2-spd cells increased remarkably when compared with that of untreated cells ($p<0.05$; Fig. 7A), while the expression of Hnrnph1 protein decreased significantly ($p<0.05$; Fig. 7B). This result coincided with what we observed in the heat-treated mouse testis in the anterior experiment (Fig. 5) and indicated that Hnrnph1 protein might participate in regulating apoptosis of GC2-spd cells.

3.6 siRNA-mediated repression of Hnrnph1 expression promoted apoptosis of GC2-spd cells

For revealing the precise role of Hnrnph1 protein on cell apoptosis, we stably transfected the GC2-spd cell line with an Hnrnph1-targeted siRNA to downregulate Hnrnph1 expression and observed the effects on protein expression, and apoptotic rate of GC2-spd cells. We found that expression of Hnrnph1-targeted siRNA resulted in a decrease in Hnrnph1 protein levels, as detected by Western blot studies (Fig. 8A). Transfected cells also exhibited a marked increase in the rate of cell apoptosis compared to cells transduced with an eGFP-target siRNA plasmid as control ($p<0.01$; Fig. 8B). Twenty-four hours after cell warming (incubation at 42°C for 3 h), the cell apoptosis increased both in Hnrnph1-targeted siRNA cells and eGFP-targeted siRNA control cells, and the apoptotic rate of Hnrnph1-targeted siRNA cells was markedly higher than that of control cells ($p<0.01$; Fig. 8B). Thus, downregulation of Hnrnph1 expression could promote cell apoptosis, which indicated that Hnrnph1 protein was an anti-apoptosis factor in GC2-spd cells. It was notable that the apoptotic rate of the transfected cells was lower than that of blank cells, both at baseline and after heat treatment. Transfection with vectors seemed to enable the cells to tolerate the heat shock by an unknown mechanism. However, the apoptotic rate of Hnrnph1-targeted siRNA cells was always higher than that of control cells markedly.

3.7 The regulatory role of Hnrnph1 protein on the expression of heat-related proteins

Using Real-time PCR, we detected the mRNA levels of 29 proteins in Hnrnph1-target siRNA cells and compared them with control eGFP-target siRNA cells. These 29 proteins were mouse homologous proteins of the human heat-related proteins screened in the aforementioned experiment. The expression trends of the 29 heat-related proteins were the same as that of the HNRNPH1 protein in the human testis,

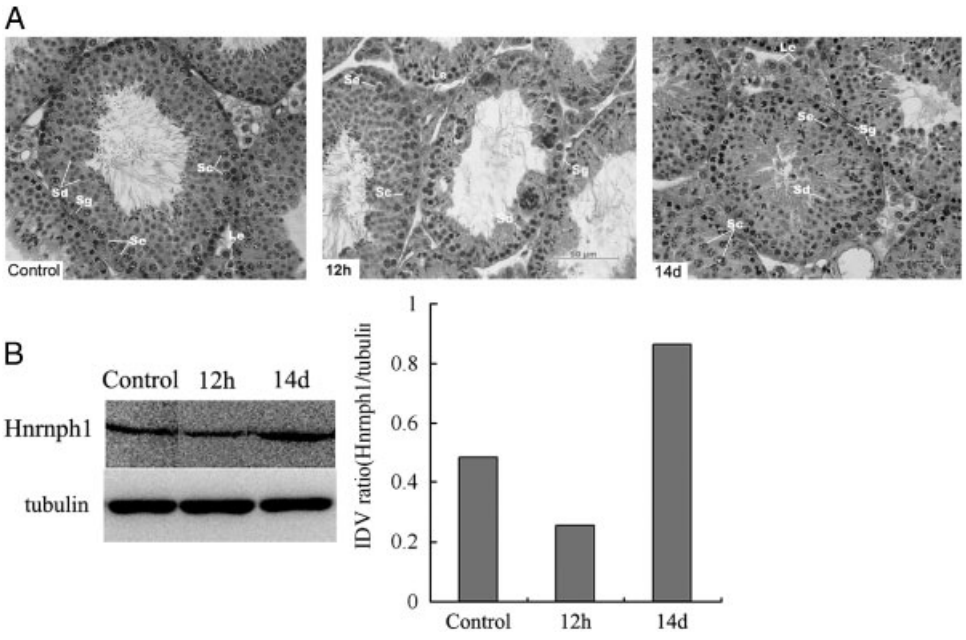

Figure 5. Expression of the Hnrnph1 protein in the mouse testes after heat treatment. (A) The morphological appearance of the mouse testes showed seminiferous epithelia began to show damage at 12 h after heat treatment, and showed seminiferous epithelia recovery at 14 days after heat treatment. (B) The expression level of the Hnrnph1 protein decreased at 12 h and increased at 14 days in the mouse testes after heat treatment by Western blot analysis. Scale bar: 50 μm.

