Am J Transl Res 2016;8(6):2776-2782
www.ajtr.org /ISSN:1943-8141/AJTR0020298

Original Article

FSCB phosphorylation regulates mouse spermatozoa capacitation through suppressing SUMOylation of ROPN1/ROPN1L

Xinqi Zhang[1], Mingrui Chen[2], Renyi Yu[2], Benli Liu[2], Zhiqiang Tian[3], Shunli Liu[2]

Departments of [1]Emergency, [2]Burns and Plastic Surgery, General Hospital of Jinan Military Region, Jinan 250031, China; [3]Institute of Immunology, PLA, Third Military Medical University, Chongqing 400038, China

Received November 22, 2015; Accepted January 1, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: Fibrous sheath CABYR binding protein (FSCB) is regulated by protein kinase A (PKA)-mediated tyrosine phosphorylation in the spermatozoa capacitation. Recently, we showed that FSCB phosphorylation activated spermatozoa motility. Nevertheless, the underlying mechanisms have not been completely elucidated. Here, we showed that FSCB phosphorylation inhibited SUMOylation of two crucial proteins ROPN1/ROPN1L that are associated with PKA/A kinase activity and spermatozoa motility. Suppression of SUMOylation of ROPN1/ROPN1L mimicked the effects of FSCB phosphorylation on spermatozoa motility. Immunoprecipitation assay showed that phosphorylated FSCB had a significantly higher affinity to ROPN1/ROPN1L than non-phosphorylated FSCB. Together, our data suggest that FSCB phosphorylation may regulate mouse spermatozoa capacitation through suppressing SUMOylation of ROPN1/ROPN1L, which sheds new light on creating a therapeutic strategy targeting FSCB phosphorylation in the study of infertility.

Keywords: Infertility, SUMOylation, phosphorylation, sperm, FSCB, ROPN1/ROPN1L

Introduction

Ejaculation of Spermatozoa freshly into the female reproductive tract is followed by a series of biochemical changes for obtaining capacity of fertilization, called spermatozoa capacitation [1-3]. Tyrosine phosphorylation is a critical regulator during spermatozoa capacitation to generate spermatozoa tail fibrous sheath as an important mechanical component of flagella that produce glycolytic enzymes important for sperm motility and for constituents of signaling cascades [4].

There are more than 20 proteins associated with the fibrous sheath, including Calcium Binding Tyrosine-(Y)-Phosphorylation Regulated (CABYR) protein, fibrous sheath CABYR binding protein (FSCB), ROPN1 (ropporin 1), ROPN1L (ROPN1-like protein, formerly known as ASP), etc [1-3]. Among these proteins, FSCB is a recently detected protein expressed specifically on the surface of the fibrous sheath of the mouse spermatozoa principal piece, which can be phosphorylated by PKA. We recently reported that FSCB is capable of being phosphorylated and binding to calcium, which enhance the spermatozoa flagellar movement, spermatozoa capacitation and activation [5]. However, the underlying mechanisms have not been completely elucidated. On the other hand, ROPN1/POPN1L are proteins associated with cAMP-dependent protein kinase (PKA)/A-kinase anchoring protein (AKAP), and are essential for murine sperm motility, phosphorylation, and fibrous sheath integrity [6, 7]. Nevertheless, the relationship between FSCB and ROPN1/POPN1L has not been determined.

SUMO is a small ubiquitin-like protein which is covalently attached to proteins through generating isopeptide bonds with specific lysine residues of target proteins [8-12]. The mammalian SUMO protein family includes 4 members (SUMO-1-4) [8-12]. SUMO targets lysine by an enzymatic cascade composed of 3 enzymes: E1 (Uba2/Aos1), E2 (Ubc9), and E3 ligases [8-12]. SUMO conjugation is initiated by formation of a thioester bond with the activating enzyme E1, a heterodimer of Aos1 and Uba2

[8-12]. Afterwards, Aos1/Uba2 transfers SUMO to the single E2-conjugating enzyme Ubc9, which is responsible for SUMOylation of the substrate [8-12]. The Ubc9 substrate recognition is substantialized by specific E3 SUMO ligases [8-12]. Of note, whether protein SUMOylation is involved in the regulation of ROPN1/POPN1L degradation has not been studied before.

