Clusterin in the mouse epididymis: possible roles in sperm maturation and capacitation

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Abstract

Clusterin (CLU) is known as an extracellular chaperone for proteins under stress, thus preventing them from aggregation and precipitation. We showed herein that CLU, expressed by principal cells of the mouse caput epididymis, was present in high amounts in the lumen. In the cauda epididymis, CLU bound tightly to the sperm head surface and its amount on total sperm was similar to that in the bathing luminal fluid. In both immotile and motile caudal epididymal sperm, CLU was localized over the entire sperm head except at the convex ridge, although in the motile sperm population, the CLU immunofluorescence pattern was distinctively mottled with a lower intensity. However, when motile sperm became capacitated, CLU was relocalized to the head hook region, with immunofluorescence intensity being higher than that on the non-capacitated counterparts. Under a slightly acidic pH of the epididymal lumen, CLU may chaperone some luminal proteins and deliver them onto the sperm surface. Immunoprecipitation of epididymal fluid proteins indicated that CLU interacted with SED1, an important egg-binding protein present in a high amount in the epididymal lumen. In a number of non-capacitated sperm, fractions of SED1 and CLU co-localized, but after capacitation, SED1 and CLU dissociated from one another. While CLU moved to the sperm head hook, SED1 translocated to the head convex ridge, the egg-binding site. Overall, CLU localization patterns can serve as biomarkers of immotile sperm, and non-capacitated and capacitated sperm in mice. The chaperone role of CLU may also be important for sperm maturation and capacitation.

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Introduction

‘Clusterin’ (CLU), first coined by Fritz and colleagues, is an 80-kDa dimeric glycosylated protein isolated from ram rete testis fluid that has the ability to cluster isolated Sertoli cells in suspension (Blaschuk et al. 1983, Fritz et al. 1983). Immunolocalization and immunoblotting reveal that CLU is expressed in ram and rat Sertoli cells and secreted into the seminiferous tubule lumen (Kissinger et al. 1982, Blaschuk et al. 1983, Blaschuk & Fritz 1984, Sylvester et al. 1984, Griswold et al. 1986, Cheng et al. 1988, Law & Griswold 1994). CLU is the same protein as SGP-2 (sulfated glycoprotein-2, a major secreted protein of Sertoli cells), TRPM-2 (testosterone-repressed prostate message-2), SP-40, 40, apoJ (apolipoproteinJ) and CLI (complement lysis inhibitor). Studies on the last three proteins indicate that CLU is also present in non-reproductive tissues/fluids (Jenne & Tschopp 1989).

Newly synthesized CLU in rat Sertoli cells and other cells contains 447 amino acids with a molecular mass of ~68–74 kDa. Upon maturation, intracellular cleavage occurs at Arg226 (numbering based on rat Sertoli cell CLU), yielding CLU-β (34 kDa, Glu22-Arg226) and CLU-α (47 kDa, Ser227-Glu447), which remain linked together by five disulfide bonds (see Supplementary Fig. 1, see section on supplementary data given at the end of this article) (Blaschuk et al. 1983, Collard & Griswold 1987, Sylvester et al. 1991). Further biochemical studies strongly indicate the roles of extracellular CLU in lipid transport, membrane remodeling during apoptosis and molecular chaperoning of proteins under physical and chemical stress conditions (Poon et al. 2002, Carver et al. 2003, Wyatt et al. 2009) as well as protective roles against oxidative stress and complement-induced cell lysis (Trougakos 2013), Jenne & Tschopp 1989, Murphy et al. 1989, Tschopp & French 1994, Mollnes & Kirschfink 2006). On the other hand, nuclear CLU isoform is a proapoptotic factor (Rohne et al. 2016).

In the male rat reproductive system, CLU is also expressed and secreted by epididymal epithelial principal cells, with the highest level in the caput region (Sylvester et al. 1984, 1991, Tung & Fritz 1985,
CLU also coats the surface of rat sperm in the descending regions of the epididymis (Hermo et al. 1991, Sylvester et al. 1991). Since the epididymis is a site of sperm maturation (Robaire & Hinton 2015), CLU in the epididymal fluid and sperm may play roles in this process (Robaire & Viger 1995) as well as the subsequent sperm capacitation event. In particular, we asked whether CLU could act as an extracellular chaperone for proteins present abundantly in epididymal fluid. SED1 is one of such proteins, which we selected for this study, as it also deposits onto epididymal sperm and its essential function in egg binding and male reproduction in mice is well documented (Ensslin & Shur 2003, Shur et al. 2006). However, since information on CLU amounts and localization in the epididymal entities (epithelial cells and fluid, and sperm) in mice was unavailable, our initial study was targeted toward this characterization. This study serendipitously indicated that CLU immunolocalization signals could also be used as biomarkers of mouse sperm capacitation.

Materials and methods

Chemicals

Goat polyclonal anti-CLU antibody, directed against the C-terminus sequence EKALQYRRKSAE of mouse CLU-α peptide and immunogen-affinity purified, was purchased from Novus Biologicals (Oakville, ON, Canada, Catalog number: NB51P06027). Immunogen-affinity purified goat polyclonal anti-SED1 (aka MFGE8) IgG antibody generated against recombinant mouse SED1 was from R&D Systems (Catalog Number: AF2805, Cedarlane, Burlington, ON, Canada). Rabbit polyclonal anti-SED1 IgG antiserum generated against recombinant His-tagged SED1 (Ensslin & Shur 2003) was provided by Dr Barry Shur, Emory University School of Medicine. A mouse monoclonal anti-phosphotyrosine IgG antibody, clone 4G-10 (catalog number: 05-321X), was from Millipore Canada. Normal goat IgG (Catalog number: SC-2028) was from Santa Cruz Biotechnology and normal rabbit IgG (Catalog number: 12-370) was from Millipore Canada.

