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·*Original Article*

Immunolocalization assessment of metastasis-associated protein 1 in human and mouse mature testes and its association with spermatogenesis

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Abstract

Aim: To investigate the stage-specific localization of metastasis-associated protein 1 (MTA1) during spermatogenesis in adult human and mouse testis. **Methods:** The immunolocalization of MTA1 was studied by immunohistochemistry and Western blot analysis. The distribution pattern of MTA1 in mouse testis was confirmed by using quantitative analysis of purified spermatogenic cells. **Results:** The specificity of polyclonal antibody was confirmed by Western blot analysis. MTA1 was found expressed in the nucleus of germ cells, except elongate spermatids, and in the cytoplasm of Sertoli cells; Leydig cells did not show any specific reactivity. MTA1 possessed different distribution patterns in the two species: in humans, the most intensive staining was found in the nucleus of round spermatids and of primary spermatocytes while in mice, the most intense MTA1 staining was in the nucleus of leptotene, zygotene and pachytene spermatocytes. In both species the staining exhibited a cyclic pattern. **Conclusion:** The present communication initially provides new evidence for the potential role of MTA1 in mature testis. In addition, its distinctive expression in germ cells suggests a regulatory role of the peptide during spermatogenesis. *(Asian J Androl 2007 May; 9: 345–352)*

Keywords: human metastasis-associated protein1; mouse metastasis-associated protein 1; spermatogenesis; nuclear remodeling and deacetylation complex; chromatin remodeling; spermiogenesis; deacetylation

1 Introduction

Metastasis-associated protein1 (*MTA1*) is representative of a family of genes that are highly conserved through evolution, and its high expression has previously been found to be associated with progression to the metastatic state in various cancers, such as breast, esophageal, colorectal, gastric and prostate carcinomas [1]. The full-length human *MTA1* cDNA sequence contains an open reading frame encoding a protein of 714 amino acid residues. The MTA1 protein contains several possible phosphorylation sites. A proline-rich amino acid stretch at the carboxyl-terminal completely matched the consensus sequence for the *src* homology 3 domain-binding motif [2]. As a part of the nuclear remodeling and

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deacetylation (NuRD) complex, it is thought to modulate transcription by influencing the status of chromatin remodeling [3]. However, MTA1 expression is not restricted to tumors [4]; several normal mouse tissues and organs also express low levels of MTA1 [5]. Interestingly, the expression of MTA1 in mouse testis is dominantly higher than that in any other normal organs [5], suggesting its potential function during mouse spermatogenesis. Therefore, the possibility of additional, yet unknown, physiological effects of this newly cloned peptide in reproduction could not be ruled out.

The aim of the present study is to reveal the presence and cellular localization of MTA1 in mammalian testis. We investigated the stage-specific localization of MTA1 during spermatogenesis in adult human and mouse testes by immunohistochemical staining method employing specific polyclonal antibodies. Furthermore, quantitative analysis was done on purified germ-cell types to confirm the cellular expression of MTA1 during mouse spermatogenesis. As there is no published evidence of *in vivo* function of MTA1 in spermatogenesis, this report might be informative for further investigations into the physiological function of MTA1 in this field.

2 Materials and methods

2.1 Tissue collection and preparation

Fourteen testes were collected from autopsy cases following accidental deaths (23–45 years old). To ensure the usability of the testes, normal tissue and spermatogenesis were identified in accordance with the criteria by Suarez-Quian *et al*. [6] and only eight testes, taken from six traffic accidents victims aged 25 to 40 years old, were selected for analysis. Some testes were frozen in liquid nitrogen. The others were immediately fixed in Bouin's acid after being taken out of the body and then processed for routine paraffin embedding. The use of the human tissue in this study was approved by the Human Research Committee of the Fourth Military Medical University for Approval of Research Involving Human Subjects. The human tissue was supplied by Department of Urology of Xijing Hospital in Xi'an, China.

