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 a 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.¹¹ 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) for in vivo 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 CO₂ asphyxiation. All the experiments were conducted according to a protocol approved by the Institute Animal Care Committee.
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. 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 32P-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^(-ΔΔCt) 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 mmol l−1 5a-dihydrotestosterone and cultured at 33°C with 5% (v/v) CO2. 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, and the siRNA targeting enhanced green fluorescent protein (EGFP, NC_011521) was used as control for sequence specificity (all-length coding sequence) with mass ratio 1:2. The sense strands of siRNAs were: C5si1 (sequence1), beginning at nt 807, 5'-CGATTTCAGCCATCTTCTTCT-3'; C5si2 (sequence2), beginning at nt 1878, 5'-GTTCCCCAGGCTGCTGCTTCTTCT-3'; C5si3 (sequence3), beginning at nt 1543, 5'-GCCACTGAGGAGAAGATGT-3' and Csi, beginning at nt 106, 5'-GCGGATGTCCTACCTTTGCGAAAG-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. The surgical procedures were performed according to a previously described method with modification. 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 × 106 transducing units of shRNA Lentivirus particles of Csi, C5si1 or C5si2 in PBS solution (viral titer was 2 × 107 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.

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 (ERKBK) medium (94.6 mmol l−1 NaCl, 25 mmol l−1 KCl, 1.71 mmol l−1 CaCl2, 1.19 mmol l−1 MgSO4, 1.19 mmol l−1 KH2PO4, 25 mmol l−1 NaHCO3, 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) warmed 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 × 106−4 × 106 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.

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 chlorotetracycline (CTC) staining according to the procedures previously described. 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), β-tubulin (SIGMA-ALDRICH, Monoclonal Anti-β-tubulin, Clone B515) was used as loading control.

Protein extraction and Western blotting of CESSA

For preparation of protein extracts from the rat cauda epididymidis, fresh tissue was homogenized in 4 ml homogenization buffer (25 mmol l−1 NaH2PO4, pH 7.2, 1.5 mmol l−1 EDTA, 10% (v/v) glycerol, 10 mmol l−1 NaM2O4, 10 mmol l−1 NaF, 2 µmol l−1 aprotonin, 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 ERKB medium warmed 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

Table 1: Primers used for Q-PCR

| Primers       | Sequence 5’-3’                  |
|---------------|---------------------------------|
| Rat GAPDH forward | TACAGGAGTGAAGAAACCGTG           |
| Rat GAPDH reverse  | GTTATATGGGCTGTGGAGTG            |
| Rat Ces5a forward | CCACCTGAGGATGAAAGTGC           |
| Rat Ces5a reverse  | CTTCAGTCTCTACCATCAGTCT         |

Q-PCR: quantitative-polymerase chain reaction
fluoride membranes, and probed with rabbit polyclonal antisera against CESSA (1:10 000). 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. 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. Each male rat was placed with two normal females in succession 4 days after injection into cauda epididymis. 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. Given the transduction efficiency and duration of gene silencing, we applied lentiviral-mediated RNAi to knock down Ces5a gene expression in the cauda epididymis and explore its role in sperm maturation and storage. According to the RNAi mechanism-based rules, 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 CESSA in the cauda region of the rat epididymis after 7 days by siRNA treatment (Figure 2a–2c). As expected, down-regulation of Ces5a expression led to a dramatic reduction of secreted CESSA proteins into the cauda lumen (Figure 2d). These results suggested that we had successfully established a CESSA knockdown rat model.

**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 CESSA 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).

**CESSA 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. 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 antibody 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 CESSA 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 CESSA 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 CESSA protein and inhibits sperm capacitation-associated protein tyrosine phosphorylation. However, it is uncertain whether the inhibition of sperm capacitation induced by HongrES2 was caused by the decrease of Ces5a expression.

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**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.
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 CESSA was the mediator between HongrES2 and sperm capacitation. Our previous results showed that the CES5A protein could not bind to the sperm cell, so the effect of CESSA 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 spermatozoa 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. 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, rat, hamster and mouse. 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. Biochemical modifications of sterols and fatty acids occurring in the epididymis have a direct influence on sperm plasma membrane architecture and dynamics. Considering that CESSA belongs to the carboxylesterase family, and recombinant CES5A protein exhibits high levels of carboxylester hydrolase activity and of cholesterol and choline esterase activity, 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 epididymis. On the other hand, combined with data on...
capacitation, we propose that CESSA 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.

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. 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 spermatozoa. 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.

However, very little evidence demonstrates the role of carboxylesterases in the male mammalian reproductive system. In the present study, we identified a function of CESSA 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 CESSA 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 contributes to spermatogenesis 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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