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ORIGINAL ARTICLE

Male Fertility

An epididymis-specific carboxyl esterase *CES5A* is required for sperm capacitation and male fertility in the rat

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Despite the fact that the phenomenon of capacitation was discovered over half century ago and much progress has been made in identifying sperm events involved in capacitation, few specific molecules of epididymal origin have been identified as being directly involved in this process *in vivo*. Previously, our group cloned and characterized a carboxyl esterase gene *Ces5a* in the rat epididymis. The CES5A protein is mainly expressed in the corpus and cauda epididymidis and secreted into the corresponding lumens. Here, we report the function of *CES5A* in sperm maturation. By local injection of *Lentivirus*-mediated siRNA in the *CES5A*-expressing region of the rat epididymis, *Ces5a*-knockdown animal models were created. These animals exhibited an inhibited sperm capacitation and a reduction in male fertility. These results suggest that *CES5A* plays an important role in sperm maturation and male fertility.
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INTRODUCTION

The epididymis is one of the most important components of the mammalian male reproductive system. It is only during transit through the epididymal luminal microenvironment that spermatozoa undergo maturation and acquire progressive motility and the ability to fertilize oocytes.[1–4] The mammalian epididymal duct can be subdivided into four morphological regions: the initial segment, the caput, corpus and cauda epididymidis, all of which are essential for sperm maturation.[5,6]

Carboxylesterases are a multi-gene family of serine-dependent enzymes (carboxyl-ester hydrolases; EC 3.1.1.1) which are localized in the endoplasmic reticulum of many tissues and hydrolyze carboxylester, amide, and thioester bonds in a variety of exogenous and endogenous compounds.[7] The comparative investigation of sex-dependent protein expression in the male reproductive-tract tissues of bivalve molluscs, fruit flies, and mammals has revealed evolutionarily stable patterns of high carboxylesterase expression in the male reproductive tract of all animals studied.[8–10] The phenomenon of conserved high carboxylesterase expression indicates similar male sex-associated functions of the enzymes.[8] However, very little are known about the functional significance of overexpressed carboxylesterase in the male reproductive system. To understand the role of carboxylesterase in the male reproductive tract and sperm quality, it is important to identify sperm-associated carboxylesterases and trace their fate at all steps during the sperm's pathway to the egg.

Our group recently cloned and characterized a new carboxylesterase gene *Ces5a*, which is expressed specifically in the rat epididymis. *CES5A* is mainly expressed in the corpus and cauda epididymidis and secreted into the corresponding lumens.[11] The recombinant CES5A proteins exhibit carboxylesterase activity hydrolyzing cholesterol ester and choline ester.[12] Moreover, cauxin, the homologous protein of *CES5A*, is found in urine of the domestic cat.[13–16] Recent study from our laboratory has shown that over-expression of *HongrES2*, a novel noncoding RNA specifically expressed in epididymis, can reduce the CES5A protein level and sperm capacitation-associated protein tyrosine phosphorylation.[17] Because small noncoding RNA might target a wide variety of genes, it is not clear whether *CES5A* is associated with sperm capacitation. Here we applied RNAi specifically to knock down CES5A protein in the rat and investigate its role in male fertility. The results indicated that the knockdown of *CES5A* led to marked inhibition of sperm capacitation and a reduction in male fertility. These data suggest that *CES5A* plays an important role in sperm maturation and male fertility.

MATERIALS AND METHODS

Animals

Healthy Sprague-Dawley (SD) rats were supplied by the Animal Center of the Chinese Academy of Sciences (Shanghai, China) and housed under controlled lighting (14 h light; 10 h darkness) at 21–22°C and were provided with water and libitum. In this study, we used 120 male rats (400–450 g) and 50 female rats (200 g for *in vitro* fertilization [IVF], 300 g for the mating test). Trichloroacetaldehyde monohydrate (6%, w/v) was used as an anesthesia for *in vivo* lentiviral procedures. The rats were killed by CO_2 asphyxiation. All the experiments were conducted according to a protocol approved by the Institute Animal Care Committee.

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RNA isolation and Northern blot analysis

Total RNA was extracted from tissue homogenates and cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Northern blot analysis was performed according to a procedure described previously.[18] Twenty microgramme of total RNA from each sample were subjected to 1.0% (*w/v*) agarose-formaldehyde gel electrophoresis, blotted onto nylon membranes by capillary transfer and hybridized with a probe which was obtained from the full *Ces5a* clone digested with BamHI at both 291 and 1612 bp sites (Gene bank, NM_001012056). The ^{32}P-labeled probe was prepared by using the prime-a-gene labeling kit (Promega, Madison, USA). An 18S rRNA hybridization signal was used as a loading control.

Quantitative real-time polymerase chain reaction

Quantitative real-time reverse transcription polymerase chain reaction (PCR) was performed by using SYBR Green Real-time PCR Mix (Toyobo, Osaka, Japan) according to the manufacturer's protocol with a Rotorgene 3000 machine. The data were analyzed by the 2^(-ΔΔ) method. Levels of mRNA were normalized to GAPDH mRNA and then normalized to the *Csi* group. The primers used for real-time PCR are in **Table 1**.

