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Original Article

Effect of a recombinant ribosomal protein L10a (rRpL10a) on mouse spermatogenesis

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Abstract

This study attempted to clarify RpL10a function in testis using mouse testis as a model. The histological method of mouse testis was used to determine the percentages of cell types in the spermatogenesis after being treated with a recombinant RpL10a protein (rRpL10a). The percentages of spermatid cells in the testis treated with 0.5, 1.0, 1.5, and 2.0 µM of rRpL10a protein were 40.78%, 49.65%, 45.03%, and 45.33%, respectively, while the untreated group was 21.72%. The spermatid cells of the experiment tissue were confirmed by WGA lectin histochemistry. *Prm2* gene expression was moderately increased, whereas the expression of *Rhau* gene decreased in rRpL10a treated testes compared to the untreated testis. Furthermore, increased cell proliferation in the testes treated with 1.0 µM rRpL10a was revealed by an intense fluorescent signal of EdU (5-ethynyl-2′-deoxyuridine) staining. Altogether, the results indicated that the rRpL10a protein induced spermatogenesis in mice and may be applicable for *in vitro* spermatogenesis induction in the future.

Keywords: rRpL10a, spermatogenesis, testis, mice

1. Introduction

Much evidence has suggested the functions of ribosomal proteins in DNA replication, and its repair, regulation of translation, and control of development (Wool, 1996). Recently, the extra functions of ribosomal proteins in the regulation of organ development were studied extensively. The ovarian maturation of mosquito was inhibited after ribosomal protein S2 (*rps2*) gene expression knock-down (Kim & Denlinger, 2010). In addition, protein synthesis and proliferation were disturbed when the *rpl24* gene was mutated (Oliver, Saunders, Tarlé, & Glaser, 2004). RPL24 protein was highly expressed in ovary and played a function in oogenesis of marine shrimp (Zhang *et al.*, 2007). Similarly, ribosomal protein S3a (*rps3a*) gene was expressed at the highest level during the early stage of ovarian maturation in shrimp (Navakanitworakul, Deachamag, Wonglapsuwan, & Chotigeat, 2013). The other member of ribosomal protein that was extensively studied is the RpL10a protein, encoded by the *rpl10a* gene. This gene was down regulated in the thymus in mice when they were treated with cyclosporine A-19 (an immunosuppressive drug). Moreover, RpL10a protein might play

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an important role during embryogenesis and organogenesis (Fisicaro *et al.,* 1995). The differential expression of *RpL10a* genes were reported to be involved in ovarian maturation (Wonglapsuwan, Phongdara, & Chotigeat, 2009) where a high level of the *rpl10a* gene in ovaries was found during early stage (stage I) maturation as well as in lymphoid tissue during shrimp ovarian development. It was also reported that the early development of ovarian explants treated with rRpL10a protein enhanced higher expression of vitellogenin related genes (Wonglapsuwan, Miyazaki, Loongyai, & Chotigeat, 20 10). The rRpL10a protein also proved to be a stimulant of shrimp ovarian development *in vivo* which promoted early stage ovarian maturation (Palasin, Makkapan, Thongnoi, & Chotigeat, 2014). Comparatively, *Drosophila* germline and follicle cells were under developed when the function of *rpl10a* gene was knocked down. In contrast, the over-expression of *rpl10a* has caused the death of germline and follicle cells (Wonglapsuwan, Chotigeat, Timmons, & McCall, 2011) which indicated that an excess of RpL10a might be toxic to the cells and the cells required only a certain amount for growth and proliferation. Recently, the activity of RpL10a during rainbow trout gonadal development was reported. The expression of the *rpl10a* gene and protein were observed in spermatogonia type A and Sertoli cells of immature testes. In mature testes, *rpl10a* mRNA and protein were expressed both in spermatogonia type A and B, while the expression was rarely detected in spermatocytes and not observed in spermatid and spermatozoa (Makkapan, Yoshizaki, Tashiro, & Chotigeat, 2014). It is possible that the RpL10a protein is involved in cell proliferation of spermatogenesis. Although rRpL10a was characterized in shrimp, fly, and fish, the conserved functions in higher animals, especially in spermatogenesis, has not been investigated. Therefore, this study aimed to investigate the effect of the rRpL10a on testicular development in mice. The cell types of spermatogenesis were divided based on a histological technique and the spermatid cells were confirmed using wheat germ agglutinin (WGA) staining. The early and mature cell stages of spermatogenesis were proven by the expression of marker genes using quantitative reverse transcription-PCR (qRT-PCR). Furthermore, the proliferating cells in treated tissues were detected using 5-ethynyl-2'-deoxyuridine (EdU) assay.

