Palmitoylated GLB1L4 transfers via exosomes to maintain sperm function in rat epididymis

Daqian Dong^{1,2,*}, Jinmeng Yang^{1,4,*}, Yining Chen¹, Guofan Peng¹, Heran Cao^{1,2}, Huihui Gao^{1,4}, Tianqi Jin¹, Fangxia Yang^{3, 4} and Wuzi Dong^{1,2}

¹College of Animal Science and Technology, Northwest A&F University, Yangling, China, ²Shaanxi Provincial Key Laboratory of Animal Genetics, Breeding and Reproduction, Northwest A&F University, Yangling, China ³College of Forestry, Northwest A&F University, Yangling, China and ⁴Shaanxi Stem Cell Engineering Research Center, Northwest A&F University, Yangling, China

Correspondence should be addressed to W Dong: dongwuzi@nwsuaf.edu.cn or to F Yang: yangfangxia@nwsuaf.edu.cn

*(D Dong and J Yang contributed equally to this study)

Abstract

Epididymal specific proteins play a crucial role in sperm maturation. Some of the post-translational modified proteins are transported from the caput to the cauda of the epididymis through exosomes which regulate the function of sperm in cauda epididymis. Rat beta-galactosidase-1-like protein 4 (GLB1L4) expressed specifically in the caput epididymis, localizes on the sperm; however, the regulatory ways in which GLB1L4 protein interacts with sperm to maintain sperm function are unclear. In this study, knockdown of rat GLB1L4 could inhibit *in vitro* capacitation of sperm in cauda epididymis and reduce the fertility of the male rats by injection of special lentivirus-shRNA into caput epididymis. Moreover, a considerable proportion of GLB1L4 proteins from rat caput epididymis were loaded on exosomes. The exosomes loaded GLB1L4 from *in vitro* primary rat caput epididymal epithelial cells could bind with spermatozoa in cauda epididymis. Further, the palmitoylation status of cysteine residues at the 12th and 15th sites of the protein molecule could significantly affect cellular localization of GLB1L4 protein. It was identified that most of GLB1L4 was palmitoylated in the presence of exosomes from primary caput epididymal cells and the level of palmitoylated GLB1L4 in the exosomes could be inhibited by 2-bromopalmitate (2-BP). These results suggested that the palmitoylated GLB1L4 from rat caput epididymis could be transported to the cauda epididymis to regulate the sperm function by exosomes.

Reproduction (2021) **161** 159–172

Introduction

The immature spermatozoa from mammalian testis acquire motility and fertilizing ability in the epididymis which is a highly coiled duct that have important roles of maturation, concentration, protection, and storage of sperm (Zhu *et al.* 2007, Snyder *et al.* 2010). The spatial and temporal specificity of the epididymal gene expression is critical for its function maintenance and follows stringent regulation (Ellerbrock *et al.* 1994, Rodríguez *et al.* 2001). It had proved that *in vivo* RNAi could be an effective way to study certain gene function but transiently (Grillo *et al.* 2007, Frka *et al.* 2009, Towne & Aebischer 2009, Chou *et al.* 2010). It can be more efficient to knockdown expression of the specific gene using shRNAs-lentiviral vector that produces the long-term and down-regulating effect *in vivo* and *in vitro* (Song *et al.* 2006, Grillo *et al.* 2007, Harper & Gonzalez-Alegre 2008, Wang *et al.* 2008, 2009, Xie *et al.* 2008, Fan *et al.* 2014).

The epithelial cells in different epididymal segments can secrete a large number of protein factors into the lumen to remodel sperm (Björkgren & Sipilä 2019). Some proteins from epithelial cells are transferred in epididymis for long-distance via exosomes (namely epididymosomes), which is characterized by nanometer-scale particle with phospholipid bilayer membrane (Zhou *et al.* 2018). It had been proved that the level of WNT2B protein was higher in the caput epididymis and the mature WNT2B was harbored in the exosomes from the epididymal lumen (Koch *et al.* 2015). Microphage migration inhibitory factor (MIF) in the epididymosomes is transferred to the intracellular dense fibers of the sperm flagellum to modulate sperm flagellar beating (Eickhoff *et al.* 2001). Both WNT2B and MIF were found to interact with sperm in mouse cauda epididymis, while they only expressed in mouse epididymal caput epithelial cells (Eickhoff *et al.* 2001, Koch *et al.* 2015).

Proteins are often subjected to a series of post-translational modification (PTM) before being secreted

Table 1 ShRNA stem-loop sequences and PCR primers.

Items	Sequence (5'–3')	Aim
shRNA-p1		GLB1L4 interference (Lv-p1)
FC	GATCCGACCAACTTTGGCTTAATTTCAAGAGATTAAGCCAAAGTTGGTGCTTTTTTG	
RCC	AATTCAAAAAAGCACCAACTTTGGCTTAATCTCTTGAAATTAAGCCAAAGTTGGTG	
shRNA-p2		GLB1L4 interference (Lv-p2)
FC	GATCCGGCGTAAAGATCCACTTCATTTTCAAGAGAATGAAGTGGATCTTTACGCCTTTTTTG	
RCC	AATTCAAAAAAGGCGTAAAGATCCACTTCATTTCTCTTGAAATGAAGTGGATCTTTACGCC	
shRNA-g		Control (Lv-g)
FC	GATCCGGCGATGCCACCTACGGCAAGTTTCAAGAGAACTTGCCGTAGGTGGCATCGCCTTTTTTG	
RCC	AATTCAAAAAAGGCGATGCCACCTACGGCAAGTTCTCTTGAAAACTTGCCGTAGGTGGCATCGCC	
GLB1L4		qPCR
F	TGTTGATGAAGGATGTGCGTGAG	
R	TGGTCGAGTGAGCGGGCTA	
β-Actin		qPCR
F	AGACAGCCGCATCTTCTTGT	
R	CTTGCCGGGTAGAGTCAT	
GLB1L4-12/15M		Primers for GLB1L4-C12/15S mutant motif
F	TGAAAGCATCCTGAGTCTGTCTTCAACCTTAGG	
R	ACAGACTCAGGATGCTTTCAGGTCTCCCCAGGAAG	

The forward (FC) and reverse complimentary chains (RCC) and sequences (F and R) are presented.

from cells (Szabo-Taylor *et al.* 2015, Moreno-Gonzalo *et al.* 2018). S-palmitoylation modification of cysteine residue is a common and reversible modification to dynamically control the hydrophobicity which increases the lipid affinity of the protein molecule (María-Eugenia & Gisou 2018). The modified proteins are led to localize in the endomembrane of cellular lipid bilayers (Dunphy & Linder 1998, Percherancier *et al.* 2001, Lanyon-Hogg *et al.* 2017, Jiang *et al.* 2018). And the S-palmitoylation proteins may be preferentially selected into the extracellular vesicles which are released outside of cells (Yang *et al.* 2010, Shen *et al.* 2011, Verweij *et al.* 2015, Shibata *et al.* 2017, Romancino *et al.* 2018).

Beta-galactosidase 1-like 4 gene (*Glb1l4*) is specifically expressed in the epithelial cells of rat caput epididymis, and the GLB1L4 can be secreted into the luminal fluid and bound on the sperm (Zhen *et al.* 2009). But it is unclear how the GLB1L4 protein regulates sperm maturation in epididymis. In this study, GLB1L4 function would be explored through *in vivo* knockdown expression of GLB1L4 by microinjection of the special shRNAs-lentivirus into rat caput epididymis. Further, post-translational modification state of GLB1L4 would be analyzed to resolve the potential relationship between GLB1L4 and sperm maturation in rat epididymis.

Materials and methods

Animals

Animals (Sprague–Dawley rats) were purchased from The Animal Center of the Chinese Academy of Sciences (Shanghai, China) and The Animal Center of Xi'an Jiaotong University (Xi'an, China). They were housed for an additional 7–10 days before manipulation. Food and water were freely available throughout the experiments. In the study, the experiments were

conducted according to a protocol approved by the Animal Care Committee of Northwest A&F University (NWAUFU-ACC-2018-2).

Reconstruction and transfection of pcDNA3.1-Wt-GLB1L4 (WT) and pcDNA3.1-Mut-GLB1L4 (C12/15S)

The pcDNA3.1-GLB1L4-Wt is a eukaryotic expression plasmid with the full-length sequence of rat GLB1L4 cDNA reconstructed previously. In order to obtain the mutant GLB1L4 protein whose 12th and 15th cysteine residues were changed to serine, the pcDNA3.1-Mut-GLB1L4 (C12/15S) plasmid was generated by site-directed mutagenesis using Hieff MutTM Site-Directed Mutagenesis Kit (Qcbio Science & Technologies Co., Ltd, Item No 11003E510) according to the instructions of the manufacturer (the mutational primers are shown in Table 1, forward primer: GLB1L4-C12/15S-M-F, reverse primer: GLB1L4-C12/15S-M-R).