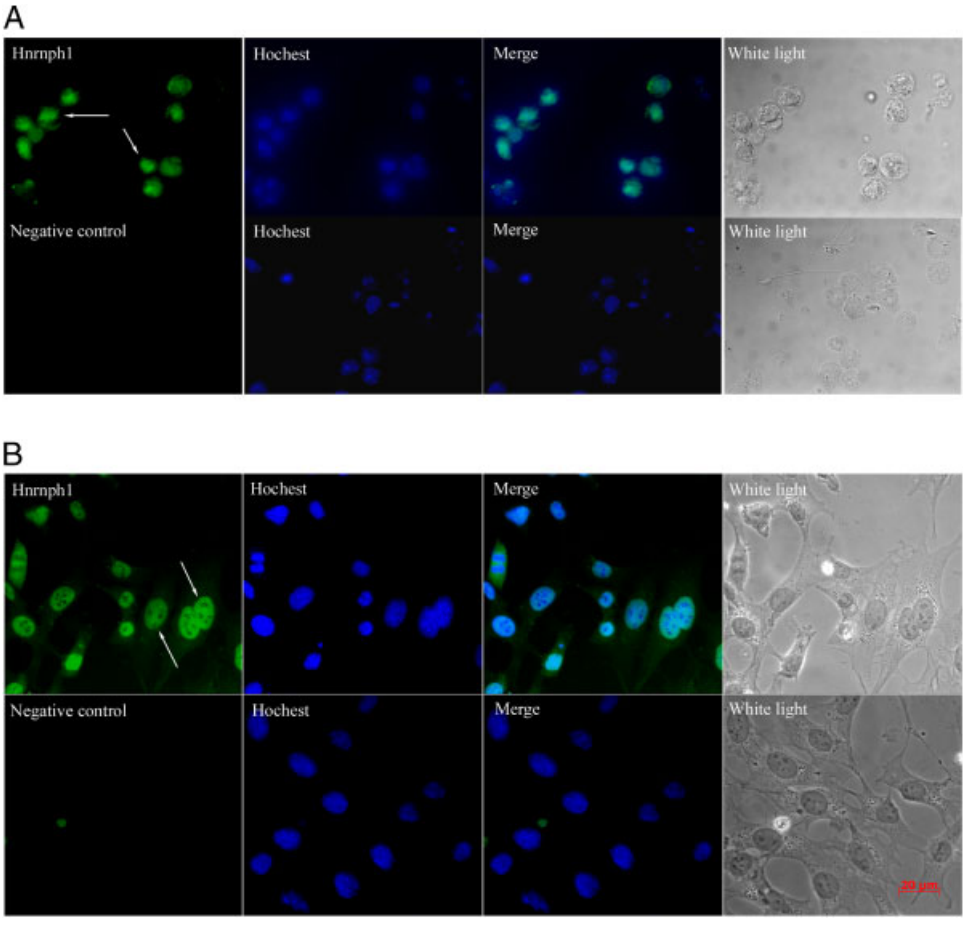

Figure 6. Expression of the Hnrnph1 protein in the isolated mouse tetraploid germ cells (A) and mouse spermatocyte cell line (B) analyzed by immunofluorescence. ''↑'' indicates the positive signal in the nucleus of mouse tetraploid germ cells and GC2-spd cells. Scale bar 20 μm.

i.e. decreased at 2 wk and increased at 9 wk after heat treatment (Fig. 1). When we compared mRNA levels of these 29 proteins in Hnrnph1-target siRNA knock down cells and control cells, we found the expression trend of ten proteins