Here, we showed that FSCB phosphorylation inhibited SUMOylation and subsequent degradation of two crucial proteins ROPN1/ROPN1L that are associated with PKA/A kinase activity and spermatozoa motility. Suppression of SUMOylation of ROPN1/ROPN1L mimicked the effects of FSCB phosphorylation on spermatozoa motility. Immunoprecipitation assay showed that phosphorylated FSCB had a significantly higher affinity to ROPN1/ROPN1L than non-phosphorylated FSCB.

Materials and methods

Mouse spermatozoa isolation and culture

Male Kunming mice (Chongqing Laboratory Animal Center, Chongqing, China) were maintained in pathogen-free conditions. All of the animal studies were approved by the Institutional Animal Care and Use Committee at the Third Military Medical University, Chongqing, China. The mice were sacrificed at 12 weeks of age, and the epididymal end was cut as a whole, and was squeezed to the end by two tweezers. White mucilage was oozed out and a syringe was used to cut the epididymis, to allow large amounts of spermatozoa to flow out into a pre-equilibrated spermatozoa medium. Two kinds of spermatozoa M_2 medium were used in the current study. HTF medium (human tubal fluid SAGE, Trumbull, CT, USA) was a capacitation medium and M_2 media (Millipore, Billerica, MA, USA) was a non-capacitation medium. Inhibitor of SUMOylation 2D08 (2-(2,3,4-Trihydroxyphenyl)-4H-chromen-4-one, Millipore) is a cell-permeable trihydroxyflavone compound that prevents the transfer of SUMO from the E2 thioester to the substrate without affecting SUMO-activating enzyme E1 or E2 thioester formation, as was used in a concentration M_2 of 100 μmol/l in vitro.

Co-immunoprecipitation assay

Spermatozoa were cultured at a density of 10^8 cells/ml in pre-equilibrated HTF or M_2 media in a CO_2 incubator at 37°C for 2 h and lysed in 200 μl Cell lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). The lysates were processed to increase the solubility of the proteins with high molecular weight, as has been previously described [5]. The liquid in the ultrafiltration tube, i.e., post-ultrafiltration spermatozoa lysates, was transferred into an EP tube. 50 μl of Protein G (Sigma-Aldrich) was washed 3 times with 0.15 mol/l NaCl, and centrifuged at 10,000 × g for 1 min. The supernatants were discarded, and the sediments were incubated with the antibody for immunoprecipitation and 480 μl of 0.15 mol/l NaCl. The mixture was shaken for 90 min at room temperature, centrifuged at 10,000 × g for 1 min, and washed 3 times with 0.15 mol/l NaCl. Then, the sediments were added to 500 μl 0.15 mol/l NaCl containing 1 mg BS_3 (Thermo Scientific, Rockford, IL, USA), and shaken for 1 h at room temperature, followed by addition of 20 μl 1 mol/l Tris.HCl of pH 7.5, with a 30 min incubation at room temperature, then 1 min centrifugation at 10,000 × g. The sediments were washed 3 times with 0.15 mol/l NaCl. The supernatants were then removed, and post-ultrafiltration spermatozoa lysates were added to the sediments. The mixture was shaken overnight at 4°C, then centrifuged for 1 min at 12,000 × g. The supernatants were removed, and the sediments were washed 3 times with a balanced solution, and centrifuged at 12,000 × g for 1 min. The sediments were washed with 250 μl Cell lysis buffer, and centrifuged at 12,000 × g for 5 min. The final supernatants were used for immunoblot assay.

Immunoblot

The above co-immunoprecipitation products with different antibodies were quantified for total protein and subjected to 4-12% SDS-PAGE, followed by immunoblot analysis. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. The antibodies used in the IP and IB are anti-SUMO-1 antibody (Santa Cruz Biotechnology, Dallas, Texas, USA), anti-PY20 (Cell Signaling, San Jose, CA, USA), anti-FSCB (Abcam, Cambridge, MA, USA), anti-ROPN1 (Abcam) and anti-ROPN1L (Abcam). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images