2. Materials and Methods

2.1 Expression and purification of the recombinant RpL10a protein

The recombinant His-RpL10a protein was produced by BL21 (DE3) *Escherichia coli* cells harboring a recombinant plasmid of pET-28a(+) containing the *rpl10a* gene (Wonglapsuwan *et al.,* 2010). Then, His-RpL10a protein purification was performed using the His-Trap FF column according to the manufacturer's instructions for the AKTA Prime system (Amersham, UK).

2.2 Treatments of testicular cultured cells by rRpL10a protein

Adult ICR male mice (*Mus musculus*) at 5-7 weeks of age with weights of 20-30 g from Southern Laboratory Animal Facility (Thailand) were used in each experiment. All animal handing procedures followed the guidelines of the Institute of Animals for Scientific Purpose Development, National Research Council of Thailand.

Male ICR mice were anesthetized by intraperitoneal injection with 40 mg/kg of thiopental sodium BP. The testes were then removed under sterile conditions. The testes were washed twice with cell culture medium before cutting into 3 mm pieces. The pieces of testes were placed in a 48-well plate containing 1 mL of Dulbecco's modified Eagle's medium (DM-EM; GIBCO, USA) pH 7.4 containing 10% FBS with rRpL-10a protein at 0, 0.5, 1, 1.5, and 2 μM under sterile conditions. The pieces of testes were incubated at 37 ^{o}C with 5% CO_2. and collected for histological observation after incubation for 4 h. Different cells of the mouse seminiferous tubules were counted, calculated into percentages of spermatogenic cell types and compared with the untreated minced seminiferous tubules (control group).

2.3 Differential cell types in spermatogenesis by histological observation

Pieces of testes after treatment with rRpL10a were fixed in 10% neutral formalin solution (10% formalin, 33 mM NaH_2PO_4, 45 mM Na_2HPO_4). Subsequently, the fixed tissues were processed by a tissue processor until embedding into paraffin blocks. Finally, the samples were sliced at 5 μm thicknesses using a rotary microtome (Leica Biosystems, Germany). The slides were stained with hematoxylin and eosin (H&E) (Bio-Optica, Italy).

To evaluate the seminiferous stage in mouse, three seminiferous tubules of different areas of nearly the same diameter as the tubules in the testis section were identified. Spermatogenic cells in tubules were divided into 4 types based on cell morphology including spermatogonia, spermatocytes, spermatids, and spermatozoa (Gartner & Hiatt, 2012; Treuting & Dintzis, 2011). The cells in different patterns were counted and calculated in percentages of spermatogenesis using this formula: (average number of cells of the same type/total cells in the seminiferous tubules) × 100%. Then, the mean percentages of spermatogenesis were calculated from the areas of seminiferous tube cross sections in four samples in each experiment and repeated in triplicate.

2.4 WGA lectin histochemistry

Since exposed *N*-acetylglucosamine sugar residues on spermatid cells were shown to bind to WGA, we used WGA lectin to confirm the stage of spermatids in the seminiferous tubules. The testes sections were dewaxed, rehydrated, and immersed in Hank's balanced salt solution (HBSS) buffer. Then, tissue sections were incubated with 5 μg/mL of Alexa Fluor 594 WGA solution and 1 μM Hoechst 33342 stain (Image-iT™ LIVE Plasma Membrane and Nuclear Labeling Kit, Invitrogen, UK) in HBSS buffer 10 min at room temperature. Tissue slides were washed with HBSS buffer for 5 min 3 times before dehydration and mounting. The fluorescent signal was detected under a fluorescence microscope (Olympus DP73, Olympus Corporation, Japan). The number of spermatid cells was counted and calculated in percentages of spermatid cells per number of total cells counted in each area.

