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Dietary cholesterol affects expression of prostatic acid phosphatase in reproductive organs of male rats

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ABSTRACT

Cholesterol homeostasis is strictly maintained to prevent abnormal biological processes that arise from excessive accumulation of cholesterol in tissues. Although dyslipidemia causes reproductive dysfunction and endocrine disruption in male rats, regulatory factors and mechanisms have not been clearly demonstrated. Therefore, the present study investigated the histology of male reproductive organs and the expression of prostatic acid phosphatase (also known as *Acpp*) that is secreted by cuboidal epithelium of the prostate gland in response to a normal diet and a high-cholesterol diet. The high cholesterol diet increased total cholesterol and low density lipoprotein (LDL) levels and decreased high density lipoprotein (HDL) levels. Histological analyses showed considerable alterations in the prostate indicating excessive papillary projections within the acinar lumen in response to the high cholesterol diet. In addition, *Acpp* expression was decreased in the penis of rats fed the high cholesterol diet and it was predominantly localized in the urethral epithelium and penile follicle that is precursor of penile spines. Moreover, *Acpp* was reduced slightly in the testes, but differential expression of *Acpp* in the prostate in response to dietary cholesterol was not detected. Furthermore, target microRNAs (miRs) of *Acpp* such as *miR-192* and *miR-215* regulated *Acpp* gene expression at the post-transcriptional levels by binding to specific sites within its 3′-UTR. These results indicate that *Acpp* plays an important role in growth and development of the penis of rats, and its expression is modulated at epigenomic levels via specific miRs.

1. Introduction

Cholesterol plays important roles in various physiological processes as a precursor molecule for biosynthesis of bile acid, vitamin D and steroid hormones for growth and development, and as an essential component of cell membranes in humans and animals [1]. Concentration of cholesterol in serum and in cells is tightly regulated to maintain cholesterol homeostasis for prevention of dyslipidemia [2]. Excessive levels of cholesterol cause multiple physiological diseases including obesity [3], cardiovascular disease [4] and Alzheimer's disease [5]. In addition, the high levels of cholesterol in plasma also known as hypercholesterolemia is associated with dysfunction of male reproductive organs and leads to male infertility [6]. However, research to define the biological functions and metabolism of cholesterol in the male reproductive system requires more studies.

For successful reproductive processes, the male reproductive system comprised of many sexual organs located outside the body and within the pelvic region must function correctly. Among the primary reproductive organs are the penis and testes, and the prostate gland is an accessory sex gland. The main function of the penis is to achieve erection for mating which requires functional corpora cavernosa, corpora spongiosum and glans penis for copulation [7]. The primary functions of the testes are spermatogenesis and production of male sex hormones, particularly testosterone in response to Luteinizing hormone from the anterior pituitary gland [8]. The prostate gland surrounds the urethra and secrets a slightly alkaline fluid appearing milky or white and accounting for a portion of the seminal fluid in which spermatozoa are suspended during ejaculation [9]. Therefore, maintenance of functions of male sexual organs is important for reproductive success of species. However, according to recent studies, elevated cholesterol levels due to high fat diets leads to an increased risk of a number of urological disorders such as penile erectile dysfunction and dysfunctional spermatogenesis, and benign prostatic hyperplasia and cancer [6,7,10].

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ACPP is well-known as a prognostic biochemical indicator for diagnosis and monitoring of progression of prostate cancer, so it is considered an essential regulator of cell growth and proliferation in the prostate. ACPP is a 100 kDa secreted glycoprotein enzyme synthesized in epithelial cells of the prostate gland in humans [11]. Its enzymatic activity requires formation of a homodimer of two catalytically inactive 50 kDa subunits by non-covalent binding [12,13]. The expression of *ACPP* mRNA is abundantly distributed in the prostate gland, and there is lower expression of *ACPP* in kidney, bladder, pancreas, small intestine, placenta, testes, lung and ovary [14,15]. On the other hand, understanding molecular mechanisms regulating *ACPP* expression is required [16]. In accordance with high fat diet, there is little information about effects of cholesterol on *ACPP* expression in the male reproductive system. Therefore, the objectives of this study were to: (1) investigate the physiological changes in reproductive organs, including penis, prostate and testes, of male rats fed a normal or a high fat diet; (2) examine effects of high fat diet on expression of *Acpp* mRNA and protein in the three selected organs of male rats; and (3) determine if *Acpp* is post-transcriptionally regulated by specific microRNAs. Results of the present study provide novel insights into the *Acpp* gene with regard to its cell- and tissue-specific expression in response to hypercholesterolemia, and regulation of its expression by target microRNAs in the reproductive organs of male rats.