PC1 cell line of mouse caput epididymis was provided by Prof Orgebin-Crist lab (the Center for Reproductive Biology Research, Tohoku University, Sendai 980-8575, Japan) and cultured in a plastic culture plate supplemented with Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS) at 32°C as described previously (Araki *et al.* 2002, Mühlbauer *et al.* 2008). The pcDNA3.1-Wt-GLB1L4 and the pcDNA3.1-Mut-GLB1L4 (C12/15S) were transfected into PC1 cells by lipofectamine (Invitrogen), respectively.

Production of ShRNA-lentivirus

Stem-loop of ShRNA double strands of GLB1L4 (ShRNA-p1 and ShRNA-p2) and EGFP (ShRNA-g) were cloned into the pLV-EGFP-C lentiviral vector (Inovogen Tech. Co., Cat. No. VL3101). The lentiviral particles were produced by co-transfection of lentiviral vector (including Lenti/ShRNA-p1, Lenti/ShRNA-p2 and Lenti/ShRNA-g), the packaging plasmids pH1 and pH2

into 293T cells, using the lipofectamine 2000 (Invitrogen). And then the lentivirus (Lv-p1, Lv-p2 and Lv-g) was harvested after 48–72 h and concentrated by ultracentrifugation (100,000 g). The virus titer (Unit titer, UT/mL) was determined via quantification of the EGFP fluorescence intensity of 293T cells by flow cytometry according to previous report (Du et al. 2016). Stem-loop of ShRNA double strands of GLB1L4 (shRNA-p1 and shRNA-p2) and EGFP (shRNA-g) were shown in Table 1.

1×10^6 UT/mL viruses (Lv-p1, Lv-p2 and Lv-g) infected the PC1 cells in six wells plate, respectively. The knockdown efficiency of GLB1L4 RNA and protein were determined by qPCR and Western blot at 48 h after infection, respectively.

Injection of lentivirus into rat caput epididymis

The rats were anesthetized with pentobarbital sodium (40 mg/kg body weight). After opening the abdominal cavity of the rat with scissors, the epididymis was exposed. In total, 50 μ L (total units titer about 2×10^6) shRNA-lentivirus (Lv-p1 or Lv-g) was injected into the stroma of caput epididymis of 60-day-old rats until the caput displayed trypan blue (TB) (Sigma) via a sharpened glass microcapillary pipette (GC100T-10, Harvard apparatus LTD Fircroft way, Edenbridge, Kent, TN86HE), respectively. Experiment 1: As an experimental group, the right epididymis of rat was injected with Lv-p1, while as a control, the left epididymis of the rat was injected with Lv-g. There were three rats in every test and each experiment was repeated three times. Experiment 2: For mating test, eight rats were injected with Lv-p1 or Lv-g into the bilateral epididymis, respectively. 30 days after injection, each male mated with two normal females.

Evaluation of sperm capacitation

To assess capacitation, sperm from the cauda epididymis was released into the semen diluent (94.6 mM NaCl, 1.19 mM KH_2PO_4 , 25 mM NaHCO_3 , 5.56 mM glucose, 10.76 mM sodium lactate and 0.5 mM sodium pyruvate, 50 mg/mL streptomycin sulfate, 75 mg/mL potassium penicillin, pH 7.4, osmolarity about 310 mOsmol/kg). After centrifugation at 800 g for 5 min, sperm was resuspended in the capacitation medium (diluent with 1.71 mM CaCl_2 , 1.19 mM MgSO_4 , 4 mg/mL BSA) and the sperm was incubated for 3 h at 37°C. The time-point of spermatozoa dilution was defined as the beginning of capacitation (0 h). The sperm pellets were suspended in RIPA with 0.1% SDS sample buffer, and total sperm protein was performed on PAGE with 12% Tris-glycine gels. The tyrosine phosphorylation of spermatozoa protein was detected by Western blot.

Sperm motility analysis

Sperm motility was analyzed according to a previous method (Zhou et al. 2008). Suspensions of spermatozoa were loaded into flat 100 μ m deep microslides (HTR1099, VitroCom Inc., Mt. Lks. NJ, USA) for CASA analysis using HTM-TOXIVOS sperm motility analyzer (Rat Head Toxicology, version 12.3A, Hamilton-Thorn Research, MA, USA). Instrument settings

were: temperature, 37°C; low VAP cut-off, 20.0; low VSL cut-off, 30.0; threshold straightness, 50%; static head size, 0.72–8.82; static head intensity, 0.14–1.84. In total, 30 frames were acquired at a frame rate of 60 Hz. During analysis, the playback feature was used to check its accuracy.

Fertility assay

30 days after injection with the same lentivirus into bilateral epididymis, male rats mated with normal female rats. They were maintained together overnight, and vaginal smears of the females were taken the next morning and examined by microscopy. The female rats were successfully mated if spermatozoa were present in vaginal smears. The mated male rats were sampled to determine the knockdown efficiency of GLB1L4. Then pregnant females that mated with the GLB1L4 knockdown male rats were housed individually. The pregnant females were subjected to hysterectomy to determine the number of fetuses.

Separation and culture of epithelial cells from rat caput epididymis

After the adult male rats (60 days old) were killed, the epididymis was removed from the rat abdominal cavity and immersed in 75% ethyl alcohol, brought into the cell culture lab immediately. After the white membrane of the epididymis was removed, the caput epididymis was cut into pieces using eye scissors in sterile PBS with penicillin and streptomycin. To remove sperm, the tissues were washed three to four times until PBS buffer was not turbid. For tubule isolation, tissue fragments were treated for 30 min at room temperature in the IMDM (Iscove's Modified Dubecco's Medium) supplemented with the antibiotics and 2 mg/mL IV type collagenase (Invitrogen) and 20 U/mL DNase I (Promega). After small pieces of tissue were collected by gravity sediment, the pellets were digested for 4 min in 0.125% trypsin-EDTA (Gibco) and filtrated to remove bulk tissues through 70 μ m nylon membrane filters. Finally, the cells were collected by centrifugation at 450 g for 5 min, washed, and suspended in serum-free IMDM medium (v/v) (Gibco) supplemented with the following nutrients and growth factors: 50 U/mL penicillin, 50 μ g/mL streptomycin, 2 mmol/L glutamine, 10 μ g/mL insulin, 10 μ g/mL transferrin, L-carnitine (1 mM), 1 μ g/mL retinoic acid, 10 ng/mL EGF, 10 ng/mL cholera toxin, 10 nM hydrocortisone, 100 nM double hydrogen testosterone. The cells were then placed in 60 mm petri dish and incubated in a humidified chamber at 32°C with 5% CO_2 . After 1 h, the unattached cells were moved to a new petri dish. To further remove fibroblasts, the cells were digested with 0.05% trypsin for 1 min after being cultured for 24 h, and the attached cells were continually cultured in whole medium, while the unattached cells (fibroblasts) were removed (Wang et al. 2010). The culture medium was changed every 48 h. The epithelial cells were digested using 0.25% trypsin to amplify sub-culturing cells. Identification of epididymal epithelial cells (>95%) was assessed using CK18 protein antibody (Sigma, No C1399, 1:200) by immunofluorescence.

Figure 1 Specific expression of rat GLB1L4 in the caput epididymis. (A) The *Glbl14* mRNA specific expression in the caput epididymis of rat by RT-PCR; (B) The GLB1L4 protein in caput, corpus and cauda epididymis by Western blot. (C) The immunolocalization of rat GLB1L4 protein in the epididymis tissues of 90-day-old male rat. Scale size: 100 μ m. (D) The GLB1L4 protein location on sperm in cauda epididymis by immunofluorescent staining. (E) The GLB1L4 protein expression of sperm in caput, corpus and cauda epididymis by Western blot. caput, corpus and cauda represented sperm protein of caput, corpus and cauda, respectively.

Isolation and characterization of exosomes

Isolation of exosomes from the epididymal lumen fluid and primary epididymal epithelial cells

To isolate the exosomes from epididymal lumen fluid, a previous method was used with slight modifications in this study (Zhen *et al.* 2009, Koch *et al.* 2015). Briefly, ten epididymal tissues from five adult rats were used per test in a tube. To avoid contamination of the damaged epididymal epithelial cell components, each epididymis was cut into pieces carefully using eye scissors, and then the ten epididymal tissues were immersed in 50 mL PBS. The tube was shaken gently for 4 h in a shaker at 4°C. After the lumen fluid was fully discharged, the samples were centrifuged at 2000 *g* for 15 min at 4°C to remove the tissue and cell debris. The supernatant was centrifuged at 5000 *g* for 15 min at 4°C to remove smaller cell debris. The supernatant was used to prepare the epididymal lumen-derived exosomes.

In like manner, the media was centrifuged at 2000 *g* for 15 min at 4°C to remove the cell debris after the epididymal epithelial cells were cultured for 72 h *in vitro*. The supernatant was then collected after centrifugation at 5000 *g* for 15 min at 4°C. The supernatant was used to isolate the epididymal epithelial cell-derived exosomes.