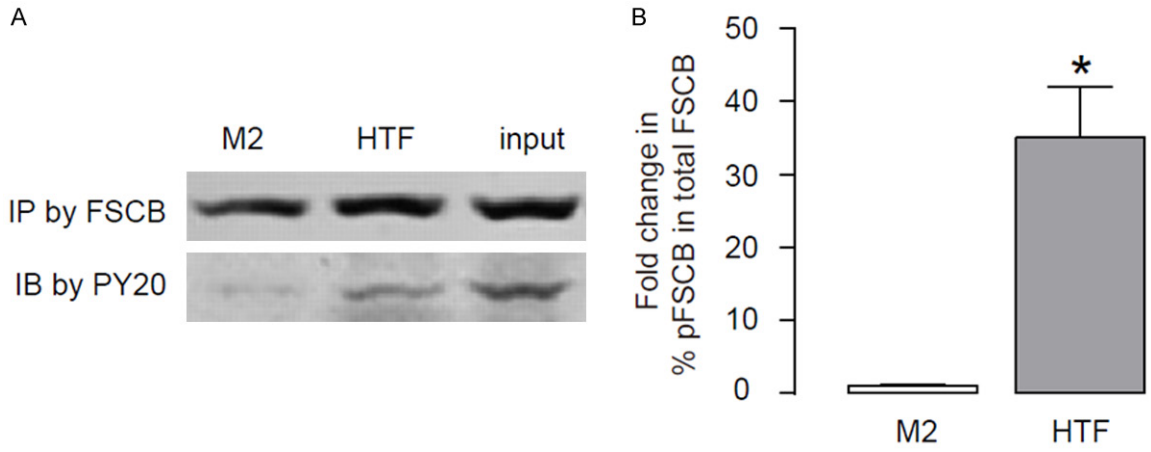

Figure 1. FSCB phosphorylation in mouse spermatozoa is induced in HTF media. The isolated mouse spermatozoa were cultured either in M_2 media (non-capacitation media) or HTF media (capacitation media). Then the protein was extracted 24 hours after culturing in the specific media, immunoprecipitated with anti-FSCB, and subsequently immunoblotted with PY20 antibody. (A, B) The results were shown by representative blots (A), and by quantification (B). pFSCB: phosphorylated FSCB. IP: immunoprecipitation. IB: immunoblot. *p<0.05. N=5.

shown in the figures were representative from 5 individuals. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were normalized to experimental controls.

Spermatozoa motility assay

Mouse spermatozoa were cultured in HTF medium, or M_2 medium, and M_2 medium containing 100 μmol/l 2D08. A 5 μl aliquot of spermatozoa was added to Microcells (Conception Technology, Benachity, India), and analyzed at 37°C at 1, 2, 5, 10, 20, 30, 60 and 120 min after capacitation using a CASA Spermatozoa Class Analyzer (Barcelona, Spain). Spermatozoa motility was classified into the following groups: class A (moving forward quickly), class B (moving forward slowly), class C (wobbling in-place), and class D (not moving). In addition, the VCL (curvilinear velocity) was all measured. (A + B)% and VCL were used and presented in this study for Spermatozoa motility.

In vitro SUMOylation assay

In vitro SUMOylation assays were performed using the SUMOylation kit (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA) according to the manufacturer's protocol. In brief, 200 nmol/l of purified recombinant human ROPN1 or ROPN1L protein was incubated with reaction mixture containing 50 mmol/Tris. HCl (pH 7.4), 2 mmol/l DTT, 5 mmol/l ATP, 10 mmol/l $MgCl_2$, Aos-1 (150 ng), His-Uba2 (400 ng), GST-Ubc9 (500 ng), and GST-SUMO-1 for 1 hour at 30°C. The control reaction was carried out in the absence of ATP. After the incubation, protein SUMOylation was identified by immunoblotting using the anti-SUMO antibody provided with the kit.

Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical significance was evaluated by Student's t test or one-way analysis of variance (ANOVA) followed by the Fisher's Exact Test, using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Results

FSCB phosphorylation in mouse spermatozoa is induced in HTF media

The isolated mouse spermatozoa were cultured either in M_2 media (non-capacitation media) or HTF media (capacitation media). Then the protein was extracted 24 hours after culturing in the specific media, immunoprecipitated with anti-FSCB, and subsequently immunoblotted with PY20 antibody, which detected all phosphorylated proteins. We detected significantly increases in the phosphorylated FSCB protein in mouse spermatozoa induced by HTF media, compared to in M_2 media (by about 35 fold increase), shown by representative blots

