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Short Communication

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Identification, characterization and expression analysis of *rLcn13*, an epididymal lipocalin in rats

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Abstract

As the essential tissue for sperm maturation and storage, the epididymis secretes a number of tissue-specific proteins to exert its functions. Among these proteins, epididymal lipocalins have been intensively studied because of their epididymis-specific expression pattern and clustered genomic organization. In this study, *rLcn13*, a member of the rat epididymal lipocalin family, is identified and elaborately characterized. The cDNA sequence of *rLcn13* consists of 719 nucleotides and encodes a 176 amino-acid protein with a predicted N-terminal signal peptide of 19 amino acids. *rLcn13* shares a similar genomic structure and predicted 3D protein structure with other lipocalin family members. A recombinant rLCN13 mature peptide of 157 amino acids is expressed and purified, which is used to raise a polyclonal antibody against rLCN13 with high specificity and sensitivity. Northern blot, western blot, and immunohistochemistry assays reveal that *rLcn13* is an epididymis-specific gene which is expressed predominantly in the initial segment and proximal caput epididymis and influenced by androgen. The rLCN13 protein is modified by N-glycosylation and secreted into the epididymal lumen, and then binds to the acrosome region of the sperm. Our data demonstrate that *rLcn13* exhibits a specific temporospatial expression pattern and androgen dependence, indicating its potential roles in sperm maturation.

Key words epididymis, lipocalin, *rLcn13*, sperm maturation

Introduction

In mammals, the spermatozoon generated from testis is morphologically complete but immotile and unable to fertilize the oocyte. It must transit through several meters of the epididymal tubule for approximately two weeks to acquire progressive motility and fertilizing ability. During its long journey in the epididymis, the spermatozoon experiences elaborate modifications when it bathes in the sequentially changing luminal environment which is formed by dynamic secretion and reabsorption of water, inorganic and organic molecules, and proteins [1]. It has been reported that as many as 732 proteins are added to and 1034 proteins are removed from the sperm when they transit through the epididymis [2]. Among these proteins, several protein families, such as beta-defensins and lipocalins, have been most intensively investigated because of their epididymis-specific expression pattern and important roles in sperm maturation [3].

The lipocalin protein family is a large group of small secreted proteins characterized by its small hydrophobic molecule binding property and conserved three-dimensional structure. Although lipocalins have an unusually low level of overall sequence conservation, their crystal structures are highly conserved and comprise a highly symmetrical all-β structure dominated by a single eight-stranded anti-parallel β-sheet closed back on itself to form a continuously hydrogen-bonded β-barrel, which encloses an internal ligand-binding site. The large cup-shaped cavity within the β-barrel can accommodate ligands with different sizes and shapes according

to the amino acid composition of the pocket and loop scaffold, as well as its overall size and conformation [4]. Because of their well-adapted structure for ligand binding, lipocalins were originally known as transport proteins. However, their roles in invertebrate cryptic coloration, prostaglandin synthesis, regulation of cell homoeostasis, modulation of the immune response, and clearance of endogenous and exogenous compounds have recently been revealed [5].

Furthermore, the possible roles of lipocalins in male reproduction are implied by the existence of the epididymis-specific lipocalin gene cluster. In mice, the epididymis-specific lipocalin gene cluster is located on A3-B of mouse chromosome 2 and includes *mLcn5*, *mLcn6*, *mLcn8*, *mLcn9*, *mLcn10*, *mLcn12* and *mLcn13*, which share similar gene structure, chromosomal order, and orientation. All these lipocalin members are specifically expressed in the epididymis in a highly restricted regionalization pattern [6]. For example, *mLcn8*, *mLcn9* and *mLcn10* are specifically expressed in the initial segment, while *mLcn5* is only present in segments 2 to 5 corresponding to the middle and distal caput epididymis. Additionally, their gene regulation patterns are diverse. For example, *mLcn8* and *mLcn9* are dependent on testicular factors, and *mLcn5* and *mLcn12* are dependent on androgens, whereas *mLcn10* gene expression is regulated by both androgens and testicular factors. Importantly, the epididymis-specific lipocalin gene cluster is also present in human chromosome 9 and rat chromosome 3 with similar gene structure, sequence, chromosomal order, and orientation. The evolutionary conservation of this cluster, together with its epididymis-specific and regionalized expression pattern, strongly suggests that the lipocalin proteins in this cluster may play an essential role in male fertility, especially in sperm maturation. In recent years, the biological functions of a few lipocalins in male reproduction have been demonstrated. For example, *mLcn5* serves as a retinoic acid transport protein in the epididymis and may be involved in the prevention of epididymitis [7]; *mLcn2* induces the elevation of intracellular pH and cAMP concentration and enhances sperm motility [8]. Recently, we revealed that *mLcn6* plays an essential role in preventing premature acrosome reactions in mouse sperm [9]. Deficiency of *Lcn8* causes epididymal sperm maturation defects in mice [10,11]. The structures and functions of several rat epididymal lipocalins have been clarified in recent years. The solution structure assay and fluorescence titration experiments indicated the high binding affinity for all-trans retinoic acid of the rLCN12 protein, implying that rLCN12 could be involved in retinoic acid transport in the epididymis [12,13]. Recombinant rLCN11 might contain multiple hydrophobic binding sites for ligand binding and work as a dimeric chemoreception protein in male reproduction [14]. *rLcn9*, a proximal caput epididymis-specific lipocalin, is regulated by luminal testicular factors and deposited onto the postacrosomal domain of caput epididymal sperm, indicating its potential roles in sperm maturation [15]

In the present study, the characteristics of *rLcn13*, another member of the epididymis lipocalin family, were elaborately demonstrated, which builds the foundation for further exploration of the functions of *rLcn13* in sperm maturation and male fertility.

Materials and Methods

Animals

Healthy male Sprague-Dawley (SD) rats and male New Zealand white rabbits (approximately 2.5 kg) were purchased from the Animal Center of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). They were housed for an additional 7–10 days before manipulation in the animal house of the institute. Food and water were freely available throughout the experiments. All experiments were conducted in accordance with the Institute Animal Care Committee of the Shanghai Institutes for Biological Sciences, with internationally accepted guidelines for the humane care and use of laboratory animals.