Cell culture and siRNA transfection

The immortalized mouse epididymal cell line PC-1 (from the proximal caput) was generously provided by Dr. Orgebin-Crist (Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN, USA). The PC-1 cell line was grown in Iscove's modified Dulbecco's medium supplemented with 10% (*v/v*) fetal bovine serum, 1 mmol l^{-1} sodium pyruvate, 0.1 mmol l^{-1} nonessential amino acids, 4 mmol l^{-1} glutamine, penicillin-streptomycin (penicillin, 0.6 μg ml^{-1}; streptomycin, 5 μg ml^{-1}), and 1 nmol l^{-1} 5α-dihydrotestosterone and cultured at 33°C with 5% (*v/v*) CO_2. All cell culture reagents were bought from Invitrogen. Three potential siRNAs of 21 nucleotides (nt) specific to *Ces5a* (NM_001012056) (*C5si1*, *C5si2* and *C5si3*) were selected empirically with a design tool incorporating standard parameters,[19] and the siRNA targeting enhanced green fluorescent protein (EGFP, NC_011521) was used as control for sequence specificity (*Csi*). The sense strands of siRNAs were: *C5si1* (sequence1), beginning at nt 807, 5'-GCATTTCTAGCCTCATTCT-3'; *C5si2* (sequence2), beginning at nt 1878, 5'-GTTTCTCCCTCAGCCATTA-3'; *C5si3* (sequence3), beginning at nt 1543, 5'-GCCACTGAGGATGAGAAGT-3'; and *Csi*, beginning at nt 106, 5'-GGCGATGCCACCTACGGCAAG-3'. To examine the silencing effect of siRNAs, psiRNA vectors (pLentiLox 3.7) producing shRNAs targeting *Ces5a* were constructed according to the kit instructions (Invitrogen), then the psiRNA constructs and a pcDNA 3.0 vector expressing *Ces5a* (all-length coding sequence) with mass ratio 1:2 were co-transfected into PC-1 cells with lipofectamine 2000 (0.4%, v/v, Invitrogen). The knockdown efficiency was determined by Northern blotting of extracts from transfected cultures 48 h after transfection.

Lentivirus particles production and injection

Lentiviral particles were produced by transient co-transfection of 293 T cells by pRNAi/Lenti vectors (HaiGene, Harbin, China), an encapsidation plasmid (Δ8.9), and a vesicular stomatitis virus (VSV-g) expression plasmid as previously described.[20] The surgical procedures were performed according to a previously described method with modification.[21,22] Briefly, after the skin covering the testis and epididymis of a male SD rat was cut open under anesthesia, the cauda epididymidis was gently squeezed out and fastened with tweezers, and 1 × 10^6 transducing units of shRNA *Lentivirus* particles of *Csi*, C5si1 or C5si2 in PBS solution (viral titer was 2 × 10^8 units ml^{-1}) were injected into the interstitial space of the cauda epididymidis at opposite sites. The lentiviral injections were performed in the cauda of both epididymides of each rat. Then the wound was sewn up carefully with a suture needle (8/3 CIRCLE). The cauda epididymidis and spermatozoa were collected for assessment of total protein 7 days after injection.

Table 1: Primers used for Q-PCR

Primers	*Sequence 5'-3'*
Rat *GAPDH* forward	TACAAGGAGTAAGAAACCGTG
Rat *GAPDH* reverse	GTTATTATGGGGTCTGGGATGG
Rat *Ces5a* forward	CCACTGAGGATGAGAAGTTGC
Rat *Ces5a* reverse	CTTTCAGTCTCCATCCAGTGCT

Q-PCR: quantitative-polymerase chain reaction

Sperm preparation and assessment of sperm motility

The cauda epididymidis was excised and freed from the fat pad, blood vessels and connective tissue. The tissue was then transferred to a dish containing 1 ml enriched Krebs-Ringer bicarbonate (EKRB) medium (94.6 mmol l^{-1} NaCl, 25 mmol l^{-1} KCl, 1.71 mmol l^{-1} $CaCl_2$, 1.19 mmol l^{-1} $MgSO_4$, 1.19 mmol l^{-1} KH_2PO_4, 25 mmol l^{-1} $NaHCO_3$, 5.56 mmol l^{-1} glucose, 10.76 mmol l^{-1} sodium lactate and 0.5 mmol l^{-1} sodium pyruvate, 0.002% (*w/v*) phenol red, 4 mg ml^{-1} bovine serum albumin; 50 mg ml^{-1} streptomycin sulfate, 75 mg ml^{-1} potassium penicillin, pH 7.4, osmolarity about 310 mOsmol kg^{-1}) prewarmed to 37°C, and cut in several places with iridectomy scissors to release the spermatozoa into the medium. After 5 min, the sperm suspension was transferred to a 5 ml centrifuge tube. The final concentration of spermatozoa was adjusted to 3 × 10^6– 4 × 10^6 cells ml^{-1} in appropriate medium and assessed in a computer-assisted semen analysis (CASA) machine (HTM-TOXIVOS; Rat Head Toxicology, version 12.3A; Hamilton-Thorn Research, MA, USA) as previously described.[22]

Evaluation of sperm capacitation

For the assessment of capacitation, spermatozoa from the whole cauda epididymidis were released into the capacitation medium (see above). Then we performed an evaluation of tyrosine-phosphorylation and chlortetracycline (CTC) staining according to the procedures previously described.[22] For tyrosine-phosphorylation detection, briefly, the sperm pellet was suspended in Laemmli buffer, and total sperm proteins were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% (*w/v*) Tris-glycine gels for western blotting (MILLIPORE, Anti-Phosphotyrosine, clone 4G10), α-tubulin (SIGMA-ALDRICH, Monoclonal Anti-α-tubulin, Clone B515) was used as loading control.

