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Expression Pattern of Acetylated α -Tubulin in Porcine Spermatogonia

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SUMMARY

Mammalian spermatogonial stem cells reside on the basement membrane of the seminiferous tubules. The mechanisms responsible for maintenance of spermatogonia at the basement membrane are unclear. Since acetylated α -tubulin (Ac- α -Tu) is a component of long-lived, stable microtubules and deacetylation of α -tubulin enhances cell motility, we hypothesized that acetylation of α -tubulin might be associated with positioning of spermatogonia at the basement membrane. The expression pattern of Ac- α -Tu at different stages of testis development was characterized by immunohistochemistry for Ac- α -Tu and spermatogonia-specific proteins (PGP 9.5, DAZL). In immature pig testes, Ac- α -Tu was present exclusively in gonocytes at 1 week of age, and in a subset of spermatogonia at 10 weeks of age. At this age, spermatogonia are migrating toward the tubule periphery and Ac- α -Tu appeared polarized toward the basement membrane. In adult pig testes, Ac- α -Tu was detected in few single or paired spermatogonia at the basement membrane as well as in spermatids and spermatozoa. Only undifferentiated (DAZL[−]), proliferating (determined by BrdU incorporation) spermatogonia expressed high levels of Ac- α -Tu. Comparison with the expression pattern of β -tubulin and tyrosinated α -tubulin confirmed that only Ac- α -Tu is specific to germ cells. The unique pattern of Ac- α -Tu in undifferentiated germ cells during postnatal development suggests that posttranslational modifications of microtubules may play an important role in recruiting and anchoring spermatogonia at the basement membrane.

INTRODUCTION

Spermatogenesis is a complex process sustained by self-renewal of spermatogonial stem cells in the stem cell niche at the basement membrane (BM) of the seminiferous tubules, differentiation of germ cells toward the tubular lumen, and ultimately formation of motile spermatozoa. In the postnatal testis, type A spermatogonia arise from gonocytes that migrate to and localize at the BM. The population of type A spermatogonia includes undifferentiated spermatogonia as well as spermatogonia committed to differentiation. The mechanisms controlling movement of gonocytes to the BM and maintenance of spermatogonia at the BM are largely unknown.

Microtubules of α , β -tubulin heterodimers are associated with many cellular functions, and both subunits of tubulin heterodimers are subject to multiple, reversible post-translational

modifications (Hammond et al., 2008). Stable microtubules are more highly modified than dynamic microtubules, and microtubule configuration changes during the cell cycle and in many differentiation pathways (Cole and Lippincott-Schwartz, 1995). Acetylation and deacetylation of α -tubulin occurs at Lys40 by the activity of an unknown acetylase and histone deacetylase 6 (HDAC6) or Sirt2 (North et al., 2003; Hammond et al., 2008; Tang and Chua, 2008). Reversible acetylation of α -tubulin at Lys40 has been implicated in regulating microtubule stability and function. Acetylation of α -tubulin is most abundant in stable microtubules but is absent from dynamic cellular structures (Hubbert et al., 2002). Acetylation of α -tubulin was also found to be associated with assembly of microtubules (Sasse and Gull, 1988). The degree of α -tubulin acetylation influences cell motility (Hubbert et al., 2002; Palazzo et al., 2003). HDAC6 is predominantly expressed in mouse testis compared to liver, heart, muscle, and kidney, and it is present in the cytoplasm of mouse spermatogenic cells including spermatogonia (Seigneurin-Berny et al., 2001).

The level of tubulin acetylation is regulated during spermatogenesis: acetylated α -tubulin (Ac- α -Tu) is present in a few microtubules in pachytene spermatocytes (Moreno and Schatten, 2000); a slight increase in acetylation is observed at the initiation of the elongation process, and tubulin becomes strongly acetylated in condensing and elongating spermatids when the flagellum is formed (Zhang et al., 2008). Ac- α -Tu status in spermatogonia has not been reported. Here, we hypothesized that α -tubulin acetylation might be involved in gonocyte migration and in positioning of spermatogonia at the BM in the mammalian testis.