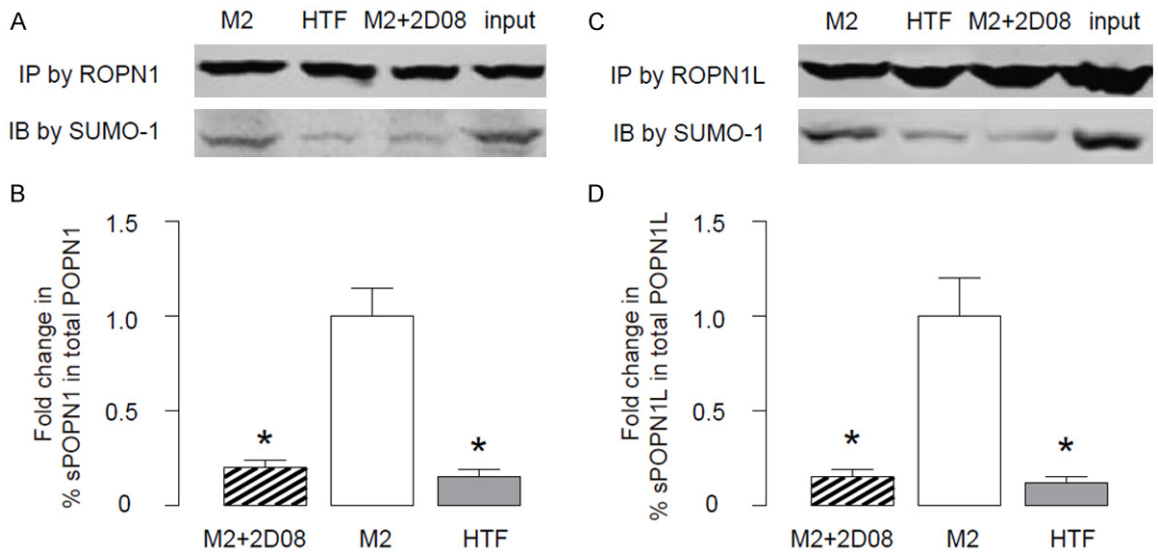

Figure 2. FSCB phosphorylation by HTF media inhibits SUMOylation of ROPN1/ROPN1L. The protein was extracted 24 hours after culturing in the M_2 media or HTF media or M_2 media with 100 μmol/l 2D08. (A, B) The protein was immunoprecipitated with anti-ROPN1, and subsequently immunoblotted with anti-SUMO1 antibody, which detected all SUMOylated proteins. The results were shown by representative blots (A), and by quantification (B). (C, D) The protein was immunoprecipitated with anti-ROPN1L, and subsequently immunoblotted with anti-SUMO1 antibody. The results were shown by representative blots (C), and by quantification (D). sROPN1: SUMOylated ROPN1. sROPN1L: SUMOylated ROPN1L. IP: immunoprecipitation. IB: immunoblot. *p<0.05. N=5.

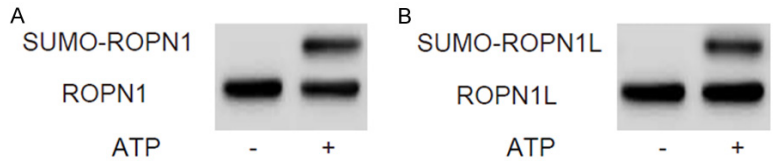

Figure 3. SUMOylation of ROPN1/ROPN1L is confirmed in in vitro SUMOylation assay. (A, B) In vitro SUMOylation assay was performed for ROPN1 (A), and for ROPN1L (B). N=5.

(**Figure 1A**), and by quantification (**Figure 1B**). Thus, FSCB phosphorylation in mouse spermatozoa is induced in HTF media, consistent with our previous reports.

FSCB phosphorylation by HTF media inhibits SUMOylation of ROPN1/ROPN1L

ROPN1/ROPN1L are two proteins associated with PKC/A kinase activity and play critical roles spermatozoa motility. Then, we analyzed the effect of FSCB phosphorylation by HTF media on the SUMOylation of ROPN1/ROPN1L. The protein was extracted 24 hours after culturing in the specific media, immunoprecipitated with anti-ROPN1, and subsequently immunoblotted with anti-SUMO1 antibody, which detected all SUMOylated proteins. We detected significantly decreases in the SUMOylated ROPN1 protein in mouse spermatozoa induced by HTF media, compared to in M_2 media (by about 80%), shown by representative blots (**Figure 2A**), and by quantification (**Figure 2B**). Moreover, the extracted protein was also immunoprecipitated with anti-ROPN1L, and subsequently immunoblotted with anti-SUMO1 antibody. Similarly, we detected significantly decreases in the SUMOylated ROPN1L protein in mouse spermatozoa induced by HTF media, compared to in M_2 media (by about 80%), shown by representative blots (**Figure 2C**), and by quantification (**Figure 2D**). Together, these data suggest that FSCB phosphorylation by HTF media inhibits SUMOylation of ROPN1/ROPN1L.