Protein extraction and Western blotting of CES5A

For preparation of protein extracts from the rat cauda epididymidis, fresh tissue was homogenized in 4 ml homogenization buffer (25 mmol l^{-1} NaH_2PO_4, pH 7.2, 1.5 mmol l^{-1} EDTA, 10% [v/v] glycerol, 10 mmol l^{-1} Na_2MO_4, 10 mmol l^{-1} NaF, 2 μmol l^{-1} aprotinin, 5 mmol l^{-1} leupeptin, 2 mmol l^{-1} PMSF). The extracts were centrifuged at 15 000 *g* for 1 h. The supernatant was assayed for protein concentration and aliquotted and stored at – 80°C. For the preparation of luminal samples, the cauda epididymidis was excised and freed of the fat-pad, blood vessels and connective tissue. The tissue was transferred to a glass dish containing 1 ml EKRB medium prewarmed to 37°C and cut in several places with iridectomy scissors to release the spermatozoa and luminal fluid into the medium. The sperm suspension was centrifuged at 8000 *g* for 2 min, and the supernatant was collected as the luminal protein sample. Protein extracts of the epididymal cauda and lumen were resolved over 8% (*w/v*) SDS-PAGE, transferred to polyvinylidene

fluoride membranes, and probed with rabbit polyclonal antisera against *CES5A* (1:10 000).[11] The bound IgG was detected with goat anti-rabbit-HRP (Calbiochem, Germany) (1:10 000) and developed with ECL Plus (Amersham). The loaded protein was assayed by probing blots with a monoclonal antibody against β-actin (SIGMA-ALDRICH, Monoclonal Anti-β-actin-Peroxidase, Clone AC-15).

Fertility assay

In vitro fertilization was performed by following a standard protocol.[23] After 48 h incubation, inseminated oocytes were examined for the presence of two-cell stage embryos as an indication of fertilization. The mating experiments were performed by following a modified protocol.[22] Each male rat was placed with two normal females in succession 4 days after injection into cauda epididymidis. They were maintained together overnight, vaginal plugs of the females were checked in the following morning. The female rats were considered mated if vaginal plugs were found, and then female rats were housed individually. The pregnant females at 18 days were subjected to hysterectomy to determine the number of embryos or fetuses.

Statistical analysis

All experiments were repeated a minimum of 3 times. The results represent the means ± standard error of the mean. Data were analyzed using one-way analysis of variance, followed by the Tukey *post hoc* test (SigmaPlot version 12.3 software; Systat Software, Richmond, CA, USA). Statistical differences were defined at $P < 0.05$, and $P < 0.01$ or $P < 0.001$.

RESULTS

Establishment of a Ces5a knockdown rat model

The strategies to investigate gene function *in vivo* include gene knockout and knockdown. However, it is difficult to establish knockout rats and hence RNAi is the best choice for functional studies of rat genes *in vivo*.[21,22,24] Given the transduction efficiency and duration of gene silencing, we applied lentiviral-mediated RNAi to knock down *Ces5a* gene expression in the cauda epididymidis and explore its role in sperm maturation and storage. According to the RNAi mechanism-based rules,[19] three siRNAs (*C5si1*, *C5si2* and *C5si3*) targeting different sites of *Ces5a* gene were designed and an siRNA (*Csi*) of EGFP was used as control for silencing specificity. *C5si1* and *C5si2* but not *C5si3*, were able to inhibit *Ces5a* expression dramatically at the mRNA level in the mouse epididymal epithelial cells (**Figure 1**). So we used these two effective siRNAs (*C5si1* and *C5si2*) targeting *Ces5a* gene for the subsequent experiments in this study. Lentiviral particles that can generate effective shRNAs were produced and locally injected into the cauda epididymidis. Western blotting indicated a 30%–40% decrease of *CES5A* in the cauda region of the rat epididiymis after 7 days by siRNA treatment (**Figure 2a**–**2c**). As expected, down-regulation of *Ces5a* expression led to a dramatic reduction of secreted CES5A proteins into the cauda lumen (**Figure 2d**). These results suggested that we had successfully established a *CES5A* knockdown rat model.

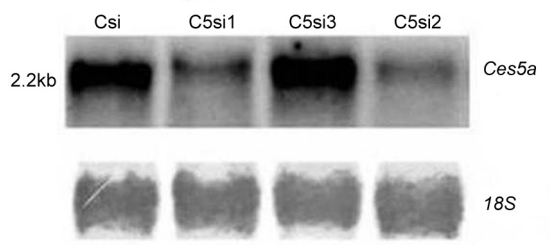

Figure 1: Identification of siRNAs targeted *Ces5a* effectively at the cellular level. Northern blotting analysis shows the expression of *Ces5a* mRNA *in vitro* 48 h after treatment with siRNAs. 18S RNA was used as loading control. This experiment was repeated 3 times. C5si1 and C5si2, two siRNAs specifically target the different sites of *Ces5a* sequence.