Twenty-five 8-week-old outbred male BALB/c mice were obtained from the Laboratory Animal Center of the Fourth Military Medical University, and were fed with standard food pellets and given water *ad libitum* throughout the experimental period. They were housed at 22ºC and a light:dark cycle of 12:12 h, five in a cage, with standard food and water provided *ad libitum*. All selected animals were housed under these conditions for less than 1 week before the experiment. The preparation was performed according to animal protocols institutionally approved by the Fourth Military Medical University. The preparation of mouse testes was performed as discussed above.

2.2 Antibody

MTA1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was raised against peptides mapping the carboxy terminus of the precursor forms of human MTA1, was recommended for the detection of precursor and mature MTA1 in mice, humans and rats.

2.3 Western blot analysis

Specificity of anti-human MTA1 goat polyclonal antibody was tested by Western blot analysis carried out by using rat mammary adenocarcinoma cell line MTLn3, normal mouse testes and specimens of human testes. Cells were lysed in phosphate-buffered saline containing 0.5% Nonidet P-40, 100 μg/mL phenylmethylsulfonyl fluoride and 2 μg/mL aprotinin. Tissues were homogenized in ice-cold modified radioimmunoprecipitation buffer (RIPA) buffer (Tris-HCl 50 mmol/L, NaCl 150 mmol/L, Triton X-100 1% [v/v], sodium deoxycholate 1% [wt/v], and SDS 0.1% [wt/v] pH 7.5) supplemented with complete proteinase-inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany). After centrifugation at 15 000 × *g* for 15 min at 4ºC, the supernatant was assayed for total protein concentration using the Bradford assay. Samples were homogenized in a ten-fold volume of boiled 25 mmol/L Tris-buffered saline (TBS, pH 7.4) containing 1% SDS using a glass homogenizer, and were then heated for 10 min at 90–95ºC to inactivate intrinsic proteases. After being cooled on ice, samples were re-homogenized with a polytron mixer, and were then centrifuged at 15 000 × *g* for 30 min. Routinely prepared samples (30 μg of proteins) were separated by modified 10% SDS-PAGE, and were then transferred to NC membranes. After blocking with 0.25% casein/PBS, membranes were incubated with primary anti-MTA1 antibody diluted (1:500) in blocking solution overnight at 4ºC. After washing with PBS containing 0.05% Tween-20 (PBST), the membranes were incubated with peroxidase conjugated rabbit anti-goat IgG diluted (1:1 000) in blocking solution for 60 min. The bands were finally detected by using an ECL kit (Amersham Biosciences, Buckinghamshire, UK) according to the

manufacturer's instructions.

2.4 Immunohistochemistry

For immunohistochemical staining, paraffin-embedded tissues were sectioned to 4 μm. The avidin–biotin–peroxidase (ABC) method was employed in the immunohistochemical assay on serial slides as previously described [7]. After endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 30 min, slides were incubated with the anti-MTA1 goat antibody (1: 150 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted in PBS at 4ºC overnight in a moist box. Biotinylated rabbit anti-goat IgG (1:500 dilution; Sigma, St. Louis, MO, USA) was incubated for 1 h at room temperature and detected with streptavidin–peroxidase complex. Peroxidases were detected with 0.7 mg/mL 3-3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) in 1.6 mg/mL urea hydrogen peroxide, 60 mmol/L Tris buffer, pH 7.6, room temperature as the chromogen and the sections were briefly counter-stained with hematoxylin. Negative control slides were incubated with PBS substituted for the primary antibody. Immunohistochemical reactions for all samples were repeated for at least five times, and typical results were illustrated.