2.5 Expression of *Rhau* and *Prm2* gene

To confirm the stage of spermatogenesis cell, the expression of gene marker of early stage and late stage were determined. The *Rhau* gene which is highly expressed in spermatogonia stem cells and primary spermatocytes were used as a marker in early stage spermatogenesis. The sperm protamine 2 *(Prm2)* gene, which is expressed in spermatids, was detected in late stage spermatogenesis. Total RNA was isolated from the rRpL10a treated testes using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Thereafter, 1 μg of total RNA was reverse transcribed to cDNA using AMV reverse transcriptase (Promega, USA).

The expression levels of *Rhau* and *Prm2* genes were determined by qRT-PCR. The reaction of real-time PCR included 12.5 μL of FastStart Universal SYBR Green Master (Rox) (Roche, Germany), 20 pM of each primer (Table 1), and 300 ng of cDNA. Thermal cycling and fluorescence detection were performed using the Mx3000P™ (Stratagene, USA). The PCR program was initiated with a denaturing step at 94 ºC for 5 min followed by 40 cycles at 94 ºC for 30 sec, 57 ºC for 30 sec, and 72 ºC for 30 sec. The results of gene expression were shown as the relative copy number of each gene normalized to that of *β-actin* gene in each sample. Data are expressed as mean±SD. The primers of these genes are shown in Table 1.

Table 1. Primer sequences used for quantitative PCR.

Primer name	Primer sequences
Forward *Rhau*	5' ATGGATGAACGTCGAGAAGAGC 3'
Reverse *Rhau*	5' ATACCCATGATCCTCAGGAGC 3'
Forward *Prm2*	5' TACCGAATGAGGAGCCCCA 3'
Reverse *Prm2*	5' TGCGGATGCCGCCTCCTGT 3'
Forward β-*actin*	5' GCTACAGCTTCACCACCACCG 3'
Reverse β-*actin*	5' GATGTCCTCGTCRCACTTCAT 3'

2.6 Determination of cell proliferation using the EdU assay

To detect DNA synthesis in proliferating cells, the EdU label was detected in the testis tissue. The minced testes were incubated with different concentrations of rRpL10a protein for 1 h followed by addition of EdU (Thermo Fisher Science, USA). After incubation for 4 h, the tissues were collected. The treated tissues were fixed in neutral formaldehyde fixative buffer, embedded in paraffin, and sectioned. The paraffin of the 5-μm tissue sections was removed by soaking in xylene two times. Then the sample slides were rehydrated by ethanol and TBS buffer. After that, the sections were stained by incubating with 10 μM Alexa Fluor® 488 azide (Thermo Fisher Science, USA) in 100 mM Tris, pH 8.5 containing 1 mM $CuSO_4$ and 100 mM ascorbic acid for 20 min. Then, the slides were washed three times with TBS with 0.1% Triton X-100. After washing, the sections were mounted with DAPI staining medium (Vector Laboratories, California, USA) (Salic & Mitchison, 2008) and observed under fluorescence microscopy (Olympus DP73, Japan).

2.7 Statistical analysis

The data were reported as mean of percentage in each stage and statistically analyzed using the R program. One-way analysis of variance was followed by Tukey's multiple comparison tests for the analysis with a significance level of $P<0.05$. Two sample *t*-tests were performed to compare the two control groups and the qRT-PCR experiment results ($P<0.05$).