Exosomes were isolated from the supernatant of the primary epithelial cells from caput epididymis or epididymal lumen fluid by gradient ultracentrifugation according to a previously described method with modifications (Gao *et al.* 2018). In each experiment, at least 50 mL of the supernatant was centrifuged at 16,000 *g* for 15 min at 4°C to remove pellets. The supernatant was filtered using 0.22 μ m filters, and the exosomes were collected by centrifugation at 120,000 *g* for 1

h at 4°C. The pellets were then washed twice with buffer (30 mM Tris, 130 mM NaCl, pH 7.6) followed by centrifugation at 120,000 *g* for 1 h at 4°C. Finally, the exosome pellets were resuspended in 0.5 mL sterile PBS.

Detection of exosomes

The exosome suspension was diluted 1000 times in the PBS. The particle size of exosomes were observed and the concentration (particles/mL) of exosomes was calculated with the NanoSight NS3000 system according to the manufacturer's protocols (Malvern Instruments, Malvern, UK). The profile of the exosomes were observed by Scan electron microscopy using previously described methods (Gao *et al.* 2018).

PKH67 labeling of exosomes

Exosomes from primary epididymal epithelial cells were resuspended in PBS and PKH67 (the final concentration is 0.01 mM) was added, and then the solution was incubated for 10 min at 37°C. Excess dye was removed by centrifugation at 120,000 *g* for 1 h at 4°C and the PKH67-labeled exosomes were resuspended with 0.5 mL semen diluent.

Test of exosomes binding sperm

Sperm collected from cauda epididymis was washed three times using the semen diluent and resuspended with 90 μ L semen diluent. Then 10 μ L PKH67-labeled exosomes were added into the solution for 1 h at 37°C. Finally, the sperm was washed three times in PBS and then dropped on slides and observed in fluorescence microscope.

Figure 2 Detection of GLB1L4 knockdown *in vitro*. (A) Expression level of *Glub1l4* mRNA after infection of viruses (Lv-p1, Lv-p2 and Lv-g) into the PC1 cell overexpressing GLB1L4 by RT-qPCR ($n=3$). (B) Expression level of GLB1L4 protein after infection of viruses (Lv-p1, Lv-p2 and Lv-g) into the PC1 cell overexpressing GLB1L4 by Western blot ($n=3$). Lv-p1, Lv-p2 and Lv-g represented the samples from the overexpressing GLB1L4 PC1 cell that had been treated with Lv-p1, Lv-p2 and Lv-g lentiviral, respectively. Con represented samples from the overexpressing GLB1L4 PC1 cell that had not been treated with any lentiviral vectors. a, b, c: The same letter means no significant difference, while the difference between different letters is significant.

Effect of 2-BP on the epididymal epithelial cells

To analyze effect of 2-BP on protein palmitoylation, the epididymal epithelial cells were cultured for 24 h in the whole serum-free medium with final concentration of 20 mM 2-BP (without 2-BP as control). The cells were observed under a microscope, and then protein of cells, the medium and exosomes from the cells were extracted for Western blot and ABE assay.

Extraction of the palmitoylated proteins of cells using acyl-biotinyl exchange (ABE) method

The ABE assay for determination of the palmitoylation level was carried out as described previously (Wan *et al.* 2007). Briefly, after incubating with N-ethylmaleimide (NEM, Thermo) to block free sulfhydryl groups on proteins, purified samples were treated with or without hydroxylamine (HA, Thermo)

Figure 3 Observation of lentivirus injected into rat caput epididymis. (A) The virus solution with trypan blue (TB) was distributed in rat caput epididymis when 50 μ L virus was injected into rat caput epididymis. (B) The green fluorescence in rat caput epididymis was observed by fluorescence stereoscopy on the second day after the virus was injected. (C and D) The green fluorescence in rat caput (C) and cauda (D) epididymis was detected by fluorescence stereoscopy on the 30th day after the virus was injected into epididymis. (E) Expression of EGFP and GLB1L4 in caput, corpus and cauda epididymis was analyzed by Western blot.

and Biotin-BMCC (Thermo) to label palmitoylation sites. The presence of Biotin on proteins was then analyzed by SDS-PAGE with HRP-conjugated anti-streptavidin (Beyotime). The detailed protocol of ABE is described subsequently.

Sealing the protein – SH using NEM

A total of 300 μ L of SDS buffer (4% SDS, 50 mM Tris, pH 7.4 and 5 mM EDTA) containing 10 mM NEM was added to the samples (medium, cells or exosomes) and the mixture was incubated for 10 min at 37°C with occasional agitation to lyse the samples. Then 900 μ L of buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM NEM, 0.2% Triton X-100,

1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.25 µg/mL each of pepstatin, leupeptin, antipain and chymostatin) was added to the sample solution and the mixture was incubated overnight at 4°C with gentle rocking. Unreacted NEM was removed by three sequential precipitations with ice-cold acetone as described previously.

Reduction of fatty acyl groups

The protein pellet was collected by centrifugation (10,000 g, 10 min), dissolved in 240 µL of SDS buffer (4% SDS, 50 mM Tris, pH 7.4 and 5 mM EDTA) and incubated at 37°C for 10 min. At this point, the sample was divided into two equal portions, one with HA (+HA sample) and the other without HA (−HA sample) in 1.5 mL screw-cap centrifuge tubes. The +HA sample was diluted five-fold with 480 µL of the +HA buffer. For the −HA sample, 480 µL of −HA buffer was used. +HA buffer: 0.7 M hydroxylamine, pH 7.4, 1 mM HPDP-biotin, 0.2% Triton X-100, 1 mM PMSF and 0.25 µg/mL each of pepstatin, leupeptin, antipain and chymostatin. −HA buffer: 50 mM Tris-HCl, pH 7.4, 1 mM HPDP-biotin, 0.2% Triton X-100, 1 mM PMSF and 0.25 µg/mL each of pepstatin, leupeptin, antipain and chymostatin.

HPDP-biotin sign

The two sample solutions were incubated at room temperature for 1 h with end-over-end rotation. After the reaction, the tubulin was precipitated three times using ice-cold acetone. Each resulting pellet was dissolved in 120 µL of SDS buffer (4% SDS, 50 mM Tris, pH 7.4 and 5 mM EDTA), diluted with 480 µL of low-HPDP-biotin buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2 mM HPDP-biotin, 0.2% Triton X-100, 1 mM PMSF and 0.25 µg/mL each of pepstatin, leupeptin, antipain and chymostatin) and then incubated at room temperature for 1 h with end-over-end rotation. To remove unreacted HPDP-biotin, both samples were precipitated three times using ice-cold acetone. Each pellet was dissolved in 1.5 mL of incubation buffer (50 mM Tris-HCl, pH 7.7, 1 mM EDTA and 0.4% Triton X-100). Twenty micrograms of sequencing

grade modified trypsin were added to each sample solution and incubated at 37°C overnight. To terminate proteolysis, PMSF was added to a final concentration of 1 mM.

Isolation of HPDP-biotinylated peptides

To perform affinity purification of the HPDP-biotinylated peptides from the digested tubulin, 0.5 mL of 50% suspension of streptavidin agarose was washed twice by resuspension in 5 mL of ice-cold PBS buffer (0.1 M phosphate, pH 7.2 and 0.15 M NaCl), followed by gravity sedimentation and decanting the supernatant. The washed streptavidin beads were resuspended in 0.25 mL of incubation buffer. To affinity-capture the HPDP-biotinylated peptides, 100 µL of the streptavidin agarose bead suspension was added to each tryptic digest. The mixture was incubated at room temperature with rotation. After 1 h, the beads were pelleted by centrifugation at 2000 g for 10 min and washed four times each with incubation buffer, wash buffer (50 mM Tris-HCl, pH 7.7, 600 mM NaCl, 1 mM EDTA and 0.4% Triton X-100) and bicarbonate buffer (5 mM ammonium bicarbonate in 20% acetonitrile). HPDP-biotinylated peptides were eluted from streptavidin agarose by incubation with 500 µL of Mguanidine hydrochloride (pH 1.5) for 30 min with gentle inversion at room temperature. Sample solutions were centrifuged to remove insoluble particulates. The supernatants were collected for the following studies.

Immunohistochemistry (IHC)

For histological analysis, epididymal tissues were fixed in Bouin's fixative overnight. After successive dehydration, the tissues were imbedded in paraffin. Sections were mounted on slides with poly-lysine pretreatment, dewaxed in a xylene bath, and then rehydrated through gradient ethanol solutions. The sections were used for IHC. After rinsing in PBS, the slides were placed in boiling citrate for heat-mediated antigen retrieval for 10 min. Slides were cooled for 40 min at room temperature and then rinsed three times with PBS before being treated with 3% H₂O₂. Non-specific protein binding was blocked for 1 h by normal goat

Figure 4 Detection of the expression level of GLB1L4 protein in the caput epididymis, lumen of epididymis and sperm on the 30th day after lentivirus (Lv-p1 and Lv-g) was injected into rat caput epididymis by Western blot ($n=3$).