2.5 Analysis of seminiferous epithelium stages of human and of mouse testis

We employed the criterion by Russell *et al.* [8] to identify the germ cell types, and the criteria by Zhang *et al.* [9] and Kurth *et al.* [10] to define the stages of seminiferous epithelium cycle in the human testis. In humans, typical germ-cell associations of fixed composition were identified with the nuclear morphology of the germ cell and the topographical arrangement of spermatids as the principal criteria. Briefly, the human seminiferous epithelium cycles were divided into six stages: stage I is characterized by the presence of two generations of spermatids and pachytene spermatocytes; stage II by the appearance that more mature spermatids (elongated spermatids) were in the process of release into the lumen moving to the luminal aspect of the seminiferous epithelium; stage III by only one generation of round spermatids; stage IV by spermatids with nuclei showing initial signs of elongation; stage V by one generation of elongating spermatids having typically pointed and deeply stained nuclei directed toward the limiting membrane; and stage VI by primary and secondary spermatocytes undergoing the first and second maturation divisions. The stages of seminiferous epithelium cycle in the mouse testis were defined following the criterion by Russell *et al.* (as shown in Figure 5) [8].

2.6 Isolation of mouse seminiferous epithelium and spermatogenic cells

Seminiferous cords and tubules were prepared from mice (The Fourth Military Medical University, Animal Research Center, Xi'an, China) by collagenase treatment. Monodispersed suspensions of spermatogenic cells were prepared from the seminiferous tubes with collagenase treatment and trypsin digestion. Type A and B spermatogonia were isolated from the testes of 8-day prepubertal mice (30 animals). Preleptotene and leptotene/zygotene spermatocytes were isolated from the testes of 17-day-old mice (30 animals). Pachytene spermatocytes and round spermatids (steps 1-8) were isolated from testes of > 60-day-old mice (20 animals). Each testis was decapsulated by making a small incision in the testis and forcing (by sterile tweezers) the content of the testis through the incision into a 15-mL Falcon (CytRx Corporation, Los Angeles, CA , USA) tube containing 5 mL ice-cold separation medium. Then, 0.25 mL collagenase (Calbiochem-Behring, La Jolla, CA, USA) from a 2 mg/mL stock solution (prepared in separation medium) was added to the tube with the decapsulated testes, and incubation was carried out for 5 min at 35–37ºC under vigorous shaking. The seminiferous cords were then allowed to sediment to the bottom of the tube while being incubated on ice. The seminiferous cords were washed twice in 10 mL separation medium, resuspended in 12 mL separation medium containing 2.5 mg/mL trypsin and 1 U/mL DNase I (Boehringer Mannheim, Mannheim, Germany), incubated for 2 min at 35–37ºC, and transferred to ice. Using a Pasteur pipette, the seminiferous cords were disintegrated into single cells and were then filtered through a 50-mm nylon mesh, washed twice with separation medium (centrifugation at 200–300 × *g*), and counted. The germ cells were separated by velocity sedimentation at unit gravity on 2%–4% BSA gradients [11]. Populations of the spermatogenic cell types were > 80% pure based on examination of cell morphology under phase contrast optics. Animals were killed by cervical dislocation. Approval for these studies was received from Institutional animal Care and Use Committee of The Fourth Military Medical University.

2.7 Quantitative analysis of MTA1 expression of differ-

ent spermatogenic cells

Total RNA was extracted from isolated germ cells, using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized with Superscript III (Rnase H-Reverse Transcriptase; Invitrogen, Carlsbad, California, USA) primed from oligo(dT), exactly according to the manufacturer's instructions and PCR was set up according to Promega's reverse transcription system protocol. Primer sequences were designed as 5'-AGC CCA ACC CAA ACC AG-3' and 5'-GGC AAT GCG TGT CAA CT-3' (GenBank accession number AF288137), 5'-GCC TCA AGA TCA GCA AT-3' and 5'-AGG TCC ACC ACT GAC ACG TT-3' (GAPDH) (GenBank accession number NM 001001303). A single denaturing step at 94ºC for 300 s was followed by the chosen number of cycles: 94ºC for 30 s; 58ºC for 30 s; 72ºC for 30 s. The PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The anticipated sizes of the amplified fragments were 482 base pairs (bp) for MTA1 and 452 bp for GAPDH. All PCR reactions for all samples were repeated for at least three times, typical results are illustrated. Results were normalized by the ratio of band density of MTA1 mRNA to GAPDH mRNA. Statistical analysis was performed with the paired *t*-test.