3. Results

3.1 Effect of rRpL10a protein on development

The testes of the experimental groups were incubated with rRpL10a protein at different dosages (0.5, 1.0, 1.5, and 2.0 μM of rRpL10a protein). After 4 h incubation, the percentages of the spermatogenic cell development in this study were observed (Figure 1). The results showed that the rRpL10a protein treated groups promoted a higher number of spermatid cells (mature stage) than the control group. The rRpL10a at 0.5, 1.0, 1.5, and 2.0 μM showed the percentages of spermatids at 40.78%, 49.65%, 45.03%, and 45.33%, respectively (Figure 1). In fact, 1 μM of rRpL10a protein induced early spermatogenic cells into elongated spermatid cells more than the other groups. The percentage of spermatid cells in the control group was 21.72% (Table 2). The results of the experimental mice were significantly different from the control group after 4-h incubation with the rRpL10a protein ($P<0.05$). However, there were no differences in the percentages among various dosages of rRpL10a protein.

The histological results of spermatogenic cell stages were further confirmed by WGA lectin histochemistry. The Alexa Fluoro 594 WGA staining detected the acrosomes of the spermatids (red fluorescent) and the Hoechst 33342 staining found chromatin in the nuclei (blue) (Figure 2A). The percentages of spermatid cells under WGA staining detection were 11.36%, 36.24%, 20.02%, and 16.64% in the 0.5, 1.0, 1.5, and 2.0 μM rRpL10a protein treated groups, respectively. Similar to the histological results above at 1 μM, the rRpL10a protein treated group had the highest percentage of spermatid

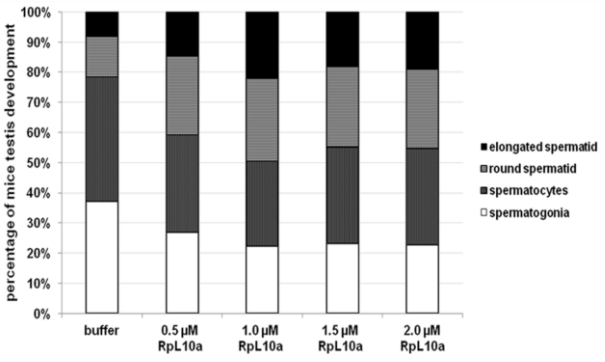

Figure 1. Effect of the recombinant His-rRpL10a protein at different dosages on testicular development in male mouse after 4-h incubation in DMEM medium. The graph shows the percentages of the different spermatogenic cells in mouse (n=4, the data performed in triplicate experiment).

Table 2. Percentages of mice testis development after different concentrations of rRpL10a protein at 4-h incubation compared with control groups (untreated testis and buffer group).

Sample	Percentages of spermatogenic cells (%)			
	spermatogonia	spermatocytes	round spermatid	elongated spermatid
Buffer	37.04	41.23	13.61	8.11
0.5 µM RpL10a	26.89	32.32	25.88	14.90
1.0 µM RpL10a	22.13	28.22	27.57	22.08
1.5 µM RpL10a	23.17	31.79	26.76	18.27
2.0 µM RpL10a	22.61	32.05	26.06	19.27

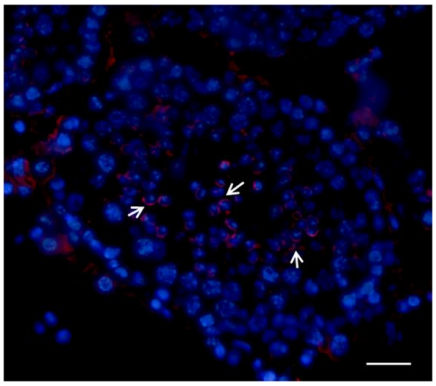

Figure 2A. Percentage of spermatid cells counted by WGA staining.