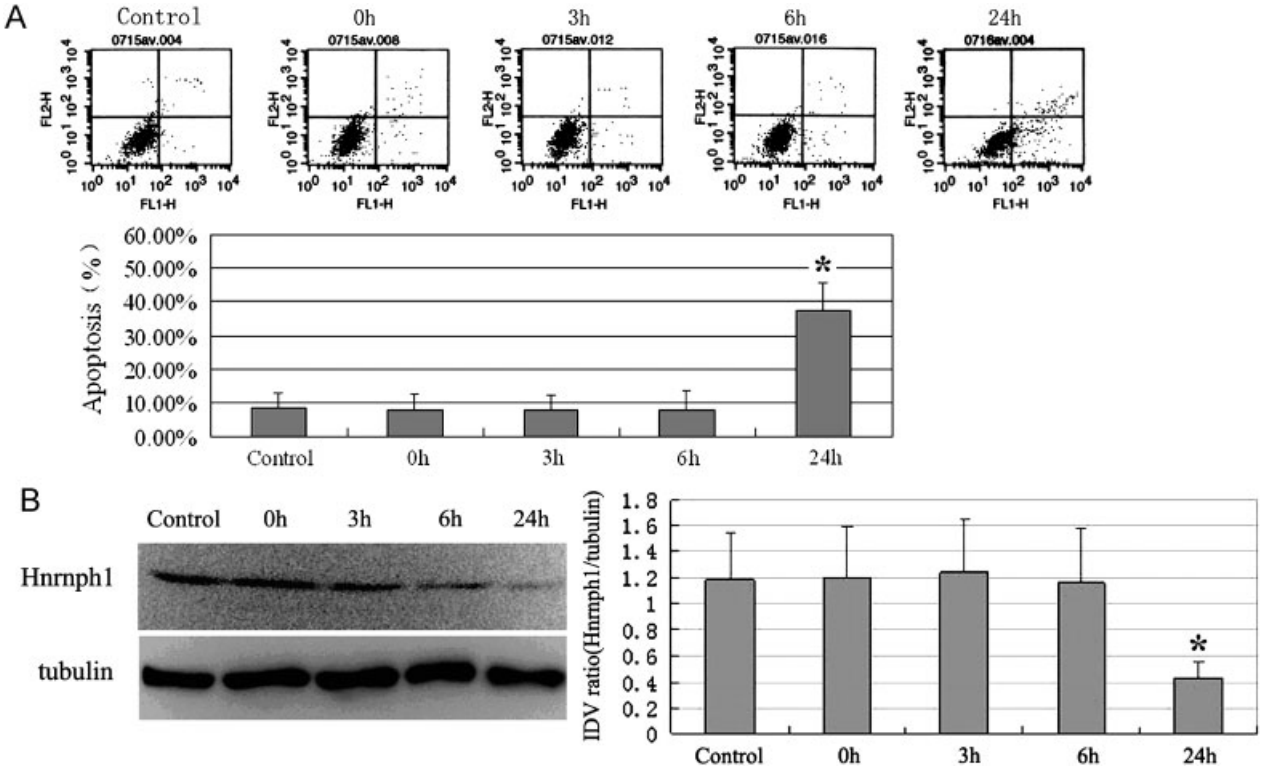

Figure 7. Relationship between apoptosis and the expression level of the Hnrnph1 protein in mouse GC2-spd cells (A) The apoptotic rate of GC2-spd cells at different times after heat incubation or without heat treatment. (B) The expression level of the Hnrnph1 protein in the GC2-spd cells at different times after heat incubation or without heat treatment. $^{*}p<0.05$.