Recombinant mouse CLU (Catalog number: 2747-HS-050) was purchased from R&D Systems (Cedarlane). It was produced in a mouse myeloma cell line as a disulfide-linked heterodimer of CLU-β and CLU-α, with an apparent molecular mass of 37kDa and 44kDa, respectively, upon subjection to reducing SDS-PAGE.

Animals and gamete collection

Male CD-1 (age: 8–10 weeks) and female CF-1 (age: 5–8 weeks) mice (from Charles River Canada) were kept in a temperature-controlled room with 14-h light and 10-h dark photoperiod. They were fed ad libitum with Purina rodent chow and water. All experiments involving the use of mice adhered to the Canadian Council on Animal Care guidelines, and the protocols followed were approved by the Committee of the Animal Care & Veterinary Service, University of Ottawa.

Collection of epididymal sperm and fluid

Media

Four types of media were used in sperm preparation and/or incubation. These included: (1) Krebs Ringer Medium containing 4mM NaHCO$_3$ buffered with HEPES (KR4B) (112.4 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 4 mM NaHCO$_3$, 21 mM HEPES, 25 mM sodium lactate, 1 mM sodium pyruvate, 5.6 mM glucose, 28 µM phenol red, pH 7.4), (2) KR0B (same ingredients as KR4B, except that NaHCO$_3$ was omitted and the NaCl concentration was adjusted to 116.4 mM), (3) KROB-BSA (same ingredients as KROB but with addition of 0.3% BSA) and (4) KR25B-BSA (same ingredients as KR4B except that HEPES was omitted and the NaHCO$_3$ concentration was increased to 25 mM). KROB and KROB-BSA should not induce capacitation, since they lacked NaHCO$_3$, an upstream activator of the capacitation-related PKA signaling pathway, whereas KR25B-BSA should support capacitation to the greatest extent due to the presence of both BSA and NaHCO$_3$, of the highest concentration (Visconti et al. 1995, Salicioni et al. 2007).

Collection of sperm from the caput and cauda epididymides

The caput and cauda epididymides were dissected out from the animal with special care to eliminate blood vessels and surrounding fat pads. For caput epididymal sperm collection, 6 caput epididymides immersed in 1 mL of KR4B were individually punctured with a 29-G needle and incubated for 15 min at 37°C for sperm to exude into the medium. The mixture of sperm and fluid was passed through a 70-µm-meshwork cup-type Filcon filter (BD Biosciences, Mississauga, ON, Canada) to remove tissue debris and cellular particulates resulting from tissue puncturing. The suspension of sperm in KR4B-diluted caput epididymal fluid was centrifuged (300g, at room temperature (RT), 5 min) to pellet sperm, which were referred to as washed caput epididymal sperm. In alternate experiments, the sperm suspension in diluted caput epididymal fluid, collected as described earlier, was further diluted with KR4B to 2 mL, and layered on top of 2 mL of 45% Percoll solution (GE Healthcare). The tube was then centrifuged (600g, RT, 30 min). The pelleted sperm were washed once with KR4B (350g, RT, 10 min) and referred to as caput epididymal sperm purified through Percoll centrifugation. On average, ~1 million purified sperm were recovered.

Hermo et al. 1991, 2000, Mattmueller & Hinton 1991, Law & Griswold 1994, Robaire & Viger 1995, Ahuja et al. 1996). CLU is indeed one of the major proteins in the epididymal lumen of several mammals (e.g., ram, pig, horse, human) (Dacheux et al. 2009). CLU in the rat epididymal lumen is exclusively a secreted product of epididymal epithelial cells, since the testicular form of CLU is endocytosed by epithelial cells of the rete testis and efferent ducts (Hermo et al. 1991). Rat epididymal CLU is in fact biochemically different from its testicular counterpart, with its molecular mass being lower than the latter (Mattmueller & Hinton 1991, Sylvester et al. 1991).
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Motile and immotile caudal epididymal sperm populations were prepared by centrifuging sperm collected in 2 mL of KR4B through a 45%/90% Percoll gradient following our previously described methods (Tanphaichitr et al. 1990, Furimsky et al. 2005). Upon resuspending the pelleted and Percoll-gradient-interfaced sperm in either KR4B or KR25B-BSA, motility of the former and latter populations was >90% and <15%, respectively. In one set of experiments, both Percoll-gradient pelletated and interfaced sperm were used without further incubation. Alternatively, Percoll-gradient pelletated sperm resuspended in KR4B or KR25B-BSA were further incubated (37°C, 1 h) under air for KR4B or under 5% CO₂ for KR25B-BSA before use for experiments.

Caudal epididymal and vas deferens sperm were also collected in 2 mL of KR0B and subjected to 45%/90% Percoll-gradient centrifugation. Likewise, motile-pelleted sperm resuspended in KR0B were either used immediately or further incubated (37°C, 1 h) under air in KR0B or KR0B-BSA before use.