SUMOylation of ROPN1/ROPN1L is confirmed in in vitro SUMOylation assay

To further confirm the occurrence of the SUMOylation of ROPN1/ROPN1L, we performed an in vitro SUMOylation assay. Slow moving immunoreactive bands of both ROPN1 was recognized by the anti-SUMO antibody. When the assay was performed without ATP as a control,

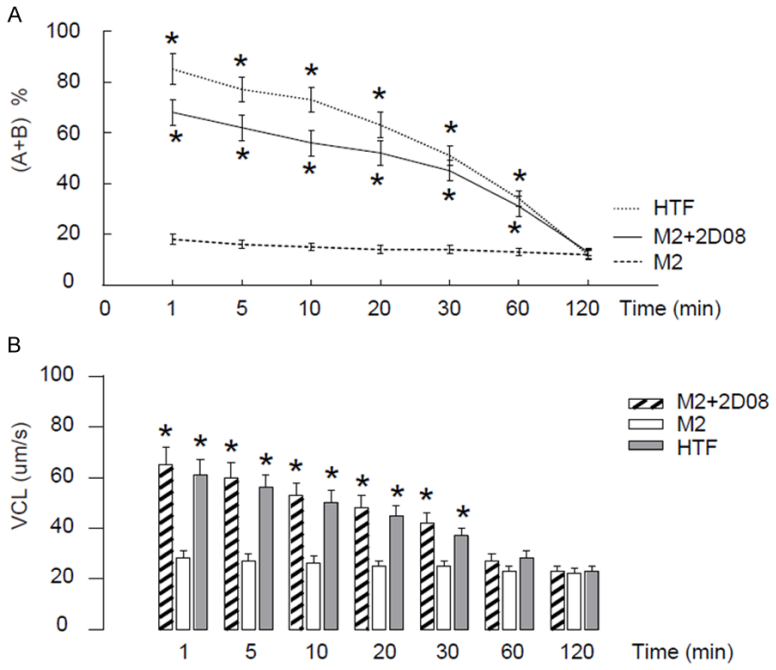

Figure 4. Suppression of SUMOylation of ROPN1/ROPN1L mimics the effects of FSCB phosphorylation on spermatozoa motility. We examined the effects of Suppression of SUMOylation of ROPN1/ROPN1L on spermatozoa motility. A. Quantification of the (A + B)% (% moving forward spermatozoa) in the HTF-cultured group, the M_2-cultured group and the M_2+2D08 group. B. Quantification of the VCL (curvilinear velocity) in the HTF-cultured group, the M_2-cultured group and the M_2+2D08 group. *p<0.05. N=5.

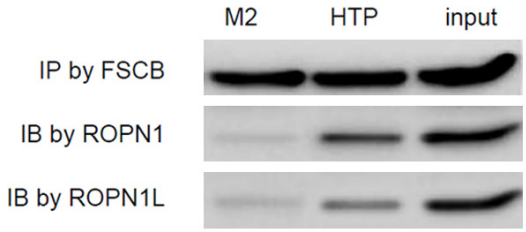

Figure 5. Phosphorylated FSCB has a significantly higher affinity to ROPN1/ROPN1L than non-phosphorylated FSCB. We cultured the isolated mouse spermatozoa either in M_2 media or HTF media. Then the protein was extracted 24 hours after culture, immunoprecipitated with anti-FSCB, and subsequently immunoblotted with either ROPN1 or ROPN1L antibody, shown by representative blots. IP: immunoprecipitation. IB: immunoblot.

no SUMOylation was detected (**Figure 3A**). Similarly, Slow moving immunoreactive bands of both ROPN1L was recognized by the anti-SUMO antibody. When the assay was performed without ATP as a control, no SUMOylation was detected (**Figure 3B**). These data thus suggest that SUMOylation of both ROPN1 and ROPN1L in vitro is not a random event, but a specific process dependent on ATP, which is required in the first step of the SUMOylation process for the activation of SUMO proteins by the E1 heterodimer AOS1-UBA2 enzyme complex.