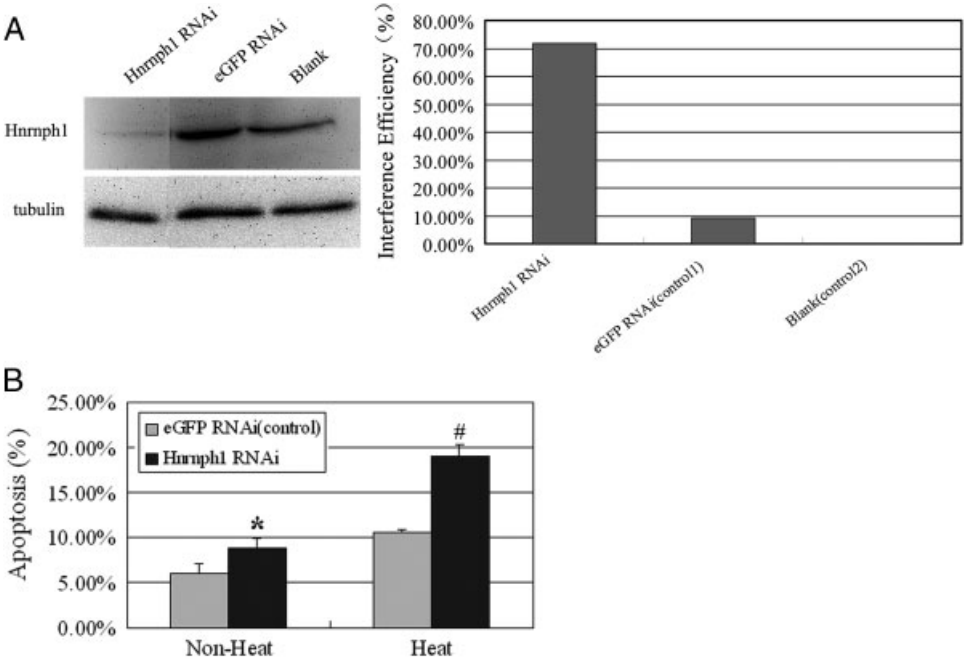

Figure 8. Downregulation of Hnrnph1 expression inhibited apoptosis of mouse GC2-spd cells. (A) Western blot analysis showed that the Hnrnph1 protein expression was markedly repressed in the Hnrnph1-targeted siRNA cells. (B) 24 h after heat treatment and without treatment, Hnrnph1-targeted siRNA cells exhibited a marked increase in the rate of cell apoptosis compared to cells transduced with eGFP-target siRNA plasmid as control. *Non-Heat $p<0.01$; ♯ Heat $p<0.01$.

was markedly decreased similar to Hnrnph1 proteins (Fig. 9). These results indicated that Hnrnph1 protein could either directly or indirectly regulate the expression of heat-related genes.

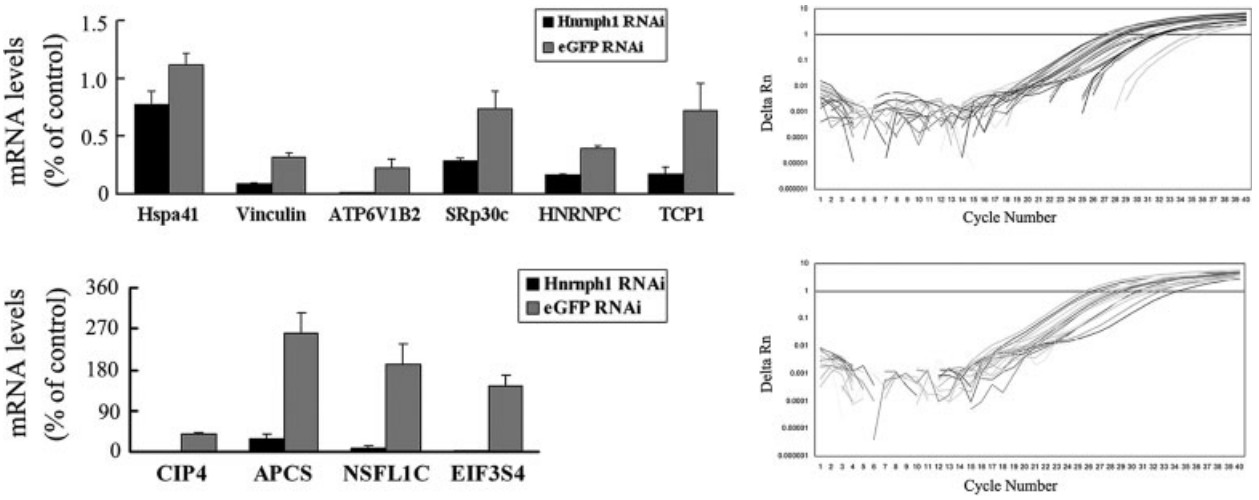

Figure 9. mRNA levels of ten heat-related proteins decreased remarkably in the Hnrnph1 RNAi cells when compared with those in the control cells ($p < 0.05$).