2. Materials and methods

2.1. Experimental animals and diets

The experimental use of rats for this study was approved by the Animal Care and Use Committee of Korea University. Sprague–Dawley (SD) rats (Samtako, Kyunggi, Korea) were exposed to a light regimen of 12 h light and 12 h dark with *ad libitum* access to water, and subjected to standard institutional guidelines for animal management. A total of 12 male SD rats were obtained at the age of 7 weeks. Animal diets were formulated on the basis of AIN-76 (American Institute of Nutrition, 1977; American Institute of Nutrition, 1980). For this study, 12 rats were assigned randomly ($n = 6$ rats/diet) as follows: (1) normal diet with phosphate buffer saline (PBS) or and (2) high cholesterol diet (accounting for 0.5% of cholesterol to normal diet) with PBS. The respective diets were fed for 5 weeks. Composition of experimental diets is provided in Supplemental Table 1.

2.2. Tissue samples

Rats ($n = 6$) in each group were euthanized with ether, then tissues were collected using liquid nitrogen for freezing tissues and fresh 4% paraformaldehyde in PBS (pH 7.4) for fixation of tissues.

2.3. RNA isolation

Total cellular RNA was isolated from frozen tissues using Trizol reagent (Invitrogen) according to manufacturer's recommendations.

2.4. Histology

For histological analyses, paraffin sections (5 μm) from isolated penis, prostate and testis of rats were deparaffinized and stained with either Masson's trichrome (Sigma) or hematoxylin (Sigma) and eosin (Thermo Fisher Scientific). The images were captured using a Leica DM3000 microscope.

2.5. Quantitative RT-PCR analysis

The level of expression of *Acpp* mRNA in the chicken oviducts and ovaries was assessed using quantitative RT-PCR as described previously [17].

2.6. In situ hybridization analysis

Location of *Acpp* mRNA in sections (5 μm) of rat penis, prostate and testis was determined by *in situ* hybridization analysis as described previously [17].

2.7. Immunohistochemistry

Immunocytochemical localization of Acpp protein was performed as described previously using a rabbit polyclonal antibody to Acpp (catalog number: 15840-1-AP, Proteintech Group, Inc.) at a final dilution of 1:100 (2.7 μg/ml). Antigen retrieval was performed using the boiling citrate method as described previously [18].

2.8. MicroRNA target validation assay

Post-transcriptional regulation of *Acpp* was determined using *in vitro* target validation assay for *miR-192* or *miR-215* as described previously [17].

2.9. Statistical analyses

Data from real-time PCR analyses are expressed as mean ± SEM unless otherwise stated. Differences in the variances between normal diet and high fat diet with respect to effect of cholesterol on rats were analyzed using the F test, and differences between means were subjected to the Student's *t*-test. Differences with a probability value of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Histological changes in reproductive organs of male rats fed the high fat diet

Histological characteristics of the penis of the rats were assessed using cross- and longitudinal-sections of the penises from rats fed a normal diet and a high fat diet (Fig. 1A). Compared with rats on the normal diet, the high fat diet with cholesterol gave no detectable histological differences based on staining with Masson's trichrome. However, histological differences were detected when prostate glands and testes were analyzed following hematoxylin and eosin staining (Fig. 1B). Histological analysis of prostate tissues revealed benign prostatic hyperplasia in response to the high fat diet and cholesterol. For rats fed the normal diet, secretory long cylinder-shaped cells (C) lined the lumen of the prostate. For rats on the high fat diet with cholesterol the glandular epithelial cells of the prostate had proliferated excessively to form papillary projections within the acinar lumen of glands. There were no detectable histological changes in testes from normal and cholesterol-treated rats.