Total protein extracts of different germ cells were prepared and Western blotting was performed as described previously. Densitometry was performed using a computerized densitometer and proteins were quantitated from the images using Sigma gel software (Sigma, St. Louis, MO, USA).

2.8 Statistical analysis

Experiments were repeated for at least five times, and one representative from at least three similar results is presented. All data were presented as mean ± SD. Data were compared by analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Specificity of anti-human MTA1 goat polyclonal antibody

Western blot analysis revealed a single band of 80 kDa in rat mammary adenocarcinoma cell line MTLn3, and in normal mouse and human testes (Figure 1). The antiserum did not cross-react with any other bands.

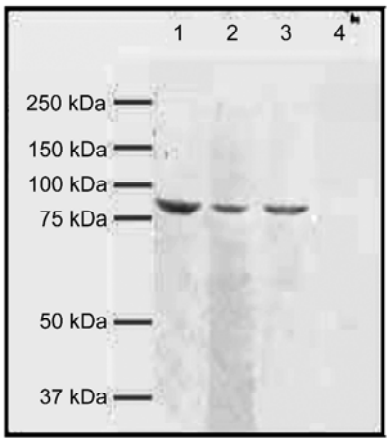

Figure 1. Western blot profile. Specimen from rat mammary adenocarcinoma cell line MTLn3 (lane 1), normal human testis (lane 2), mouse testis (lane 3) and mouse testis exposed to the secondary antibody alone (lane 4). The position of the molecular weight markers (10^{-3}) is indicated.

3.2 Immunolocalization of MTA1 in human testis

By immunohistochemical analysis, a widely distributed staining for MTA1 in human and mouse testes was observed. In particular, it was found that the staining for human MTA1 protein was stronger in germ cells except elongate spermatids, and was not detectable in the testicular interstitium. Weak staining was observed in the nuclei of spermatogonia and in the cytoplasm of Sertoli cells. The most intensive staining was shown in the nucleus of round spermatids and of primary spermatocytes (Figure 2). In particular, the staining for MTA1 protein was consistent with the appearance of round spermatids and primary spermatocytes. MTA1 first appeared and reached the highest intensity at stages I–II, declining to weak at stage III. When the spermatids began to elongate at stage IV, MTA1 appeared in spermatocytes and reached the highest intensity in the spermatocytes nuclei at stage V, with weak staining at stage VI (Figure 3).

3.3 Immunolocalization of MTA1 in mouse testis

In mouse testis, MTA1 first appeared in the nucleus of spermatogonia at stage I, with increased staining in leptotene and zygotene spermatocytes at stages II–VIII. We observed the most intense MTA1 staining in the nucleus of leptotene, zygotene and pachytene spermatocytes at stages IX and X. Staining was declining at stages