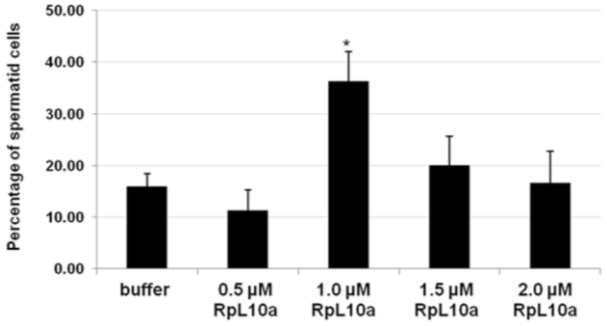

Figure 2B

Figure 2. WGA lectin histochemistry confirmed the spermatid cells in seminiferous tubules. (A) The testis explants were stained with Alexa Fluor 594 WGA (Red) and Hoechst 33342 (blue). The acrosomal spermatids were detected (pointed by white arrows), original magnification ×400, scale bars = 20 µm. (B) The percentages of spermatid cells under WGA histochemistry observation are presented. Significant differences were compared by one-way ANOVA test (mean±SD, n=4 and P<0.05).

cells compared with the other doses and the buffer group (Figure 2B). WGA detection is based on the sensitivity of WGA to sialic acid of spermatids while the H&E histological staining method is based solely on cell observation by the researcher. Therefore, both of the techniques were used to confirm the results. Although the percentages of spermatids evaluated by WGA detection were not equal to the H&E assessment, the results showed that the percentages of spermatid cells counted by WGA staining were similar to the counting results of the H&E staining.

3.2 Determination of early and late stage genes expression of spermatogenesis in rRpL10a treated mice

To confirm the percentages of spermatogenesis in mouse testis treated with rRpL10a protein, the expression of genes in early and late stages of spermatogenesis were evaluated. The 1 µM rRpL10a was the best concentration for spermatogenesis as demonstrated in the experiment mentioned above. Therefore, 1 µM rRpL10a was used to treat spermatogenesis in mice and the early and late stages of gene expression were determined. RNA helicase associated with AU-rich element or *Rhau* mRNA, which is highly expressed in spermatogonia stem cell and primary spermatocytes, was selected as a marker for early stage gene expression of spermatogenesis. Another gene used to detect the mature stage of spermatogenesis was protamine 2 or the *Prm2* gene. Relative mRNA expression from the qRT-PCR analysis showed that the levels of *Rhau* gene expression were significantly lower in the testis treated with 1 µM rRpL10a compared with the control group ($P<0.05$) (Figure 3). In contrast, the relative expression of *Prm2* transcript was higher in the 1 µM rRpL10a protein treated group compared with the control group. The results suggested that the rRpL10a protein treated group effectively induced transformation of early spermatogenic cells to become spermatids. This was in agreement and supported spermatogenic cell development by histological methods.

3.3 rRpL10a protein enhanced proliferation of spermatogonia cells

The EdU assay was used to determine cell proliferation in mouse testis after rRpL10a protein incubation (Figure 4). We found that EdU was strongly incorporated into the

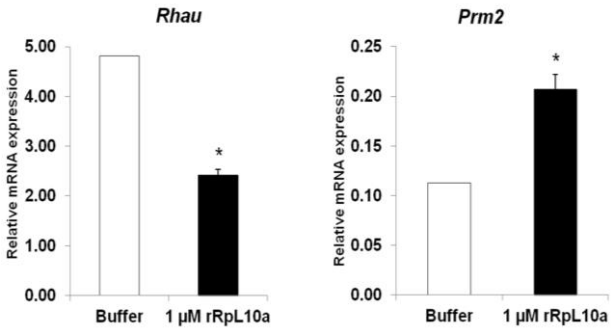

Figure 3. Effect of the recombinant RpL10a protein on the levels of *Rhau* and *Prm2* mRNA on mouse testis explants. The relative mRNA expression levels of *Prm2* and *Rhau* in the testis explants normalized with *β-actin* after incubation with the rRpL10a protein at 1µM (mean±SD, n=2 and P<0.05). Significant differences were compared by *t*-test.