RESULTS

α -Tubulins (access number in NCBI) were found to be highly conserved between mouse (NP_035783.1), pig (NP_001038009.1), and monkey (Q4R538) with an identical domain flanked by Lys40, representing the acetylation site (Fig. 1).

Spermatogonia were identified based on expression of protein gene product 9.5 (PGP 9.5, also named ubiquitin carboxyl-terminal hydrolase L1, UCH-L1) that is expressed in spermatogonia located at the BM (Tokunaga et al., 1999; Luo et al., 2006; Wang et al., 2006).

Compared to the ubiquitous expression of β -tubulin and tyrosinated α -tubulin (Tyr- α -Tu) in testicular cells, the distribution of Ac- α -Tu in the testis appeared to be developmental stage dependent, especially in germ cells (Figs. 2 and 3). In the immature postnatal testis containing gonocytes (1 week pig), Ac- α -Tu was prominent in all germ cells (Figs. 2 and 3); however, at later stages of postnatal testis development when primary germ cells are spermatogonia (10 weeks pig), expression of Ac- α -Tu level is stronger in spermatogonia expressing PGP 9.5 than in Sertoli cells and PGP 9.5-negative spermatogonia (Figs. 2 and 3). Interestingly, expression of Ac- α -Tu was polarized toward the BM of seminiferous tubules in pig spermatogonia at 10 weeks of age when germ cells are migrating to the BM (Figs. 2 and 3).

In adult testes, Ac- α -Tu was clearly detected in few single or paired spermatogonia localized at the BM that also expressed PGP 9.5, as well as in spermatids and spermatozoa (Figs. 2 and 3).

To further characterize cell-type specificity of Ac- α -Tu expression, we employed double immunolabeling for stage-specific germ cell markers. In the adult pig testis, spermatogonia expressing high levels of Ac- α -Tu did not express DAZL (Fig. 4a), a protein essential for meiotic differentiation of spermatogonia (Ruggiu et al., 1997; Saunders et al., 2003). This indicated that undifferentiated spermatogonia maintain a high level of Ac- α -Tu expression. In addition, BrdU pulse labeling demonstrated that these undifferentiated spermatogonia

with high expression of Ac- α -Tu were actively dividing, thereby ruling out the possibility that these cells were undergoing apoptosis (Fig. 4b). Therefore, expression of Ac- α -Tu in pre-meiotic germ cells in adult testes appears limited to undifferentiated spermatogonia.

DISCUSSION

Here we report for the first time that posttranslational modification of α -tubulin by acetylation is restricted to gonocytes and undifferentiated spermatogonia among the premeiotic germ cells in mammalian testis. Since Ac- α -Tu is mainly associated with long-lived, stable microtubules *in vivo*, and deacetylation of tubulin enhances cell motility (Hubbert et al., 2002; Palazzo et al., 2003), a high level of Ac- α -Tu maintained in gonocytes and undifferentiated spermatogonia might play a role in maintaining their orientation and limiting their movement which may aid in directing gonocytes migration by Sertoli cells and stabilizing spermatogonia at the BM. It can be speculated that manipulation of the degree of tubulin acetylation could influence germ cell activity. Recently, it was reported that mice deficient in HDAC6 showed hyperacetylation of tubulin in all differentiation stages examined, but no obvious spermatogenic defects were observed (Zhang et al., 2008). However, it is possible that subtle defects in spermatogonial function in the absence of HDAC6 were not noticed, or that redundancy exists in the acetylation/deacetylation machinery given the parallel requirements expected during formation of the axoneme.

While it is suggested that acetylation of α -tubulin contributes to microtubules stability, the exact role of the acetylation in α -tubulin remains unclear. Ac- α -Tu is required for interaction with membrane Na⁺, K⁺-ATPase and inhibition of enzyme activity (Casale et al., 2003). Interaction of acetylated tubulin with membrane ATPase may trigger the activation of signal pathways that regulate growth and differentiation (Casale et al., 2003). Tubulin acetylation also plays a role in controlling motor protein kinesin-1 trafficking (Reed et al., 2006).