4 Discussion

The overall goal of our study applying transient heat to the testes was to identify testis specific molecules regulating spermatogenesis that can be used as target for future male contraceptive development. In a recent study, we found that short-term exposure of the testis to heat induced reversible spermatogenesis suppression in Chinese men [11]. Although the physiological and cellular responses of the testes to heat treatment have been well documented, the molecular mechanisms through which these responses are directed, especially the molecular mechanism behind the germ cell apoptosis induced by heat treatment remain largely unknown. Gene expression following increased scrotal temperature has been described in mice [2], only limited proteomic information is available regarding testicular heat shock. In the present study, using proteomic analyses, we attempted to delineate the human testicular cell response to the heat treatment, and to elucidate the underlying molecular mechanism involved in heat-induced germ cell apoptosis and accordingly the reversible suppression of spermatogenesis.

4.1 Testicular hyperthermia affects the ''proliferation,'' ''differentiation,'' ''apoptosis'' and ''cell survival'' processes in human testes

We employed global proteomic analyses of the human testis at baseline and at 2 and 9 wk after heat administration. Forty-five protein spots were found with significant differential expression at 2 wk after heat treatment. By comparing their peptide mass fingerprints to the SwissProt/TrEMBL sequence databases, 31 known proteins were identified and were subsequently used to characterize the cellular and molecular events in the testes when spermatogenesis was disturbed by heat treatment. Thirty-six protein spots were found with significant differential expression at 9 wk after heat treatment, and 26 known proteins were identified and were subsequently used to characterize the cellular and molecular events in the testes when spermatogenesis began to recover after heat treatment.

The PathwayStudio software enabled us to create a functional network of these differential proteins that could provide valuable information on specific protein functions in the cellular process. At 2 wk after heat treatment, 25 proteins were observed to participate in the complex functional network, and most of them were involved in the events relating to ''proliferation,'' ''differentiation,'' ''apoptosis'' and ''cell survival'' (Fig. 3A). Most proteins playing the role of mitigating apoptosis or promoting proliferation and cell survival, such as ERP57 [17] and PRPF [18], showed decreased expression, whereas proteins functioning as the pro-apoptosis or anti-proliferation factors, such as Ppia [19] and Lamin A/C [20], were increased at this time point. At 9 wk after heating, we identified 17 proteins in the complex functional network with ''proliferation,'' ''differentiation,'' ''cell survival'' and ''apoptosis'' as predominant events (Fig. 3B). At this time point, the expression levels of proteins which could induce apoptosis or inhibit cell survival, such as NPM [21, 22] and Vinculin [23] were decreased, and proteins suppressing apoptosis or promoting proliferation and differentiation, such as TCP1 [24], RBP1 [25] and Alad [26] were increased. Thus, the changing protein expression might contribute to the spermatogenic disturbance at 2 wk and spermatogenic repair at 9 wk after heat treatment.

It has been demonstrated that ''proliferation'' [27–29], ''differentiation'' [27, 30], ''apoptosis'' [31–33] and ''cell survival'' [31, 34] are all important categories of events involved in the process of spermatogenesis. Thus, heat treatment induced the reversible suppression of spermatogenesis by changing the expression of proteins related to proliferation, differentiation, apoptosis and cell survival. These differential

proteins were the key molecular targets of heat treatment in human testis. At 2 wk post-treatment, the changed expression of a series of proteins could promote apoptosis or suppress proliferation and cell survival. At 9 wk post-treatment, the changed expression of proteins could promote cell proliferation, differentiation and survival, but resist cell apoptosis.