Sperm motility of Ces5a gene knockdown rats

Although sperm acquire motility during transit through the caput epididymidis, we still examined if the sperm motility was affected during transit through the cauda region, where CES5A proteins had a 20%–30% decrease, by CASA. Although there was no obvious change in the percentage of motile and progressive sperm cells and their beat cross frequency (**Figure 3a** and **3b**), straight-line velocity (VSL) and curvilinear velocity (VCL) of spermatozoa from *Ces5a* siRNA-treated male rats increased dramatically (**Figure 3c**).

CES5A contributes to sperm capacitation

We explored whether *Ces5a* knockdown affected sperm capacitation. It has been shown that capacitation is characterized by a spontaneous, time-dependent increase of tyrosine phosphorylation of sperm proteins.[25] Hence, protein tyrosine phosphorylation patterns were analyzed by western blotting by using an anti-phosphotyrosine antibody. As shown in **Figure 4a**, the level of sperm phospho-tyrosine protein decreased dramatically in the *Ces5a* siRNA-treated group when spermatozoa were collected and incubated for 3 h. For confirmation, we performed CTC staining, which is another method for evaluating capacitation. CTC is a fluorescent antibiotic whose distribution in the sperm cell changes during the transition from the noncapacitated to the capacitated state and then to the acrosome-reacted state, as shown in **Figure 4b**. The results of CTC staining revealed obvious changes with the percentage of uncapacitated spermatozoa (F pattern) increasing in the *Ces5a* siRNA-treated group at 1 h and 3 h (**Figure 4c**). On the contrary, the percentage of capacitated patterns (B pattern) markedly decreased at the 0 h time point, and this status was maintained as time progressed (1, 3 and 5 h) (**Figure 4d**). The percentage of spontaneous acrosome-reacted spermatozoa (AR pattern) showed no obvious changes (**Figure 4e**). Consistent with the result of tyrosine phosphorylation, CTC staining indicated that the sperm capacitation was inhibited in the *Ces5a* knockdown rat.

Reduced fertility of CES5A knockdown rats in vitro and in vivo

Capacitation indicates the completion of sperm maturation that confers on the mammalian sperm fertilization competence. It is widely accepted that sperm capacitation is a prerequisite for fertilization. In view of the inhibition of sperm capacitation in the *Ces5a* knockdown rat, we conducted IVF and *in vivo* matings. IVF showed that the fertilization by the spermatozoa from *Ces5a* siRNA-treated rats was much lower than that of the control groups (**Figure 5a**). Finally, we performed mating experiments to evaluate the fertility of *Ces5a* knockdown male rats. As shown in **Figure 5b**, the numbers of normal fetuses were considerably reduced in the litters from receptive female rats mated with specific siRNA-treated male rats, compared with the numbers in the control groups. These data confirm that *CES5A* is important for sperm capacitation and male fertility.

DISCUSSION

A recent study by our group showed that over-expression of the epididymis-specific *HongrES2* reduces CES5A protein and inhibits sperm capacitation-associated protein tyrosine phosphorylation.[17] However, it is uncertain whether the inhibition of sperm capacitation induced by *HongrES2* was caused by the decrease of *Ces5a* expression.

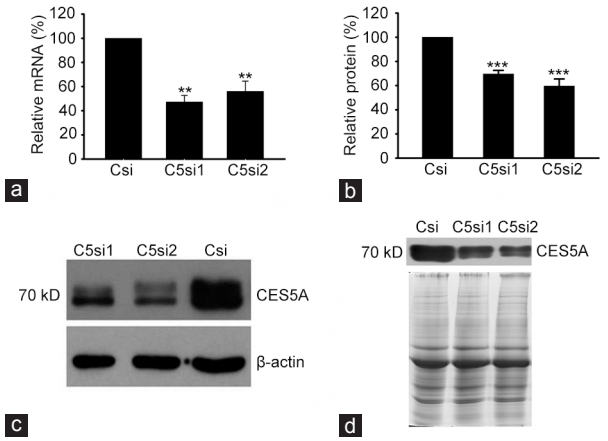

Figure 2: Establishment of a *Ces5a* knockdown rat model. (**a**) Expression of *Cea5a* in cauda epididymidal tissue of the rat at the mRNA level. (**b**) Expression of *CES5A* in cauda epididymidal tissue of the rat at the protein level. Densitometry of *CES5A* and its corresponding β-actin band were performed by Multi Gauge (Fujifilm, version 3.0). The densitometry ratio of *CES5A* to ACTIN was calculated, and this ratio was calibrated to the nontreated sample to provide the relative protein (%). (**c**) Detection of CES5A protein in the cauda epididymidis by western blotting. β-actin was used as loading control. The western blot is a representative of 5 independent experiments. (**d**) Detection of secreted CES5A protein in the cauda lumen by western blotting. Coomassie blue staining was used as internal control. Ten microgramme of total protein were loaded to sodium dodecyl sulfate polyacrylamide gel electrophoresis. In (**a**–**c**): *Csi*, EGFP siRNA control; C5si1 and C5si2, two siRNAs specifically targeting the different sites of *Ces5a* sequence. Results represent the means ± standard error of mean of 5–7 independent experiments and were analyzed by one-way analysis of variance and subsequently by the Tukey *post hoc* test (SigmaPlot version 12.3 software; Systat Software, Richmond, CA, USA). $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the respective *Csi* group.

