Expression and localization of alpha-tubulin N-acetyltransferase 1 in the reproductive system of male mice

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Abstract. The structure of microtubules is essential for the fertilizing ability of spermatozoa. Acetylation of α-tubulin plays an important role in flagellar elongation and spermatozoa motility. Previous reports have suggested that alpha-tubulin N-acetyltransferase 1 (ATAT1) is the main acetyltransferase involved in the acetylation of α-tubulin. Although ATAT1 is reported to express in the testis, no information is available regarding its expression in elongated spermatids, epididymis, and mature spermatozoa. Hence, it remains unclear whether ATAT1 is involved in spermatozoa maturation and capacitation. Therefore, we evaluated the expression of ATAT1 in the mouse male reproductive system using immunostaining and western blotting. Our results showed that ATAT1 was expressed in spermatids during spermogenesis in mouse testes, but its expression varied according to the seminiferous tubule stage. We observed ATAT1 in the cytoplasm of round spermatids, the flagella of elongated spermatids, and in the cytoplasm of step 16 spermatids, just before its release into the lumen. In addition, ATAT1 was expressed in epithelial cells of the epididymis. In spermatozoa of the cauda epididymis, ATAT1 expression was primarily observed in the midpiece of the spermatozoa. The localization of ATAT1 protein in the male germline was observed during spermiogenesis as well as during spermatozoa maturation. Our results suggest that ATAT1 may be involved in the formation of flagella and in the acetylation process, which has attracted attention in recent years regarding male infertility.

Key words: Alpha-tubulin N-acetyltransferase 1, Epididymis, Spermatogenesis, Spermatozoa

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investigate the expression and localization of ATAT1 during mouse spermatogenesis to determine the relationship between ATAT1 and flagellar elongation.

The acetylation level of α-tubulin is reported to increase in the epididymis during spermatozoa maturation [18]. Since spermatozoa are mostly transcriptionally and translationally silent, the luminal environment of the epididymis is responsible for spermatozoa functional changes [19–25]. Epididymal vesicles containing various types of proteins, lipids, and non-coding RNAs [2, 26–28] are secreted from epididymal cells into the lumen and transported to spermatozoa [29, 30], thereby facilitating the functional maturation of spermatozoa. It is possible that some acetyltransferases, such as ATAT1, are secreted from epididymal cells and are involved in spermatozoa maturation via α-tubulin acetylation. However, it remains unclear which acetyltransferases are present in the vesicles to regulate this modification in the epididymis and none of the studies have analyzed the epididymis in ATAT1-deficient mice [15].

After ejaculation, spermatozoa must undergo capacitation, a series of physiological modifications in the female reproductive tract that allow spermatozoa to fertilize an oocyte [31]. Interestingly, the acetylation of lysine residues in mouse spermatozoa is enhanced by capacitation, suggesting that lysine acetylation may be a modulator of spermatozoa capacitation [32]. These findings indicate that spermatozoa microtubules are acetylated during epididymal transit and capacitation. However, it remains unknown whether ATAT1, the major acetyltransferase in mice, is involved in the acetylation process in spermatozoa.

In the present study, we examined ATAT1 expression patterns in mouse testes and epididymis to gain new insights into the possible roles of ATAT1 during spermiogenesis, sperm maturation, and sperm capacitation.

Materials and Methods

Animals

C57BL/6N mice were purchased from Japan SLC (Shizuoka, Japan). Testes and epididymides were collected from four male mice (aged 12–24 weeks), which were euthanized in accordance with the Guide for the Care and Use of Laboratory Animals published by Tohoku University. All animals were cared for and experimental procedures were conducted in accordance with the Regulations for Animal Experiments and Related Activities at Tohoku University. The study was approved by the Tohoku University Institutional Animal Care and Use Committee (approval number: 2016AgL-005).