Suppression of SUMOylation of ROPN1/ROPN1L mimics the effects of FSCB phosphorylation on spermatozoa motility

We next examined whether the alteration of SUMOylation of ROPN1/ROPN1L by HTF-induced FSCB phosphorylation may have effects on spermatozoa motility. We used a specific SUMOylation inhibitor, 2D08, in the M_2 media to suppress SUMOylation. We found that the 2D08 significantly decreased the SUMOylation of both ROPN1 and ROPN1L (**Figure 2A-D**). Next, we examined the effects of Suppression of SUMOylation of ROPN1/ROPN1L on spermatozoa motility. Our data showed that the (A + B)% (% moving forward spermatozoa) was significantly higher in the HTF-cultured group than the M_2-cultured group (**Figure 4A**), while the adding of 2D08 in the M_2 medium significantly increased the (A + B)%, and thus mimicked the effects of HTF media, which represented FSCB phosphorylation (**Figure 4A**). Similarly, the VCL (curvilinear velocity) was significantly higher in the HTF-cultured group than the M_2-cultured group (**Figure 4B**), while the adding of 2D08 in the M_2 medium significantly increased the VCL, and thus mimicked the effects of HTF media, which represented FSCB phosphorylation (**Figure 4B**). Together, these data suggest that HTF-induced FSCB phosphorylation may increase spermatozoa motility through suppression of SUMOylation of ROPN1/ROPN1L.

Phosphorylated FSCB has a significantly higher affinity to ROPN1/ROPN1L than non-phosphorylated FSCB

Based on the data, we hypothesized that the FSCB phosphorylation may increase the affinity

of FSCB to ROPN1/ROPN1L to inhibit their SUMOylation and subsequent protein degradation, resulting in the increases in spermatozoa motility. To prove it, we cultured the isolated mouse spermatozoa either in M_2 media or HTF media. Then the protein was extracted 24 hours after culture, immunoprecipitated with anti-FSCB, and subsequently immunoblotted with either ROPN1 or ROPN1L antibody. We detected significantly increases in the co-immunoprecipitated ROPN1 and ROPN1L protein with FSCB protein in mouse spermatozoa induced by HTF media, compared to in M_2 media (**Figure 5**). Since significant phosphorylated FSCS was in the HTF-treated mouse spermatozoa, compared to M_2-treated mouse spermatozoa, our data suggest that phosphorylated FSCB has a significantly higher affinity to ROPN1/ROPN1L than non-phosphorylated FSCB.

Discussion

The regulation of spermatozoa capacitation involves more than 20 associated proteins, e.g. AKAP3, AKAP4, TAKAP-80, GAPDS, HK1-S, GSK3β, ALDOA, LDHA, SFEC, triose phosphate isomerase, GAPDH, pyruvate kinase, LDH-C, sorbitol dehydrogenase, GSTM5, FS39, Ropporin, Rhophilin, SP17, PDE4A, FSIP1 and FSIP2, ASP, and CABYR [5]. In addition, FSCB is a CABYR binding protein, while ROPN1 and ROPN1L are known to bind AKAP3 [5]. However, an association between FSCB and ROPN1/ROPN1L has not been appreciated in the previous studies.

SUMOylation is a major mechanism for protein degradation. However, the regulation of ROPN1 and ROPN1L has not been studied before. Here, we not only showed that the phosphorylation of FSCB increased the affinity of FSCB to ROPN1 and ROPN1L, but also showed that this association between FSCB and ROPN1/ROPN1L seemed to protect ROPN1/ROPN1L from being SUMOylated, which may result in the increases in both proteins to favor the mobility of the mouse spermatozoa.

We previously reported that phosphorylation of FSCB occurred as early as 1 min after mouse spermatozoa capacitation, which increased over time and remained stable after 60 min [5]. We also used immunoprecipitation to show that the tyrosine and Ser/Thr phosphorylation of FSCB occurred during spermatozoa capacitation [5]. The extent of phosphorylation and was closely associated with the PKA activity and spermatozoa motility. FSCB phosphorylation could be induced by PKA agonist DB-cAMP, but was blocked by PKA antagonist H-89 [5]. Then, we conclude that FSCB contributes to spermatozoa capacitation in a tyrosine-phosphorylated manner. To summarize both studies, we think that the FSCB may be phosphorylated during the mouse spermatozoa capacitation, which induces the mobility of mouse spermatozoa through two mechanisms. First, FSCB phosphorylation may activate CABYR. Second, FSCB phosphorylation may increase ROPN1/ROPN1L through suppression protein SUMOylation-mediated degradation.