Bioinformatics analysis

The homology of the nucleic acid and amino acid sequences of the *Lcn13* gene (accession number: NC_051338) among mice, rats and humans was determined using the Clustal W program of DNAStar software (DNASTAR, Madison, USA). The signal peptide cleavage sites, N-glycosylation sites and phosphorylation sites were predicted at the website (http://www.cbs.dtu.dk/services/) by SiganlP.4.1 Server, NetNGlyc 1.0 Server and NetPhos 2.0 server, respectively. The 3D structure prediction was conducted at the website (http://swissmodel.expasy.org). Briefly, the mature rLCN13 protein sequence excluding the signal peptide was used to search the most homologous protein whose structure had been solved, and the human orthologue protein of rLCN13, odourant binding protein OBPIIa (4run.1. A) was used as the templates for structure remodeling.

RNA isolation and Northern blot analysis

Total RNA was extracted from rat tissues using Trizol (Invitrogen, Grand Island, USA) according to the manufacturer's instructions. Northern blot analysis was performed as described previously [16]. Briefly, 10 μg of total RNA was separated by 1.2% agarose gel, blotted to Hybond N+ (Amersham Biosciences, Piscataway, USA) by the capillary method and probed with [^{32}P]-labelled *rLcn13* cDNA fragment (225–871 bp). The 18S rRNA hybridization signal was used as the internal control.

Reverse-transcribed-quantitative PCR (RT-qPCR)

Total RNA (1 μg) was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (FSQ-301; TOYOBO, Tokyo, Japan). The reverse-transcription product (1 μL) was used to amplify *Lcn13* by SYBR Green Real-time PCR Master Mix (QPK-201; TOYOBO). *Gapdh* was used as the reference gene. The primers of *Lcn13* and *Gapdh* were shown as follows: *Lcn13* forward primer 5′-CTACATTTTCTACTGCGAAGGACTG-3′, and reverse primer 5′-TTTCTTAAATTCTTCCATAGCCTCC-3′; *Gapdh* forward primer 5′-TACAAGGAGTAAGAAACCGTGGAC-3′, and reverse primer 5′-GTTATTATGGGGTCTGGGATGG-3′. The values of the threshold cycle were analyzed using the $2^{-\Delta\Delta CT}$ method.

Castration and androgen replacement

Castration and androgen replacement were performed as described previously [17]. Normal adult male SD rats were castrated bilaterally under sodium pentobarbital anesthesia. Animals were divided into 9 groups (3 rats per group) and sacrificed on days 0, 1, 3, 5 and 7 postcastration and 1, 3, 5 and 7 days after the initial testosterone propionate injection. Androgen supplementation began on the 7th day after castration, and rats were injected with testosterone propionate (3 mg/kg body weight) every 2 days. Serum testosterone concentrations were measured by radio immunoassay

(RIA) as previously described [17].

Preparation of anti-rLCN13 polyclonal antisera

The specific antibody to rLCN13 was prepared as described by Zhu *et al.* [17]. The cDNA fragment for the *rLcn13* mature peptide of 157 amino acids excluding the signal peptide was amplified by PCR and was inserted into a pET28 (a) vector (Novagen, Gibbstown, USA). The expression of recombinant protein was induced by isopropylthiogalactoside (IPTG) after the expression vector was transformed into the *Escherichia coli* BL21 Codon Plus RP strain (Novagen). The purification of the recombinant protein from inclusion bodies was performed as described previously [18]. The antisera were obtained according to our modified immunization methods [19]. Subcutaneous injection of six hundred micrograms of antigen into rabbits was performed on days 1, 3, and 28. On the 35th day, the antisera were harvested from the arteriae carotis. The preimmune sera of the rabbits were reserved in advance before immunization as the negative control for the subsequent experiments.

Western blot analysis

Total protein extracts were prepared as described previously [20]. The tissues were dissected and homogenized in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS) supplemented with 1 × protease inhibitor cocktail (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride (PMSF). After incubation at 4°C for 30 min and centrifugation at 4°C for 10 min, total proteins were extracted into the supernatant. For the epididymal luminal fluid and sperm protein extraction, the different epididymal regions were finely minced in phosphate-buffered saline (PBS) and incubated for 10 min at 37°C. The supernatant containing spermatozoa and luminal contents was further centrifuged at 500 *g* for 5 min, and the final supernatant was collected as luminal fluid protein extract. The sperm pellets were washed three times with cold PBS and lysed with 2% (w/v) SDS supplemented with a protease inhibitor cocktail (Roche) for 30 min and then centrifuged at 12,000 *g* for 15 min. The supernatants were collected as sperm protein extracts.

A total of 30 μg protein for each sample was separated on 15% SDS-PAGE gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Piscataway, USA). The blocked membranes were incubated overnight at 4°C with the polyclonal anti-sera against rLCN13 at 1:10,000, followed by incubation with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotech, Santa Cruz, USA) at 1:10,000 for 1 h at room temperature. The protein bands were finally detected using an Enhanced Chemiluminescence (ECL) kit (GE Amersham, Pittsburgh, USA).

Immunohistochemical staining

Tissue section preparation and immunohistochemical staining were performed as described previously [17]. Briefly, epididymides dissected from adult male rats were fixed in Bouin's fluid overnight at 4°C and cut into 5-μm-thick sections. Primary and secondary antibodies were diluted in PBS containing 10% (v/v) normal goat serum. The 1:200 diluted anti-rLCN13 antiserum was applied to the tissues and incubated overnight at 4°C. After the sections were washed, 1:200 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG was applied and incubated for 1 h at room temperature. As a negative control, serial sections were subject to the same treatment with normal rabbit serum instead of the primary antibody.