Mice spermatozoa preparation

The cauda epididymis from each animal was placed in 1 ml of phosphate-buffered saline (PBS; Nacalai Tesque, Kyoto, Japan) in a 1.5 ml plastic tube. The medium containing spermatozoa was incubated at 37°C for 30 min for the swim-up under a 5% CO₂ humidified atmosphere. Following this, 600 µl of the upper layer were collected and washed three times using centrifugation (5,000 rpm, 5 min, 25°C). The supernatant was discarded and the samples for western blotting were stored at –80°C. All subsequent experiments were performed using spermatozoa samples pooled from four mice.

Western blot analysis

Western blot analysis was performed to determine the expression levels of ATAT1 in the testes and spermatozoa. The samples were homogenized in Radioimmunoprecipitation (RIPA) Buffer (Nacalai Tesque) containing a protease inhibitor cocktail (Nacalai Tesque), centrifuged (10,000 rpm, 5 min, 4°C), and the supernatants were collected. The samples were suspended in an equivalent volume of 2 × sample buffer (Nacalai Tesque), sonicated, and incubated at 100°C for 5 min. The sample concentration was adjusted to 1 µg/µl and equal amounts of protein were separated via 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck, Darmstadt, Germany). Nonspecific binding sites on the membranes were blocked by incubating membranes in 3% Bovine serum albumin (BSA) in PBS for 1 h at 25°C. After blocking, the membranes were incubated with a rabbit polyclonal anti-ATAT1 antibody (1:3,000; Novus Biologicals; Centennial, CO, USA) overnight at 4°C. For the antibody pre-absorption test, the primary antibody was preincubated with a 10-fold molar excess of the epitope-blocking peptide (Novus Biologicals) overnight at 4°C and used as a negative control. The membranes were washed three times with PBS containing 0.1% Tween-20 (PBS-T) and treated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibodies (1:2,000; Promega, Madison, WI, USA) for 2 h at 25°C. Following three additional washes with PBS-T, the membranes were treated with Chemi-Lumi-One (Nacalai Tesque), and images were obtained using a LAS-3000-mini Lumino Image Analyzer (Fujifilm, Tokyo, Japan). Following image analysis, the membranes were incubated in WB Stripping Solution (Nacalai Tesque) for 30 min at 25°C to remove antibodies and, subsequently, membrane blocking was performed as described above. The membranes were incubated with mouse monoclonal anti-α-tubulin antibody (1:5,000; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. The membranes were washed and incubated for 2 h at 25°C with secondary horseradish peroxidase-conjugated anti-mouse immunoglobulin G antibody (1:2,000; Promega). The membranes were developed, imaged, and analyzed as described above. Each protein was detected and quantified using ImageGauge V4.22 software (Fujifilm).

Immunohistochemistry

The epididymis and testes were dissected and fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. The fixed tissues were dehydrated and embedded in paraffin. Paraffin-embedded sections (4 µm) were mounted on glass slides. The sections were deparaffinized using xylene, dehydrated using ethanol, and subsequently incubated with HistoVT One (Nacalai Tesque) at 90°C for 30 min to promote antigen retrieval. The sections were then washed with distilled water, incubated with 3% BSA in PBS at 25°C for 1 h, and subsequently incubated with rabbit polyclonal anti-ATAT1 antibody (1:200; Novus Biologicals) overnight at 4°C. For the antibody pre-absorption test, the primary antibody was preincubated with a 10-fold molar excess of synthetic peptide (Novus Biologicals) at 4°C overnight and used as a negative control in immunofluorescent analysis. After incubation, the sections were incubated with Alexa Fluor 488-labeled anti-rabbit and Alexa Fluor 568-labeled anti-mouse secondary antibodies (1:500; Thermo Fisher Scientific; Waltham, MA, USA) at 4°C for 2 h. Nuclei were counterstained with Hoechst 33342 (1:5,000; Nacalai Tesque) or/and propidium iodide (PI, 1:5,000; Fujifilm Wako Pure Chemical, Japan).
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Osaka, Japan). Images were captured using a BZ-X710 fluorescence microscope (Keyence; Osaka, Japan) and analyzed using BZ-X Viewer software. In each seminiferous tubule, the development stage of spermatids was classified into stages I–VI, VI–VIII, and IX–XII based on the nuclear morphology according to the criteria described previously [33].