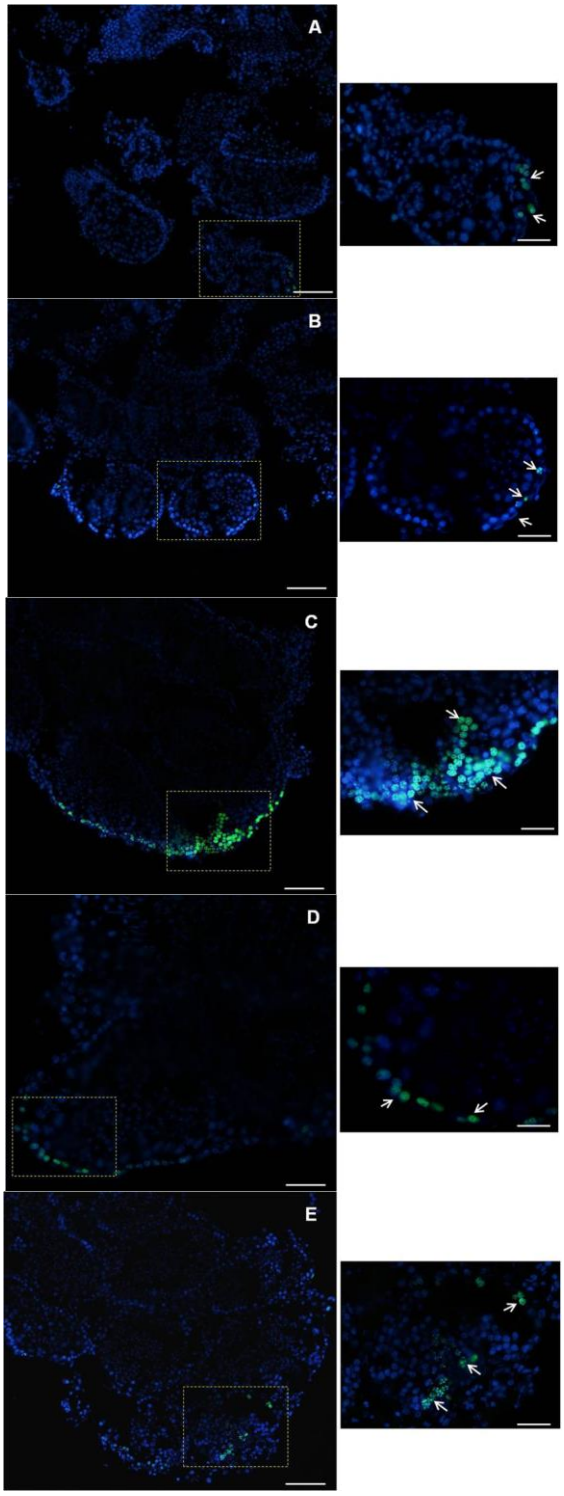

Figure 4. Effect of rRpL10a protein on cell proliferation. The testis explants were incubated with EdU and stained with Alexa Fluoro 488 (green) and DAPI (blue) staining. The mouse testis tissue was incubated with different doses of rRpL10a protein: (A) buffer as control, (B) 0.5 μM, (C) 1.0 μM, (D) 1.5 μM, and (E) 2.0 μM. Original magnification ×200, scale bars = 50 μm. In the magnified inserts, original magnification ×400, scale bars = 20 μm.

DNA of the proliferating spermatogenic cells in mice testis treated with 1 μM of rRpL10a protein. The 1.5 and 2.0 μM doses had some intensity of DNA labeling, whereas the 0.5 μM and buffer group had weak signals of EdU staining. The results revealed that the rRpL10a protein, particularly at 1 μM, promoted spermatogonia proliferation.