Figure 5 Influence of knockdown expression of GLB1L4 protein on sperm capacitation and male fertility. (A) The tyrosine phosphorylation of sperm proteins was analyzed by induction of sperm capacitation *in vitro* with BSA and Ca^{2+} , the semen samples were from the cauda epididymis of the male rats which was injected with the Lv-p1 lentivirus into bilateral caput epididymis for 30 days. (B) The average number of fetuses of female rats mated with the GLB1L4 knockdown male rats with same lentivirus (Lv-p1 or Lv-g) injected into bilateral epididymis.

serum before incubating with the GLB1L4 antibody (Zhen *et al.* 2009, 1:200) overnight in a damp box. Sections were washed with PBS and incubated for 30 min with biotinylated anti-rabbit IgG (Solarbio, SHB134, 1:100). Tissues slides were stained by diaminobenzidine (DAB) according to the manufacturer's instructions before counterstaining with hematoxylin. Images were obtained using a microscope (Carl Zeiss Company Ltd). As controls, the primary antibodies were replaced by PBS.

Immunofluorescence

For immunostaining, the cells were cultured in 48-well plate at 32°C for 24 h, fixed in 4% paraformaldehyde for 15 min. The fixed cells were then treated with PBS (pH 7.2) containing

0.3% (v/v) Triton X-100 for 10 min at room temperature. The blocking of non-specific antigens was incubated with 10% (w/v) goat serum in PBS for 1 h at 37°C. Then the cytokeratin-18 (CK-18) antibody mouse MAB (Sigma, No C1399, 1:200) was performed for 12 h at 4°C. The secondary antibody employed was fluorescein isothiocyanate (FITC)-goat anti-mouse IgG (sino-American Biotechnology Co., Luoyang, 1:300). The nuclear was stained with DAPI (Sigma, cat. no. D9542, 1:1000). Control cells received the same treatment except that the primary antibody was replaced with PBS. Immunofluorescence-stained samples were examined with Axiophot microscope equipped fluorescence (Carl Zeiss Company Ltd). Images were captured with a CCD camera using SPOT RT software version 3.1 (Diagnostic Instruments Inc, Sterling Heights, Mich) and then edited with Adobe Photoshop 6.0.

RNA Isolation and PCR

Total RNA of different samples was isolated with Trizol reagent (Invitrogen) following the manufacturer's recommendations. Total RNA isolated was reverse transcribed using an oligo dT (20) primer by M-MLV Reverse Transcriptase (Promega) according to the manufacturer's recommendations.

PCR was subsequently done to identify the presence of transcriptional GLB1L4. PCR amplification was done using the following program: 94°C for 5 min, 35 cycles of 95°C for 30 s; 57°C for 30 s, 72°C for 30 s; 72°C for 5 min for the addition of poly-A overhangs and cooled to 4°C. PCR products were then separated on a 1% agarose gel and visualized with ethidium bromide using a Fluor-S Multi-Imager densitometer (Bio-Rad Laboratories). The qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-02), and the PCR program performed as described previously. β -actin was an interior control. Primers of *Glb1l4* and β -actin are shown in Table 1.

Protein extract and Western blot

Total protein from sample was extracted with RIPA buffer containing protease inhibitor cocktail (Pierce) and phosphatase inhibitor cocktail (Sigma). Total protein extract (about 30 μg) for each sample was electrophoresed on 12% polyacrylamide gels (SDS-PAGE). Resolved protein were transferred to Hybond-P membranes (Amersham Pharmacia Biotech), immunoreacted and visualized with the ECL analysis system (Western blot chemiluminescence Reagent Plus, Amersham Pharmacia Biotech). Anti- β -actin (Sigma), anti-tyrosine phosphorylation antibody (Bio-Techne, MAB16761, 1:2000), anti-CD63 antibody (Abcam, ab217345, 1:2000), anti-EGFP (Abcam, ab6556, 1:2000) and anti-GLB1L4 antiserum (Zhen *et al.* 2009; 1:5000) were used as primary antibody. The second antibodies (1:10,000) were the horseradish peroxidase (HRP)-conjugated anti-rabbit (Calbiochem) and anti-mouse IgG (Abcam, ab131368).

Statistical analysis

The data from the experiments are presented as mean $\pm$ S.D. Data of the groups were compared using independent samples one way ANOVA by SPSS statistics 17.0. The *P* values less than

Figure 6 Identification of GLB1L4 protein in epididymal exosomes. (A) The extracellular vesicles from epididymal luminal fluid were observed by S.E.M. (B) Density and sizes of exosomes from epididymal luminal fluid were measured by Nanosight system. (C) GLB1L4 protein in exosomes (Exo), luminal fluid free of exosomes (Exo-free lumen), and whole lumen fluid (Lumen) was analyzed by Western blot.

0.05 were considered statistically significant. Experiments were repeated at least three times and each time in triplicate.

Results

Analysis of GLB1L4 in the rat epididymis

The results of RT-PCR pointed out that the *Glb1l4* mRNA expressed specifically in the caput epididymis (Fig. 1A), while the GLB1L4 protein distributed in caput, corpus and cauda epididymis in 90-day-old male rats by Western blot analysis (Fig. 1B). Meanwhile, localization of GLB1L4 was further confirmed in caput epididymis and in lumen of corpus and cauda epididymis by immunohistochemistry (Fig. 1C). In addition, it was demonstrated that there existed a low level of GLB1L4 protein of sperm from cauda epididymis by immunofluorescence and Western blot (Fig. 1D and E).

Influence of GLB1L4 knockdown on sperm function

Lentivirus production and GLB1L4 knockdown efficiency detection

According to rat *Glb1l4* and *Egfp* gene sequences, stem-loop structure shRNA sequences were designed (shRNA-p1, shRNA-p2 and shRNA-g) (Table 1) and synthesized. The level of *Glb1l4* mRNA (Fig. 2A) and GLB1L4 protein (Fig. 2B) were reduced significantly compared with control group (Lv-g) when the recombinant lentivirus (Lv-p1 and Lv-p2) were transferred into the PC1 cells overexpressing GLB1L4 protein, respectively. The Lv-p1 was more efficient than Lv-p2 (Fig. 2). Therefore, we mainly used the Lv-p1 lentivirus for subsequent studies.

Distribution of lentivirus injected into rat caput epididymis

The virus solution was rapidly transferred to the rat caput epididymis when 50 μ L virus (Lv-p1) with Trypan blue (TB) injected into rat caput epididymis (Fig. 3A). On the second day, after the virus was injected into caput epididymis, the green fluorescence of caput epididymis was detected by fluorescence stereoscopy (Fig. 3B). 30 days after injection of viruses, EGFP protein was detected in caput, corpus and cauda and the strong green fluorescence was mainly in caput and cauda epididymis (Fig. 3C, D and E). Further, the level of GLB1L4 protein was downregulated significantly in all section of the epididymis (Fig. 3E). It indicated that the lentivirus could work at least 30 days in rat epididymis.

GLB1L4 expression in different parts in 30 days after injection of lentivirus

To analyze the expression of GLB1L4 30 days after lentiviral (Lv-p1) injection into rat caput epididymis, tissues of caput epididymis, epididymal lumen liquid and sperm in cauda epididymis were collected, respectively. The results showed that the expression level of GLB1L4 was reduced by at least 50% in the caput epididymis, epididymal lumen liquid and sperm by Western blot, respectively (Fig. 4).

Influence of GLB1L4 knockdown expression on sperm in vitro capacitation and male fertility

Motility of sperm in rat cauda epididymis was detected by CASA. Compared with the control (Lv-g), CASA results showed that parameters related to spermatozoa hyperactivity, such as VAP (average path velocity), VSL

Figure 7 Characterization of the exosomes from the primary epithelial cells of rat caput epididymis and binding to sperm. (A) Morphology of the primary epididymal epithelial cells in the serum-free medium IMDM was displayed. (B) CK8 positive epithelial cells screened out by immunofluorescence. Scale bar: 50 μ m. (C) The exosomes from the epididymal epithelial cells was observed by S.E.M. (D) Density and sizes of exosomes from the epididymal epithelial cells was measured by Nanosight system. (E) Expression of GLB1L4 protein was analyzed by Western blot in the epididymal epithelial cells (cell), the whole medium (medium) and exosomes from the medium (Exo). (F) PKH67 labeled exosomes (PKH67-Exo) from the medium band with outer membrane of sperm from cauda epididymis. PKH67-Exo shows green fluorescence.

(straight-line velocity), VCL (curvilinear velocity) and ALH (amplitude of lateral head) displacement were significantly decreased in the knockdown group after 3 h *in vitro* capacitation ($P < 0.05$), although the indexes was not different before capacitation (Table 2). To examine sperm capacitation, tyrosine phosphorylation of sperm was analyzed during BSA inducing sperm *in vitro* capacitation by Western blot. On the 30th day after injection of Lv-p1 lentivirus, the level of sperm tyrosine phosphorylation significantly decreased compared with the control (Lv-g) after *in vitro* capacitation at 3 h (Fig. 5A).