3.2. Cell-specific expression of Acpp in the rat penis

Quantitative RT-PCR analyses revealed that *Acpp* mRNA expression was decreased by 72% ($P < 0.001$) in penises of rats fed the high fat diet with cholesterol as compared to the normal diet (Fig. 2A). The cell-specific localization of *Acpp* mRNA and protein was the urethral epithelium (UE), penile epithelium (PE) and penile follicles (PF) of rats fed the normal diet (Fig. 2B and C).

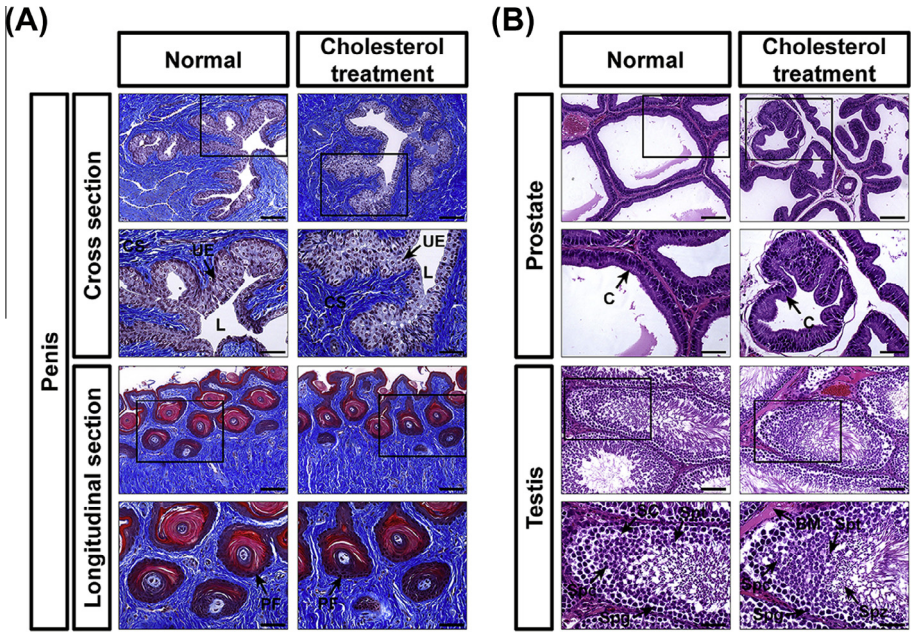

Fig. 1. Histological evaluation of penises, prostate glands and testes of rats on normal and high fat diets. (A) The cross- and longitudinal-sections of penises were observed following Masson's trichorome staining. (B) The cross sections of prostate glands and testes were observed following staining with hematoxylin and eosin (H&E). Legend: BM, basement membrane; C, cylinder-shaped cells; CS, corpus spongiosum; L, lumen; PF, penile follicle; SC, Sertoli cell; Spc, spermatocyte; Spg, spermatogonium; Spt, spermatid; Spz, spermatozoa; UE, urethral epithelium. *Scale bar* represents 10 μm (the first- and third-horizontal panels) and 5 μm (the second- and fourth-horizontal panels).