4. Discussion

In this study, the rRpL10a protein was examined as a stimulator for spermatogenesis in mice. It appears that rRpL10a protein was able to induce the spermatogenesis *in vitro* from spermatogonia cells into spermatid cells within 4 h. The results also showed evidence that the rRpL10a protein triggered spermatogonial proliferation which occurred in the early stage of spermatogenesis. The results agreed with the results of treatment of the shrimp's ovarian explants at 1 μM of rRpL10a protein for 4 h. After rRPL10a incubation, the genes involved in early vitellogenesis, including *sop*, *tctp*, and *hsp70* gene, were upregulated in ovarian tissues at their maximal level at 4 h (Wonglapsuwan *et al.*, 2010). In addition, the optimal concentration of rRpL10a protein was required. From the previous results, the excess rpl10a in *Drosophila* led to the death of cells in the germline and the eyes (Wonglapsuwan *et al.*, 2011). Beyond the function in the ribosomes, many ribosomal proteins played secondary functions (Wool, 1996). Therefore, it is possible that RpL10a protein might play the extra function in triggering spermatogenesis in mice.

Lectins could bind to the surface of spermatogenic cells because of the surface modification process in conjunction with the change of the intracellular glycoprotein during germ cell differentiation (Koehler, 1981). Therefore, lectins are useful tools for testicular spermatozoa inactivation detection. Lee and Damjanov (1984) reported that WGA reacted with all mouse spermatogenic cells, but its intensity increased in the late stage of spermatogenesis, which was apparently detected in the surface of spermatid cells. WGA was also positive in acrosomal granules of Golgi-phase spermatids (Arya & Vanha-Perttula, 1986). Similarly, Söderström, Malmi, and Karjalainen (1984) reported that WGA stained the acrosomes of the late spermatids after formalin and permeabilization. The acrosomes of spermatids contain N-acetyl-D-glucosamine sugar residues that were specifically bound with WGA lectin (Lee & Damjanov, 1984). In addition, increasing the intensity of WGA binding in spermatids indicated that the WGA reactive proteins were continuously integrated to the sperm surface formed during spermatogenesis.

RNA helicase associated with AU-rich element (*Rhau*) gene was reported to be required for spermatogonial differentiation. Gao *et al.* (2015) reported that *Rhau* gene was up-regulated in mouse testis much higher than in other tissues. They also reported that *Rhau* is expressed at a high level in spermatogonial stem cells (SSC) and primary spermatocytes, but expressed at a low level in spermatid cells. In the *Rhau* knockout testes, the percentages of primary spermatocytes apparently decreased which resulted in abnormal cell proliferation during spermatogonial differentiation. Therefore, our results also provided evidence that *Rhau* plays an important role in spermatogonial differentiation.

Protamines are the major proteins in the nucleus of sperm that are associated with late chromatin condensation in the spermatogenesis. There are two types of protamines in

mice which are protamine 1 (Prm1) and protamine 2 (Prm2). Both protamine mRNAs were detected in the round spermatid stage and translated during the elongating spermatid stage (Bower, Yelick, & Hecht, 1987). When the mouse *Prm1* or *Prm2* gene is interrupted, it leads to the reduction of specific protein quantity and Prm2 aberration. Furthermore, Prm2 defective sperm were dramatically affected which resulted in blastocyst development of mouse eggs. The results indicated that *Prm2* plays an important role in sperm chromatin condensation which is crucial during fertilization and early embryonic development (Cho *et al.*, 2003). Alteration of *Prm1* and *Prm2* gene expressions and mutation of protamine genes have caused infertility in man (Oliva, 2006). Moreover, deficiency of protamine has greatly affected the decrease of number, motility, and spermatozoa morphology (Akmal, Widodo, Sumitro, & Purnomo, 2016). These reports strongly indicated that the *Prm2* gene is an essential gene in the late stage of spermatogenesis.

In our study *Rhau* gene was used as a marker gene in the early stage of spermatogenesis. A 4-h treatment using rRpL10a protein in mouse testes decreased the expression of *Rhau* gene compared with the control buffer. Similarity, the results also indicated that after incubating with rRpL10a protein for 4 h, the expression of *Prm2* gene was upregulated in contrast to the *Rhau* gene which was downregulated. In addition, the rRpL10a protein apparently affected the proliferation of spermatogonia cells in a way that rRpL10a protein stimulates the efficiency of spermatogonia replication.

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