To analyze the influence of *in vivo* knockdown expression of GLB1L4 protein on male fertility, the number of fetuses was determined after normal female rats mated with male rats with same lentivirus (Lv-p1 or Lv-g) in bilateral epididymis. After the normal female rats mated with the male rats with same lentivirus (Lv-p1) in bilateral epididymis, the average number of fetuses (12

fetuses) was significantly low compared with control groups (16 fetuses) ($P < 0.05$) (Fig. 5B).

Palmitoylated GLB1L4 protein from caput epididymis transports through exosomes in rat epididymal lumen

To exploit whether GLB1L4 protein was loaded on epididymal exosomes, the extracellular vesicles from epididymal luminal fluid was isolated by gradient ultracentrifugation. The extracellular vesicles were membranous vesicles with 50–100 nm in diameter by S.E.M. (Fig. 6A). The average particle size was 97 nm and the concentration of exosomes in rat epididymal lumen was about 7.4×10^{10} particles/mL by Nanosight system (Fig. 6B). Western blot showed that this exosome-enriched fraction harbored plenty of GLB1L4 protein, while the GLB1L4 protein seemed to be rare in the exosome-free lumen fraction (Fig. 6C). The results displayed that most GLB1L4 protein from rat

Table 2 Comparison of effect of Lv-p1 and Lv-g on sperm motility indexes and kinetics parameters in BSA inducing sperm capacitation for 3 h. Results are given as average mean $\pm$ S.D. ($n = 3-5$). Values within columns with different letters represent significant difference ($P < 0.05$).

Virus types CT	Lv-p1		Lv-g	
	0 h	3 h	0 h	3 h
MS (%)	51 $\pm$ 12.4	54 $\pm$ 10.3	50 $\pm$ 6.7	56 $\pm$ 3.6
PS (%)	48.7 $\pm$ 7.6	46.4 $\pm$ 7.4 ^A	45.5 $\pm$ 5.4	38.3 $\pm$ 5.4 ^B
VAP (μ m/s)	89 $\pm$ 5.2	92 $\pm$ 5.2	92 $\pm$ 7.3	107 $\pm$ 7.3
VSL (μ m/s)	86.1 $\pm$ 5.2	92.1 $\pm$ 5.2 ^B	92 $\pm$ 4.2	108 $\pm$ 4.2 ^A
VCL (μ s)	102 $\pm$ 8.2	113 $\pm$ 5.1 ^B	112 $\pm$ 10.2	132 $\pm$ 10.2 ^A
ALH (μ m)	7.3 $\pm$ 0.4	7.8 $\pm$ 0.4 ^B	7.6 $\pm$ 0.3	8.6 $\pm$ 0.3 ^A
BCF (Hz)	19.6 $\pm$ 0.6	19 $\pm$ 0.6 ^B	20 $\pm$ 0.7	22.3 $\pm$ 1.4 ^A

Lv-g refers to sperm collected from the rats injected with the lentivirus Lv-g; Lv-p1 refers to the sperm collected from the rats injected with the lentivirus Lv-g.

ALH, amplitude of lateral head displacement; BCF, beat cross-frequency; CT, capacitation time; MS, number of motile sperm; PS, number of progressive sperm; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

caput epididymis was loaded onto exosomes in epididymal lumen.

To demonstrate whether the exosomes loaded GLB1L4 protein comes from caput epididymis, the primary epithelial cells of rat caput epididymis were isolated and cultured in serum-free medium IMEM (Fig. 7A and B) and the extracellular vesicles were separated by gradient ultracentrifugation (Fig. 7C and D). The GLB1L4 protein was detected in exosomes (Fig. 7E) which could be banded with outer membrane of sperm from cauda epididymis (Fig. 7F). It suggested that exosomes loaded GLB1L4 from *in vitro* culturing caput epithelial cells could bind to sperm in cauda epididymis.

Further, it was predicted that the GLB1L4 protein is a potential palmitoylated protein (Fig. 8A). The 12th cysteine residue (C12) of GLB1L4 protein was predicted to be the most likely palmitoylation site and the 15th cysteine residue (C15) as the next highest possible site of palmitoylation.

2-BP, a palmitoylation inhibitor, is widely used to inhibit palmitoylation modification (Webb *et al.* 2000, Resh 2006, Jennings *et al.* 2009, Hannoush & Sun 2010). Compared with the control, the level of GLB1L4 protein in the caput epithelial cells was not significantly changed (Fig. 8C). However, the level of GLB1L4 protein in exosomes from the medium was significant inhibited by 2-BP (Fig. 8C). Especially, the level of palmitoylated GLB1L4 protein in the caput epithelial cells was also significantly reduced when the 2-BP was added into the medium by ABE assay (Fig. 8D).

The preferential palmitoylation of specific cysteine sites is thought to regulate protein membrane binding and subcellular localization (Milligan *et al.* 1995, Resh 1999). In the PC1 cells transfected with the pcDNA3.1-GLB1L4-Wt plasmid (GLB1L4-Wt), the GLB1L4 protein appeared dotted cluster (Fig. 8E). By contrast,

the GLB1L4 protein in PC1 cells with mutant GLB1L4 (C12/15S) was more diffuse in whole cells (Fig. 8F). The results indicated that the mutant cysteine at the 12th and 15th site of GLB1L4 protein significantly affected the localization of GLB1L4 protein in the cells.

Discussion

The variation of gene expression in different epididymal regions of mammals promotes sperm maturation. Many proteins in caput epididymis involve in the initiative movement of sperm, as well as the functional maintenance (Xu *et al.* 2010). It has been reported that the *Glb1l4* mRNA was expressed specifically in the caput epididymis of rats and the GLB1L4 protein localized in the epithelial cells of rat caput epididymis (Zhen *et al.* 2009), which were confirmed in our study. Further, we found that the protein was also present in lumen of cauda epididymis and sperm, although the level of GLB1L4 protein was lower in cauda epididymis and sperm than in caput epididymis. It suggested that GLB1L4 protein from epithelial cells of the caput epididymis might be transported to cauda epididymis in lumen.

The main function of genes in mammalian epididymis is promoting sperm maturation (Zhou *et al.* 2018). It is very difficult to study the function of epididymis-specific genes *in vitro* because of the specificity of mammal epididymal structure, while the *in vivo* conditional knockout is an effective way (Xie *et al.* 2013, Koch *et al.* 2015). It was proved that *in vivo* RNAi could be an effective way to study certain gene (Grillo *et al.* 2007, Frka *et al.* 2009, Towne & Aebischer 2009, Chou *et al.* 2010). Injection of siRNA into cauda epididymis with electrotransfection had been used to study the function of HongrES1 (Zhou *et al.* 2008). But it had identified that siRNA can only knockdown target gene expression transiently. ShRNA-lentivirus system is an alternative way which can knockdown target gene expression long-term (Song *et al.* 2006, Grillo *et al.* 2007, Harper & Gonzalez-Alegre 2008, Wang *et al.* 2008, 2009, Xie *et al.* 2008, Fan *et al.* 2014). Lentivirus vectors are potential gene delivery vehicles because of their ability to incorporate into genomic DNA with high efficiency in cells (Yu *et al.* 2007, Day *et al.* 2009, van Hooijdonk *et al.* 2009). So, the lentivirus system was usually used in animal transgenes (Coudmoul & Deng 2006, van Hooijdonk *et al.* 2009) and gene therapy (Copreni *et al.* 2004, Nguyen *et al.* 2005, Oh *et al.* 2006). In this study, the shRNA-lentivirus was used to knockdown the level of GLB1L4 *in vitro* or *in vivo*. The results illustrated that the level of GLB1L4 expression was downregulated significantly after shRNA-lentivirus was injected into the caput epididymis of rats for 1 month (Fig. 4).