Polarized distribution of Ac- α -Tu towards the BM in migrating spermatogonia suggests that Ac- α -Tu might associate with polarized protein trafficking, which is critical for cell morphogenesis and function (Horton and Ehlers, 2003; Lecuit and Pilot, 2003). Spermatogonia with a high level of Ac- α -Tu are actively cycling, therefore tubulin acetylation might play a role during germ cell proliferation through polarized protein trafficking.

Taken together, a high level of α -tubulin acetylation in gonocytes and spermatogonia might play a role in migration of gonocytes to and maintenance of spermatogonia at the BM.

MATERIALS AND METHODS

Alignment of α -Tubulin Amino Acid Sequences in Three Species

The amino acid sequences of α -tubulin were compared between pig, mouse, and rhesus monkey using the Clustal W program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

Animals and Testis Tissues

Testes were obtained from 1-week-old, 10-week-old, and adult Yorkshire cross pigs. Testis pieces were rinsed in PBS without calcium and immediately fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) overnight at 4°C followed by three changes of PBS, or fixed in Bouin's solution (Sigma-Aldrich) overnight at room temperature followed by three changes of 70% ethanol before being processed for paraffin sections.

Antibodies and Immunofluorescence

After deparaffinization and rehydration, tissue sections were subjected to antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 30 min and cooled down to room temperature. Sections were washed three times with PBS for 5 min each followed by exposure to 0.5% triton $\times$ 100 in PBS for 10 min at room temperature. After washing, tissues were blocked with 3% BSA for 1 hr at room temperature. For double immunofluorescence staining, two primary antibodies raised in different species were mixed in 3% BSA and incubated with tissues at 4 °C overnight or for 3 hr at room temperature. Sections were washed with PBS (five times for 5 min each) and incubated with Alexa Fluor 555 (488) donkey anti-mouse IgG (H+L) and Alexa Fluor 488 (555) donkey anti-rabbit IgG (H+L) (Invitrogen Corporation, Carlsbad, CA) (each 4 μ g/ml in 3% BSA) for 1 hr at room temperature. Slides were washed and mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) for imaging. The primary antibodies used were: rabbit anti- β -tubulin (1:100, Abcam, Inc., Cambridge, MA), rabbit anti-PGP 9.5 (1:300, AbD Serotec, Raleigh, NC), mouse anti-Ac- α -Tu, clone 6-11B-1 (1:200, Sigma-Aldrich), rat anti-Tyr- α -Tu (1:200, Abcam, Inc.), rabbit anti-DAZL (1:200, a kind gift from Dr. F. Kent Hamra, University of Texas Southwestern Medical Center). Immunofluorescent images were captured with digital imaging systems (Image Pro, Axiovision) on Leica or Zeiss fluorescence microscopes.

BrdU Labeling and Staining

BrdU labeling of proliferative cells was performed essentially as described (Zeng et al., 2006, pp. 527–533). For double immunostaining of BrdU and Ac- α -Tu on sections, the protocol was modified from the literature (Zeng et al., 2006, pp. 527–533) by mixing primary antibodies of sheep anti-BrdU (1:100, Abcam, Inc.) and mouse anti-Ac- α -Tu, clone 6-11B-1 (1:200). Alexa Fluor 555 donkey anti-sheep IgG (H+L) (Invitrogen Corporation) and Alexa Fluor 488 donkey anti-mouse IgG (H+L) were used as secondary antibodies.

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MRECISIHVGQAGVQIGNACWELYCLEHGIQPDGQMPSDETTIGGGDDSFNTFFSETGAGK 60
MRECISIHVGQAGVQIGNACWELYCLEHGIQPDGQMPSDETTIGGGDDSFNTFFSETGAGK 60
MRECISIHVGQAGVQIGNACWELYCLEHGIQPDGQMPSDETTIGGGDDSFNTFFSETGAGK 60

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HVPRAVFVDLEPTVIDEVRTGTYSRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLD 120
HVPRAVFVDLEPTVIDEVRTGTYSRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLD 120

