Ran-binding protein M is associated with human spermatogenesis and oogenesis

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Abstract. The aim of the present study was to explore the underlying mechanism and diagnostic potential of Ran-binding protein M (RanBPM) in human spermatogenesis and oogenesis. RanBPM expression in human testis and ovaries was analysed using polymerase chain reaction (PCR) and western blotting, and immunofluorescence was performed on testis and ovary tissue sections during different developmental stages of spermatogenesis and oogenesis using RanBPM antibodies. Interactions with a variety of functional proteins were also investigated. RanBPM mRNA and protein expression levels were determined by PCR and western blotting in the tissue sections. Results revealed that the mRNA expression levels were highest in the testis followed by the ovary. The RanBPM protein was predominantly localized in the nucleus of germ cells, and the expression levels were highest in pachytene spermatocytes and cells surrounding spermatids in testis tissue. In ovary cells, RanBPM was localized in the nucleus and cytoplasm. In conclusion, the results suggested that RanBPM may have multiple roles in the regulation of germ cell proliferation during human spermatogenesis and oogenesis. This research may provide a novel insight into the underlying molecular mechanism of RanBPM and may have implications for the clinical diagnosis and treatment of human infertility.

Introduction

Human infertility is a major worldwide issue affecting 15% of couples of reproductive age. Human infertility is associated with genetic and non-genetic causes. Chromosomal abnormalities and gene mutations are frequently found in infertile men, particularly in those with a low sperm count, and also in women with low quality oocytes (1-3). Gametogenesis is a complex biological process that involves producing cells for sexual reproduction via both meiotic and mitotic cell division steps. The feature of meiosis is the reduction of the DNA content by half through two cell divisions, resulting in germ cells. At the molecular level, spermatogenesis is highly organized and involves the expression and interaction of numerous overexpressed specific genes. Abnormal translocation carriers may result in disorders of gene expression, meiotic arrest, failure of spermatogenesis and infertility (4-6). During this process, proteins and protein interactions may have a universal role in gametogenesis. Scaffold proteins act as important modulators of a variety of physiological functions based on protein interactions. Ran-binding protein M (RanBPM), also termed Ranbp9, is a scaffold protein belonging to the ran-binding proteins (ranBPs) (7). RanBPM is a multimodular protein containing a consensus SPRY domain, a CRA domain, a lissencephaly type-1-like homology (LisH) motif, a C-terminal to LisH (CTLH) domain and a proline-rich SH3-binding module. SPRY domains are protein-protein interaction modules that were initially discovered in the ryanodine receptor (8,9). The CRA motif is also involved in protein-protein interactions (10), whereas the LisH motif is involved in protein dimerization and microtubule binding (11-13). The CTLH domain function is unknown and is frequently found adjacent to the LisH domain in proteins involved in microtubule dynamics, cell migration,

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nucleokinesis and chromosome segregation, and has been previously identified in various proteins associated with RanBPM (14).

RanBPM is component of a large protein complex in which it functions as an adaptor scaffold protein (15-17). The full-length RanBPM cDNA was determined and predicted to encode a protein of 729 amino acids. The human sequence shares 95% sequence identity with the mouse RanBPM sequence. RanBPM was identified and mapped to human chromosome 6. It is a member of the RAS superfamily of proteins. The identification of the different domains of RanBPM may provide important clues in understanding the underlying molecular mechanisms of this protein in human infertility. Several previous studies have also suggested that RanBPM interacts with various proteins and participates in numerous cellular processes including neuronal morphogenesis (18-20), cell growth and cell migration signalling (21-24), regulation of gene transcription (18,25), apoptosis and apoptotic pathways (21,26,27). RanBPM-deficient mice study revealed a role for the protein in gametogenesis, and RanBPM is essential for normal gonad development, as both male and female RanBPM$^{-/-}$ mice are sterile (7). However, additional defects resulting from RanBPM deficiency remain to be investigated. Although RanBPM has a role in mouse gametogenesis, the exact expression pattern, cellular localization and physiological function in human testis and ovaries remain unclear.