Indirect immunofluorescence staining

Indirect immunofluorescence staining was performed as described previously [20]. The spermatozoa were fixed in 4% (w/v) paraformaldehyde at room temperature for 10 min, placed on polylysine-coated slides and air-dried. The slides were blocked for 1 h at room temperature with 10% (v/v) goat serum in PBS. They were then incubated with polyclonal anti-rLCN13 serum (diluted 1:200 in PBS containing 10% goat serum) overnight at 4°C, with preimmune rabbit serum as the control. After three times wash with PBST (PBS containing 0.2% Tween-20), the corresponding secondary antibody (TRITC-conjugated anti-rabbit IgG, 1:500 diluted in PBS containing 10% goat serum; Abcam, Boston, USA) was applied. Alexa Fluor 488-conjugated peanut agglutinin (PNA; Abcam) and DAPI (Abcam) were used to stain the acrosome region and the nuclei, respectively. The slides were washed three times with PBST and mounted in 80% (v/v) glycerol. Slides were examined with an Olympus BX-52 microscope (Olympus, Tokyo, Japan).

Results

The *rLcn13* gene belongs to the epididymis-specific lipocalin cluster

The *rLcn13* gene is located on rat chromosome 3p13 which is known as the epididymis-specific lipocalin cluster. This region also includes *Lcn5*, *Lcn6*, *Lcn8*, *Lcn9*, *Lcn10* and *Lcn12*, whose orthologues in mice are only expressed in the epididymis. The *rLcn13* gene spans 4.3 kb and includes 7 exons and 6 introns, which is consistent with the consensus lipocalin gene structure (Figure 1A). The cDNA corresponding to *rLcn13* is 719 bp, with a very short 4 bp 5′UTR that needs to be further investigated by 5′RACE, a relatively complete 3′UTR that contains the stop codon-TAG, polyadenylation signal-AATAAA and polyA, and a predicted 531 bp open reading frame (ORF) that encodes a precursor protein of 176 amino acids. Similar to other lipocalin proteins, the rLCN13 precursor contains a 19-amino acid signal peptide at its N-terminus, and the cleavage of this peptide would produce a mature protein of 157 amino acids with a calculated molecular mass of 18.3 kDa and a calculated isoelectric point of 5.28. The predicted existence of one N-glycosylation site at N42 and seven phosphorylation sites at S31, S110, T89, T103, T109, Y30, and Y115 in the mature rLCN13 protein sequence indicates the possible posttranslational modifications of this protein (Figure 1B).

Although the amino acid sequence identity between rLCN13 and other rat lipocalins is extremely low, it contains conserved lipocalin motifs (G-X-W, T, and K/R) as well as two cysteine residues that are involved in the formation of a disulfide bond to stabilize the protein conformation (Figure 2A). It is well known that lipocalin family members share high similarities in their three-dimensional structures despite the very low overall sequence conservation. To investigate whether rLCN13 adopts the conserved conformation as other lipocalin family members, the human homolog of rLCN13, whose three-dimensional structure has been determined by X-ray crystallography [21], was used for rLCN13 structure remodeling. The results demonstrated that, like other lipocalins, the rLCN13 protein consists of an eight-stranded antiparallel β-barrel forming a cup-shaped ligand-binding pocket (Figure 2B). Compared with its

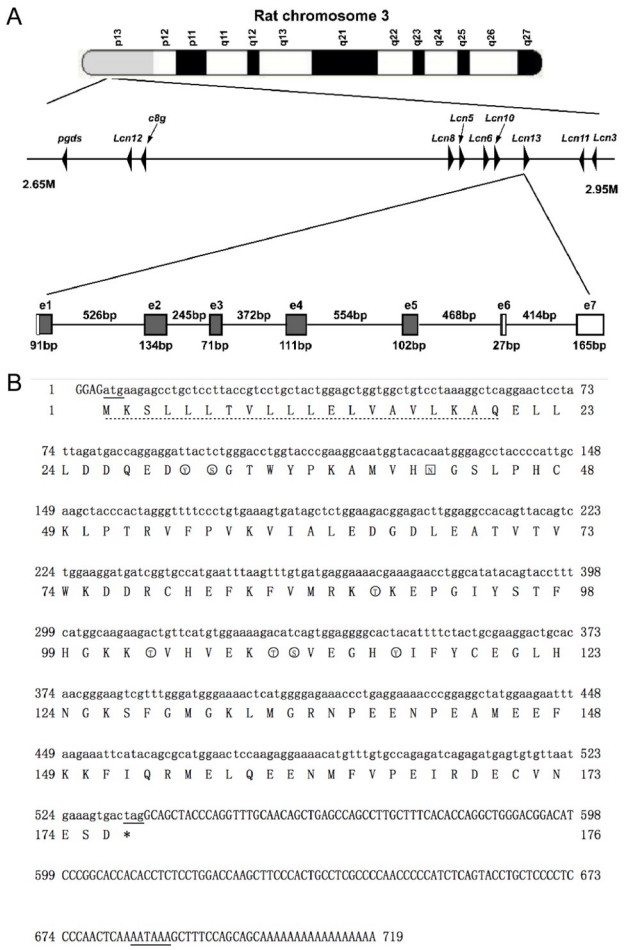

Figure 1. The gene location, structure, cDNA and protein sequence analysis of *rLcn13* (A) Location and orientation of the lipocalin gene cluster on rat chromosome 3 and intron-exon structure of *rLcn13*. Each triangle represents a lipocalin gene and indicates the gene orientation. Other genes in this area are not shown. Closed boxes represent coding regions, and open boxes indicate noncoding regions. The numbers indicate the intron and exon length. (B) The *rLcn13* open reading frame (ORF) contains 531 bp coding for a precursor protein containing 176 amino acids with a 19 amino acid signal peptide (indicated by "……") at its N-terminus. The mature rLCN13 protein contains one putative N-glycosylation site (N42) indicated by "□" and seven putative phosphorylation sites (S31, S110, T89, T103, T109, Y30, Y115) indicated by "○". The initial codon "ATG", terminal codon "TAG" and polyadenylation signal "AATAAA" are indicated by "__".