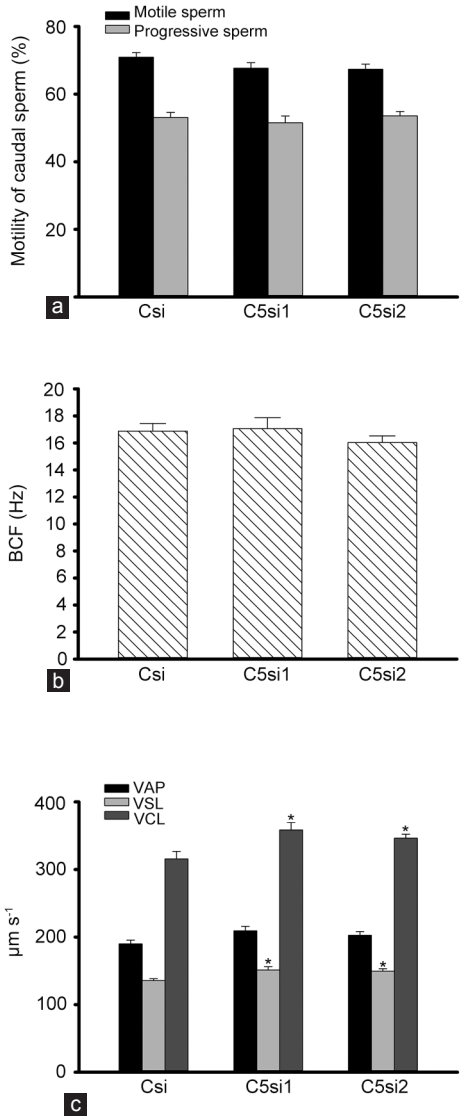

Figure 3: Sperm motility assay after *Ces5a* knockdown. (**a**) The percentage of total and progressive motility of spermatozoa from the rat cauda epididymidis treated with *Ces5a* siRNA. (**b**) Beat/cross frequency of spermatozoa from the rat cauda epididymidis after *Ces5a* expression had been downregulated. (**c**) Average path velocity, straight-line velocity and curvilinear velocity of spermatozoa from the rat cauda epididymidis after *Ces5a* expression had been downregulated. In (**a**–**c**): *Csi*, EGFP siRNA control; C5si1 and C5si2, two siRNAs specifically targeting the different sites of *Ces5a* sequence. Motility data were collected immediately after spermatozoa were released into the incubation medium. Results represent the means ± standard error of mean of 9 independent experiments and were analyzed by one-way analysis of variance and subsequently by the Tukey *post hoc* test (SigmaPlot version 12.3 software; Systat Software, Richmond, CA, USA). $^{*}P < 0.05$ compared with the respective *Csi* group.

In this study, we showed that *Ces5a* knockdown resulted in inhibited sperm capacitation and subsequent subfertility both *in vitro* and *in vivo*. Therefore, we concluded that *CES5A* was the mediator between *HongrES2* and sperm capacitation. Our previous results showed that the CES5A protein could not bind to the sperm cell,[11] so the effect of *CES5A* on sperm function is not direct.

From the phenotype of the *CES5A* knockdown rat, it is important to search for the underlying mechanisms. The regulation of cholesterol and lipid homeostasis in the epididymis is a crucial point to ensure the normal function of male gametes in the last steps of the fertilization process. The cholesterol/phospholipid (C/PL) ratio of the sperm plasma membrane determines the capacitation state of the cell. A freshly ejaculated spermatozooan has a high C/PL ratio, and during capacitation, cholesterol moves from the sperm membrane to soluble protein acceptors or phospholipid moves into the sperm membrane.[26,27] The modification of the sperm cholesterol content during epididymal maturation has been investigated in several mammals, and an about 50% decrease has been reported in ram,[28] rat,[29] hamster[30] and mouse.[31] Moreover, the fatty acid composition of the sperm cells is also highly modified during epididymal transit, with an increase in the relative proportion of polyunsaturated fatty acids, other important factors contributing to the membrane fluidity of these cells.[32] Biochemical modifications of sterols and fatty acids occurring in the epididymis have a direct influence on sperm plasma membrane architecture and dynamics.[33] Considering that *CES5A* belongs to the carboxylesterase family, and recombinant CES5A protein exhibits high levels of carboxylester hydrolase activity and of cholesterol and choline esterase activity,[12] it is very likely that the regulation of lipid environment by CES5A protein in the epididymal lumen is vital for sperm maturation and storage and experiments are in progress to check this.