**Sperm motility assessment**

A 5-µL aliquot of the sperm suspension was pipetted into a CellVision CV 1020-2cv slide (CellVision Technologies, Heerhugowaard, The Netherlands), which was then video-imaged for motility at 30 frames/second under a Nikon TMS inverted microscope using a 10× objective with a Celestron HD Digital Microscope Imager (Torrance, CA, USA, containing a 30× magnifier lens) attached to an eyepiece.

**Immunocytochemical localization of CLU on mouse epididymis sections**

Epididymides were fixed in Bouin’s fixative solution and processed for paraffin embedding following a standard protocol. For immunocytochemical detection of CLU, sections (5µm thick) of the embedded tissue were deparaffinized. Successive washing of the sections in PBS containing 0.1% Tween-20 was performed at all steps below when changing the incubation solution. After pre-blocking for non-specific staining in PBS containing 4% BSA (RT, 30 min), the sections were incubated (RT, 1.5 h) with 5 µg/mL anti-CLU IgG in PBS-2% BSA, and then with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (H+L) antiserum (Bio-Rad) at 1:150 dilution in PBS-2% BSA. The antibody–antigen recognition was detected using a Vector peroxidase ABC kit with diaminobenzidine as a substrate (Vector Laboratories Inc, Burlingame, CA, USA). The tissues were counterstained with Mayer’s hematoxylin (Sigma-Aldrich Canada Ltd) and 1% methylene blue (Thermo Fisher Scientific) followed by stepwise dehydration in ethanol and finally in xylene before mounting with Permount (Thermo Fisher Scientific). Images were captured on a Zeiss Axioskop (Carl Zeiss Canada) using a Nikon D5000 digital Camera. In a control experiment, epididymis sections were subjected to the same procedures except that 5 µg/mL normal goat IgG was used in place of anti-CLU IgG.

**Immunofluorescence localization of CLU and SED1 on sperm**

Sperm from various preparations were washed (500 g, 5 min) twice in PBS-0.1% polyvinylpyrrolidone (PVP) and then fixed (RT, 15 min) in 4% paraformaldehyde in PBS. Washing sperm twice with PBS-0.1% PVP was performed at this step and other steps described later when changing the incubation solution. After pre-blocking (RT incubation for 30 min) from non-specific binding with 5% normal rabbit serum made in PBS-0.1% PVP, sperm were incubated with anti-CLU IgG (5 µg/mL in PBS-2% BSA) and then with secondary antibody, Alexa Fluor-488 conjugated rabbit anti-goat IgG (H+L) (Life Technologies, 1:200 dilution in PBS-2% BSA). Incubation for both antibodies was for 45 min at RT. Sperm were then resuspended in PBS+Fluoroshield solution (Sigma-Aldrich Canada Ltd, used as per the manufacturer instruction) and applied onto a microscope slide with a coverslip for imaging under a Zeiss Axio Observer epifluorescence microscope, using excitation and emission wavelengths of 450–490 nm and 520 nm, respectively. Approximately 200 sperm were counted for various patterns of CLU signals in each replicate experiment. In an alternate experiment, immunofluorescence of CLU was performed on sperm that were bound to the zona(e) pellucida(e) (ZP) layer coated onto the surface of a glass slide. ZP used in this experiment were prepared from mouse ovaries as previously described (Xu et al. 2012) and heat solubilized in 0.2 M sodium phosphate buffer, pH 6.8 at two ZP/µL. The solubilized ZP solution was plated into the middle of the well of a Thermo Scientific 8-well Diagnostic glass slide (Thermo Fisher Scientific) and allowed to air-dry. After two gentle washes with PBS, the ZP layer was incubated (5 min, 37°C) with 20 µL of sperm pre-incubated for 1 h in KR25B-BSA (prepared as described earlier, but washed once and resuspended in KR4B to a concentration of 10⁶/mL). The zona layer on the glass slide was then washed twice with PBS and semi-dried under air. Sperm bound to the ZP layer were then subjected to CLU immunofluorescence. Approximately 100 sperm were evaluated for CLU signal patterns in each of the duplicate experiments. Double immunofluorescence for CLU and SED1 was performed on Percoll-gradient pelletated caudal epididymal sperm, which were immediately resuspended in KR4B or further capacitated (37°C, 5% CO₂) in KR25B-BSA for another hour. Sperm were fixed and first subjected to CLU immunofluorescence, following the same protocol as described earlier. However, donkey anti-goat IgG (H+L) conjugated with Alexa Fluor 488 (Life Technologies, 1:200 dilution in PBS-2% BSA) was used as the secondary antibody for CLU signal detection in this experiment. After completing immunofluorescence for CLU, the same sperm samples were...
subjected to immunofluorescence for SED1, using the rabbit anti-SED1 IgG antiserum (1:100 dilution) as the primary antibody and goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 594 (Life Technologies, Catalog Number: 14600-070) as the secondary antibody. Accordingly, 5% donkey serum (blocking step) and 5% bovine serum (binding step) were used in the blocking step (1 h, RT) of the immunofluorescence for CLU and SED1, respectively. Aldehyde fixed sperm incubated with normal goat IgG and normal rabbit IgG in place of anti-CLU IgG and anti-SED1 IgG, respectively, and subjected to the same processing served as negative controls for CLU and SED1 immunofluorescence localization.

**Flow cytometry**

Relative amounts of CLU on sperm that were subjected to immunofluorescence as described earlier were analyzed by flow cytometry. Sperm were gated based on forward scatter and side scatter profiles in order to exclude debris, and fluorescence was measured in FL1 (510/20 nm band-pass filter). For each sample, a total of 10,000 events were acquired using a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Data were analyzed with FCS Express 4.0 software (De Novo Software, Thornhill, ON, Canada).