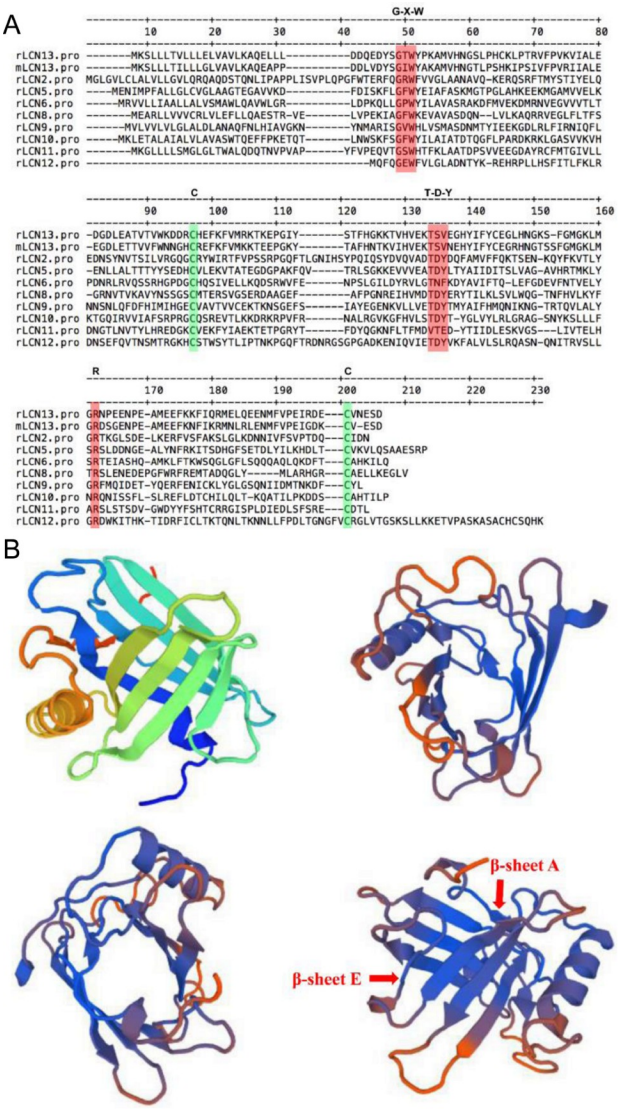

Figure 2. Amino acid sequence alignment of rat lipocalins (rLCN13, rLCN2, rLCN5, rLCN6, rLCN8, rLCN9, rLCN10, rLCN11, and rLCN12) and mouse LCN13 and 3D structure modeling of rLCN13 (A) rLCN13 shares the conserved lipocalin motifs (G-X-W, T-D-Y, R and C) indicated by red and green colors. Dashes show alignment gaps. (B) rLCN13 adopts the conserved 3D structure as other lipocalins. On the top left is the dissected 3D structure of human LCN13 downloaded from ExPASy (https://www.expasy.org). The other three are predicted 3D structures of rLCN13 displayed at different angles. The blue color represents a good structural match between human and rat LCN13, and the red color indicates structural differences between these two proteins. The red arrows indicate discontinuous "β-sheet A" and unstructured "β-sheet E".

homolog protein, human LCN13 which is also known as OBP2a, the three-dimensional structure of the rLCN13 protein has its own characteristics: (i) β-sheet A is not continuous; (ii) "β-sheet E" is not typical; and (iii) the loops between sheets of these two proteins are poorly merged. These characteristics indicate that although rLCN13 is the homolog of human LCN13, it may have its own properties in ligand binding, receptor recognition and biological functions.

rLcn13 mRNA is predominantly expressed in the epididymis and mainly regulated by androgen

Based on its location in the epididymis-specific lipocalin cluster locus, we speculate that *rLcn13* may be exclusively expressed in the epididymis. To confirm this hypothesis, total RNA from the caput (including the initial segment), corpus, cauda epididymis and 14 other tissues was extracted and subject to Northern blot analysis. As shown in Figure 3A, a specific band of approximately 750 nt was detected only in the caput epididymis. To examine the relative mRNA expression level of *rLcn13* in different epididymis regions, the rat epididymis was further dissected into the initial segment, caput, corpus and cauda, and RT-qPCR was performed. The results demonstrated that *rLcn13* mRNA is predominantly expressed in the initial segment and that the caput epididymis also has weak

expression (Figure 3B). Although all the members of the epididymis-specific lipocalin cluster are exclusively expressed in the epididymis, their regulation patterns by androgens are different from each other. A castration and androgen replacement rat model were applied to investigate whether *rLcn13* expression is regulated by androgen. As shown in Figure 3C, *rLcn13* mRNA in the epididymis was dramatically decreased in abundance concomitant with the drastic decrease in serum testosterone after castration. After the administration of testosterone to rats that had been castrated for 7 days, *rLcn13* mRNA gradually returned to nearly normal levels in parallel with serum testosterone restoration, which indicated that *rLcn13* expression is upregulated by androgen.

The native status of the rLCN13 protein in the epididymis

To characterize the protein distribution pattern of rLCN13, a rabbit polyclonal antiserum against the full-length rLCN13 protein was prepared. Proteins from the epididymis and 12 other tissues were extracted, and rLCN13 protein was detected by western blot analysis. The results demonstrated that the rLCN13 protein is specifically expressed in the epididymis but not in the testis or other tissues examined (Figure 4A), which is consistent with the tissue distribution pattern of *rLcn13* mRNA. The rat epididymis was further dissected into the initial segment, caput, corpus and cauda, and the rLCN13 protein was detected. The initial segment showed the strongest rLCN13 expression, followed by the caput epididymis, and the signals were very weak in the corpus and cauda epididymis (Figure 4B). As mentioned above, the rLCN13 protein harbors one putative N-glycosylation site. To validate whether the rLCN13 protein is glycosylated in the epididymis, the protein extracts from the initial segment were deglycosylated by N-glycosidase. After deglycosylation, the band shifted to a smaller size, which indicated glycosylation of the rLCN13 protein in the epididymis (Figure 4C).