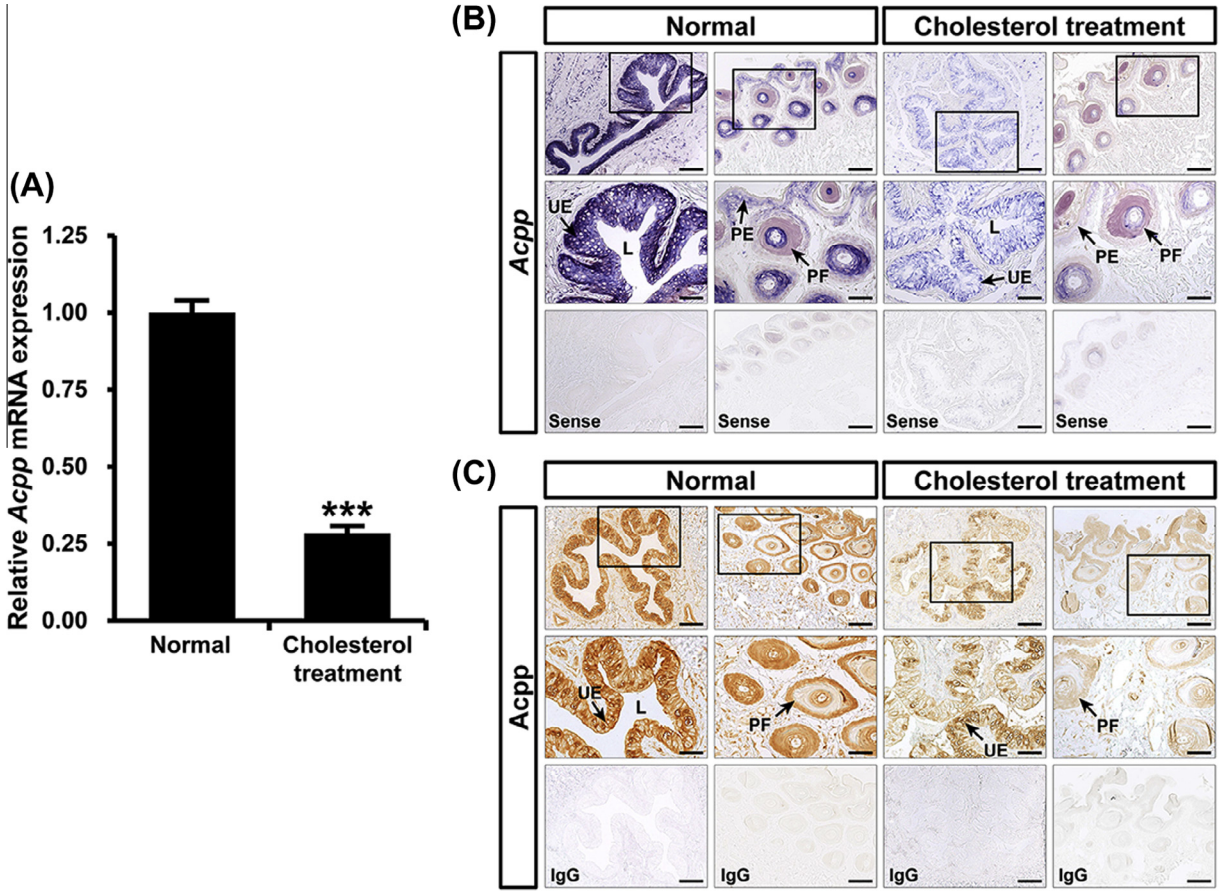

Fig. 2. Distribution and localization of *Acpp* mRNA and protein in penises of rats. (A) Quantitative RT-PCR analyses were performed using cDNA templates from on normal and high fat diets (mean ± SEM; $^{***}P < 0.001$). (B) *In situ* hybridization analyses indicated cell-specific expression of *Acpp* mRNA in penises from cholesterol-treated and non-treated rats. Cross- and longitudinal-sections of penises treated with cholesterol or vehicle were hybridized with antisense or sense rat *Acpp* cRNA probes. (C) Immunoreactive Acpp protein in penises from cholesterol-treated and non-treated rats. Sections were not counterstained. Legend: CS, corpus spongiosum; L, lumen; PE, penile epithelium; PF, penile follicle; UE, urethral epithelium. *Scale bar* represents 10 μm (the first horizontal panels, sense and IgG) and 5 μm (the second horizontal panels).

3.3. Effects of high fat diet with cholesterol on Acpp expression in the prostate gland

Next, we examined the effects of the high fat diet with cholesterol on *Acpp* expression in rat prostate glands. As illustrated in Fig. 3A, differences of *Acpp* mRNA expression were only 10% less for rats fed the high fat diet. *In situ* hybridization analyses indicated that *Acpp* was expressed mainly in the cylinder-shaped cells (C) of prostate glands from rats fed normal and high fat diets. *Acpp* mRNA and protein were abundant in cylinder cells of the prostate of rats fed normal and high fat diets. The rabbit IgG used as a negative control did not detect Acpp.