In the present study, it was demonstrated that RanBPM was highly expressed in human testis and ovary tissue, and differentially expressed during spermatogenesis and oogenesis. In addition, the RanBPM protein was localized in both the nucleus and cytoplasm of germ cells, especially primary spermatocytes and follicle cells. RanBPM was found to interact with a variety of functional proteins in germ cells, suggesting multiple roles in the regulation of germ cell proliferation during human spermatogenesis and oogenesis. These results provide additional support to expand the understanding of infertility in men, and future studies on spermatogenesis in individuals that are RanBPM mutation carriers may improve the understanding of the underlying mechanisms of fertility.

Materials and methods

Tissue collection and ethics statement. Testicular and ovary tissue samples were obtained from men with obstructive azoospermia and infertile women, at the Peking University Third Hospital. The muscle, heart and kidney tissues were also obtained from men (aged 25-38 years) at the Peking University Third Hospital. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals issued by the Peking University Third Hospital in Beijing. All participants gave their written informed consent, and the study was approved by the Ethics Commission of the Medical Faculty of the Peking University Third Hospital. The protocol was approved by the Institutional Care and Use Committee of the Peking University Third Hospital (protocol no. 2013SZ021). From August to September 2013, 15 patients (aged 25 weeks of fetal-38 years old) and 9 patients (aged 25 weeks of fetal-38 years old) agreed to further tests. The participants or close relatives signed consent forms permitting the collection and use of all samples

used in this study. Chemicals were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

RNA isolation and semi-quantitative polymerase chain reaction (PCR). Total RNA was extracted from human testicular tissue and ovaries using an RNeasy Mini kit (QiagenGmbH, Hilden, Germany) and treated with DNase I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol (28-30). First-strand cDNA was generated by reverse transcription using the Superscript III first-strand synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) and random hexamer oligonucleotides. Primers were designed based on sequences in the human genome database. Subsequent PCR analysis was performed with ExTaqHot-Start DNA polymerase (cat. no. RR006A; Takara Biotechnology Co., Ltd., Dalian, China). All experiments were performed semi-quantitatively at three different escalation cycles, and representative images are shown in the results. For PCR, reaction mixture was first heated at 94˚C for 2 min. 24 cycles were then carried out with the following parameters: Denaturing at 94˚C for 30 sec, annealing at 58˚C for 30 sec, extension at 72˚C for 45 sec. Reaction was finished with a final extension at 72˚C for 5 min.

The primer pairs: Forward, 5'-AAGGTCGACACATGA ATAGACTACCAGGTTGG-3' and reverse, 5'-CGCAAGCTT TTCAAATCAGCAGAGCTAGTC-3', were used to detect the mRNA expression levels of RanBPM. The products of PCR were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Relative amounts of cDNA were normalized against β-actin (forward, 5'-GATGGTGGGAAT GGGTCAGAA-3' and reverse, 5'-TGGCGTGAGGGAGAG CATAGC-3').

Protein extract preparation and western blotting. Ovary and testicular tissue samples were homogenised and lysed in radioimmunoprecipitation assay lysis buffer [50 mMTris-HCl (pH 7.5), 150 mMNaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitors], as previously described (31). Human tissues were homogenized in lysis buffer containing 20 mMTris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA (pH 8.0) and 1% Nonidet-P40 (NP-40). After centrifuged at 12,000 x g for 30 min at 4˚C, supernatants were diluted with sample buffer A from NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) containing 0.5% NP-40, 1 mM DTT and 1X protease inhibitor (PI) and boiled. A total of 10 μg protein was separated by 7.5% SDS-PAGE and transferred to a nitrocellulose (cat. no. 10401196; Sigma-Aldrich; Merck KGaA) or polyvinylidene difluoride (cat. no. RPN303F; GE Healthcare, Chicago, IL, USA) membranes. Membranes were blocked at room temperature with 5% (w/v) skimmed milk for 30 min. After 5 washes with 0.1% PBS with Tween-20, membranes were incubated with primary antibodies at room temperature for 1 h. Rabbit anti-RanBPM (1:2,000; cat. no. ab64275; Abcam, Cambridge, UK) was used as primary antibody, after 3 washes with 0.1% PBST, horseradish peroxidase-conjugated (1:2,000; cat. no. N100; Thermo Fisher Scientific, Inc) was incubated for 1 h at room temperature. β-actin antibody (1:5,000; cat. no. ab8226; Abcam) was used as a loading control. Protein

expression levels were analysed using Image-Pro plus software version 5.1 (Media Cybernetics, Inc., Rockville, MD, USA) and ECL reagent (cat. no. RPN2209; GE Healthcare).