**Co-immunoprecipitation of SED1 and CLU**

SED1 and CLU present in the mouse caudal epididymal fluid were co-immunoprecipitated using anti-SED1 IgG captured and cross-linked to paramagnetic Dynabeads Protein G, provided in a Novex Immunoprecipitation Kit (Life Technologies, Catalog Number: 10007D). Sperm + luminal fluid was squeezed out from two sides of the cauda epididymis into 1 mL of PBS + PIC (Roche Diagnostics). Following sperm removal by centrifugation (1000 g, 5 min, RT), the supernatant was centrifuged at a high speed (14,000 g, 5 min, RT) to remove all particulates. Epididymal fluid in the final supernatant was used in this co-immunoprecipitation experiment. Following methods provided by the manufacturer, 40 µL of prewashed Dynabeads was incubated with 2 µg of goat anti-SED1 IgG, and the Protein G-anti-SED1 IgG complexes obtained were covalently linked using a BS3 linker (Life Technologies). Control beads were also made using normal goat IgG in place of anti-SED1 IgG. These Dynabeads containing protein G-anti-SED1 IgG complexes or protein G-normal goat IgG complexes were then incubated (45 min, RT, with rotation) with 150 µg of caudal epididymal fluid proteins (input fraction) in 700 µL of Modified Dulbecco’s PBS. Unbound proteins were washed off from the beads thrice, each in 200 µL of Washing Buffer (provided in the kit). For each wash, the paramagnetic beads were captured to the wall surface of the tube placed in the holding magnet. Finally, caudal epididymal proteins captured on the anti-SED1-Protein G-Dynabeads were eluted into solution by treatment (95°C, 10 min) with Laemmli’s SDS-PAGE sample buffer containing 80 mM DTT. After the beads were removed by the magnetic force, eluted proteins (bound fraction) were subjected to SDS-PAGE and immunoblotting, which was sequentially performed on the same membrane, i.e., first with anti-CLU and then with anti-SED1 antibody.

**Protein quantification, SDS-PAGE and immunoblotting**

Whole proteins in caput and caudal epididymal fluid were quantified by the Bradford assay (Bio-Rad Laboratories), following the manufacturer’s instruction. Aliquots of these samples were treated with Laemmli’s sample buffer with or without 80 mM DTT or 1% mercaptoethanol for SDS-PAGE, which was performed on an 8% or 10% polyacrylamide gel following the method described (Laemmli 1970). To extract proteins from caput and caudal epididymal sperm for CLU immunoblotting, sperm were treated with Laemmli’s sample buffer, with sonication, followed by centrifugation at 12,000 g to pellet the particulates; the supernatants were then subjected to SDS-PAGE. Extraction of caudal epididymal sperm proteins for phosphotyrosine immunoblotting was performed as previously described (Navarrete et al. 2015). Following electrophoresis, the proteins were electro-transferred (100V, 1 h) (Towbin & Gordon 1984) onto a nitrocellulose (Bio-Rad Laboratories) or PVDF (GE Healthcare Life Science) membrane (both with a 0.45-µm pore size) for CLU and phosphotyrosine immunoblotting, respectively. In all steps described later, TBS containing 0.1% Tween-20 (TBST) was used as a washing solution (RT, three 5-min washes) for the membrane when changing the incubation solution. After pre-blocking (RT, 1 h) in 5% powdered skim milk in TBST for CLU immunoblotting or in 20% fish skin gelatin (Sigma-Aldrich Canada Ltd) in TBST for phosphotyrosine immunoblotting, the membrane was incubated (overnight, 4°C) with anti-CLU antibody (0.25 µg/mL in TBST containing 5% skim milk) and anti-phosphotyrosine (monoclonal 4G10, 0.1 µg/mL in TBST), and then with a secondary antibody (1 h, RT) obtained from BioRad (HRP-conjugated rabbit anti-goat IgG, 1:5000 dilution in TBST-5% skim milk for CLU detection, and HRP-conjugated goat anti-mouse IgG, 1:10,000 dilution in TBST for tyrosine phosphate detection). Reactivity of antigen with the antibody was revealed by enhanced chemiluminescence (ECL) on an Amersham Hyperfilm (GE Healthcare Life Sciences), using a Pierce ECL Western blotting Substrate (Thermo Scientific). CLU-α reactive bands were subjected to densitometric analyses using Alphalager Software (AlphaEase version 5.5, Alpha Innotech Co., San Leandro, CA, USA). Amounts of CLU-α were then determined from the standard curve of rec CLU (3–4 concentrations loaded into the same gel as the samples, and immunoblotted and densitometrically analyzed in parallel).

Similar conditions of immunoblotting for CLU-α as described earlier were used in the co-immunoprecipitation experiments except that anti-CLU antibody was used at 0.5 µg/mL. SED1 was probed using anti-SED1 antibody (1 µg/mL, overnight incubation at 4°C) and HRP-conjugated rabbit anti-goat IgG (1:5000, 1 h, RT). Detection of antigen–antibody reactivity was by chemiluminescence using a regular ECL substrate (as described earlier) and a SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific).