Immunocytochemistry for spermatozoa

Epididymal spermatozoa were suspended in PBS, collected via centrifugation at 5,000 rpm for 5 min at 4°C, and fixed with 2% paraformaldehyde and 0.1% Triton X-100 in PBS for 1 h at 4°C. The samples were washed twice with PBS and blocked with 3% BSA in PBS for 60 min at 4°C. The suspensions were incubated overnight with the indicated primary antibody at 4°C [rabbit polyclonal anti-ATAT1 antibody (1:200; Novus Biologicals) and mouse monoclonal anti-acetylated-α-tubulin antibody (1:200; Santa Cruz Biotechnology)]. The antibody pre-absorption test for the primary antibody was performed as described above. After incubation, the sections were incubated with Alexa Fluor 488-labeled anti-rabbit and Alexa Fluor 568-labeled anti-mouse secondary antibodies (1:500; Thermo Fisher Scientific) at 4°C for 2 h. Nuclei were counterstained with Hoechst 33342 (1:5,000). Images were captured using a BZ-X710 fluorescence microscope and analyzed using the BZ-X Viewer software.

Results

Expression of ATAT1 in testes

Western blotting analysis revealed a band at the molecular weight (47 kDa) corresponding to ATAT1, which was abolished by incubating the anti-ATAT1 antibody with the blocking peptide, suggesting that the anti-ATAT1 antibody specifically bound to ATAT1 (Fig. 1A). In the immunostained sections of the testes, signals from the anti-ATAT1 antibody were observed in the seminiferous tubules and interstitium. However, ATAT1 signals were almost absent in pre-absorbed sections incubated with the anti-ATAT1 antibody and the blocking peptide, except for the interstitium and the basal compartment of the seminiferous tubules (Figs. 1B, B’, C, C’). At the cellular level, the ATAT1 signals in spermatids entirely disappeared after pre-absorption with the blocking peptide, whereas ATAT1 staining was observed in some cells of the basal compartment of the seminiferous tubules and interstitium (Figs. 1D, E), suggesting that the immunostaining signals of ATAT1 in spermatids reflected the bona fide localization of ATAT1.

Next, we focused on ATAT1 localization in round and elongating spermatids and during spermiogenesis. The seminiferous tubule stages can be roughly classified into three groups (stages I–VI, VII–VIII, and IX–XII) (Figs. 2A–C). We detected weak ATAT1 signals in round spermatids at stages I–VI; a slight increase in the fluorescent intensity from ATAT1 was detected in round spermatids as they moved to stages VII–VIII (Figs. 2A’, B’). In the beginning of stages IX–XII in elongating spermatids, ATAT1 signals were observed mainly in the region between the nucleus (Hoechst+) and flagellum (acetylated tubulin+) (Fig. 2C’). Along the length of the flagellum, ATAT1 distribution was observed throughout stages I–VIII (Figs. 2A”, B”). Additionally, ATAT1 signals were enhanced just before the release of spermatozoa into the lumen and could be observed as small round-shaped particles around the nuclei of step 16 spermatids at stages VII–VIII (Fig. 2B’). These results suggest that ATAT1 is continually expressed in round and elongating spermatids, whereas their intracellular localization changes dynamically during spermiogenesis.

ATAT1 expression in epididymis

Next, we evaluated the localization of ATAT1 in the epididymis using immunostaining. A strong ATAT1 signal was detected in the cytoplasm of epithelial cells of the caput, corpus, and cauda regions in the epididymis (Figs. 3A, A’, B, B’, C, C’), whereas these signals were almost absent in pre-absorbed sections (Figs. 3D–F). The intensity of the ATAT1 signal in the epithelia of the corpus and cauda regions was higher than that in the epithelium of the caput region (Figs. 3A, A’, B, B’, C, C’). Strong spot-like signals were observed occasionally on the apical side of epithelia in the caput region (Figs. 3A, A’). Interstitial cells in the caput and corpus epididymis were negative for ATAT1; however, some interstitial and peritubular cells were positive for ATAT1 in the cauda epididymis (Figs. 3C, C’). In the lumen of epididymal tubules, which showed weak staining for ATAT1, we could not determine whether ATAT1 was localized in the spermatozoa using low magnification immunohistochemical images (Fig. 3).