It should be noted that some sperm-related parameters (VSL and VCL) were promoted after *Ces5a* knockdown. On the one hand, because the motility parameters presented in this study reflect the status before capacitation, it is possible that *CES5A* suppresses sperm motility in the cauda epididymidis. On the other hand, combined with data on

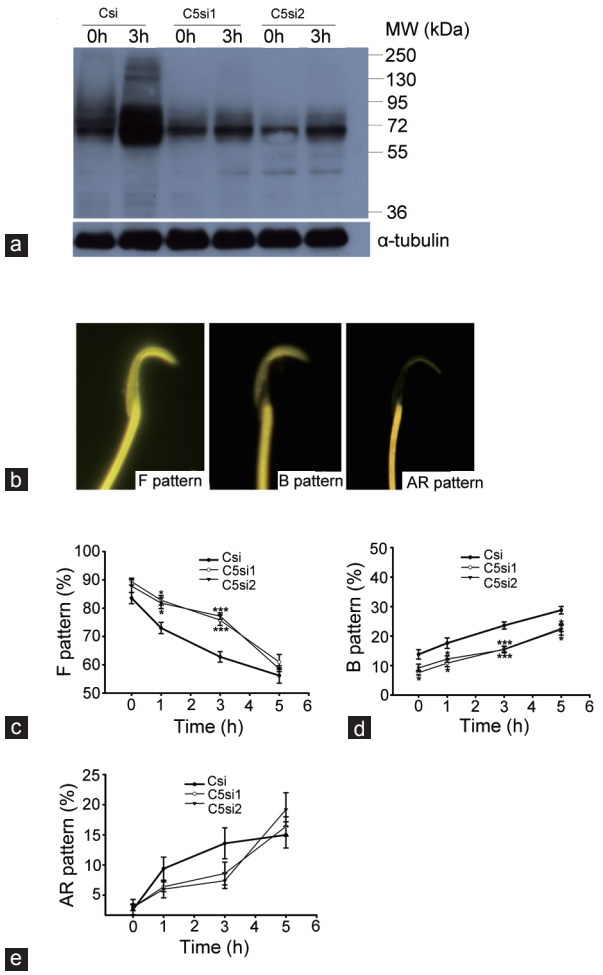

Figure 4: Assessment of sperm capacitation of *Ces5a* knockdown male rats. (**a**) The pattern of protein tyrosine phosphorylation of spermatozoa after *Ces5a* expression is inhibited. α-tubulin was used as loading control. Spermatozoa were incubated for 3 h and then collected for analysis. (**b**) Distinct chlortetracycline fluorescence staining patterns in an uncapacitated spermatozoon with uniform bright fluorescence over the head, F pattern, capacitated spermatozoon with a dark band in the postacrosomal region, B pattern, and acrosome-reacted spermatozoon with dark head except for the tip, which retains some weak fluorescence, AR pattern. (**c**–**e**) The changes in the percentage of uncapacitated spermatozoa, or F pattern (**c**), capacitated spermatozoa, or B pattern (**d**), and acrosome-reacted spermatzoa, or AR pattern (**e**) after *Ces5a* expression had been inhibited. In (**a**–**d**): *Csi*, EGFP siRNA control; C5si1 and C5si2, two siRNAs specifically targeting the different sites of *Ces5a* sequence. Results represent the means ± standard error of mean of 5 independent experiments and were analyzed by one-way analysis of variance and subsequently by the Tukey *post hoc* test (SigmaPlot version 12.3 software; Systat Software, Richmond, CA, USA). $^{*}P < 0.05$; $^{***}P < 0.001$ compared with the respective *Csi* group.

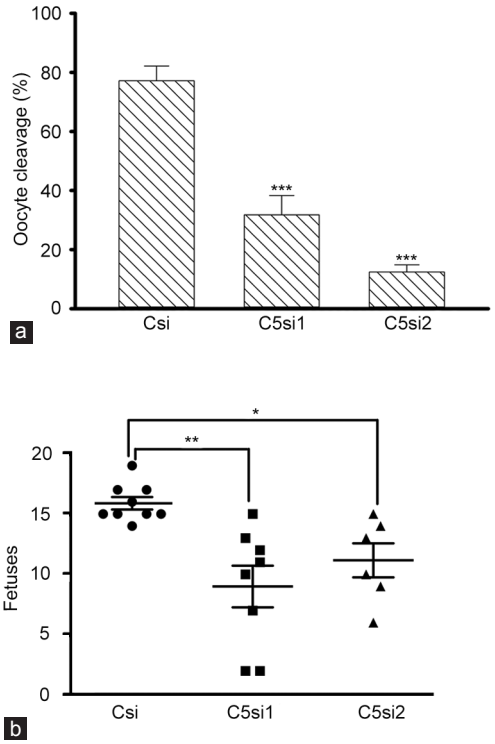

Figure 5: Fertility assay of *Ces5a* knockdown male rats. (**a**) *In vitro* fertilization by spermatozoa from *Ces5a* knockdown male rats. (**b**) Numbers of normal fetuses from normal female rats mated with control (*Csi*) and knockdown (C5si1 and C5si2) male rats. *Csi*, EGFP siRNA control; C5si1 and C5si2, two siRNAs specifically targeting the different sites of *Ces5a* sequence. Results represent the means ± standard error of mean of 4–9 independent experiments and were analyzed by one-way analysis of variance and subsequently by the Tukey *post hoc* test (SigmaPlot version 12.3 software; Systat Software, Richmond, CA, USA). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the respective *Csi* group.