**Results**

**Expression of CLU by epididymal epithelial cells**

Mouse Sertoli cells showed immunoreactive signals for CLU in their cytoplasm (Supplementary Fig. 2). However,
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Fig. 2, left panel), whereas caput epididymal sperm showed strong immunofluorescence CLU signals on the sperm head and tail (Fig. 2B, left panel), whereas caput epididymal sperm that had been centrifuged through a 45% Percoll solution exhibited only a trace signal of CLU (Fig. 2B, right panel), a result indicating that CLU was loosely bound to caput epididymal sperm.

Immunoblotting of caudal and caput epididymal fluid and caudal sperm, performed on non-reducing SDS-PAGE, revealed CLU bands of a dimeric form, with a molecular mass of 80, 72 and 72 kDa, respectively (Fig. 2C). A slightly lower mass of CLU in caudal epididymal fluid and sperm suggested protein processing occurring in this epididymal area.

Immunoblotting results (Fig. 2A and C) indicated that the CLU level decreased in caudal epididymal fluid, relative to that in the caput fluid. Quantitative analyses indicated that this decrease was 8-fold, from 6.67 ± 1.01 µg in the caput to 0.82 ± 0.02 µg in the cauda in one side of the epididymis. This decrease in the absolute CLU amount together with an increase in total proteins in the caudal epididymal fluid, compared with those in the caput region, further lowered the percentage of CLU over total proteins to only 0.22%, a value 18-fold lower than the corresponding percentage in the caput fluid (3.91%) (Fig. 2D). However, the amounts of CLU in sperm and luminal fluid in one side of the cauda epididymis were of the same range (0.51 ± 0.04 and 0.82 ± 0.02 µg) (Fig. 2D).

**Presence of CLU in epididymal lumen and on epididymal sperm**

Immunoblotting indicated that CLU was a component of the epididymal luminal proteins in both the caput and cauda epididymides (Fig. 2). Under reducing conditions (Fig. 2A), CLU-α present in the lumen of both epididymal regions possessed a similar apparent molecular mass, i.e., an average of 40 kDa, which was significantly different from that of CLU-α secreted by mouse Sertoli cells in culture (i.e., 44 kDa, Supplementary Fig. 2C). In the caudal epididymal fluid, a faint anti-CLU reactive band of 50 kDa was also observed, possibly reflecting a more heavily glycosylated form of CLU-α, whereas the additional 37-kDa band may be a processed product of the main CLU-α bands (42 kDa and 37 kDa). Caudal epididymal sperm that were centrifuged through a 45% Percoll solution also showed an immunoreactive CLU band of a 40-kDa molecular mass, a result corroborating their positive CLU immunofluorescence staining (Fig. 2B). In contrast, caput epididymal sperm processed in parallel showed only a trace amount of immunoreactivity (Fig. 2A). This observation did not agree with the immunocytochemistry results, indicating strong anti-CLU reactivity on sperm inside the caput epididymal lumen (Fig. 1B). Immunofluorescence results of caput epididymal sperm shown in Fig. 2B explained this disparity. Sperm retrieved from the caput epididymis and simply washed in PBS by centrifugation showed strong immunofluorescence CLU signals on the sperm head and tail (Fig. 2B, left panel), whereas caput epididymal sperm that had been centrifuged through a 45% Percoll solution exhibited only a trace signal of CLU (Fig. 2B, right panel), a result indicating that CLU was loosely bound to caput epididymal sperm.

**Immunofluorescence of CLU in mouse caudal epididymal sperm**

**Different CLU localization patterns between immotile and motile sperm**

Percoll-gradient centrifugation of caudal epididymal sperm collected in KR4B generated gradient-interfaced sperm and gradient-pelleted sperm fractions, which were mainly immotile and motile, respectively, upon resuspending in KR4B. The majority of both sperm populations exhibited CLU fluorescence signals but with different patterns, although 12% of immotile sperm and 7% of motile sperm showed no anti-CLU signals (Fig. 3A). In immotile sperm, ~90% showed strong CLU immunofluorescence signals in the head region with 72% displaying the signal over the entire head except at the convex ridge. Another 17% of immotile sperm had the CLU signal at the post-acrosomal region as well as in the concave side of the sperm head hook (Fig. 3A, panels a, b).

Approximately 94% of motile sperm showed positive CLU immunofluorescence signals with mottled patterns. In ~57% of these motile sperm, the mottled pattern appeared to be over the entire sperm head except at the convex ridge, whereas in the remaining 37%, the mottled staining was more confined in the upper part of the sperm head toward the hook region (Fig. 3A, panels a, b).
Figure 1 Immunocytochemistry of CLU in adult mouse epididymis sections. Note different anti-CLU reactivity in various regions of the epididymis. Panel A: The two proximal initial segment tubules (arrowheads) showed no CLU signals, whereas the four distal initial segment tubules (arrows) revealed positive CLU signals (brown color). Panel B: All caput epididymis sections possessed CLU. Panels C and D: Proximal and distal caudal epididymal sections, respectively, also had positive reactivity with anti-CLU. In panel D, some regions of the distal caudal epididymal tubules showed low CLU signals (exemplified by the area under the brackets). Sperm in all epididymis tubules with their epithelial cells containing CLU also showed CLU staining. Zoomed-in images were shown for the proximal initial segment (panel E), the distal initial segment (panel F), the caput epididymis (panel G) and proximal cauda epididymis (panel H). Blue arrows in panels F and G point to the supranuclear reticular network, likely corresponding to the Golgi apparatus, whereas red arrows indicate vesicular structures, some of which were likely secretory vesicles, beneath the apical cell membrane. Black arrows in panel H were small vesicular structures, which could be endosomes. Sections of all epididymis areas, when incubated with normal goat IgG instead of anti-CLU, showed no CLU signals (data not shown). Bar = 30 µm. Results shown are representative of two replicate experiments.
panels d, e). Interestingly, flow cytometry indicated that the CLU immunofluorescence intensity of motile caudal epididymal sperm was overall lower than that of the immotile sperm population (Fig. 3B).