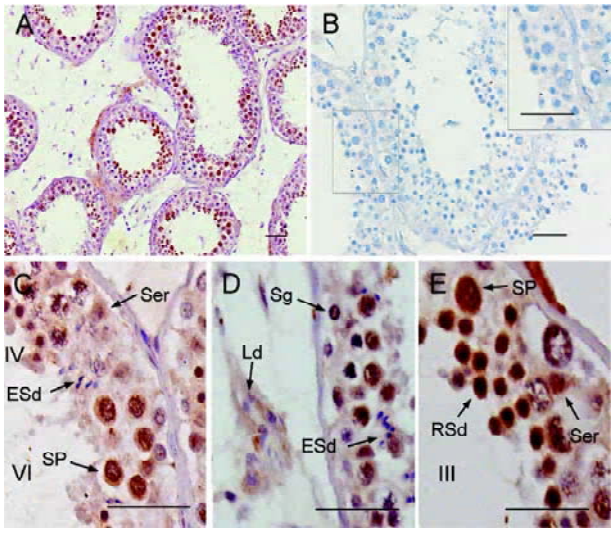

Figure 2. Immunohistochemical assay to show the distribution of MTA1 protein in normal adult human testis. To identify specific cell types, all sections were briefly counter-stained with hematoxylin. (A): Staining for MTA1 protein was strong in round spermatids and primary spermatocytes, weak in spermatogonia and Sertoli cells and not detectable in Leydig cells. Original magnification ×100. (B): Negative control incubated with a substituting PBS instead of primary antibody. Low-magnification (× 200) and high-magnification (× 400) (C), (D) and (E) High-magnification (× 400) views were provided to highlight the positive germ cells. Arrows identify specific germ cell types. Sg, spermatogonia; SP, spermatocytes; RSd, round spermatids; ESd, elongated spermatids; Ser, Sertoli cell; Ld, Leydig cell. Studies in (A), (C), (D) and (E) were representative of five independent experiments, and studies in C, D and E were representative of ten histological sections used for each of the antibody and negative controls. Scale bar = 50 μm.

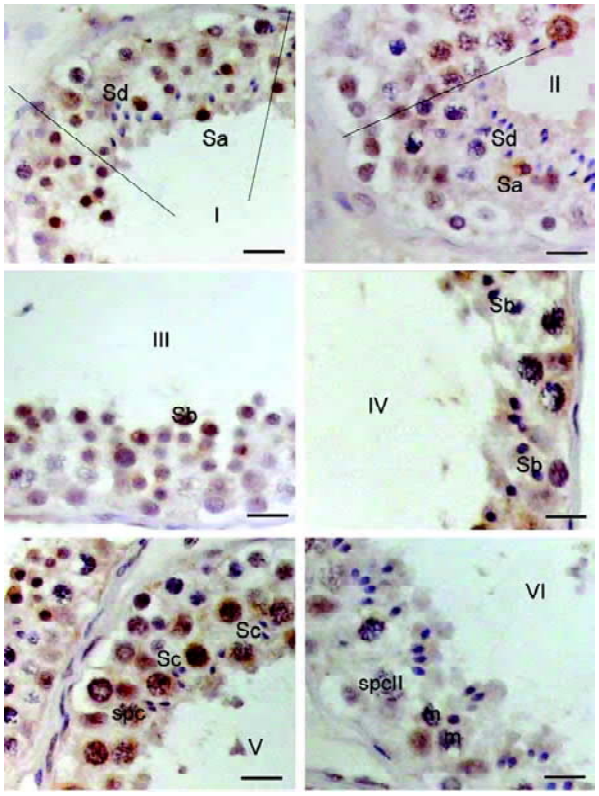

Figure 3. A careful examination of the staining pattern revealed a stage-specific pattern of MTA1 expression in adult human testes. The staining for MTA1 protein was consistent with the appearance of round spermatids and primary spermatocytes. MTA1 first appeared in the nucleus of round spermatids and reached the highest intensity at stages I–II, declining to weak at stage III. When the spermatids began to elongate at stage IV, MTA1 appeared in spermatocytes and reached the highest intensity in the spermatocytes nuclei at stage V, weak staining at stage VI. There was no distinct positive staining in elongate spermatids and interstitium. Sa, very early round spermatids; Sd, mature elongated spermatids; Sb, spermatids entering the beginning of the cap-phase steps in development; Sc, elongating spermatids; spc, primary spermatocytes and m, meiotic figures. Original magnification × 400. Scale bar = 25 μm.

XI and XII. The positive staining could be found in spermatogonia at all the stages (Figure 4). Some round spermatids and Sertoli cells (cytoplasm) were stained but the positive reaction was weak. In contrast, elongate spermatids or Leydig cells, did not exhibit any MTA1-specific immunostaining (Figure 5). Negative control showed no reactivity.

3.4 MTA1 expression of purified germ cells

We conducted the expression analysis against the extract of testicular cells from 8-day-old, 17-day-old and > 60-day-old mice. These ages correspond to the onset of different germ cells during testis development [11]. Quantitative analysis revealed that mouse MTA1 was most highly expressed in leptotene, zygotene and pachytene spermatocytes (Figure 6).