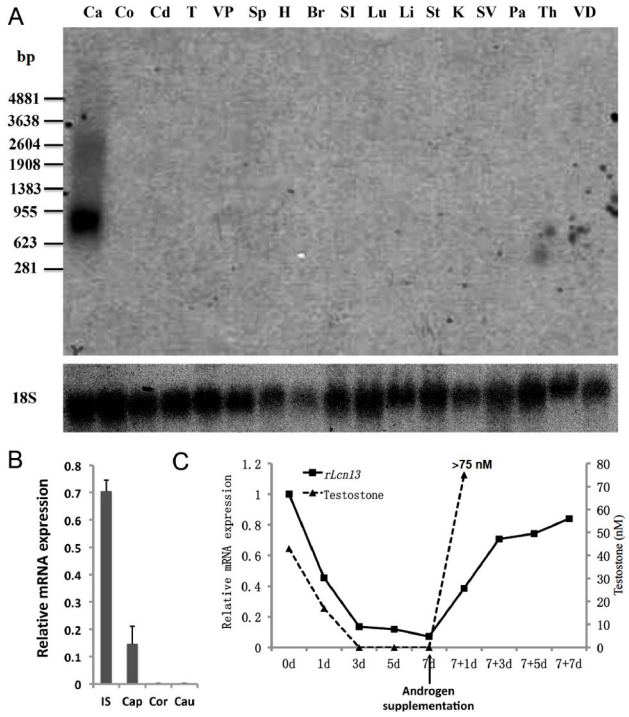

Figure 3. Initial segment epididymis predominant and androgen-dependent expression of *rLcn13* mRNA (A) Northern blot analysis shows that *rLcn13* mRNA is highly expressed in the caput (including the initial segment) region of the rat epididymis. Ca, caput; Co, corpus; Cd, cauda; T, testis; Vp, ventral prostate; Sp, spleen; H, heart; Br, brain; SI, small intestine; Lu, lung; Li, liver; St, stomach; K, kidney; Sv, seminal vesicle; Pa, pancreas; Th, thyroid; VD, vas deference. (B) *rLcn13* mRNA is mainly expressed in the initial segment of the epididymis. (C) The *rLcn13* mRNA expression levels in the epididymis relative to the serum androgen concentration.

Regional, temporal, and cell-specific expression of *rLcn13* in the epididymis

To investigate the expression of *rLcn13* mRNA and protein during development, total RNA and protein were extracted from the epididymis of rats on postnatal days 1, 7, 15, 30, 45, 60, 90 and 730 and subject to RT-qPCR and western blot analysis, respectively. As shown in Figure 5, *rLcn13* mRNA was detected at high levels at postnatal day 30 (Figure 5A), and the rLCN13 protein could be detected at postnatal day 45 (Figure 5B). Both the *rLcn13* mRNA and rLCN13 protein reached their maximum levels at postnatal day 60 and were maintained at this level during the rest of the rat's life.

The epididymis from the adult rat was mounted, sectioned and subjected to immunohistochemical analysis. The rLCN13 protein was restricted to the initial segment and the proximal caput region of the rat epididymis, consistent with the mRNA and protein distribution detected by RT-qPCR and western blot analysis (Figure 6A). As shown in the enlarged images in Figure 6B, rLCN13 also exhibits a cell-specific expression pattern, implying that not all of the epithelial cells express rLCN13 and that the epithelium has a

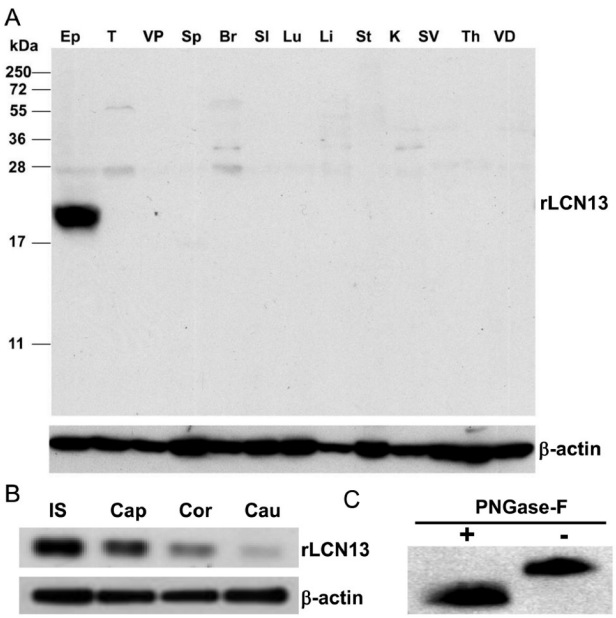

Figure 4. Initial segment epididymis predominant expression and glycosylation of rLCN13 protein (A) Protein extracts (30 μg) from each tissue were analyzed by western blot analysis, and β-actin was used as the internal control. Ep, epididymis; T, testis; Vp, ventral prostate; Sp, spleen; Br, brain; SI, small intestine; Lu, lung; Li, liver; St, stomach; K, kidney; Sv, seminal vesicle; Th, thyroid; and VD, vas deference. (B) The rLCN13 protein is predominantly expressed in the initial segment epididymis. C. A total of 30 μg of protein extract from the initial segment was treated with PNGase-F or with buffer only, and then subjected to western blot analysis. The change in the molecular mass of rLCN13 after PNGase-F treatment indicates the glycosylation of rLCN13 in the epididymis.