There are some novel questions based on the results from the current study. First, how does FSCB phosphorylation regulate SUMOylation of ROPN1/ROPN1L. Since our immunoprecipitation experiment showed that phosphorylated FSCB has a significantly higher affinity to ROPN1/ROPN1L than non-phosphorylated FSCB, the association of the phosphorylated FSCB to ROPN1/ROPN1L may inhibit the association of those two proteins with enzymes that catalyze their SUMOylation. In future, further approaches should be taken to verify this hypothesis. Second, what is the contribution of FSCB/CABYR and of FSCB/ ROPN1/ROPN1L to the increases in the mobility of mouse spermatozoa. Inhibition of either pathway followed by comparative analyses may help to find the answer.

In summary, our data suggest that suppression of SUMOylation of ROPN1 and ROPN1L partially mediate the effects of FSCB phosphorylation on the mobility of mouse spermatozoa. Hence, our study sheds new light on a therapeutic strategy targeting FSCB phosphorylation in the study of infertility.

Acknowledgements

This work was supported by the National Natural Science Foundation Project (No. 8140-1250).

Disclosure of conflict of interest

None.

Address correspondence to: Shunli Liu, Department of Burns and Plastic Surgery, General Hospital of Jinan Military Region, 25 Shifan Road, Jinan 250031, China. Tel: +86053151665333; E-mail: liushunli5678@163.com

References

[1] Dumpala PR, Parker HM and McDaniel CD. Similarities and differences between the sperm quality index and sperm mobility index of broiler breeder semen. Poult Sci 2006; 85: 2231-2240.

[2] Froman DP, Bowling ER and Wilsont JL. Sperm mobility phenotype not determined by sperm quality index. Poult Sci 2003; 82: 496-502.

[3] Froman DP, Pizzari T, Feltmann AJ, Castillo-Juarez H and Birkhead TR. Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, Gallus gallus domesticus. Proc Biol Sci 2002; 269: 607-612.

[4] Li YF, He W, Jha KN, Klotz K, Kim YH, Mandal A, Pulido S, Digilio L, Flickinger CJ and Herr JC. FSCB, a novel protein kinase A-phosphorylated calcium-binding protein, is a CABYR-binding partner involved in late steps of fibrous sheath biogenesis. J Biol Chem 2007; 282: 34104-34119.

[5] Liu SL, Ni B, Wang XW, Huo WQ, Zhang J, Tian ZQ, Huang ZM, Tian Y, Tang J, Zheng YH, Jin FS and Li YF. FSCB phosphorylation in mouse spermatozoa capacitation. BMB Rep 2011; 44: 541-546.

[6] Fiedler SE, Dudiki T, Vijayaraghavan S and Carr DW. Loss of R2D2 proteins ROPN1 and ROPN1L causes defects in murine sperm motility, phosphorylation, and fibrous sheath integrity. Biol Reprod 2013; 88: 41.

[7] Lan J, Zhao J and Liu Y. Molecular cloning, sequence characterization, polymorphism and association analysis of porcine ROPN1 gene. Mol Biol Rep 2012; 39: 2739-2743.

[8] Gareau JR, Reverter D and Lima CD. Determinants of small ubiquitin-like modifier 1 (SUMO1) protein specificity, E3 ligase, and SUMO-RanGAP1 binding activities of nucleoporin RanBP2. J Biol Chem 2012; 287: 4740-4751.

[9] Chung TL, Hsiao HH, Yeh YY, Shia HL, Chen YL, Liang PH, Wang AH, Khoo KH and Shoei-Lung Li S. In vitro modification of human centromere protein CENP-C fragments by small ubiquitin-like modifier (SUMO) protein: definitive identification of the modification sites by tandem mass spectrometry analysis of the isopeptides. J Biol Chem 2004; 279: 39653-39662.

[10] Kurepa J, Walker JM, Smalle J, Gosink MM, Davis SJ, Durham TL, Sung DY and Vierstra RD. The small ubiquitin-like modifier (SUMO) protein modification system in Arabidopsis. Accumulation of SUMO1 and -2 conjugates is increased by stress. J Biol Chem 2003; 278: 6862-6872.

[11] Sternsdorf T, Jensen K, Reich B and Will H. The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. J Biol Chem 1999; 274: 12555-12566.

[12] Desterro JM, Rodriguez MS, Kemp GD and Hay RT. Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. J Biol Chem 1999; 274: 10618-10624.