capacitation, we propose that *CES5A* performs differently on sperm motility and capacitation. This phenomenon has also been observed in a previous study. For example, calmodulin (CaM) inhibitors differentially affect capacitation-associated protein tyrosine phosphorylation of a subset of sperm components, and hyperactivated motility.[34]

So far many carboxylesterases have been identified in the male reproductive system. In the *Mytilus galloprovincialis* reproductive system, the male-associated polypeptide (MAP), a member of the carboxylesterase family, has been identified and characterized. The functional significance of a high MAP concentration in male mussel gonads is unclear at present.[9] *Est-6*, the major β-carboxylesterase of *Drosophila melanogaster*, is present in male seminal fluid and exerts its function in the female reproductive duct by affecting both the storage and utilization of spermaozoa.[8,35–39] In mice, up to 70 esterase isoforms have been identified in male reproductive tissues and fluids by two-dimensional gel electrophoresis. Moreover, studies of the male reproductive tract in rats have yielded several other esterase isoforms associated with the testis, epididymis and spermatozoa.[9] However, very little evidence demonstrates the role of carboxylesterase in the male mammalian reproductive system. In the present study, we identified a function of *CES5A* in the rat epididymis *in vivo*. To the best of our knowledge, this is the first evidence *in vivo* that one of the carboxylesterases specifically expressed in the epididymis affects sperm maturation. This knowledge will be helpful for understanding the roles of carboxylesterases in the maintenance or protection of the male gonad and spermatogenesis.

As previously mentioned, we are trying to explore the mechanism whereby CES5A proteins influence sperm capacitation by study of C/PL ration of sperm and global protein profile of the cauda lumen. However, no apparent clue (either in the C/PL of spermatozoa and global protein profiles of the cauda lumen) was given to explain the conclusion in our research. Possibly these unexpected data arise from the inefficiency of

RNAi and the poor substrate specificity of carboxylesterase. In order to compensate for the defects of the RNAi approach mentioned above, we are developing *Ces5a* knockout mouse to determine the exact mechanisms whereby CES5A proteins contribute to sperm maturation and storage by sustaining a 'healthy' environment of the epididymis.

AUTHOR CONTRIBUTIONS

YFR, YCZ and YLZ conceived and designed the experiments; YFR, HMX, ZMN and DX performed the experiments; YFR and YCZ analyzed the data; YFR, YCZ and YLZ wrote the paper.

COMPETING INTERESTS

The authors declare no competing financial interests.