3.4. Effects of high fat diet with cholesterol on Acpp expression in the rat testes

Tissue specific expression of *Acpp* mRNA decreased 23% ($P < 0.01$) in testes of rats fed a high cholesterol compared to a normal diet (Fig. 3D). *In situ* hybridization and immunohistochemistry analyses revealed that Acpp was expressed predominantly in Sertoli cells (SC), spermatogonia (Spg), spermatocytes (Spc), spermatids (Spt) and spermatozoa (Spz) of testes fed a normal diet, whereas expression of Acpp was weakly detected in those components of testes of rats fed a high fat diet (Fig. 3E and F).

3.5. Post-transcriptional regulation of microRNAs affecting Acpp

To investigate the possibility that expression of *Acpp* is regulated at the post-transcriptional level by microRNAs (miRNAs), we selected highly ranked and putative binding sites for *miR-192* and *miR-215* within the 3′-UTR of *Acpp* (Fig. 4A). Next, a fragment of the 3′-UTR of *Acpp* was cloned downstream of the GFP open reading frame (ORF) sequence leading to establishment a fluorescent reporter for the *Acpp* 3′-UTR region whereas target miRNAs were inserted between the red fluorescent protein ORF sequence and poly A as illustrated in Fig. 4B. For analyses of the GFP intensities and the percentage of GFP-expressing cells, eGFP-3′-UTR and DsRed-miRNA were co-transfected into 293FT cells and estimated using fluorescence activated cell sorting (FACS) and fluorescence microscopy. Results of the present study indicated that binding of a predicted *miR-192* and *miR-215* to their specific target sequences in the *Acpp* 3′-UTR reduced the intensity and percentage of GFP-*Acpp*-expressing cells 49.4% and 61.8%, respectively as compared to a control (Fig. 4C and D).