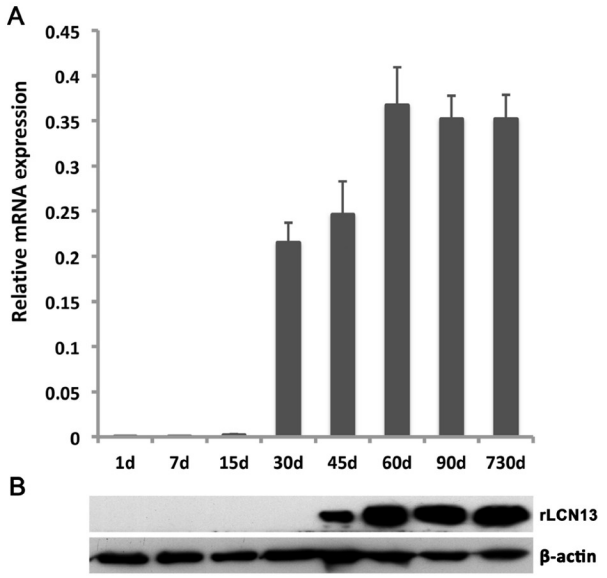

Figure 5. Postnatal expression of the *rLcn13* gene (A) Relative expression of *rLcn13* mRNA in the rat epididymis at different developmental stages from birth to approximately 2 years old. *GAPDH* was used as the internal control. (B) Relative expression of rLCN13 protein in the rat epididymis at different developmental stages from birth to approximately 2 years old. β-actin was used as the internal control.

checkerboard protein staining pattern.

Immunolocalization of rLCN13 in rat sperm

As a secretory protein, rLCN13 may be secreted to the lumen of the epididymal tubule and interact with the sperm bathing in the lumen fluid. Spermatozoa from different regions of the epididymis were subject to indirect immunofluorescent staining with anti-rLCN13 antibody. As shown in Figure 7A,B, rLCN13 binds to the spermatozoa from the initial segment, caput and corpus epididymis, and the fluorescence signal is colocalized with the PNA488 signal, which indicates the acrosome. In contrast, the fluorescence signal is very faint on the head of cauda spermatozoa, which indicates that rLCN13 is segregated from them when they arrive at the cauda epididymis. Western blot analysis of proteins extracted from the spermatozoa of different epididymal regions further confirmed the immunofluorescence results (Figure 7C). To determine the binding affinity of rLCN13 to the sperm surface, spermatozoa from the caput epididymis were incubated with PBS, 0.5 M NaCl or 0.1% Triton X-100, and the supernatants were subjected to western blot analysis to detect the rLCN13 protein segregated from the sperm surface. As shown in Figure 7D, part of the rLCN13 protein segregates from the sperm surface even in PBS and in 0.5 M NaCl solution, and rLCN13 could be easily extracted from the sperm surface with 0.1% Triton X-100. These results indicate that rLCN13 noncovalently interacts with the sperm.

Discussion

As one of the important gene clusters identified in the mouse and human genomes involved in reproduction and immune surveillance, the lipocalin family is thought to be indispensable for epididymis functions. It is clear that at least six lipocalins are specifically expressed in the epididymis with region-specific expression patterns. For example, *Lcn5* is expressed in the mid/distal caput epididymis, and *Lcn8* and *Lcn9* are expressed only in the initial segment, while *Lcn10* is expressed both in the initial segment and in the upper margin of the distal caput [6]. In the present study, the sequence characteristics and expression patterns of r*Lcn13*, another member of the epididymis-specific lipocalin cluster, were elaborately investigated. The analysis of the DNA, RNA and protein sequences and predicted 3D structure of *rLcn13* confirmed that it is a *bona fide* lipocalin gene. Its initial segment epididymis-specific expression pattern and its interaction with the acrosome region of the sperm head indicate that it may be involved in the process of sperm maturation. These findings will serve as an important reference for further investigation of the function and regulation of epididymis-specific lipocalin genes.

Although our results and a previous study revealed that both rat and mouse *Lcn13* are specifically expressed in the epididymis [6], recent reports revealed that mouse *Lcn13* is also synthesized in the liver, muscle and pancreas and can be secreted into the serum and regulate glucose and lipid metabolism [22,23]. Additionally, the essential role of liver-secreted LCN13 in regulating glucose metabolism was confirmed by another group [24]. However, the abundance of LCN13 in the serum, liver, muscle and pancreas must be extremely low because the LCN13 protein in these tissues can only be detected after enrichment by immunoprecipitation [22,23]. A recent study on transcriptomic and proteomic profiling in the mouse main olfactory epithelia and vomeronasal organ revealed that *Lcn13* is among the most abundant genes, as its expression tags account for 14.1% of all sequencing reads in the vomeronasal organ [25]. These reports indicate that the epididymis is not the only tissue expressing LCN13 or that LCN13 is predominantly but not exclusively expressed in the epididymis.

Since rLCN13 is predominantly expressed in the initial segment epididymis and secreted into the lumen, and then binds to the acrosome region of the spermatozoa, it is of great importance to uncover the biological effects of rLCN13 on male reproduction. Perhaps it can promote sperm motility like BIN1B [26], transport bioactive chemicals into the spermatozoa like LCN2 [27], control acrosome reactions like LCN6 [9], and modulate sperm energy metabolism. To clarify these issues, knockout models, especially epididymis-specific conditional knockout animal models, are needed. Thanks to the recently emerged CRISPR-Cas9 technology [28,29], it is convenient to produce a gene knockout rat in a very short time. Moreover, dissecting the conformational characteristics of the rLCN13 protein and determining the molecules that it can bind with and its receptor on the sperm surface will also greatly help us to understand its functions and mechanisms in sperm maturation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