Relocalization of CLU during sperm capacitation

Upon incubation for one hour in KR25B-BSA, motile Percoll-gradient pelleted sperm became fully capacitated, as revealed by their hyperactivated whiplash motility patterns and very high levels of protein tyrosine phosphorylation (Supplementary Fig. 3). In the majority (83%) of these fully capacitated sperm, CLU immunofluorescence patterns were markedly different from those that were not capacitated (resuspended in KR4B without further incubation, Supplementary Fig. 3 and Video 1). Namely, CLU was present specifically to the head hook region, mostly on the concave side (Fig. 4A, panel b and Fig. 5, bottom plot). The CLU immunofluorescence intensity in these sperm was also higher than that in non-capacitated sperm (Fig. 4A and B). Flow cytometry indicated that an increase in the CLU signal intensity occurred within 30 min of sperm incubation in KR25B-BSA, and reached a plateau at 60 min (Fig. 4B). Only 8% of sperm incubated in KR25B-BSA for 1 h showed an absence of CLU signals, whereas another 7% still possessed mottled CLU localization patterns in the sperm head typical of those in non-capacitated sperm (Fig. 5, bottom plot). However, fully capacitated sperm that bound to mouse ZP coated on the slide showed CLU signals only in the head hook area (Fig. 6).
Video 1

Video records of motility of sperm incubated in four Krebs Ringer media. These included KR0B (containing no NaHCO3), KR0B-BSA (containing no NaHCO3 but supplemented with BSA), KR4B (containing 4 mM NaHCO3) and KR25B-BSA (containing 25 mM NaHCO3 and 0.3% BSA). Video recording was performed at 0 h of incubation time. The data shown were representative of ten video clips recorded for each sample in one of the three replicate experiments performed on different days.

When sperm were incubated in KR4B for 1 h, ~20% of them gained hyperactivated whiplash motility patterns (Video 2) as well as a slight increase in their protein tyrosine phosphate level (Supplementary Fig. 3).
Interestingly, 33% of these KR4B-incubated sperm showed CLU localization in the head hook region, concurrently with a decrease in the population with mottled CLU signals (from 94% at 0 h to 62% at 1 h of incubation) (Fig. 5, third plot from the top). In contrast, when Percoll-gradient pelleted (motile) sperm were prepared from caudal epididymal and vas deferens sperm collected in KR0B and further incubated for 1 h in KR0B or KR0B-BSA, CLU localization in the majority (~85%) of these two motile sperm samples was of the same patterns (Fig. 5, first and second plots from the top) as that in motile sperm resuspended in KR4B (0 h incubation) (Figs 3A, panels d and e, Fig. 4A, panel a), i.e., as mottled patterns in either the entire or part of the sperm head except at the convex ridge. Notably, these two motile sperm samples remained non-capacitated, showing no hyperactivated motility patterns and a residual level of protein tyrosine phosphorylation (Supplementary Fig. 3).

**Video 2**

Video records of motility of sperm incubated in four Krebs Ringer media. These included KR0B (containing no NaHCO3), KR0B-BSA (containing no NaHCO3 but supplemented with BSA), KR4B (containing 4 mM NaHCO3), and KR25B-BSA (containing 25 mM NaHCO3 and 0.3% BSA). Video recording was performed at 1 h of incubation time. The data shown were representative of ten video clips recorded for each sample in one of the three replicate experiments performed on different days.

**Relationship between CLU and SED1**

SED1 is present abundantly in the epididymal lumen, with a fraction depositing onto sperm transiting through the epididymis. In solution, SED1 has a propensity
to undergo oligomerization (Ensslin & Shur 2003, Raymond et al. 2009, Kongmanas et al. 2015), which could lead to precipitation under the slightly acidic pH of the epididymal lumen (Levine & Kelly 1978). Therefore, we asked whether CLU through its chaperone property could interact with SED1, thus preventing SED1 from precipitation. Our immunoprecipitation results indicated that CLU and SED1 in the epididymal fluid were associated with each other. CLU in the epididymal fluid was captured to the paramagnetic beads covalently conjugated with anti-SED1 IgG (Fig. 7, lane 3). The major anti-CLU reactive band had a molecular mass of ~40 kDa, as expected under reducing SDS-PAGE (Fig. 2). The other two minor anti-CLU reactive bands detected in the 'bound' fraction were of 50- and 35-kDa molecular mass. All these anti-CLU bands (Fig. 7, lane 3, arrowheads) were present in the 'input' epididymal fluid (Fig. 7, lanes 1 & 2). When the same protein blot was reprobed with anti-SED1, three intense reactive bands of 74, 60 and 55 kDa were observed in both bound and input fractions (Fig. 7, lane 6 vs lanes 4 & 5). While the 55-kDa band was as expected based on the known apparent molecular mass of mouse epididymal SED1 (Ensslin & Shur 2003), the identity of the 74- and 60-kDa bands was unclear. They may represent complexes of SED1 with other epididymal fluid proteins, which were resistant to treatment with the SDS-PAGE sample buffer. When the control beads containing protein-G-normal goat IgG complexes were used for incubation with the same epididymal fluid input (Fig. 7, lane 7), neither SED1 nor CLU was pulled down (Fig. 7, lane 8).