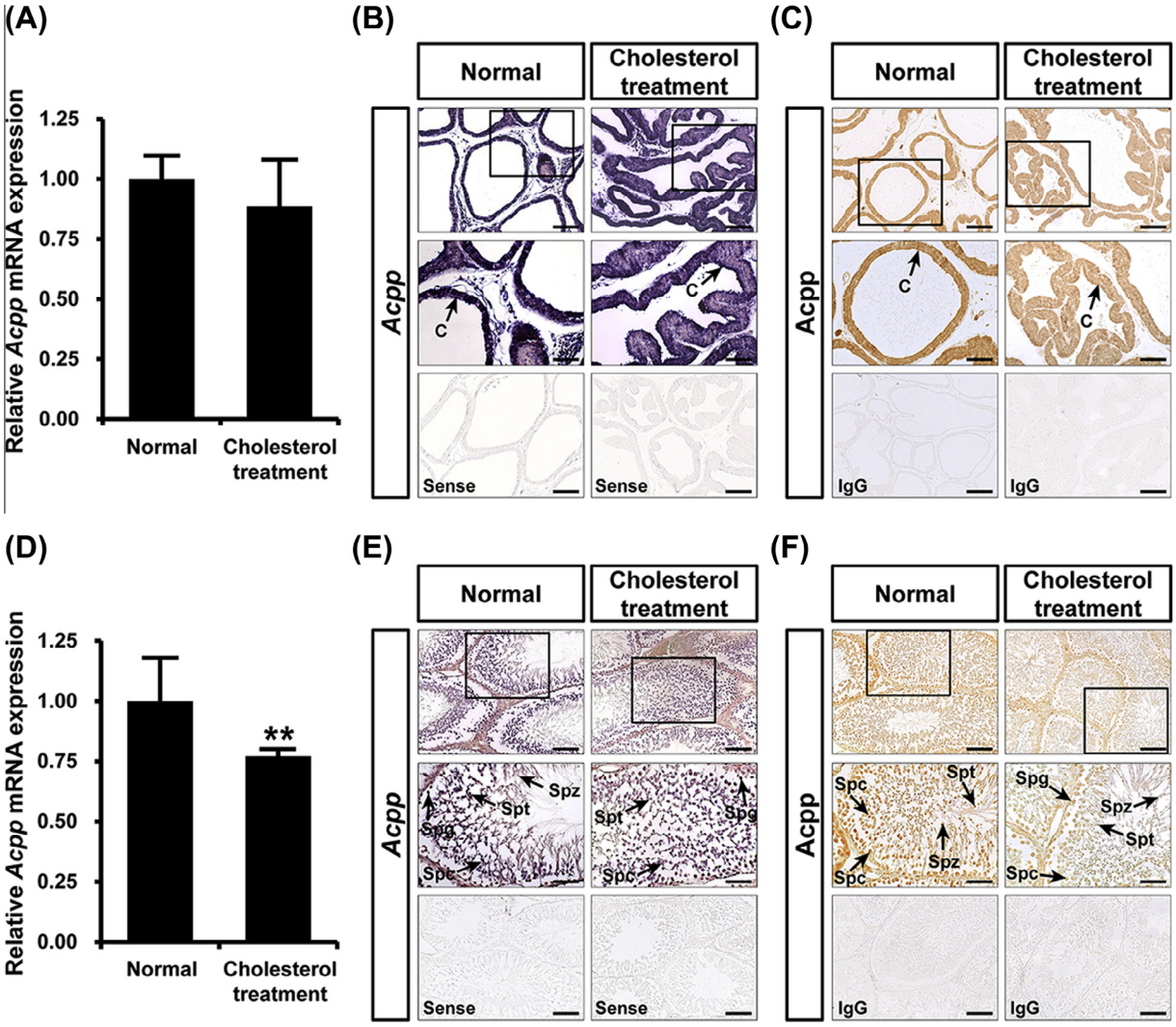

Fig. 3. Effect of cholesterol on cell-specific expression of Acpp in prostate glands and testes of rats. (A-C) Expression of *Acpp* in prostate glands from cholesterol-treated and non-treated rats was analyzed using quantitative RT-PCR (A), *in situ* hybridization (B) and immunohistochemistry (C). (D and E) Expression of *Acpp* in testes from cholesterol-treated and non-treated rats was analyzed using quantitative RT-PCR (D), *in situ* hybridization (E) and immunohistochemistry (F). *Scale bar* represents 10 μm (the first horizontal panels, sense and IgG) and 5 μm (the second horizontal panels).