At a higher magnification, we found both weak and strong ATAT1 signals in the cauda epididymis. Most images showed very weak ATAT1 signals around the Hoechst- and PI-positive spermatozoa nuclei, whereas the other images showed strong ATAT1 signals with Hoechst-negative/PI-positive parts, both of which became negative after pre-absorption (Fig. 4).

ATAT1 expression in spermatozoa

Western blotting confirmed the ATAT1 expression in spermatozoa (Fig. 5A). Immunocytochemistry demonstrated the ATAT1 localization along the midpiece of spermatozoa (Fig. 5B). Pre-absorbed samples incubated with the anti-ATAT1 antibody and blocking peptide exhibited no immunoreactivity as determined via western blot analysis and immunocytochemistry (Figs. 5A, B). These results suggest that ATAT1 is localized in the midpiece of matured spermatozoa in the cauda epididymis.

Discussion

In this study, we showed the localization of ATAT1 in the testes and epididymis. We observed weak staining for ATAT1 in round spermatids, along the length of the flagellum, whereas stronger ATAT1 signals were observed around the head region of step 16 spermatids and in the epididymal epithelium. Additionally, we demonstrated the presence of ATAT1 in the midpiece of matured spermatozoa in the cauda epididymis. Collectively, our findings indicate that ATAT1 is continuously expressed in male mouse germ line cells, from the differentiation steps of round spermatids to mature spermatozoa and in the epididymal epithelium.

Our findings showed that ATAT1 is continually localized in the flagella of elongating spermatids. Earlier reports showed that spermatozoa from ATAT1-deficient mice were morphologically abnormal...
Fig. 1. Detection of alpha-tubulin N-acetyltransferase 1 (ATAT1) expression in mice testes. A: ATAT1 detected using western blotting analysis. B, B', D: Immunohistochemistry image showing ATAT1 localization in seminiferous tubules. C, C', E: preabsorbed sections of testis. Blue: Hoechst 33342; green: ATAT1; scale bars = 200 μm (B, B', C, C') or 50 μm (D, E). E-Sp: elongated spermatid; R-Sp: round spermatid; asterisk: interstitium.

Fig. 2. A–C: Immunohistochemical analysis of alpha-tubulin N-acetyltransferase 1 (ATAT1). Expression patterns for each seminiferous tubule stage. A', A'', B', B'', and C': Enlarged figures of the white square above. Blue: Hoechst 33342; green: ATAT1; red: acetylated-α-tubulin; scale bars = 200 μm.
and exhibited relatively short tails [15], suggesting that ATAT1 plays an important role in spermiogenesis. However, the expression of ATAT1 in elongating spermatids has not been revealed in mouse testes [14]. In the current study, by using an ATAT1-specific antibody, we succeeded in performing a detailed immunohistochemical analysis of spermatids at a higher magnification and showed ATAT1 expression in round and elongating spermatids. From the data presented in this study and a previous deficient mice study [15], we surmise that acetylation of α-tubulin via ATAT1 in spermatids is important for the formation of a normal spermatozoa tail during spermiogenesis.

The highly dense spot-like signals of ATAT1 around the nuclei of step 16 spermatids imply that ATAT1 is present in the cytoplasmic droplet, called the residual body. Since most of the cytoplasmic droplets are removed from spermatozoa during spermatogenesis [33], it is possible that the majority of ATAT1 may also be removed after spermatogenesis. However, our results indicate that ATAT1 is continuously localized in the midpiece of spermatozoa residing in the cauda epididymis. N-acetyltransferases utilize acetyl coenzyme A (acetyl-CoA) as an acetyl donor [34, 35]. Therefore, it is likely that ATAT1 functions as an N-acetyltransferase in the midpiece of spermatozoa, which is where mitochondria, the source of acetyl-CoA, are also localized. Although previous studies have indicated that ATAT1 is expressed in testicular germ cells [14, 15, 17] and is involved in flagella formation in spermatozoa [15], this study provides the first confirmation of the ATAT1 expression in the flagellum of elongated spermatids and mature spermatozoa after spermatogenesis.