Generation of antibodies and immunofluorescence. Immunohistochemistry was performed using standard procedures, as previously described (32,33). Bouin's fixed human testicular tissue and ovaries were dehydrated and embedded in paraffin at room temperature. Human testis and ovary tissue were fixed immediately in 4% paraformaldehyde in PBS for 15 min at room temperature. Then, 8 μm sections of testicular tissue and ovaries were incubated in citrate buffer for 30 min at 99˚C for antigen retrieval and incubated in 1% H_2O_2 for 10 min at room temperature. Non-specific binding was blocked with 20% normal goat serum in TBS for 30 min to eliminate background. Sections were washed 3 times, 5 min each, in TBS (0.05 M Tris, 0.15 M NaCl, pH 7.6) plus 0.1% Tween-20 (TBST). After deparaffinization and rehydration, the sections were incubated with a rabbit anti-RanBPM antibody (1:50; cat. no. ab64275; Abcam) in TBS plus 5% horse serum or 5% pre-immune rabbit serum (used instead of the primary antibody as a negative control) for 1 h at room temperature. Sections were washed 3 times, 5 min each in TBST between the incubation with antibodies. A secondary antibody (1:300; cat. no. a2556; monoclonal mouse anti-rabbit IgG; Sigma-Aldrich; Merck KGaA) and a third antibody (1:300; cat. no. A0216; HRP-labelledgoat anti-mouse IgG; Beyotime Institute of Biotechnology, Haimen, China) in TBS plus 5% horse serum, was added to the sections for 45 min at room temperature. After washing the section in TBST, the protein of interest was revealed using an VECTASTAINABC kit (cat. no. PK-6101; Vector laboratories, Ltd., Burlingame, CA, USA) for 30 min and diaminobenzidine substrate solution (cat. no. P0018; Beyotime). Sections were examined under a laser confocal microscope (Zeiss GmbH, Jena, Germany).

Results

RanBPM mRNA and protein expression levels in human testes and ovaries. Human RanBPM encodes a protein of 729 amino acids and consists of a CTLH domain, LisH domain, a SPRY domain, a pro-rich domain and a CRA domain (Fig. 1A and B). RanBPM mRNA and protein expression was investigated in various male and female tissues by PCR (Fig. 2A) and western blotting (Fig. 2B). RanBPM mRNA was detected in 5 tissues of adult (Fig. 2A) and 5 patients (aged 25-38 years) of testis were performed (Fig. 2C). The RanBPM protein consists of 729 amino acids and had an apparent molecular mass of 78 kDa based on western blotting. RanBPM mRNA expression was detected in testis tissue (Fig. 2A), and the RanBPM protein displayed a similar expression pattern RanBPM protein expression was not abundant in the testis shortly after birth, but the levels were higher in adult testis (Fig. 2B), suggesting RanBPM may be involved in male germ cell development and proliferation. Furthermore, RanBPM protein was also abundant in adult ovaries (Fig. 2B).

RanBPM is localized to the nucleus and cytoplasm of testis. Immunostaining of RanBPM in human testis tissue sections was performed to determine the distribution in different germ cell stages (Fig. 3). Immunostaining revealed the localization of RanBPM in the nucleus and cytoplasm of germ cells. RanBPM expression was detected in both spermatogonia and sertoli cells that support the spermatogenic process (Fig. 3B).

RanBPM is expressed in human ovaries. Oogenesisis a complex developmental process that involves sophisticated regulation of gene expression in ovaries. To investigate the expression of RanBPM in oogenesis, immunostaining was performed on adult ovary sections (Fig. 4). The RanBPM protein was found to be present in the primordial follicle (Fig. 4A) of ovary sections RanBPM appeared to be localized the nucleus of follicles (Fig. 4B).

Discussion

Gametogenesis is a complex process by which diploid cells differentiate into haploid gametes during sexual reproduction. During this process, a large number of genes and proteins involved in fertility are highly regulated in males and females (31,32). Gametes are generated from diploid germ cells through meiosis, during which homologous chromosomes must align, pair, synapse, recombine and separate to form haploid cells. This complicated process involves a series of highly regulated molecular events that are dependent on complex interactions between a number of distinct genes and proteins (33,34). Gametogenesis is highly organized and involves the expression and interaction of numerous genes (28,31,33); however, mammalian gametogenesis remains to be elucidated at the molecular level. The targeting of candidate genes with proposed roles in gametogenesis has provided valuable information (29,32). For example, many genes involved in spermatogenesis in mice are X-linked (31,33,34) and are expressed exclusively in males.