Numerous proteins are identified to be involved in sperm maturation in the epididymis (Myles & Primakoff 1984, Myles *et al.* 1987, Vreeburg *et al.* 1990, Hunnicutt *et al.* 1997, Sipilä *et al.* 2006). Secretory proteins from

Figure 8 Analysis of the palmitoylated GLB1L4 protein. (A) The potential palmitoylation position of four cysteine residues of rat GLB1L4 protein was predicted by CSS at website <http://csspalm.biocuckoo.org/online.php>. (B) The morphology of the epithelial cells of caput epididymal was shown when the cells was cultured for 24 h in the medium with 20 mM 2-BP or without 2-BP (Control), respectively. Scale bar: 80 μ m. (C) GLB1L4 protein in cells, whole medium, and exosomes was analyzed by Western blot when the caput epididymal epithelial cells was cultured for 24 h in the medium with 20 mM 2-BP or without 2-BP (Control), respectively. The level of GLB1L4 protein in the caput epithelial cells was not significantly changed. However, the level of GLB1L4 protein in exosomes from the medium was significant inhibited by 2-BP. (D) The level of palmitoylated GLB1L4 protein in the caput epithelial cells was also significantly reduced when the 2-BP was added into the medium by ABE assay. (E) Localization of GLB1L4 protein in PC1 cells transfected pcDNA3.1-GLB1L4-WT plasmid showed clustered. Scale bar: 20 μ m. (F) Localization of GLB1L4 protein in PC1 cells transfected mutant pcDNA3.1-GLB1L4-C12/15S plasmid was more diffuse in the PC1 cells. Scale bar: 20 μ m.

caput initiate sperm motility (Xu *et al.* 2010) and the proteins from cauda regulate sperm motility (Dungdung & Majumder 2003, Das *et al.* 2010, Ghosh *et al.* 2018) or affect sperm capacitation (Roberts *et al.* 2003, Roberts *et al.* 2006, Ni *et al.* 2009, Krapf *et al.* 2012, Saewu *et al.* 2017). In this study, the sperm hyperactivation indexes (such as VAP, VSL, VCL and ALH) and the level of sperm protein phosphorylation decreased significantly after sperm capacitation of the knockdown GLB1L4 rats was

induced by BSA and Ca^{2+} . The average number of fetuses of female rats mated with the GLB1L4 knockdown male rats was significantly low compared with the control group ($P < 0.05$). It was inferred that GLB1L4 protein might regulate hyperactivation of capacitation sperm to maintain the normal physiological function of sperm in epididymis.

As sperm pass through the epididymis, some components of the epididymal fluid bind to the surface

of the sperm membrane to promote sperm maturation (Young & Goodman 1982, Cooper 1998, Jones 1998). The epididymosomes (namely exosomes), as cargo carriers, play an important role in transferring secretory proteins in the epididymal fluid onto the sperm surface for sperm maturation (Sullivan 2016, Sullivan & Mieusset 2016), meanwhile, epididymosomes loading critical molecules are transported from caput to cauda to maintain sperm function during storage of sperm in cauda epididymis (Frenette *et al.* 2006, Sullivan *et al.* 2007). In this study, we found that the GLB1L4 is involved in sperm capacitation and the GLB1L4 is released by binding on exosomes in the caput epididymis.

Exosomes, are a kind of nanoparticle with phospholipid bilayer membranous structure, and can selectively load some post-translational modification protein molecules when released from cells (Szabo-Taylor *et al.* 2015, Moreno-Gonzalo *et al.* 2018). Palmitoyl modification is an important protein modification mode, and the palmitoylation protein can be preferentially selected into exosomes by anchoring to the inner side of the bilayer membrane (Chamberlain & Shipsto 2015). The cysteine residues at the 12th and 15th sites of GLB1L4 protein molecule might be two key palmitoylation modification sites (Fig. 8A), and the level of palmitoylated GLB1L4 in caput epithelial cells *in vitro* could be inhibited by 2-BP (Fig. 8B, C, D and E). Further, the palmitoylated GLB1L4 could be released to the medium from epithelial cells *in vitro* and GLB1L4 in medium was mainly loaded on exosomes (Fig. 8C). The results indicated that palmitoylated GLB1L4 could be selected into exosomes in epithelial cells of rat caput epididymis and transported to cauda epididymis of rat. In rat cauda epididymis, the exosome could bind with sperm via the affinity of the lipid bilayer of exosome with the sperm membrane, which could result in GLB1L4 being loaded on the exosomes and added into sperm. It implied that GLB1L4 was transferred from epithelia of caput epididymis to sperm in cauda epididymis by exosomes in order to maintain the level of GLB1L4 in sperm.

In summary, GLB1L4 expression was down-regulated by lentivirus injection into the caput epididymis, and the function of GLB1L4 protein could be related to sperm capacitation. Moreover, the palmitoylated GLB1L4 loaded on exosomes could be a main form to regulate the function of sperm in rat epididymis. It will be necessary to further exploit the palmitoyl modified mechanism of GLB1L4 protein and to resolve how the palmitoylated GLB1L4 protein regulates sperm function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Fund of the Agriculture Sci-Tech Project of Shaanxi Province (No. 2014K01-20-01) and the National Natural Science Foundation China (NSFC) (No. C170104-31172205).

Author contribution statement

Wuzi Dong was the corresponding author who designed the overall studies. Guofan Peng and Huihui Gao performed bio-statistical analyses. Daqian Dong, Yining Chen and Wuzi Dong performed to inject lentivirus into rat and analyze the morphology of rat. Daqian Dong, Jinmeng Yang and Tianqi Jin performed to Western blot and ABE assay. Heran Cao and Tianqi Jin did the experimental verification. Daqian Dong, Jinmeng Yang and Wuzi Dong wrote and edited the manuscript. Fangxia Yang performed isolation and analysis of exosomes and revised manuscript. All authors discussed the results and commented on the manuscript.

References

- Araki Y, Suzuki K, Matusik RJ, Obinata M & Orgebin-Crist MC 2002 Immortalized epididymal cell lines from transgenic mice overexpressing temperature-sensitive simian virus 40 large T-antigen gene. *Journal of Andrology* **23** 854–869. (<https://doi.org/10.1002/j.1939-4640.2002.tb02344.x>)
- Björkgren I & Sipilä P 2019 The impact of epididymal proteins on sperm function. *Reproduction* **158** R155–R167. (<https://doi.org/10.1530/REP-18-0589>)
- Chamberlain LH & Shipsto MJ 2015 The physiology of protein S-acylation. *Physiological Reviews* **95** 341–376. (<https://doi.org/10.1152/physrev.00032.2014>)
- Chou MI, Hsieh YF, Wang M, Chang JT, Chang D, Zoual Mi & Tsay GJ 2010 *In vitro* and *in vivo* targeted delivery of IL-10 interfering RNA by JC virus-like particles. *Journal of Biomedical Science* **17** 51. (<https://doi.org/10.1186/1423-0127-17-51>)
- Cooper TG 1998 Interactions between epididymal secretions and spermatozoa. *Journal of Reproduction and Fertility: Supplement* **53** 119–136. (<https://doi.org/10.1023/A:1022582422932>)
- Copreni E, Penzo M, Carrabino S & Conese M 2004 Lentivirus-mediated gene transfer to the respiratory epithelium: a promising approach to gene therapy of cystic fibrosis. *Gene Therapy* **11** (Supplement 1) S67–S75. (<https://doi.org/10.1038/sj.gt.3302372>)
- Coumoul X & Deng CX 2006 RNAi in mice: a promising approach to decipher gene functions *in vivo*. *Biochimie* **88** 637–643. (<https://doi.org/10.1016/j.biochi.2005.11.010>)
- Das S, Saha S, Majumder GC & Dungdung SR 2010 Purification and characterization of a sperm motility inhibiting factor from caprine epididymal plasma. *PLoS ONE* **5** e12039. (<https://doi.org/10.1371/journal.pone.0012039>)
- Day CP, Carter J, Bonomi C, Esposito D, Crise B, Ortiz-Conde B, Hollingshead M & Merlino G 2009 Lentivirus-mediated bifunctional cell labeling for *in vivo* melanoma study. *Pigment Cell and Melanoma Research* **22** 283–295. (<https://doi.org/10.1111/j.1755-148X.2009.00545.x>)
- Du J, Wang L, Wang Y, Shen J, Pan C, Meng Y, Yang C, Ji H & Dong W 2016 Autophagy and apoptosis induced by Chinese giant salamander (*Andrias davidianus*) Iridovirus (CGSIV). *Veterinary Microbiology* **195** 87–95. (<https://doi.org/10.1016/j.vetmic.2016.09.011>)
- Dungdung SR & Majumder GC 2003 Isolation and identification of a novel motility-inhibiting factor from goat cauda sperm plasma membrane. *Cellular and Molecular Biology* **49** 413–420.
- Dunphy JT & Linder ME Signalling functions of protein palmitoylation 1998. *Biochimica et Biophysica Acta* **1436** 245–261. ([https://doi.org/10.1016/s0005-2760\(98\)00130-1](https://doi.org/10.1016/s0005-2760(98)00130-1))
- Eickhoff R, Wilhelm B, Renneberg H, Wennemuth G, Bacher M, Linder D, Bucal Ra, Seitz J & Meinhardt A 2001 Purification and characterization