4.2 HNRNPH1, a key protein regulated by heat treatment, is an anti-apoptosis factor during spermatogenesis

Further studies on differentially expressed proteins after heat exposure may help to clarify the mechanism of testicular hyperthermia induced suppression of spermatogenesis. By comparing of the differential proteins between 31 proteins in 2-wk group and 26 proteins in 9-wk group, we found that HNRNPH1 expression level was decreased when spermatogenesis was disturbed at 2 wk but began to increase when seminiferous epithelia was undergoing recovery at 9 wk after heat treatment. This result indicated HNRNPH1 was correlated with spermatogenesis. Aligning the sequences of human and mouse HNRNPH1 showed that mouse Hnrnph1 protein was highly homologous to human HNRNPH1 protein (Supporting Information Fig. 2). We also verified that the expression level of Hnrnph1 protein decreased at the stage of spermatogenic damage, and increased when spermatogenesis was recovering in the mouse testis after heat treatment (Fig. 5), just same as what we have observed in human heat-treated testes (Fig. 2). Thus, in the present study the functional study on HNRNPH1 protein was performed using the mouse model.

Expression and localization of Hnrnph1 protein in the mouse testis from birth to mature adult were studied. We found that concurrent with the initiation of spermatogenesis, Hnrnph1 protein was gradually expressed in the germ cells and mainly in the nucleus of spermatocytes, which was similar to the localization characteristic of HNRNPH1 protein in the human testis. These results implied Hnrnph1 protein was involved in the development and possibly the functions of spermatocytes. Thus, the mouse spermatocyte cell line (GC2-spd cell) was selected for the functional study on Hnrnph1 protein *in vitro*.

Using the model of cell warming (GC2-spd cells were incubated at 42°C for 3 h then came back to 37°C for incubation), we observed the relation between apoptosis and Hnrnph1 expression. Along with the increase of cell apoptosis after heat treatment, the expression level of Hnrnph1 protein decreased markedly ($p < 0.05$). Furthermore, we observed the influence of siRNA-mediated inhibition of Hnrnph1 on cell apoptosis and found that down expression of Hnrnph1 resulted in the increase of apoptosis both at baseline and after heat treatment. These results indicated Hnrnph1 protein was an anti-apoptosis factor, which could explain why Hnrnph1 (HNRNPH1) expression decreased in the testis when germ cell apoptosis was activated and increased when recovery of spermatogenesis occurred.

HNRNPH1 protein is a member of the heterogeneous nuclear ribonucleoprotein (HNRNP) protein family [35]. HNRNP proteins are nucleus-enriched proteins and play important roles in RNA processing, including mRNA stabilization, transport and splicing, *etc*. [36–38]. It has been shown that Hnrnph/h' protein is responsible for the mRNA levels of some proteins such as HUMMK, OCACE3 and HSFOLA [39]. Thus, the changed expression of Hnrnph1 protein might influence the mRNA levels of other proteins. This conjecture was verified in the present study. We found the Hnrnph1 expression maintained mRNA levels of some heat-related proteins. When the expression of Hnrnph1 protein was reduced in the Hnrnph1-target siRNA cells, mRNA levels of ten heat-related proteins decreased markedly (Fig. 9). Furthermore, expression of these ten proteins followed the trend of HNRNPH1 protein in the heat-treated human testis. Taken together, our results indicate that HNRNPH1 protein is an important regulatory protein and may be a key protein involved in the suppression of spermatogenesis induced by heat treatment.

In summary, by proteomic analysis, we showed that heat treatment induced the reversible suppression of spermatogenesis by changing the expression of a series of proteins related to proliferation, differentiation, apoptosis and cell survival. These differentially expressed proteins were the key molecular targets of heat treatment related to spermatogenesis. We demonstrated that HNRNPH1 protein expression changed with the state of apoptosis after heat exposure. Further studies on other proteins will provide more information about the exact mechanisms mediating the effect of transient elevation of temperature on germ cell apoptosis and spermatogenesis. At the same time, it may provide new avenues for the management of human infertility and novel targets for contraceptive development.

The study was supported by NSFC (No. 30772347, 30630030), the 973 program (2006CB504002), the Contraceptive Research and Development Program and the Mellon Foundation (MFG-02-64, MFG-03-67), the General Clinical Research Center Core Laboratory at Harbor-UCLA Medical Center (MO1-RR00425) and Program of Changjiang Scholars and Innovative Research Team in University (IRT0631).

The authors have declared no conflict of interest.

5 References

[1] Mieusset, R., Bujan, L., Testicular heating and its possible contributions to male infertility: a review. *Int. J. Androl.* 1995, *18*, 169–184.