We found that ATAT1 was expressed in the midpiece of epididymal spermatozoa. Mammalian spermatozoa acquire motility in the epididymis [22–25] and a major goal of spermatozoa maturation is to modify flagellar function to provide motility. It has been reported that the spermatozoa motility is affected by the acetylation level of α-tubulin in spermatozoa tails [18]. Additionally, some infertile men exhibit low levels of α-tubulin acetylation in spermatozoa tails [16]. These findings suggest that α-tubulin acetylation is a

Fig. 3. Detection of alpha-tubulin N-acetyltransferase 1 (ATAT1) expression in mice epididymis. A, A’, B, B’, C, C’: caput, corpus, and cauda epididymis. D–F: preabsorbed sections of caput, corpus, and cauda epididymis. Blue: Hoechst 33342; green: ATAT1; scale bars = 100 μm.
key requirement for spermatozoa motility. Moreover, spermatozoa from ATAT1-deficient mice show lower progressive motility [15]. It was predicted that ATAT1 is responsible for α-tubulin acetylation in spermatozoa tails to modulate flagellar function. It is known that α-tubulin is acetylated in spermatozoa tails during epididymal transit in rats [18, 36]. Furthermore, we found that ATAT1 was present in the epididymal epithelium. Epididymosomes, which are secreted from epididymal epithelial cells, contain several proteins critical to spermatozoa maturation and fertilization, such as those belonging to the zona pellucida family and disintegrin and metalloproteinase (i.e.,
ADAMs) family [37, 38]. Recently, acetyltransferases were reported to be present within epididymosomes [39]. Therefore, ATAT1 is presumed to be included in these epididymosomes. Supporting our hypothesis, we observed strong ATAT1 signals occasionally in the Hoechst negative/PI-positive parts of the cauda epididymis, which are considered to be rich in RNAs [40]. It is possible that the RNA-rich luminal content of epididymis either contains cytoplasmic droplets from the testes or secreted material from epididymal epithelial cells. Thus, ATAT1 may be functional during epididymal transit, even if most testicular spermatozoa ATAT1 is removed, along with the cytoplasmic droplet during spermiogenesis. We propose that ATAT1 localizes in spermatozoa, is expressed in epididymal epithelial cells, and plays a role in spermatozoa maturation in the epididymis. Therefore, the functional role of ATAT1+ particles in the epididymal lumen in the maturation process of spermatozoa should be further explored in the future.

Immunocytochemical analyses revealed that ATAT1 was present in the midpiece of epididymal spermatozoa. A previous study showed that the acetylation level of lysine in the midpiece of mouse spermatozoa was increased via capacitation treatment, suggesting that lysine acetylation is one of the modulators of spermatozoa capacitation [32]. However, it is not clear which acetyltransferases are involved in this process. It is well established that ATAT1 is a major acetyltransferase that regulates the acetylation levels of α-tubulin lysine 40 in mammals, including mice [9–15]. Therefore, ATAT1 may play a role during spermatozoa capacitation by modulating lysine acetylation in the midpiece of the spermatozoa tail. ATAT1-deficient mice are not completely infertile but show a decline in litter size [15], which is consistent with our hypothesis that ATAT1 is involved in promoting spermatozoa function, such as spermatozoa capacitation, and is necessary for an optimal fertilization. Other acetyltransferases, such as chromodomain Y-like proteins, are also present in mature spermatozoa [36]. Therefore, further research is required to clarify the relationship between these acetyltransferases and spermatozoa capacitation. Even with the current technology available, the specific control of acetyltransferases in spermatozoa is difficult. If a new technique to examine the regulation of protein activity in spermatozoa could be developed, a more detailed analysis would be possible.

In conclusion, we showed that ATAT1 is expressed in the male germline and in the epididymal epithelium in mice, implying that ATAT1 is an important regulator of spermatozoa function. Future research is necessary to clarify the molecular function of ATAT1 regarding the dynamic changes in α-tubulin acetylation in germ cells during spermatogenesis, epididymal transit, and spermatozoa capacitation.

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