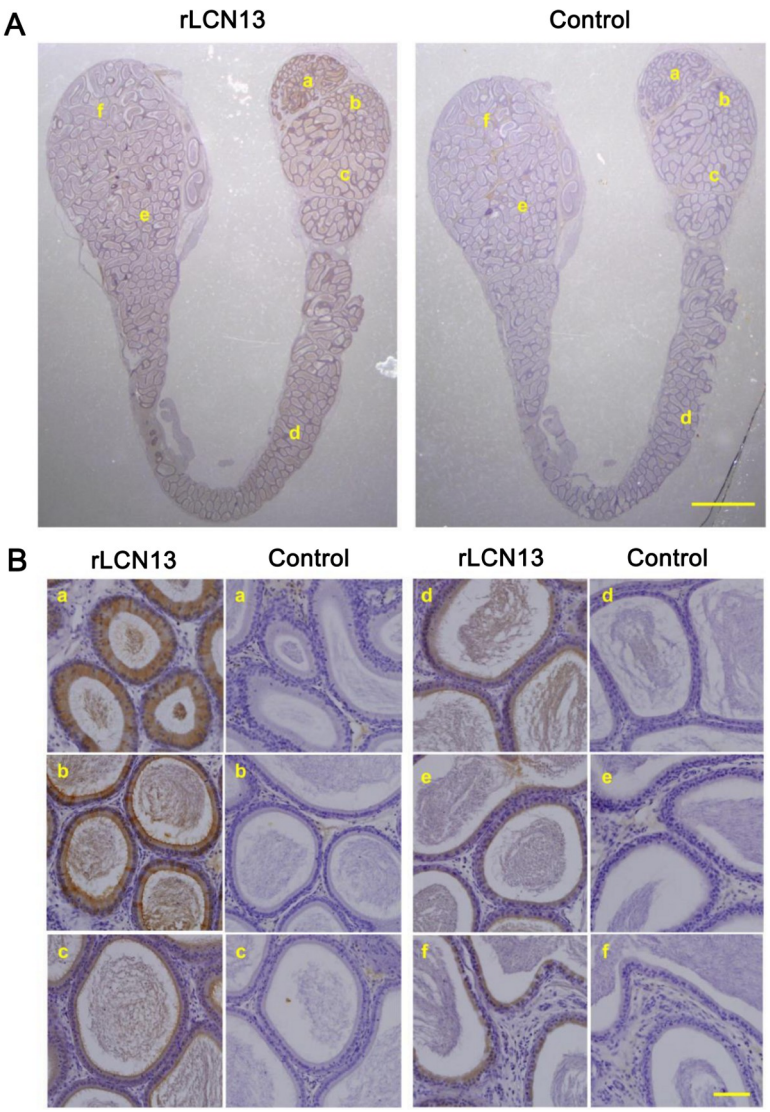

Figure 6. The region- and cell-specific expression of rLCN13 in the rat epididymis (A) Localization of rLCN13 in the whole adult rat epididymis. Scale bar = 4 mm. (B) Magnified photographs of the indicated field of (A): initial segment (a); proximal caput (b); distal caput (c); corpus (d); proximal cauda (e); and distal cauda (f). Scale bar = 100 μm.

References

1. Dacheux JL, Dacheux F. New insights into epididymal function in relation to sperm maturation. *Reproduction* 2014, 147: R27–R42
2. Skerget S, Rosenow MA, Petritis K, Karr TL. Sperm proteome maturation in the mouse epididymis. *PLoS ONE* 2015, 10: e0140650
3. Belleannée C, Thimon V, Sullivan R. Region-specific gene expression in the epididymis. *Cell Tissue Res* 2012, 349: 717–731
4. Flower DR, North ACT, Sansom CE. The lipocalin protein family: structural and sequence overview. *Biochim Biophys Acta* 2000, 1482: 9–24
5. Flower DR. The lipocalin protein family: structure and function. *Biochem J* 1996, 318: 1–14
6. Suzuki K, Lareyre JJ, Sánchez D, Gutierrez G, Araki Y, Matusik RJ, Orgebin-Crist MC. Molecular evolution of epididymal lipocalin genes localized on mouse chromosome 2. *Gene* 2004, 339: 49–59
7. Ong DE, Newcomer ME, Lareyre JJ, Orgebin-Crist MC. Epididymal retinoic acid-binding protein. *Biochim Biophys Acta* 2000, 1482: 209–217
8. Lee YC, Liao CJ, Li PT, Tzeng WF, Chu ST. Mouse lipocalin as an enhancer of spermatozoa motility. *Mol Biol Rep* 2003, 30: 165–172
9. Yin Q, Shen J, Wan X, Liu Q, Zhou Y, Zhang Y. Impaired sperm maturation in conditional Lcn6 knockout mice. *Biol Reprod* 2018, 98: 28–41
10. Wen Z, Liu D, Zhu H, Sun X, Xiao Y, Lin Z, Zhang A, *et al.* Deficiency for Lcn8 causes epididymal sperm maturation defects in mice. *Biochem Biophys Res Commun* 2021, 548: 7–13
11. Gong Q, Dou Z, Wang X, Zhang K, Chen H, Gao J, Sun X. Epididymal initial segment-specific Cre recombinase activity in Lcn8-Cre knock-in mice. *Mol Biol Rep* 2021, 48: 6015–6023
12. Peng Y, Liu J, Liu Q, Yao Y, Guo C, Zhang Y, Lin D. Conformational and biochemical characterization of a rat epididymis-specific lipocalin 12 expressed in *Escherichia coli*. *Biochim Biophys Acta* 2010, 1804: 2102–2110
13. Peng Y, Zhang X, Liu J, Liu Q, Guo C, Zhang Y, Lin D. Solution structure of the protein lipocalin 12 from rat epididymis. *Proteins* 2011, 79: 2316–2320
14. Gu Y, Liu Q, Chen P, Guo C, Liu Y, Zhao Y, Zhang Y, *et al.* Characterization of the oligomerization and ligand-binding properties of recombinant rat lipocalin 11. *Biochim Biophys Acta* 2013, 1834: 1–7