[2] Rockett, J. C., Mapp, F. L., Garges, J. B., Luft, J. C. *et al.*, Effects of hyperthermia on spermatogenesis, apoptosis,

gene expression, and fertility in adult male mice. *Biol. Reprod.* 2001, *65*, 229–239.

[3] Hand, J. W., Walker, H., Hornsey, S., Field, S. B., Effects of hyperthermia on the mouse testis and its response to X-rays, as assayed by weight loss. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* 1979, *35*, 521–528.

[4] Chowdhury, A. K., Steinberger, E., A quantitative study of the effect of heat on germinal epithelium of rat testes. *Am. J. Anat.* 1964, *115*, 509–524.

[5] Lue, Y. H., Sinha Hikim, A. P., Swerdloff, R. S., Im, P. *et al.*, Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. *Endocrinology* 1999, *140*, 1709–1717.

[6] Lue, Y., Wang, C., Liu, Y. X., Hikim, A. P. *et al.*, Transient testicular warming enhances the suppressive effect of testosterone on spermatogenesis in adult cynomolgus monkeys (Macaca fascicularis). *J. Clin. Endocrinol. Metab.* 2006, *91*, 539–545.

[7] Barth, A. D., Bowman, P. A., The sequential appearance of sperm abnormalities after scrotal insulation or dexamethasone treatment in bulls. *Can. Vet. J.* 1994, *35*, 93–102.

[8] Mieusset, R., Quintana Casares, P., Sanchez Partida, L. G., Sowerbutts, S. F. *et al.*, Effects of heating the testes and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. *J. Reprod. Fertil.* 1992, *94*, 337–343.

[9] Mieusset, R., Bujan, L., The potential of mild testicular heating as a safe, effective and reversible contraceptive method for men. *Int. J. Androl.* 1994, *17*, 186–191.

[10] Kandeel, F. R., Swerdloff, R. S., Role of temperature in regulation of spermatogenesis and the use of heating as a method for contraception. *Fertil. Steril.* 1988, *49*, 1–23. Review.

[11] Wang, C., Cui, Y. G., Wang, X. H., Jia, Y. *et al.*, Transient scrotal hyperthermia and levonorgestrel enhance testosterone-induced spermatogenesis suppression in men through increased germ cell apoptosis. *J. Clin. Endocr. Metab.* 2007, *92*, 3292–3304.

[12] Zhu, Y. F., Cui, Y. G., Guo, X. J., Wang, L. *et al.*, Proteomic analysis of effect of hyperthermia on spermatogenesis in adult male mice. *J. Proteome Res.* 2006, *5*, 2217–2225.

[13] Cui, Y. G., Zhu, H., Zhu, Y. F., Guo, X. J. *et al.*, Proteomic analysis of testis biopsies in men treated with injectable testosterone undecanoate alone or in combination with oral levonorgestrel as potential male contraceptive. *J. Proteome Res.* 2008, *7*, 3984–3993.

[14] Armstrong, D. J., Hiscott, P., Batterbury, M., Kaye, S., Corneal stromal cells (keratocytes) express thrombospondins 2 and 3 in wound repair phenotype. *Int. J. Biochem. Cell Biol.* 2002, *34*, 588–593.

[15] Grdeň, M., Podgórska, M., Szutowicz, A., Pawełczyk, T., Altered expression of adenosine receptors in heart of diabetic rat. *J. Physiol. Pharmacol.* 2005, *56*, 587–597.

[16] Abukawa, H., Tomi, M., Kiyokawa, J., Hori, S. *et al.*, Modulation of retinal capillary endothelial cells by Müller glial cell-derived factors. *Mol. Vis.* 2009, *15*, 451–457.

[17] Hetz, C., Russelakis-Carneiro, M., Walchli, S., Carboni, S. *et al.*, The disulfide isomerase Grp58 is a protective factor against prion neurotoxicity. *J. Neurosci.* 2005, *25*, 2793–2802.

[18] Mahajan, K. N., Mitchell, B. S., Role of human Pso4 in mammalian DNA repair and association with terminal deoxynucleotidyl transferase. *Proc. Natl. Acad. Sci. USA* 2003, *100*, 10746–10751.