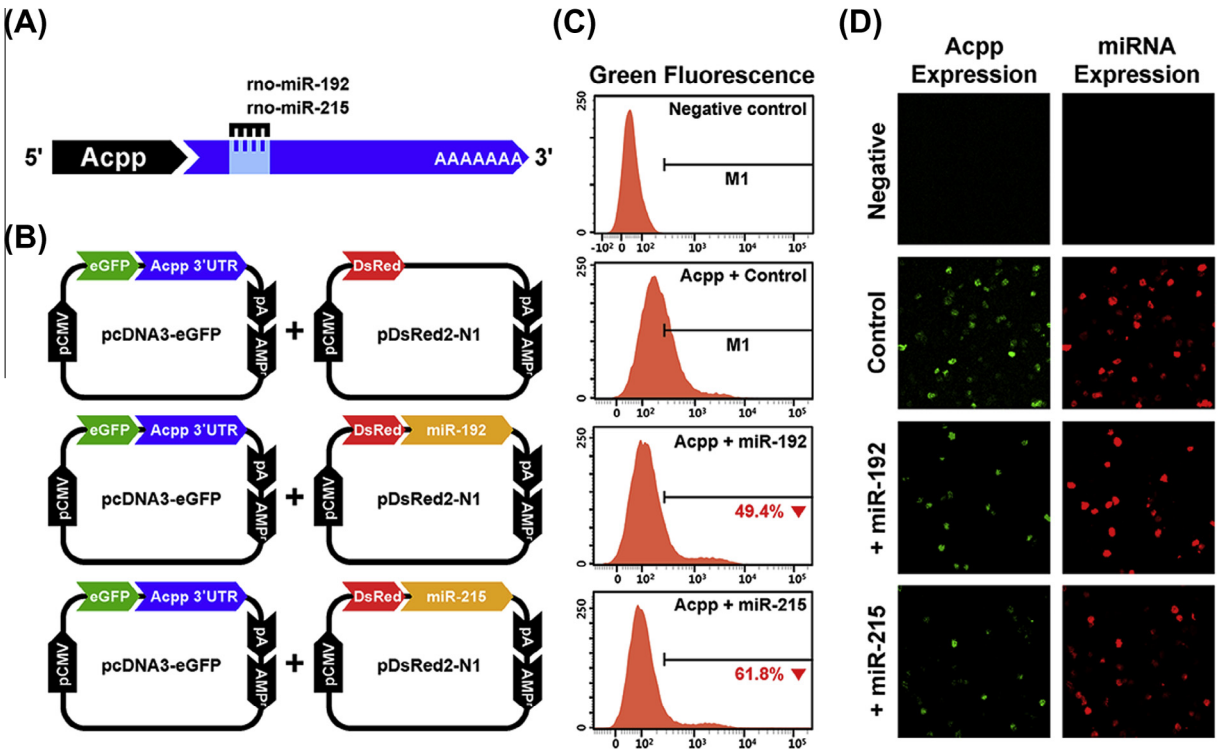

Fig. 4. *In vitro* target assay and quantitation of microRNAs regulating *Acpp* transcripts. (A) Diagram of *miR-192* and *miR-215* binding site in *Acpp* 3′-UTR. (B) Expression vector maps for eGFP with *Acpp* 3′-UTR and Ds-Red with *miR-192* and *miR-215*. The 3′-UTR of the *Acpp* transcript was subcloned between the eGFP gene and the polyA tail to generate the fusion construct of the GFP transcript following the miRNA target 3′-UTR (pcDNA-eGFP-3′-UTR) (left panel) and the miRNA expression vector was designed to co-express DsRed and *miR-192* and *miR-215* (pcDNA-DsRed-miRNA) (right panel). (C and D) After co-transfection of pcDNA-eGFP-3′UTR for the *Acpp* transcript and pcDNA-DsRed-miRNA for the *miR-192* and *miR-215*, the fluorescence signals of GFP and DsRed were detected using FACS (C) and fluorescent microscopy (D).

4. Discussion

Results of the present study reveal cell-specific expression of *Acpp* in male reproductive organs of rats, particularly penis, prostate gland and testes with respect to effects of a high fat diet. Also, our results indicated that expression of *Acpp* is regulated by specific target miRNAs including *miR-192* and *miR-215* at the post-transcriptional level through binding directly to their target sequences in the 3′-UTR of the *Acpp* gene. These results support our hypothesis that *Acpp* might be involved in dysfunction of the male reproductive organs from rats in response to a high-fat diet and that its expression is controlled by post-transcriptional regulation via specific miRNAs in rats.

Most previous studies focused on the erectile dysfunction in males due to hypercholesterolemia that was linked to deficiencies in the vascular supply to the corpus cavernosum penis in response to excitatory stimuli. However, the physiological mechanisms responsible for erectile dysfunction due to hypercholesterimia are not well known. To investigate regulators for male reproduction affected by concentrations of cholesterol in serum, we established the rat as an animal model for comparing effects of a normal diet and a diet containing 5% cholesterol. Supplemental Table 2 shows that concentrations of total and LDL cholesterol were significantly increased in rats fed a high fat diet with cholesterol compared to a normal diet. On the other hand, HDL cholesterol decreased in response to feeding the hypercholesterolemic diet. Several previous studies revealed high risks of erectile dysfunction due to increases in total and LDL cholesterol and a decrease in HDL cholesterol [19,20].