The association between CLU and SED1 on caudal epididymal sperm was further investigated by double indirect immunofluorescence. Approximately 90% of non-capacitated motile caudal epididymal sperm showed reactivity with both anti-CLU and anti-SED1. The SED1 signals were mottled and localized over the entire sperm head except at the convex ridge (Fig. 8A, middle column), similar to the patterns observed for CLU (Fig. 3A, panels d, e, and Fig. 4A, panel a). Notably, SED1 and CLU co-localized spots were present in 50% of non-capacitated sperm (Fig. 8A, orange/yellow spots in the ‘merged’ column), although in these sperm, there were a number of CLU and SED1 signal spots that did not overlap. However, when these motile caudal epididymal sperm were capacitated upon 1-h incubation in KR25B-BSA, ~50% of capacitated sperm showed the SED1 signal mainly in the convex ridge (Fig. 8B, middle column), whereas in the remaining 50%, SED1 signals remained unmovied in the sperm head with the same pattern as in non-capacitated sperm (data not shown). In contrast, ~80% of capacitated sperm possessed the CLU signal at the sperm hook region (Fig. 8B), similar to results described in Fig. 4. In essence, the CLU and SED1 signals were well separated in the majority of capacitated sperm (Fig. 8B). When motile caudal epididymal sperm were incubated with normal goat IgG (control IgG for anti-CLU IgG) or normal rabbit IgG (control IgG for anti-SED1) and subsequently Alexa

Figure 7 Co-Immunoprecipitation of SED1 and CLU. Paramagnetic beads covalently conjugated with goat anti-SED1 IgG were used in the immunoprecipitation experiment to capture SED1 and CLU from epididymal fluid. Proteins bound to the anti-SED1 beads on were first probed by immunoblotting with anti-CLU (left panel). The same blot was then reprobed with anti-SED1 (middle panel). A parallel aliquot of total epididymal fluid proteins used in this immunoprecipitation experiment (input) was also loaded into the same gel for immunoblotting with anti-CLU and anti-SED1. The blot was developed first using a regular (normal) ECL substrate and then post-developed with a Femto Maximum Sensitivity substrate. See more details in Materials and methods section. The normal and Femto ECL signals of the same ‘input’ lane for both anti-CLU and anti-SED1 reactivities were then placed next to one another in the left (lanes 1 and 2) and middle (lanes 4 and 5) panels for comparison. Arrowheads in the left and middle panels indicate anti-CLU reactive bands, whereas arrows in the middle panel show anti-SED1 bands. Paramagnetic beads conjugated with normal goat IgG (negative controls) were also incubated with total epididymal fluid proteins under the same conditions as those used for anti-SED1 beads. Note that proteins eluted from these rabbit IgG beads (labeled as ‘bound’) showed no anti-CLU or anti-SED1 reactive bands (right panel). Results shown are representative of two replicate experiments. IP = immunoprecipitation.
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FLuor 488-conjugated secondary antibody (for goat IgG) or Alexa Fluor 594-conjugated secondary antibody (for rabbit IgG), no fluorescence signals were detected (Fig. 8C).

Discussion

Presence of CLU in epididymal lumen and sperm: its possible role as a chaperone protein

CLU, a major luminal protein secreted by Sertoli cells and epididymal epithelial cells in various animal species (see Introduction section), is shown herein for the first time for its differential amounts in the mouse caput and caudal epididymis. As well, deposition of CLU from epididymal fluid to only mouse caudal epididymal sperm with appreciable affinity was demonstrated. The level of CLU in the caudal epididymal fluid was 8× less than that in the luminal fluid of the caput region (the site of its secretion) and was reaching an equilibrium with the amount of CLU on the surface of total sperm in the cauda epididymis. The decrease in CLU levels in the cauda epididymis might be regulated by endocytic activities of CLU by caudal epididymal epithelial cells, as implicated from the immunocytochemical results (see Fig. 1, panel h).

The high amounts of CLU in the epididymal lumen suggest that it is involved in sperm maturation. Purified CLU is known for its property as an extracellular chaperone preventing precipitation and/or aggregation of proteins under stress (e.g., high temperature or acidic pH and low fluid volume) (Poon et al. 2002, Carver et al. 2003, Wyatt et al. 2009, 2011, Wyatt & Wilson 2010). Since the milieu of the epididymal lumen is slightly acidic (pH 6.5) (Levine & Kelly 1978), the chaperone property of epididymal CLU would be important in preventing luminal proteins that exist in abundance from precipitation. SED1 is one of such proteins secreted by epididymal epithelial cells, which tends to oligomerize and/or form complexes with other proteins in solution (Shur et al. 2006, Kiedzierska et al. 2007, Raymond et al. 2009). A fraction of SED1 in epididymal fluid deposits onto transiting sperm and functions in ZP binding during sperm–egg interaction (Ensslin & Shur 2003, Shur et al. 2006). Herein, we demonstrated that SED1 and CLU were associated with each other, and fractions of these two proteins were co-localized on non-capacitated sperm. All these results suggested that CLU may chaperone a pool of epididymal SED1 onto epididymal sperm. ApoE receptor 2 is likely one of the CLU receptors on the mouse sperm surface (Andersen et al. 2003, Leeb et al. 2014, Riaz et al. 2017). CLU also binds to lipids (Gelissen et al. 1998, Wilson & Easterbrook-Smith 2000, Nuutinen et al. 2009, Mishra et al. 2011, Trougakos 2013) which may serve as anchoring molecules during deposition of free CLU and CLU–SED1 complexes onto the sperm surface. SED1 may also have its own receptor(s), and the interaction between CLU receptors and SED1 receptors and
CLU-SED1 complexes would give synergy to the deposition of both proteins onto the sperm surface. However, the reallocation of CLU and SED1 to separate sites on the sperm head following capacitation, i.e., the head hook region for CLU and the convex ridge (the site for ZP interaction) for SED1, would free SED1 to exert its function in ZP binding (see Fig. 9 for a model of the chaperone role of CLU during sperm maturation and capacitation). Notably, association of CLU with two other epididymal luminal proteins, SPAM1 and EPPIN, which deposit onto transiting sperm like SED1, has been documented (Wang et al. 2007, Griffiths et al. 2009), although it is not clear whether CLU is co-localized with these two proteins on epididymal sperm.