A malfunction in the expression of genes such as RanBPM may result in spermatogenic disorders, although the diagnostic potential of this gene and its underlying mechanism of action in human gametogenesis and reproduction are poorly understood. In the present study, RanBPM was demonstrated to be abundantly expressed in human tissues (such as kidney, heart and muscle), especially in the nuclei of testes and ovaries. Although RanBPM was generally localized within nuclei, its specific localization in the cytoplasm of round spermatids and sertoli cell suggested a possible involvement in gametogenesis in male patients. In addition, the RanBPM protein was present in the primordial follicle in ovary sections. PCR also revealed that RanBPM was expressed in follicles in ovary sections. These results suggested that RanBPM may be involved in human spermatogenesis and oogenesis.

RanBPM is a scaffold protein belonging to the Ran-binding protein family, members of which contain a SPRY domain, CRA domain, LisH motif and CTLH domain. Scaffold proteins act as important modulators of a variety of protein-mediated physiological functions. RanBPM is involved in the regulation of the immune system (35). RanBPM is associated with cell cycle proteins and regulates the progression of the cell cycle. Additionally, RanBPM may inhibit protein ubiquitination and acts as a tumour suppressor. For example, it interacts with the Met tyrosine kinase receptor and in doing so facilitates activation of the RAS-extracellular regulated kinase pathway (36).
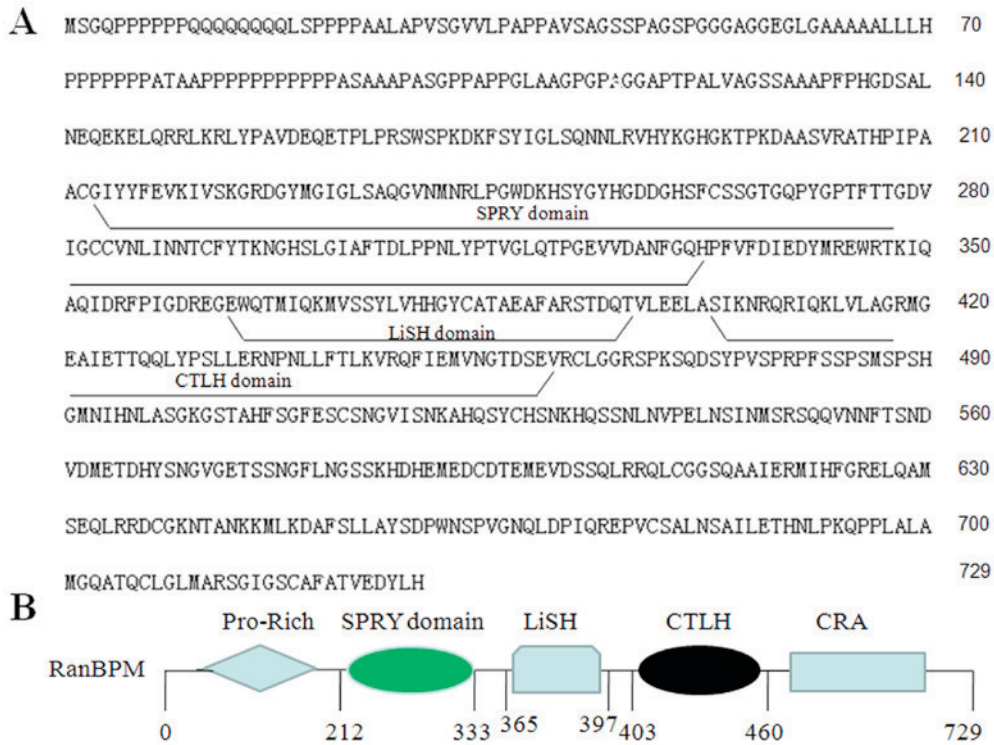
Figure 1. Protein sequence of RanBPM and its structural domains. (A) RanBPM cDNA encodes 729 amino acids. (B) RanBPM is a member of the RAS superfamily and includes a CTLH domain, LisH domain, SPRY domain, pro-rich domain and a CRA domain. RanBPM, Ran-binding protein; LisH, lissencephaly type-1-like homology.
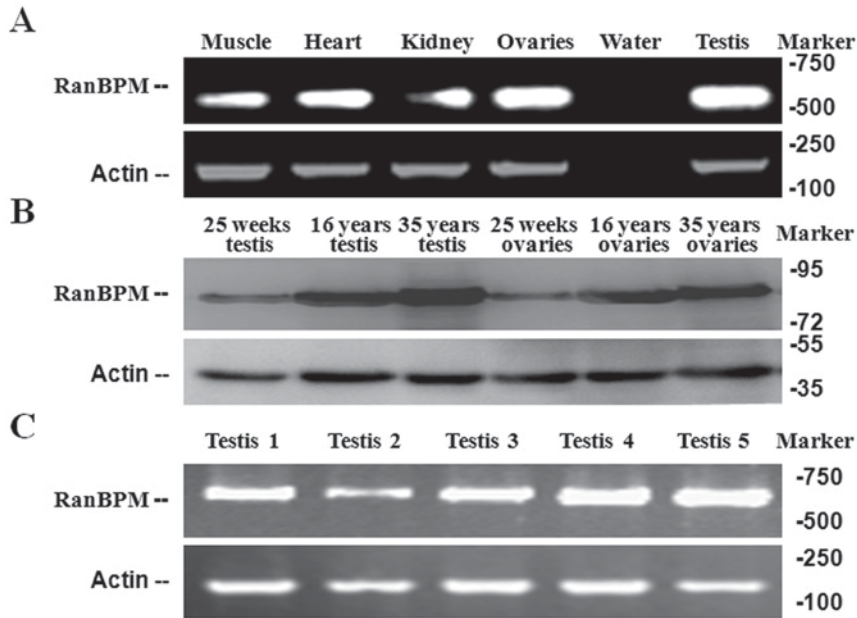