In humans, *ACPP* is abundant in seminal fluid. In the present study, *Acpp* expression was decreased significantly in penises of rats fed a high-cholesterol diet as compared to a normal diet. And its expression was mainly localized to the urethral epithelium of the rat penis (see Fig. 2). In rodents, the urethra can be separated into the pelvic and penile portions [21]. Penile urethra is surrounded by the corpus spongiosum of the penis, and transports urine as well as semen. Epithelial cells of urethra are characterized based on their position within the urethra to include pseudostratified columnar epithelium between pelvic and penile urethra and stratified squamous epithelium at the external orifice of the urethra on the glans penis [22]. *Acpp* was expressed predominantly in the penile epithelium (PE) and penile follicles (PF). Numerous PFs are characterized as dermic papillae that form penile spines consisting of cone-like keratinized structures and mesenchymal cells around the penile skin of rodents. The keratinized projections of penile spins stimulate the vagina during copulation that results in ovulation. Moreover, they support fertility at a subsequent ejaculation by removal vaginal plugs deposited previously before ejaculation [23,24]. From the present results, a high fat diet with cholesterol down-regulates the *Acpp* gene in the penis and affects dysfunction of the male reproductive system.

Histological analysis of prostatic tissues in rats indicated excessive cells and glands when feeding a high cholesterol diet whereas rats on a normal diet had round regular acini with intact basement membranes [25]. Especially, in the rats fed the high fat diet, there were increased numbers of cells with irregular acinar growth and distribution associated with atypical hyperplasia of prostate tissues lined by cylinder cells with villous projections (see Fig. 1B). However, the hyperplastic changes were not accompanied by differential expression of *Acpp* due to diet (Fig. 3). *ACPP* is known as a biomarker for benign prostate hyperplasia and prostate cancer that are most common pathophysiological diseases of prostate glands in individuals at advanced ages [26], and its expression is abundant in normal, cancerous prostate tissues [15]. Previous

studies revealed that the incidence of prostate cancer is associated with cholesterol levels indicating that high concentrations of total cholesterol and LDL increase the risk for prostate cancer [27]. These results suggest that a high-cholesterol diet induces benign prostate hyperplasia with abundant expression of *Acpp* in prostate tissue.

The accumulation of cholesterol increases the incidence of impaired testicular steroidogenesis due to alterations in the endocrine system through effects on steroid receptors and transcription factors [28]. In addition, a cholesterol-enriched diet influences sperm quality, quantity and fertilizing potential. Also, it particularly decreases kinetics of the acrosome reaction in spermatozoa [29]. For maintenance of cellular cholesterol homeostasis, liver X receptors (*LXR*) alpha and beta, proliferating peroxisomal activated receptors (*PPARs*), liver receptor homolog 1 (*LRH1*) and steroidogenic factor 1 (*SF1*) play important roles as transcription factors regulating differentiation of germ cells, Sertoli cells and Leydig cells [30]. Moreover, *TNF-α* inhibits steroidogenesis of Leydig cells by preventing expression of steroidogenic acute regulatory protein (*StAR*) [31]. *TNF-α* also regulates *ACPP* gene expression by modulating the promoter activity of *ACPP* [32]. In this study, *Acpp* expression decreased in the testes of rats fed 0.5% cholesterol as compared with a normal diet. Therefore, the results from current study indicated that *Acpp* expression may be affected by endocrine and transcriptional factors in response to a high-cholesterol diet.

MicroRNAs (miRNAs) play important roles in various biological processes such as development, differentiation and immune response. Especially, miRNAs have emerging roles in lipid metabolism suggesting potential effects for development of therapeutics [33]. For examples, *miR-33a* and *-b* were identified as key regulators for cholesterol and fatty acid metabolism modulating sterol regulatory element-binding transcription factor 1 (*SREBF1*) and *SREBF2* genes [34–36]. Repression of *miR-33* stimulates the ATP-binding cassette, sub-family A, member 1 (*ABCA1*) expression leading to increased biogenesis of HDL and transposed cholesterol metabolism [37]. In the current study, we investigated the role of miRNAs as regulators for the *Acpp* gene in rats fed a normal and a hypercholesterolemic diet using miRNA target validation assay. Results of the present study indicated that the 3′-UTR of rat *Acpp* gene contains *miR-192* and *miR-215* binding sites, and the two miRNAs attenuate intensity of GFP-*Acpp* expression in cells by directly binding to the their specific sites at the 3′-UTR. Therefore, our results provide evidence that *miR-192* and *miR-215* are closely associated with regulation of *Acpp* gene expression in response to lipid metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.100.

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