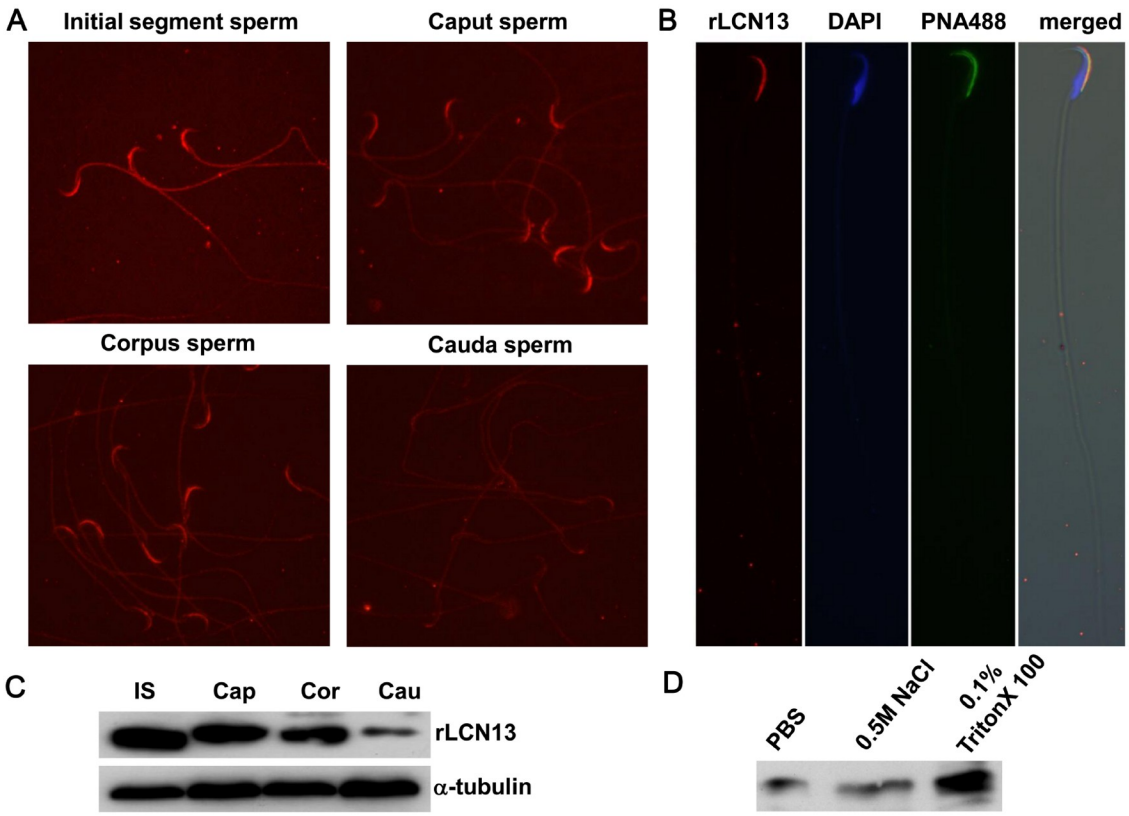

Figure 7. rLCN13 weakly interacts with sperm head (A) Immunolocalization of rLCN13 protein (TRITC-labelled, red) on sperm by indirect immunofluorescence assays. (B) Coimmunostaining of rLCN13 (TRITC-labelled, red), acrosome marker-lectin PNA (Alexa Fluor 488-labelled, green) and DAPI (blue). The merged micrograph demonstrates the colocalization of rLCN13 and lectin PNA, which implies the localization of rLCN13 on the acrosome region of the rat sperm head. (C) Western blot analysis confirmed the binding of rLCN13 to the initial segment, caput and corpus sperm. (D) Western blot analysis of rLCN13 protein in the supernatant after treatment of caput sperm with PBS, 0.5 M NaCl or 0.1% Triton X-100 for 30 min.

15. Li X, Zhan X, Liu S, Hu S, Zhu C, Hall SH, French FS, *et al.* Cloning and primary characterizations of rLcn9, a new member of epididymal lipocalins in rat. *Acta Biochim Biophys Sin* 2012, 44: 876–885
16. Li P, Chan HC, He B, So SC, Chung YW, Shang Q, Zhang YD, *et al.* An antimicrobial peptide gene found in the male reproductive system of rats. *Science* 2001, 291: 1783–1785
17. Zhu CF, Liu Q, Zhang L, Yuan HX, Zhen W, Zhang JS, Chen ZJ, *et al.* RNase9, an androgen-dependent member of the RNase A family, is specifically expressed in the rat epididymis1. *Biol Reprod* 2007, 76: 63–73
18. Hu ZH, Li B, Liu Q, Zhang YL. Expression, purification of the recombinant protein fragment of the monkey epididymis expressed gene ESc-615 and preparation of polyclonal antisera to it. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* 2002, 34: 753–756
19. Hu YX, Guo JY, Shen L, Chen Y, Zhang ZC, Zhang YL. Get effective polyclonal antisera in one month. *Cell Res* 2002, 12: 157–160
20. Hu SG, Du H, Yao GX, Zhang YL. Molecular cloning and identification of mouse epididymis-specific gene mHong1, the homologue of rat HongrES1. *Asian J Androl* 2012, 14: 626–634
21. Schiefner A, Freier R, Eichinger A, Skerra A. Crystal structure of the human odorant binding protein, OBP_{IIa}. *Proteins* 2015, 83: 1180–1184
22. Cho KW, Zhou Y, Sheng L, Rui L. Lipocalin-13 regulates glucose metabolism by both insulin-dependent and insulin-independent mechanisms. *Mol Cell Biol* 2011, 31: 450–457
23. Sheng L, Cho KW, Zhou Y, Shen H, Rui L. Lipocalin 13 protein protects against hepatic steatosis by both inhibiting lipogenesis and stimulating fatty acid beta-oxidation. *J Biol Chem* 2011, 286: 38128–38135
24. Ekim Üstünel B, Friedrich K, Maida A, Wang X, Krones-Herzig A, Seibert O, Sommerfeld A, *et al.* Control of diabetic hyperglycaemia and insulin resistance through TSC22D4. *Nat Commun* 2016, 7: 13267
25. Kuntová B, Stopková R, Stopka P. Transcriptomic and proteomic profiling revealed high proportions of odorant binding and antimicrobial defense proteins in olfactory tissues of the house mouse. *Front Genet* 2018, 9: 26
26. Zhou CX, Zhang YL, Xiao L, Zheng M, Leung KM, Chan MY, Lo PS, *et al.* An epididymis-specific β-defensin is important for the initiation of sperm maturation. *Nat Cell Biol* 2004, 6: 458–464
27. Elangovan N, Lee YC, Tzeng WF, Chu ST. Delivery of ferric ion to mouse spermatozoa is mediated by lipocalin internalization. *Biochem Biophys Res Commun* 2004, 319: 1096–1104
28. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol* 2013, 31: 233–239
29. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, *et al.* RNA-guided human genome engineering via Cas9. *Science* 2013, 339: 823–826