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Original Article

Polarized epithelium-sperm co-culture system reveals stimulatory factors for the secretion of mouse epididymal quiescin sulfhydryl oxidase 1

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Abstract. Spermatozoa acquire fertilization ability through post-translational modifications. These membrane surface alterations occur in various segments of the epididymis. Quiescin sulfhydryl oxidases, which catalyze thiol-oxidation reactions, are involved in disulfide bond formation, which is essential for sperm maturation, upon transition and migration in the epididymis. Using castration and azoospermia transgenic mouse models, in the present study, we showed that quiescin sulfhydryl oxidase 1 (QSOX1) protein expression and secretion are positively correlated with the presence of testosterone and sperm cells. A two-dimensional *in vitro* epithelium-sperm co-culture system provided further evidence in support of the notion that both testosterone and its dominant metabolite, 5α-dihydrotestosterone, promote epididymal QSOX1 secretion. We also demonstrated that immature caput spermatozoa, but not mature cauda sperm cells, exhibited great potential to stimulate QSOX1 secretion *in vitro*, suggesting that sperm maturation is a key regulatory factor for mouse epididymal QSOX1 secretion. Proteomic analysis identified 582 secretory proteins from the co-culture supernatant, of which 258 were sperm-specific and 154 were of epididymal epithelium-origin. Gene Ontology analysis indicated that these secreted proteins exhibit functions known to facilitate sperm membrane organization, cellular activity, and sperm-egg recognition. Taken together, our data demonstrated that testosterone and sperm maturation status are key regulators of mouse epididymal QSOX1 protein expression and secretion.

Key words: Epididymis, Epithelium, Fertility, Quiescin sulfhydryl oxidase, Spermatozoa

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Epididymal maturation and transition are prerequisite steps for the functional competence of testicular sperms. Since spermatozoa are considered transcriptionally and translationally inactive, acquisition of additional components from the epididymal epithelium allows for post-testicular surface rearrangements and/or attachments of fertility-essential proteins, lipids, and small RNAs prior to ejaculation [1, 2]. Accumulating evidence supports that the epididymis exhibits a segmental-specific cellular signature, with distinct gene expression patterns, which enables the creation and maintenance of a stage-wise and maturation-requiring microenvironment [3–5]. For example, it is known that testicular sperm cells are immotile; however, upon epididymal migration from the caput toward the cauda, activation or inhibition of specific signaling pathways and protein post-translational modifications trigger sperm motility [6]. Therefore, a better understanding of sperm-epididymal epithelial interactions would contribute to fertilization success.

The epididymis is a continuous and convoluted duct, with a single layer of epithelial cells surrounding the lumen. Upon sperm transition in the epididymis, spermatozoa progressively change their surface compositions and maturation status along with alternations in the epididymal microenvironment [4, 5]. All these changes in the sperm membrane surface are thought to be driven primarily by interactions between spermatozoa and the complex components bathed within the epididymal lumen [7]. Disulfide bonds are formed between sulfur atoms of pairs of cysteine residues (thiol groups) within or across proteins. An earlier study showed that among post-spermiogenesis modifications, the formation of disulfide bonds is essential for the stabilization of sperm structure [8]. In addition, the oxidation of thiol groups is also critical for stabilizing various sperm structures, such as the chromatin, sperm midpiece, and tail [8]. Evidence from previous publications have demonstrated that an increased amount of disulfide bonds detected on the sperm membrane, from the caput toward the cauda epididymis, coincide with the advances in sperm maturation status, demonstrating that disulfide bond formation is part of the maturation process during sperm epididymal transition [9]. Among

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the different maturation processes, the increased potential for forward motility is the most obvious alteration. Scientists have demonstrated that sulfhydryl oxidation is important for the stabilization of sperm tail structure and maintenance of motility wave patterns [10]. For example, outer dense fiber protein 1 (ODF1), a flagellar protein, is oxidized to form disulfides during the epididymal transition and is associated with the bending torque of the tail. Hetherington et al. also demonstrated that sperm cells from ODF1-knockout mice exhibit thinner filament fibers and fail to fertilize oocytes, owing to defective sperm movement [11].