4 Discussion

In the present study, evidence is provided for the identification of a metastasis associated protein of relative molecular mass 80 kDa in mature testis. The data comprise three main observations. First, the antibody against the denatured MTA1 only detected the 80 kDa protein in rat mammary adenocarcinoma cell line MTLn3, and in normal mouse and human testes. Immunoblotting analysis ensured the specificity of the following immu-

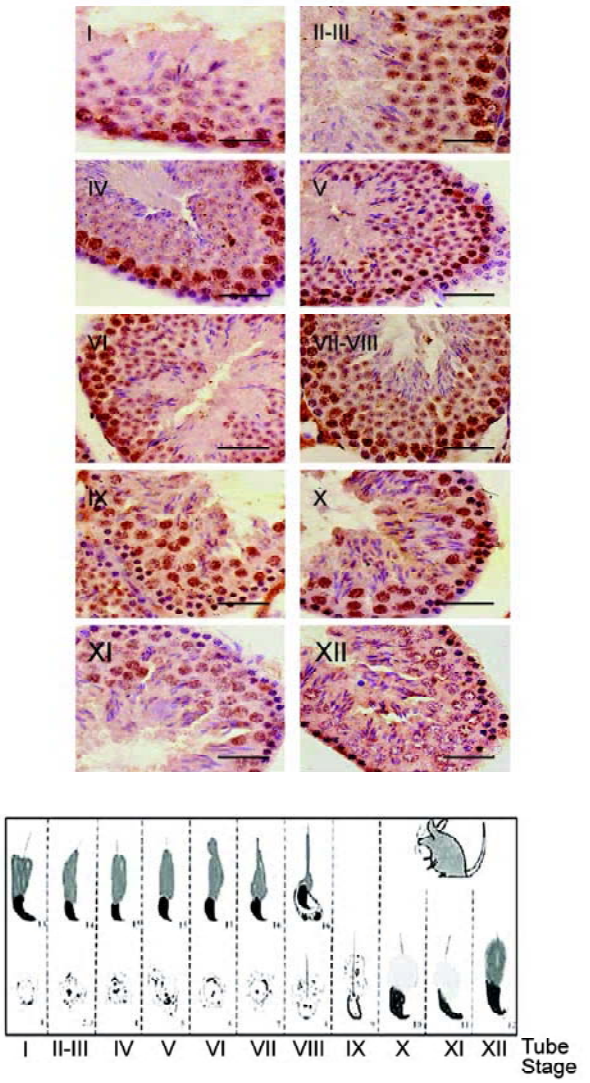

Figure 4. A careful examination of the staining pattern revealed a stage-specific pattern of MTA1 expression in adult mouse testes. MTA1 first appeared in the nucleus of spermatogonia at stage I, and staining was increased in leptotene and zygotene spermatocytes at stage VIII. We observed the most intense MTA1 staining in the nucleus of leptotene, zygotene and pachytene spermatocytes at the stages IX and X. Staining was declining at stages IX and X. The immunoreactant could be found in spermatogonia at all the stages. Some round spermatids and Sertoli cells were stained but the positive reaction was weak. In contrast, elongate spermatids or Leydig cells, did not exhibit any MTA1-specific immunostaining. Original magnification × 40. Scale bar = 25 μm. The original graph was adopted from Russel *et al.* [8].