Since the proper transfer of epididymal lumen proteins that are relevant to fertilization to the sperm surface is physiologically important, proteins additional to CLU may also be functioning in this process. Apolipoprotein E with sequence and functional homology to CLU is present in the epididymal lumen (Olson et al. 1994, 1995, Law et al. 1997) and may be one of these proteins, which can act as a CLU backup. This may explain why Clu null male mice are still fertile, although when placed under heat stress, they have an enhanced rate of the formation of apoptotic testicular germ cells and a delay of their clearance (Bailey et al. 2002).

In addition to its chaperone role, the protective property of CLU against oxidative stress (Schwochau et al. 1998, Trougakos 2013) and complement-induced cell lysis (Jenne & Tschopp 1989, Murphy et al. 1989, Tschopp & French 1994, Mollnes & Kirschfink 2006) would likely be beneficial to sperm in the epididymal lumen, which contains reactive oxygen species and complements (Harris et al. 2006, Tremellen 2008, Aitken et al. 2014).

**CLU immunolocalization patterns as potential biomarkers of sperm motility and capacitation**

Three main CLU localization patterns belonging to sperm of different physiological properties were described in this report. The first pattern belonging to immotile caudal epididymal sperm exhibited strong CLU immunofluorescence signals in the head except at the convex ridge. The majority of these immotile sperm, which sediment at the Percoll-gradient interface following Percoll-gradient centrifugation, are known for their abnormal morphology. Their head surface contains membrane vesicles (Tanphaichitr et al. 1990), to which CLU would adhere due to its ability to bind to lipids (Gelissen et al. 1998, Wilson & Easterbrook-Smith 2000, Nuutinen et al. 2009, Mishra et al. 2011, Trougakos 2013). In contrast, motile sperm sedimenting as a pellet after Percoll-gradient centrifugation do not have these membrane vesicles, thus explaining their weaker CLU signals as compared with immotile sperm. Our results are in accordance with previous findings that human and stallion sperm of lesser quality have higher amounts of CLU (O’Brien et al. 1994, Novak et al. 2010, Mucciaccia et al. 2012). The increased CLU levels might be for the purpose of protecting these abnormal sperm from further damage.

The majority of non-capacitated motile caudal epididymal sperm exhibited unique mottled patterns of CLU immunofluorescence in the head region. However, CLU immunofluorescence patterns and intensity changed markedly following sperm capacitation. CLU was exclusively localized to the sperm head hook region especially in the concave side in capacitated sperm. In addition, the intensity of CLU immunofluorescence signals in capacitated sperm was higher than that in the non-capacitated counterparts. Since sperm are relatively translationally inactive, the increase in the CLU immunofluorescence signal in capacitated Percoll-gradient-centrifuged sperm was likely from the better exposure of CLU on the sperm surface to the added antibody. This could be a consequence of the separation of CLU from SED1 or other sperm surface proteins that CLU was chaperoning prior to capacitation. Since capacitated sperm bound to the ZP coated on a slide also showed CLU localized to the sperm head hook region, this CLU localization pattern would be a potential biomarker for sperm capacitation.

![Figure 9 Postulated roles of CLU in epididymal spermatogenesis and sperm capacitation. As an extracellular chaperone, CLU interacts with certain proteins, which tend to aggregate/multimerize, thus preventing them from precipitation under a slightly acidic condition of the epididymal luminal fluid. SED1, a major ZP-binding protein present in the epididymal fluid, is likely one of such proteins and its association with CLU was shown in this report. SED1–CLU complexes then deposit onto the sperm head mainly in the post-convex ridge area. Following capacitation, all SED1 is released from CLU interaction, trafficking to the convex ridge for the highest efficiency in ZP binding. Free CLU then moves to the sperm head hook region.](https://www.reproduction-online.org)
mouse sperm with egg-binding ability. The mechanisms of how CLU is separated from proteins that it has chaperoned and how it moves to the sperm head hook region are matters for further investigation. Regardless of these unknown aspects, our results described herein suggest that the unique localization patterns of CLU along with the CLU signal intensity can be used as biomarkers at a single cell level of mouse immotile sperm, motile non-capacitated sperm and motile capacitated sperm. The distribution of immotile sperm and motile non-capacitated and capacitated sperm can be revealed in a single immunofluorescence microscopic and flow cytometry analysis of CLU. In addition, the analysis of sperm of various motility and capacitation states can be conveniently conducted on sperm stored in an aldehyde fixative.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0518.

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

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