- of macrophage migration inhibitory factor as a secretory protein from rat epididymis: evidences for alternative release and transfer to spermatozoa. *Molecular Medicine* **7** 27–35. (<https://doi.org/10.1007/BF03401836>)
- Ellerbrock K, Pera I, Hartung S & Ivell R 1994 Gene expression in the dog epididymis: a model for human epididymal function. *International Journal of Andrology* **17** 314–323. (<https://doi.org/10.1111/j.1365-2605.1994.tb01262.x>)
- Fan X, Rai A, Kambham N, Sung JF, Singh N, Petitt M, Dhal S, Agrawal R, Sutton RE, Druzin ML *et al.* 2014 Endometrial VEGF induces placental sFLT1 and leads to pregnancy complications. *Journal of Clinical Investigation* **124** 4941–4952. (<https://doi.org/10.1172/JCI76864>)
- Frenette G, Girouard J & Sullivan R 2006 Comparison between epididymosomes collected in the intraluminal compartment of the bovine caput and cauda epididymidis. *Biology of Reproduction* **75** 885–890. (<https://doi.org/10.1095/biolreprod.106.054692>)
- Frka K, Facchinello N, Del Vecchio C, Carpi A, Curtarello M, Venerando R, Angelin A, Parolin C, Bernardi P, Bonaldo P *et al.* 2009 Lentiviral-mediated RNAi *in vivo* silencing of Col6a1, a gene with complex tissue specific expression pattern. *Journal of Biotechnology* **141** 8–17. (<https://doi.org/10.1016/j.jbiotec.2009.02.013>)
- Gao H, Gao Y, Pang W, Pa Cn, Ji H & Dong W 2018 Iridoviral infection can be reduced by UCHL1-loaded exosomes from the testis of Chinese giant salamanders (Andrias davidianus). *Veterinary Microbiology* **224** 50–57. (<https://doi.org/10.1016/j.vetmic.2018.08.025>)
- Ghosh P, Mukherjee S, Bhoumik A & Dungdung SR 2018 A novel epididymal quiescence factor inhibits sperm motility by modulating NOS activity and intracellular NO-cGMP pathway. *Journal of Cellular Physiology* **233** 4345–4359. (<https://doi.org/10.1002/jcp.26275>)
- Grillo CA, Tamashiro KL, Piroli GG, Melhorn S, Gass JT, Newsom RJ, Reznikov LR, Smith A, Wilson SP, Sakai RR *et al.* 2007 Lentivirus-mediated downregulation of hypothalamic insulin receptor expression. *Physiology and Behavior* **92** 691–701. (<https://doi.org/10.1016/j.physbeh.2007.05.043>)
- Hannoush RN & Sun J 2010 The chemical toolbox for monitoring protein fatty acylation and prenylation. *Nature Chemical Biology* **6** 498–506. (<https://doi.org/10.1038/nchembio.388>)
- Harper SQ & Gonzalez-Alegre P 2008 Lentivirus-mediated RNA interference in mammalian neurons. *Methods in Molecular Biology* **442** 95–112. (https://doi.org/10.1007/978-1-59745-191-8_8)
- Hunnicuttt GR, Koppel DE & Myles DG 1997 Analysis of the process of localization of fertilin to the sperm posterior head plasma membrane domain during sperm maturation in the epididymis. *Developmental Biology* **191** 146–159. (<https://doi.org/10.1006/dbio.1997.8700>)
- Jennings BC, Nadolski MJ, Ling Y, Baker MB, Harrison ML, Deschenes RJ & Linder ME 2009 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation *in vitro*. *Journal of Lipid Research* **50** 233–242. (<https://doi.org/10.1194/jlr.M800270-JLR200>)
- Jiang H, Zhang X, Chen X, Aramsangtienchai P, Tong Z & Lin H 2018 Protein lipidation: occurrence, mechanisms, biological functions, and enabling technologies. *Chemical Reviews* **118** 919–988. (<https://doi.org/10.1021/acs.chemrev.6b00750>)
- Jones R 1998 Plasma membrane structure and remodelling during sperm maturation in the epididymis. *Journal of Reproduction and Fertility: Supplement* **53** 73–84. (<https://doi.org/10.1023/A:1022582422932>)
- Koch S, Acebron SP, Herbst J, Hatiboglu G & Niehrs C 2015 Post-transcriptional Wnt signaling governs epididymal sperm maturation. *Cell* **163** 1225–1236. (<https://doi.org/10.1016/j.cell.2015.10.029>)
- Krapf D, Ruan YC, Wertheimer EV, Battistone MA, Pawlak JB, Sanjay A, Pilder SH, Cuasnicu P, Breton S & Visconti PE 2012 cSrc is necessary for epididymal development and is incorporated into sperm during epididymal transit. *Developmental Biology* **369** 43–53. (<https://doi.org/10.1016/j.ydbio.2012.06.017>)
- Lanyon-Hogg T, Faronato M, Serwa RA & Tate EW 2017 Dynamic protein acylation: new substrates, mechanisms, and drug targets. *Trends in Biochemical Sciences* **42** 566–581. (<https://doi.org/10.1016/j.tibs.2017.04.004>)
- María-Eugenia Z & Gisou VDF 2018 The molecular era of protein S-acylation: spotlight on structure, mechanisms, and dynamics. *Critical Reviews in Biochemistry and Molecular Biology* **53** 420–451. (<https://doi.org/10.1080/10409238.2018.1488804>)
- Milligan G, Parenti M & Magee AI 1995 The dynamic role of palmitoylation in signal transduction. *Trends in Biochemical Sciences* **20** 181–187. ([https://doi.org/10.1016/s0968-0004\(00\)89004-0](https://doi.org/10.1016/s0968-0004(00)89004-0))
- Moreno-Gonzalo O, Fernandez-Delgado I & Sanchez-Madrid F 2018 Post-translational add-ons mark the path in exosomal protein sorting. *Cellular and Molecular Life Sciences* **75** 1–19. (<https://doi.org/10.1007/s00018-017-2690-y>)
- Mühlbauer M, Cheely AW, Yenugu S & Jobin C 2008 Regulation and functional impact of lipopolysaccharide induced Nod2 gene expression in the murine epididymal epithelial cell line PC1. *Immunology* **124** 256–264. (<https://doi.org/10.1111/j.1365-2567.2007.02763.x>)
- Myles DG & Primakoff P 1984 Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *Journal of Cell Biology* **99** 1634–1641. (<https://doi.org/10.1083/jcb.99.5.1634>)
- Myles DG, Koppel DE, Cowan AE, Phelps BM & Primakoff P 1987 Rearrangement of sperm surface antigens prior to fertilization. *Annals of the New York Academy of Sciences* **513** 262–273. (<https://doi.org/10.1111/j.1749-6632.1987.tb25014.x>)
- Nguyen TH, Bellodi-Privato M, Aubert D, Pichard V, Myara A, Trono D & Ferry N 2005 Therapeutic lentivirus-mediated neonatal *in vivo* gene therapy in hyperbilirubinemic Gunn rats. *Molecular Therapy* **12** 852–859. (<https://doi.org/10.1016/j.ymthe.2005.06.482>)
- Ni Y, Zhou Y, Chen WY, Zheng M, Yu J, Li C, Zhang Y & Shi QX 2009 HongrES1, a cauda epididymis-specific protein, is involved in capacitation of guinea pig sperm. *Molecular Reproduction and Development* **76** 984–993. (<https://doi.org/10.1002/mrd.21063>)
- Oh TK, Li MZ & Kim ST 2006 Gene therapy for diabetes mellitus in rats by intramuscular injection of lentivirus containing insulin gene. *Diabetes Research and Clinical Practice* **71** 233–240. (<https://doi.org/10.1016/j.diabres.2005.08.005>)
- Percherancier Y, Planchenault T, Valenzuela-Fernandez A, Virelizier JL, Arenzana-Seisdedos F & Bachelier F 2001 Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. *Journal of Biological Chemistry* **276** 31936–31944. (<https://doi.org/10.1074/jbc.M104013200>)
- Resh MD 1999 Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochimica et Biophysica Acta* **1451** 1–16. ([https://doi.org/10.1016/s0167-4889\(99\)00075-0](https://doi.org/10.1016/s0167-4889(99)00075-0))
- Resh MD 2006 Use of analogs and inhibitors to study the functional significance of protein palmitoylation. *Methods* **40** 191–197. (<https://doi.org/10.1016/j.ymeth.2006.04.013>)
- Roberts KP, Wamstad JA, Ensrud KM & Hamilton DW 2003 Inhibition of capacitation-associated tyrosine phosphorylation signaling in rat sperm by epididymal protein Crisp-1. *Biology of Reproduction* **69** 572–581. (<https://doi.org/10.1095/biolreprod.102.013771>)
- Roberts KP, Ensrud KM, Wooters JL, Nolan MA, Johnston DS & Hamilton DW 2006 Epididymal secreted protein Crisp-1 and sperm function. *Molecular and Cellular Endocrinology* **250** 122–127. (<https://doi.org/10.1016/j.mce.2005.12.034>)
- Rodríguez CM, Kirby JL & Hinton BT 2001 Regulation of gene transcription in the epididymis. *Reproduction* **122** 41–48. (<https://doi.org/10.1530/rep.0.1220041>)
- Romancino DP, Buffa V, Caruso S, Ferrara I, Raccosta S, Notaro A, Campos Y, Noto R, Martorana V, Cupane A *et al.* 2018 Palmitoylation is a post-translational modification of Alix regulating the membrane organization of exosome-like small extracellular vesicles. *Biochimica et Biophysica Acta: General Subjects* **1862** 2879–2887. (<https://doi.org/10.1016/j.bbagen.2018.09.004>)
- Saewu A, Kadunganattil S, Raghupathy R, Kongmanas K, Díaz-Astudillo P, Hermo L & Tanphaichitr N 2017 Clusterin in the mouse epididymis: possible roles in sperm maturation and capacitation. *Reproduction* **154** 867–880. (<https://doi.org/10.1530/REP-17-0518>)
- Shen B, Wu N, Yang JM & Gould SJ 2011 Protein targeting to exosomes/microvesicles by plasma membrane anchors. *Journal of Biological Chemistry* **286** 14383–14395. (<https://doi.org/10.1074/jbc.M110.208660>)
- Shibata T, Hadano J, Kawasaki D, Dong X & Kawabata SI 2017 Drosophila TG-A transglutaminase is secreted via an unconventional Golgi-independent mechanism involving exosomes and two types of fatty acylations. *Journal of Biological Chemistry* **292** 10723–10734. (<https://doi.org/10.1074/jbc.M117.779710>)