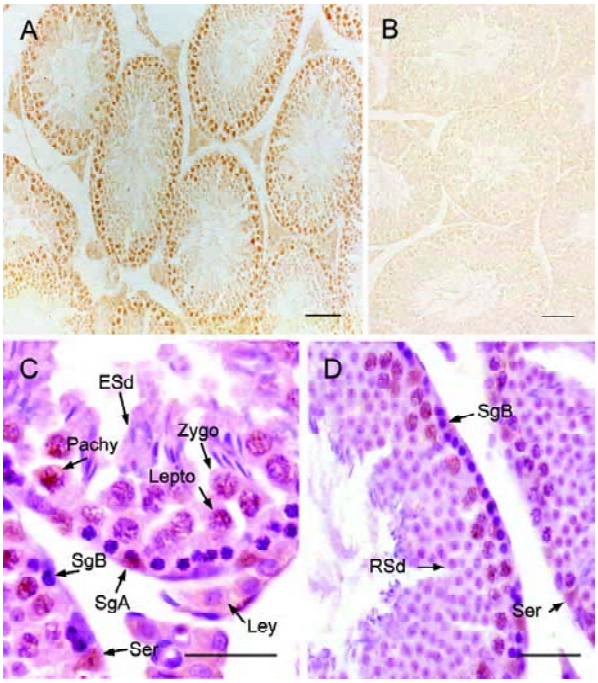

Figure 5. Immunohistochemical assay to show the distribution of MTA1 protein in normal adult mouse testis. (A): A low-magnification (× 200) view was shown. Staining for MTA1 protein was strong in primary spermatocytes, weak in spermatogonia, Sertoli cells and round spermatids, and not detectable in Leydig cells. (B): Negative control incubated with a substituting PBS instead of primary antibody. Original magnification × 200. (C) and (D): High-magnification (× 400) views were provided to highlight the positive germ cells. Arrows identify specific germ cell types. SgB, type B spermatogonia; SgA, type A spermatogonia; RSd, round spermatids; Ser, Sertoli cell; Pachy, pachtene spermatocyte; Lepto, leptotene spermatocyte; Zygo, zygotene spermatocyte. Specific staining for MTA1 protein was not detectable in Leydig cells. Scale bar = 50 μm.

nohistochemical results. Second, immunolocalizational analysis on the adult testes demonstrated distinctive expression patterns of MTA1. In human, the most intense staining was found in round spermatids and primary spermatocytes, while in mice, it was found in leptotene, zygotene and pachytene spermatocytes and spermatogonia, suggesting its regulatory role during both post-meiosis and meiosis phases. The distribution pattern in mouse testis was confirmed by the quantitative analysis on purified germ cells. MTA1 possessed different expression patterns in human and mouse testes, suggesting its species-specific distribution during spermatogenesis. Moreover, MTA1 expression in both species exhibited a seminiferous cycle, indicating that its distribution may be stage-specific. The physiological significance of these observations warrants further investigation.

The transcription regulation during spermatogenesis

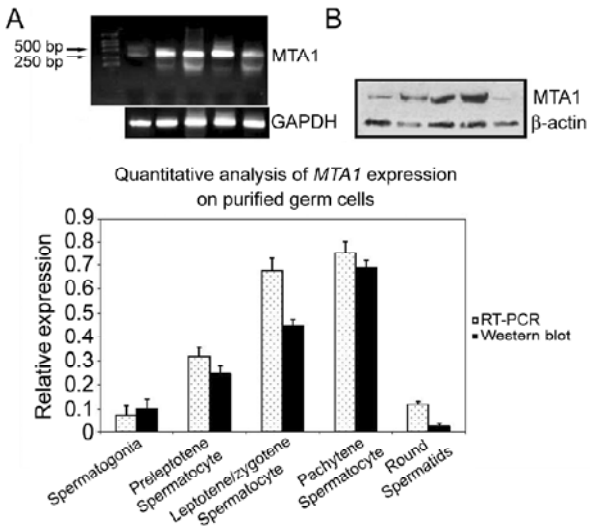

Figure 6. Analysis of MTA1 expression of extract against differentiating mouse germ cells by semi-quantitative RT-PCR (A) and Western blot analysis (B). The products of a representative semi-quantitative RT-PCR were subjected to electrophoresis on a 1.5% agarose gel. Forty micrograms of total protein of each sample were resolved on a 15% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with goat anti-MTA1. In RT-PCR, the density of MTA1 was normalized by that of GAPDH; in Western blot, the density of MTA1 was normalized by that of β-actin. The relative amount of MTA1 was reported as mean ± SD according to the results from three independent experiments. Comparison of the relative densities between groups was performed by ANOVA and $P < 0.05$ was considered significant. The results showed that mouse MTA1 is most highly expressed in leptotene, zygotene and pachytene spermatocytes.