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RIRKLADQCTGLQGFLVFHSFGGGTSGGFTSLLMERLSVDYGGKSKLEFSIYPAPQVSTA 180
RIRKLADQCTGLQGFLVFHSFGGGTSGGFTSLLMERLSVDYGGKSKLEFSIYPAPQVSTA 180

VVEPYNSILTTHTLEHSDCAFMDNEATYDICRRNLDIERPTYTNLNRISQIVSSITA 240
VVEPYNSILTTHTLEHSDCAFMDNEATYDICRRNLDIERPTYTNLNRISQIVSSITA 240
VVEPYNSILTTHTLEHSDCAFMDNEATYDICRRNLDIERPTYTNLNRISQIVSSITA 240
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SLRFDGALNVDL TEFQTNLVPYPRIHFPATYAPVISAEEKAYHEQLSVAEITNACFEPAN 300
SLRFDGALNVDL TEFQTNLVPYPRIHFPATYAPVISAEEKAYHEQLSVAEITNACFEPAN 300
SLRFDGALNVDL TEFQTNLVPYPRIHFPATYAPVISAEEKAYHEQLSVAEITNACFEPAN 300

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QMVKCDPRHGKMACCLLYRGDVVVPKDVNAAIATIKTKRTIQFVDWCPTGFKVGINYQPP 360
QMVKCDPRHGKMACCLLYRGDVVVPKDVNAAIATIKTKRSIQFVDWCPTGFKVGINYQPP 360
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TVVPGGDLAKVQRAVCHLSNTATAAEAWARLDHKFDLMYAKRAVFVHWYVGEEMEEGEFSE 420
TVVPGGDLAKVQRAVCHLSNTATAAEAWARLDHKFDLMYAKRAVFVHWYVGEEMEEGEFSE 420
TVVPGGDLAKVQRAVCHLSNTATAAEAWARLDHKFDLMYAKRAVFVHWYVGEEMEEGEFSE 420

AREDMAALEKDYEEVGVD SVEGESEEEGEEY 451   Pig/NP_001038009.1
AREDMAALEKDYEEVGVD SVEGESEEEGEEY 451   Mouse/NP_035783.1
AREDMAALEKDYEEVGVD SVEGESEEEGEEY 451   Monkey/Q4R538

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Figure 1.

Alignment of α -tubulin amino acid sequences from monkey, pig, and mouse. Lys40 (indicated by box) is the site of acetylation/deacetylation. Amino acids different among the three species are underlined and marked by arrowheads. Species name/NCBI protein ID numbers are indicated.

Figure 2.

Double immunolabeling of β -tubulin (**a**) or tyrosinated atubulin (Tyr- α -tubulin; **b**) and acetylated α -tubulin (Ac- α -Tu) in testes of 1-week-old, 10-week-old, and adult pigs. Spermatogonia with high expression of Ac- α -Tu are marked with arrows. Nuclei are stained with DAPI (blue). Basement membranes are highlighted by dotted lines. Bars=50 μ m in (a) and lower panels in (b), 20 μ m in top panels of (b).

Figure 3.

Double immunolabeling of PGP 9.5 and Ac- α -Tu in pig testes at different developmental ages. Ac- α -Tu was present in all gonocytes (1 week pig), the majority of spermatogonia in the pubertal testis (10 weeks pig), and few spermatogonia located on BM in the adult testis. Polarization of Ac- α -Tu to BM is evident in some pig spermatogonia at 10 weeks of age. Arrows point to representative spermatogonia with a high level of Ac- α -Tu. Arrowheads mark spermatogonia without Ac- α -Tu in the 10-week-old pig testis. Basement membranes are highlighted by dotted lines. Bars=50 μ m.

Figure 4. Acetylated α -tubulin in proliferating, undifferentiated pig spermatogonia. **a:** Spermatogonia with a high level of Ac- α -Tu do not express DAZL, a factor necessary for differentiation. A_{single} and A_{paired} spermatogonia are marked by an arrowhead and an arrow, respectively. **b:** Spermatogonia with a high level of Ac- α -Tu incorporated BrdU (1 hr pulse labeling; arrows). Basement membranes are highlighted with dotted lines. Bars= 50 μm .