Figure 2. Human RanBPM was determined by PCR and western blotting. (A) PCR was performed on human tissues. RanBPM mRNA was expressed in the testis, ovary, kidney, heart and muscle tissues. (B) RanBPM was expressed in testis and ovary tissues (aged 25 weeks of fetal, 16 and 35 years of men) using western blotting. (C) RanBPM mRNA was different expression in 5 adult testis (aged 25-38 years). PCR, polymerase chain reaction; RanBPM, Ran-binding protein.

In addition, RanBPM has been associated with citron kinase, possibly affecting the rate of mitosis during the production of pyramidal neurons (19). It also binds to the kelch repeat protein muscle in and affects cell morphology (23). In the immune system, it interacts with the 2-integrin lymphocyte function-associated antigen-1 (35), the neural cell adhesion molecule L1, and the neurotrophic receptor, tropomyosin receptor kinase B (37,38). Human sperm membrane protein-1 and RanBPM complex localize to the microtubule-organizing centre and interact with tubulins, and thereby may modulate microtubule assembly and/or activity (39). RanBPM has been demonstrated to interact with >50 different proteins, consistent with a number of important roles in multiple human tissues.
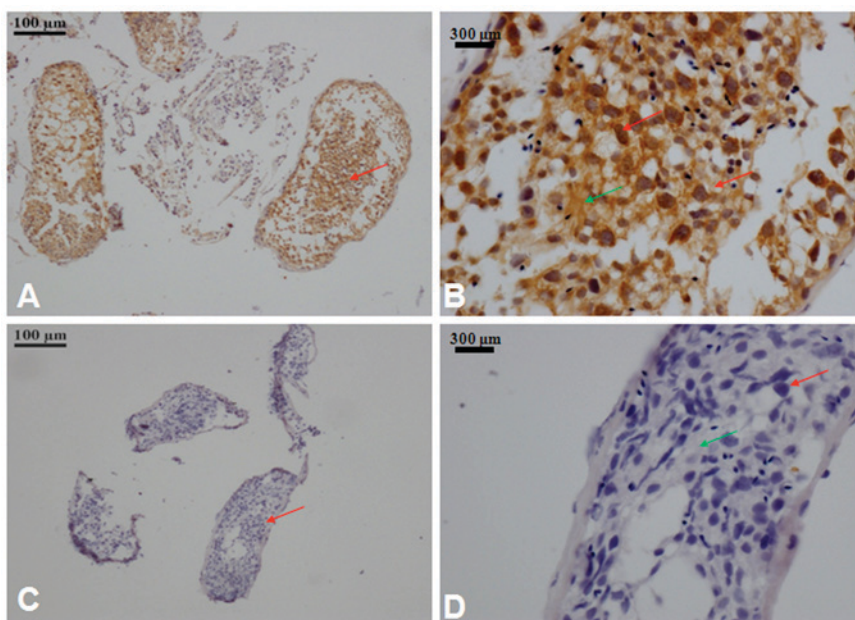
Figure 3. RanBPM expression was determined by immunostaining of testis sections. (A) The RanBPM was localized to the nucleus (red arrow shows) in human testis. (B) It was localized to the cytoplasm of testis cells (green arrow shows) too. RanBPM was present in both the nucleus and cytoplasm of testis cells, also expressed in the nucleus of primary spermatocytes, and is diffusely dispersed in the cytoplasm of round spermatids in testis of men aged 25-38 years. (C and D) Control testis tissues in men aged 25-38 years. RanBPM, Ran-binding protein.