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REFERENCES

1 Cornwall GA. New insights into epididymal biology and function. *Hum Reprod Update* 2009; 15: 213–27.
2 Jones RC. To store or mature spermatozoa? The primary role of the epididymis. *Int J Androl* 1999; 22: 57–67.
3 Bedford JM, Nicander L. Ultrastructural changes in the acrosome and sperm membranes during maturation of spermatozoa in the testis and epididymis of the rabbit and monkey. *J Anat* 1971; 108: 527–43.
4 Orgebin-Crist MC. Sperm maturation in rabbit epididymis. *Nature* 1967; 216: 816–8.
5 Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, *et al*. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* 1995; 121: 1139–50.
6 Yanagimachi R. The Physiology of Reproduction. New York: Raven Press; 1994.
7 Satoh T, Hosokawa M. The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* 1998; 38: 257–88.
8 Mikhailov AT, Torrado M. Carboxylesterase overexpression in the male reproductive tract: a universal safeguarding mechanism? *Reprod Fertil Dev* 1999; 11: 133–45.
9 Mikhailov AT, Torrado M, Korochkin LI, Kopantzeva MA, Méndez J. Male-predominant carboxylesterase expression in the reproductive system of molluscs and insects: immunochemical and biochemical similarity between *Mytilus* male associated polypeptide (MAP) and *Drosophila* sex-specific esterase S. *Comp Biochem Physiol B Biochem Mol Biol* 1997; 118: 197–208.
10 Mikhailov AT, Torrado M, Paz M, Korochkin LI. Gonad recruitment of carboxylesterase genes during evolution of the reproductive system. Conserved male-specific overexpression in mussels, fruitflies, and mammals. *Ann N Y Acad Sci* 1999; 870: 389–91.
11 Zhang L, Hu Z, Zhu C, Liu Q, Zhou Y, *et al*. Identification and characterization of an epididymis-specific gene, Ces7. *Acta Biochim Biophys Sin (Shanghai)* 2009; 41: 809–15.
12 Zhang L, Liu Q, Zhou Y, Zhang Y. Baculo-expression and enzymatic characterization of CES7 esterase. *Acta Biochim Biophys Sin (Shanghai)* 2009; 41: 731–6.
13 Li G, Janecka JE, Murphy WJ. Accelerated evolution of CES7, a gene encoding a novel major urinary protein in the cat family. *Mol Biol Evol* 2011; 28: 911–20.
14 Miyazaki M, Miyazaki T, Toyonaga M, Tsutsui T, Taira H, *et al*. Characterisation of the carboxylesterase enzyme cauxin in the seminal fluid of the cat. *Vet J* 2011; 190: 378–82.
15 Miyazaki M, Yamashita T, Hosokawa M, Taira H, Suzuki A. Species-, sex-, and age-dependent urinary excretion of cauxin, a mammalian carboxylesterase. *Comp Biochem Physiol B Biochem Mol Biol* 2006; 145: 270–7.
16 Ecroyd H, Belghazi M, Dacheux JL, Miyazaki M, Yamashita T, *et al*. An epididymal form of cauxin, a carboxylesterase-like enzyme, is present and active in mammalian male reproductive fluids. *Biol Reprod* 2006; 74: 439–47.
17 Ni MJ, Hu ZH, Liu Q, Liu MF, Lu MH, *et al*. Identification and characterization of a novel non-coding RNA involved in sperm maturation. *PLoS One* 2011; 6: e26053.
18 Zhou X, Wang J, Liu Q, Shen J, Zheng M, *et al*. Identification of the gene imds-60 in mouse epididymis. *Acta Biochim Biophys Sin (Shanghai)* 2007; 39: 939–46.
19 Birmingham A, Anderson E, Sullivan K, Reynolds A, Boese Q, *et al*. A protocol for designing siRNAs with high functionality and specificity. *Nat Protoc* 2007; 2: 2068–78.
20 Zennou V, Serguera C, Sarkis C, Colin P, Perret E, *et al*. The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. *Nat Biotechnol* 2001; 19: 446–50.
21 Zhao Y, Diao H, Ni Z, Hu S, Yu H, *et al*. The epididymis-specific antimicrobial peptide ß-defensin 15 is required for sperm motility and male fertility in the rat (*Rattus norvegicus*). *Cell Mol Life Sci* 2011; 68: 697–708.
22 Zhou Y, Zheng M, Shi Q, Zhang L, Zhen W, *et al*. An epididymis-specific secretory protein HongrES1 critically regulates sperm capacitation and male fertility. *PLoS One* 2008; 3: e4106.
23 Berger T, Miller MG, Horner CM. *In vitro* fertilization after *in vivo* treatment of rats with three reproductive toxicants. *Reprod Toxicol* 2000; 14: 45–53.
24 Ma L, Yu H, Ni Z, Hu S, Ma W, *et al*. Spink13, an epididymis-specific gene of the Kazal-type serine protease inhibitor (SPINK) family, is essential for the acrosomal integrity and male fertility. *J Biol Chem* 2013; 288: 10154–65.
25 Luconi M, Barni T, Vannelli GB, Krausz C, Marra F, *et al*. Extracellular signal-regulated kinases modulate capacitation of human spermatozoa. *Biol Reprod* 1998; 58: 1476–89.
26 Cross NL. Role of cholesterol in sperm capacitation. *Biol Reprod* 1998; 59: 7–11.
27 Lin Y, Kan FW. Regionalization and redistribution of membrane phospholipids and cholesterol in mouse spermatozoa during *in vitro* capacitation. *Biol Reprod* 1996; 55: 1133–46.
28 Parks JE, Hammerstedt RH. Development changes occurring in the lipids of ram epididymal spermatozoa plasma membrane. *Biol Reprod* 1985; 32: 653–68.
29 Aveldaño MI, Rotstein NP, Vermouth NT. Lipid remodelling during epididymal maturation of rat spermatozoa. Enrichment in plasmenylcholines containing long-chain polyenoic fatty acids of the n-9 series. *Biochem J* 1992; 283 (Pt 1): 235–41.
30 Awano M, Kawaguchi A, Mohri H. Lipid composition of hamster epididymal spermatozoa. *J Reprod Fertil* 1993; 99: 375–83.
31 Rejraji H, Sion B, Prensier G, Carreras M, Motta C, *et al*. Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation. *Biol Reprod* 2006; 74: 1104–13.
32 Saez F, Ouvrier A, Drevet JR. Epididymis cholesterol homeostasis and sperm fertilizing ability. *Asian J Androl* 2011; 13: 11–7.
33 Jones R. Plasma Membrane Composition and Organisation During Maturation of Spermatozoa in the Epididymis. New York: Kluwer Academic/Plenum Publishers; 2002.
34 Zeng HT, Tulsiani DR. Calmodulin antagonists differentially affect capacitation-associated protein tyrosine phosphorylation of mouse sperm components. *J Cell Sci* 2003; 116: 1981–9.
35 Gilbert DG, Richmond RC. Studies of esterase 6 in *Drosophila melanogaster*. VI. ejaculate competitive abilities of males having null or active alleles. *Genetics* 1981; 97: 85–94.
36 Mane SD, Tompkins L, Richmond RC. Male esterase 6 catalyzes the synthesis of a sex pheromone in *Drosophila melanogaster* females. *Science* 1983; 222: 419–21.
37 Meikle DB, Sheehan KB, Phillis DM, Richmond RC. Localization and longevity of seminal-fluid esterase-6 in mated female *Drosophila melanogaster*. *J Insect Physiol* 1990; 36: 93–101.
38 Smith GM, Rothwell K, Wood SL, Yeaman SJ, Bownes M. Specificity and localization of lipolytic activity in adult *Drosophila melanogaster*. *Biochem J* 1994; 304 (Pt 3): 775–9.
39 Clark AG, Aguadé M, Prout T, Harshman LG, Langley CH. Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. *Genetics* 1995; 139: 189–201.