- Sipilä P, Pujianto DA, Shariatmadari R, Nikkilä J, Lehtoranta M, Huhtaniemi IT & Poutanen M 2006 Differential endocrine regulation of genes enriched in initial segment and distal caput of the mouse epididymis as revealed by genome-wide expression profiling. *Biology of Reproduction* **75** 240–251. (<https://doi.org/10.1095/biolreprod.105.047811>)
- Snyder EM, Small CL, Bomgardner D, Xu B, Evanoff R, Griswold MD & Hinton BT 2010 Gene expression in the efferent ducts, epididymis, and vas deferens during embryonic development of the mouse. *Developmental Dynamics* **239** 2479–2491. (<https://doi.org/10.1002/dvdy.22378>)
- Song Y, Zhang Z, Yu X, Yan M, Zhang X, Gu S, Stuart T, Liu C, Reiser J, Zhang Y *et al.* 2006 Application of lentivirus-mediated RNAi in studying gene function in mammalian tooth development. *Developmental Dynamics* **235** 1334–1344. (<https://doi.org/10.1002/dvdy.20706>)
- Sullivan R 2016 Epididymosomes: role of extracellular microvesicles in sperm maturation. *Frontiers in Bioscience* **8** 106–114. (<https://doi.org/10.2741/s450>)
- Sullivan R & Miesusset R 2016 The human epididymis: its function in sperm maturation. *Human Reproduction Update* **22** 574–587. (<https://doi.org/10.1093/humupd/dmw015>)
- Sullivan R, Frenette G & Girouard J 2007 Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian Journal of Andrology* **9** 483–491. (<https://doi.org/10.1111/j.1745-7262.2007.00281.x>)
- Szabo-Taylor K, Ryan B, Osteikoetxea X, Szabo TG, Soda Br, Holub M, Nemeth A, Paloczi K, Pallinger É, Winyard P *et al.* 2015 Oxidative and other posttranslational modifications in extracellular vesicle biology. *Seminars in Cell and Developmental Biology* **40** 8–16. (<https://doi.org/10.1016/j.semcdb.2015.02.012>)
- Towne C & Aebischer P 2009 Lentiviral and adeno-associated vector-based therapy for motor neuron disease through RNAi. *Methods in Molecular Biology* **555** 87–108. (https://doi.org/10.1007/978-1-60327-295-7_7)
- van Hooijdonk LW, Ichwan M, Dijkmans TF, Schouten TG, de Backer MW, Adan RA, Verbeek FJ, Vreugdenhil E & Fitzsimons CP 2009 Lentivirus-mediated transgene delivery to the hippocampus reveals sub-field specific differences in expression. *BMC Neuroscience* **10** 2. (<https://doi.org/10.1186/1471-2202-10-2>)
- Verweij FJ, De Heus C, Kroeze S, Cai H, Kieff E, Piersma SR, Jimenez CR, Middeldorp JM & Pegtel DM 2015 Exosomal sorting of the viral oncoprotein LMP1 is restrained by TRAF2 association at signalling endosomes. *Journal of Extracellular Vesicles* **4** 26334. (<https://doi.org/10.3402/jev.v4.26334>)
- Vreeburg JT, Holland MK, Cornwall GA & Orgebin-Crist MC 1990 Secretion and transport of mouse epididymal proteins after injection of 35S-methionine. *Biology of Reproduction* **43** 113–120. (<https://doi.org/10.1095/biolreprod43.1.113>)
- Wan J, Roth AF, Bailey AO & Davis NG 2007 Palmitoylated proteins: purification and identification. *Nature Protocols* **2** 1573–1584. (<https://doi.org/10.1038/nprot.2007.225>)
- Wang F, Chen L, Mao ZB, Shao JG, Tan C & Huang WD 2008 Lentivirus-mediated short hairpin RNA targeting the APRIL gene suppresses the growth of pancreatic cancer cells *in vitro* and *in vivo*. *Oncology Reports* **20** 135–139. (<https://doi.org/10.3892/or.20.1.135>)
- Wang YH, Wang ZX, Qiu Y, Xiong J, Chen YX, Miao DS & De W 2009 Lentivirus-mediated RNAi knockdown of insulin-like growth factor-1 receptor inhibits growth, reduces invasion, and enhances radiosensitivity in human osteosarcoma cells. *Molecular and Cellular Biochemistry* **327** 257–266. (<https://doi.org/10.1007/s11010-009-0064-y>)
- Wang Z, Luo J, Wang W, Zhao W & Lin X 2010 Characterization and culture of isolated primary dairy goat mammary gland epithelial cells. *Sheng Wu Gong Cheng Xue Bao* **26** 1123–1127.
- Webb Y, Hermida-Matsumoto L & Resh MD 2000 Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *Journal of Biological Chemistry* **275** 261–270. (<https://doi.org/10.1074/jbc.275.1.261>)
- Xie SM, Fang WY, Liu Z, Wang SX, Li X, Liu TF, Xie WB & Yao KT 2008 Lentivirus-mediated RNAi silencing targeting ABCG2 increasing the sensitivity of a human nasopharyngeal carcinoma cell line against cisplatin. *Journal of Translational Medicine* **6** 55. (<https://doi.org/10.1186/1479-5876-6-55>)
- Xie S, Xu J, Ma W, Liu Q, Han J, Yao G, Huang X & Zhang Y 2013 Lcn5 promoter directs the region-specific expression of cre recombinase in caput epididymidis of transgenic mice. *Biology of Reproduction* **88** 71. (<https://doi.org/10.1095/biolreprod.112.104034>)
- Xu W, Zhang X, Chen W, Fok KL, Rowlands DK, Chui YL & Chan HC 2010 Immunization with Bin1b decreases sperm motility with compromised fertility in rats. *Fertility and Sterility* **93** 0-9580. (<https://doi.org/10.1016/j.fertnstert.2008.10.066>)
- Yang W, Di Vizio D, Kirchner M, Steen H & Freeman MR 2010 Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. *Molecular and Cellular Proteomics* **9** 54–70. (<https://doi.org/10.1074/mcp.M800448-MCP200>)
- Young LG & Goodman SA 1982 Analysis of lipid and protein components of ejaculated bull sperm surface and seminal plasma. *Gamete Research* **6** 281–291. (<https://doi.org/10.1002/mrd.1120060310>)
- Yu ZY, McKay K, Van Asperen P, Zheng M, Fleming J, Ginn SL, Kizana E, Latham M, Feneley MP, Kirkland PD *et al.* 2007 Lentivirus vector-mediated gene transfer to the developing bronchiolar airway epithelium in the fetal lamb. *Journal of Gene Medicine* **9** 429–439. (<https://doi.org/10.1002/jgm.1039>)
- Zhen W, Li P, He B, Guo J & Zhang YL 2009 The novel epididymis-specific beta-galactosidase-like gene Glib14 is essential in epididymal development and sperm maturation in rats. *Biology of Reproduction* **80** 696–706. (<https://doi.org/10.1095/biolreprod.108.071589>)
- Zhou Y, Zheng M, Shi Q, Zhang L, Zhen W, Chen W & Zhang Y 2008 An epididymis-specific secretory protein HongrES1 critically regulates sperm capacitation and male fertility. *PLoS ONE* **3** e4106. (<https://doi.org/10.1371/journal.pone.0004106>)
- Zhou W, De Iuliis GN, Dun MD & Nixon B 2018 Characteristics of the epididymal luminal environment responsible for sperm maturation and storage. *Frontiers in Endocrinology* **9** 59. (<https://doi.org/10.3389/fendo.2018.00059>)
- Zhu CF, Liu Q, Zhang L, Yuan HX, Zhen W, Zhang JS, Chen ZJ, Hall SH, French FS & Zhang YL 2007 RNase9, an androgen-dependent member of the RNase A family, is specifically expressed in the rat epididymis. *Biology of Reproduction* **76** 63–73. (<https://doi.org/10.1095/biolreprod.106.054635>)

Received 29 July 2020

First decision 20 August 2020

Revised manuscript received 21 November 2020

Accepted 4 December 2020