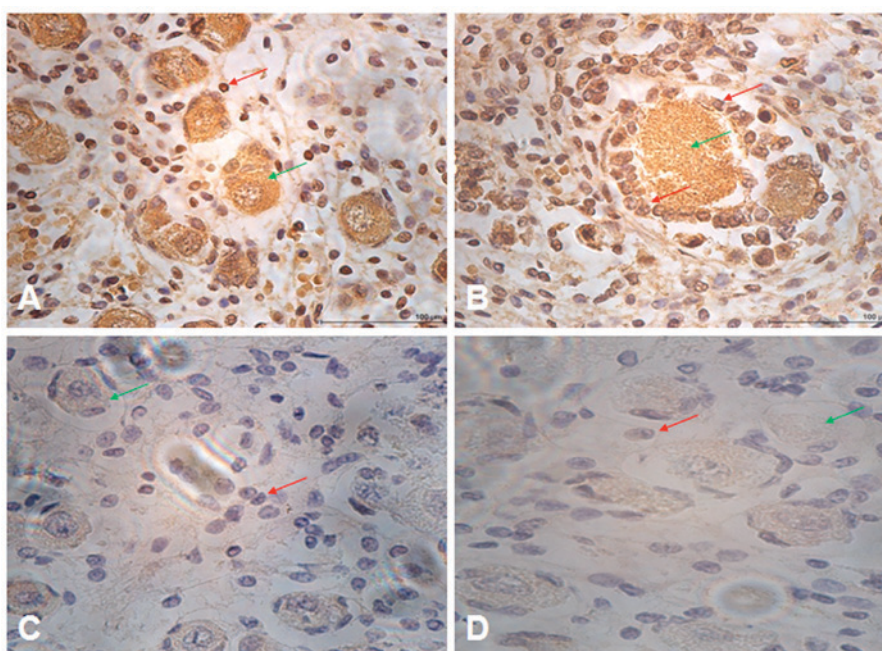

Figure 4. Localization of RanBPM protein was detected in the developing female ovary. (A) The red arrow shows nuclear RanBPM expression and the green arrow shows cytoplasmic RanBPM expression. RanBPM was present in the nucleus and cytoplasm of ovary cells in women aged 15-28 years. (B) RanBPM was also evident in the nucleus of follicle cells, and was diffusely dispersed in the cytoplasm of granule cells. (C and D) Control ovary tissues in females aged 25-38 years. RanBPM, Ran-binding protein.

RanBPM is a scaffold protein due to its ability to associate with a wide range of proteins (37). In the present study, RanBPM was localized to the cytoplasm of round spermatids and the nuclei of sertoli cells. Therefore, it appears to be a nuclear protein in spermatocytes, but a cytoplasmic protein in round spermatids, suggesting a role in spermatid cell proliferation. In conclusion, the present study examined the expression and localization of RanBPM in human testis and ovary tissues. The results may suggest that RanBPM has an involvement in spermatogenesis and oogenesis. RanBPM may be a gametogenesis-associated protein that is abundantly expressed in the testis and ovary. Given its unique expression pattern and localization, RanBPM may have multiple roles in the regulation of gametogenesis. These results also expand on understanding of the underlying mechanism of spermatogenesis and oogenesis, and may aid development of treatments for gene expression-based infertility disorders in men.

Acknowledgements

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