[19] Jin, Z. G., Lungu, A. O., Xie, L., Wang, M. *et al.*, Cyclophilin A is a proinflammatory cytokine that activates endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 2004, *24*, 1186–1191.

[20] Ivorra, C., Kubicek, M., Gonzalez, J. M., Sanz-Gonzalez, S. M. *et al.*, A mechanism of AP-1 suppression through interaction of c-Fos with lamin A/C. *Genes Dev.* 2006, *20*, 307–320.

[21] Kurki, S., Peltonen, K., Latonen, L., Kiviharju, T. M. *et al.*, Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. *Cancer Cell* 2004, *5*, 465–475.

[22] Rahman-Roblick, R., Roblick, U. J., Hellman, U., Conrotto, P. *et al.*, p53 targets identified by protein expression profiling. *Proc. Natl. Acad. Sci. USA* 2007, *104*, 5401–5406.

[23] Subauste, M. C., Perta, O., Adamson, E. D., Turner, C. E. *et al.*, Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. *J. Cell Biol.* 2004, *165*, 371–381.

[24] Liu, X., Lin, C. Y., Lei, M., Yan, S. *et al.*, CCT chaperonin complex is required for the biogenesis of functional Plk1. *Mol. Cell Biol.* 2005, *25*, 4993–5010.

[25] Boerman, M. H., Napoli, J. L., Cellular retinol-binding protein-supported retinoic acid synthesis. Relative roles of microsomes and cytosol. *J. Biol. Chem.* 1996, *271*, 5610–5616.

[26] Kelada, S. N., Shelton, E., Kaufmann, R. B., Khoury, M. J., Delta-aminolevulinic acid dehydratase genotype and lead toxicity: a HuGE review. *Am. J. Epidemiol.* 2001, *154*, 1–13.

[27] Eddy, E. M., Male germ cell gene expression. *Recent Prog. Horm. Res.* 2002, *57*, 103–128.

[28] Amann, R. P., The cycle of the seminiferous epithelium in humans: a need to revisit? *J. Androl.* 2008, *29*, 469–487.

[29] Bergmann, M., Spermatogenesis-physiology and pathophysiology. *Urologe A* 2005, *44*, 1131–1132, 1134–1138.

[30] Gong, X. D., Li, J. C., Leung, G. P., Cheung, K. H. *et al.*, A BK(Ca) to K(v) switch during spermatogenesis in the rat seminiferous tubules. *Biol. Reprod.* 2002, *67*, 46–54.

[31] Print, C. G., Loveland, K. L., Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays* 2000, *22*, 423–430.

[32] Matsui, Y., Regulation of growth and survival of germ cells. *Hum. Cell* 1997, *10*, 63–68.

[33] Shaha, C., Modulators of spermatogenic cell survival. *Soc. Reprod. Fertil. Suppl.* 2007, *63*, 173–186.

[34] Toppari, J., Suominenf, J. S., Yan, W., The role of retinoblastoma protein family in the control of germ cell proliferation, differentiation and survival. *APMIS* 2003, *111*, 245–251 (Review).

[35] Honore, B., Rasmussen, H. H., Vorum, H., Dejgaard, K. *et al.*, Heterogeneous nuclear ribonucleoproteins H, H-prime, and F are members of a ubiquitously expressed subfamily of related but distinct proteins encoded by genes mapping to different chromosomes. *J. Biol. Chem.* 1995, *270*, 28780–28789.

[36] Dreyfuss, G., Structure and function of nuclear and cytoplasmic ribonucleoprotein particles. *Annu. Rev. Cell Biol.* 1986, *2*, 459–498.

[37] Drevfuss, G., Matunis, M. J., Pinol-Roma, S., Burd, C. G., hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.* 1993, *62*, 289–321.

[38] Chou, M. Y., Rooke, N., Turck, C. W., Black, D. L., hnRNP H is a component of a splicing enhancer complex that activates a c-src alternative exon in neuronal cells. *Mol. Cell. Biol.* 1999, *19*, 69–77.

[39] Arhin, G. K., Boots, M., Bagga, P. S., Milcarek, C. *et al.*, Downstream sequence elements with different affinities for the hnRNP H/H′ protein influence the processing efficiency of mammalian polyadenylation signals. *Nucleic Acids Res.* 2002, *30*, 1842–1850.