is complex and unique, and several mechanisms have been demonstrated to be involved in this process. One major strategy contributing to local and specific transcription has so far been identified. Acetylation of histone proteins opens up the chromatin structure and leads to transcriptional activation; conversely, deacetylation of histone proteins condenses the chromatin structure and is associated with transcriptional repression [12]. The MTA1 complex contains histone deacetylases (HDAC1/2) and can be further separated, resulting in a core MTA1-HDAC complex, showing that the histone deacetylase activity and transcriptional repression activity are integral properties of the MTA1 complex [13]. Thus, our results raised the possibility that MTA1 might contribute to the deacetylation of histones during male gametogenesis.

The waves of histone acetylation occurring throughout spermatogenesis have been studied thoroughly in mice. Hazzouri *et al.* [12] reported that no acetylated histones were observed throughout meiosis in leptotene or pachytene spermatocytes. Histones remained unacetylated in most round spermatids. Acetylated forms of H2A and H2B, H3 and H4 reappeared in step 9 to 11 elongating spermatids, and disappeared later in condensing spermatids. Our results are consistent with this conclusion. MTA1 was not expressed in the most condensed germ cells, namely, elongated spermatids and spermatozoa in the testis of both humans and mice, suggesting that acetylated core histones may be replaced by transition proteins without being previously deacetylated.

Interestingly, MTA1 expression patterns were different in developing germ cells among the two species, implying that the possible function of MTA1 was different in human and mouse testes. When a mammalian cell divides it must rapidly synthesize large amounts of histone variants during the brief S-phase, increasing 35-fold as cells enter S-phase and decreasing again at the end of S-phase. The unique properties of the histone variants are critical for this regulation [13]. Therefore we believe that this species differences do exist and they might be the results of the different combination of histone variants. Indeed, disparate data from the published literature suggest that the massive incorporation of histone variants is associated with the induction of different types of histone modifications [14]. In mouse, there are two waves of histone synthesis during the meiotic prophase of germ cells; first during pre-leptotene and then during pachytene. The tH2A, tH2B and ssH2B could start to accumulate during these courses. Macro H2A1.2 is also found at high concentrations in mice testis. While in humans, tH2B first appears in spermatogonia, is maximal in round spermatids, and then gradually disappears during the elongation of spermatids [15]. These observations may account for the species differences of histone variants and succedent deacetylating modifications in two species.

Furthermore, in addition to hormonal control by gonadotrophins and testosterone, a number of locally produced growth factors (such as EGFs and TGFs) and other paracrine mediators have been suggested to play an important role in the regulation of testicular function [16]. The immunostaining of EGF in germ cells has been observed in pachytene spermatocytes, round spermatids and Sertoli cells [17]. *In vitro*, growth factor stimulation could induce the overexpression of MTA1 and lead to the enhanced anchorage-independent growth

and hormone independence of certain malignant cells [18]. Mouse histone deacetylase 1 was also identified as a growth factor-inducible gene [19]. Taken together, these results may explain why the cytoplasm of Sertoli cells was weakly stained in both species in the current study, and raises the possibility that the function of MTA1 might be regulated by growth factor stimulation.

Although we did not examine the function of MTA1 in this study, the evidence for stage-specific expression and localization in mouse meiotic spermatogenic cells suggested the possible roles of MTA1 in spermatogenic cell development and spermatocyte-Sertoli cell interactions. The distinctive staining of round spermatids and primary spermatocytes, but not elongated ones, of the human testis also suggested a possible role of MTA1 in spermatogenic differentiation. These possibilities give a functional importance to MTA1, which needs further attention in the future.

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