Quiescin sulfhydryl oxidase (QSOX) catalyzes the thiol-oxidation reaction of $2R\text{-}SH + O_2 \rightarrow R\text{-}SS\text{-}R + H_2O_2$. The hypothetical functions of QSOX include generation of disulfide bonds within seminal plasma proteins or maturating spermatozoa, preservation of sperm membrane integrity, antimicrobial activity (through the release of H_2O_2), and protection of spermatozoa against the harmful effects of thiols after ejaculation [12, 13]. Two splice variants (QSOX1a, b) of human *QSOX1* gene have been reported. QSOX1a contains a complete transmembrane domain and is translated by the whole sequence of 3314 nucleotide bases that encodes 747 amino acids; QSOX1b, on the other hand, encodes a peptide of 604 amino acids, is a spliced variant of QSOX1a, and is known to be a secretory form of QSOX1 protein in the absence of the transmembrane domain [14, 15]. Similar to that in humans, the mouse QSOX1 family contains four splice variants, QSOX1a to d; mouse QSOX1a consists of a transmembrane domain (748 amino acids), whereas all other isoforms (QSOX1b to d) are truncated forms without a transmembrane domain, due to alternative splicing variants (661, 568, and 154 amino acids for mouse QSOX1b, c, and d, respectively) [16, 17].

We have previously described that the secretory form of the mouse QSOX1 protein, QSOX1c, is detected mainly in the epididymal lumen and exhibits a region-specific distribution in the epididymis. Luminal detection of QSOX1c suggested that this protein is secreted into the lumen by the epididymal epithelium and likely plays a role at different maturation stages of spermatozoa [18]. In an earlier study, we showed that the expression and secretion of mouse epididymal QSOX2 is regulated by a fine coordinated mechanism between testosterone and glutamate [19]. Although we also demonstrated that mouse QSOX1c could aggregate defective human and mouse sperm *in vitro* and *in vivo* [20], the regulatory mechanism directing QSOX1c secretion remains unknown. Thus, in this study, we aimed to apply different mouse models with a two-dimensional polarized co-culture system, to investigate potential regulatory factors responsible for epididymal QSOX1 protein secretion.

Materials and Methods

Chemicals, reagents, and antibodies

Chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Rabbit polyclonal anti-QSOX1, which detects all splicing variants, was purchased from Abcam (catalog no. Ab82713, Cambridge, UK). To specifically investigate QSOX1 variant c (secretory QSOX1), anti-QSOX1c anti-serum was generated and kindly provided by Dr. S.H. Li from the Mackay Memorial Hospital (Taiwan), as previously described [20, 21]. For use as the western blot loading control, rabbit polyclonal anti-eukaryotic elongation factor 2 antibody (catalog no. Ab40812) was purchased from Abcam. For indirect immunofluorescence, rabbit polyclonal anti-calreticulin antibody (catalog no. Ab92516, Abcam), which is an endoplasmic reticulum (ER) marker, was obtained from Abcam. The mouse monoclonal anti-enhanced green fluorescent protein (eGFP) antibody was purchased from Invitrogen (catalog no. MA5-15256; Waltham, MA, USA). All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Animals and hormone manipulations

Ten-week-old male Institute of Cancer Research (ICR) mice were obtained from the National Laboratory Animal Center, Taiwan, and acclimatized (3 mice/cage) for 1 week prior to the experiments. To examine the effect of sperm cells on QSXO1 protein expression, the *Cre/loxP* system was used to generate sperm-null knockout mice. As described earlier, *Vasa* was used as a germ-line specific promoter and *Elp1* (also known as inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein [*IKBKAP*] in mice), a critical gene in the meiosis process, was flanked by two *loxP* sites [22]. After recombination, spermatogenesis was disrupted in *Vasa-Cre:Elp*$^{-/-}$ animals and characterized in terms of the absence of sperm cells in the epididymis, as described earlier [22]. The animal housing room was maintained at a constant temperature (22–24°C) with a 12 h/12 h alternating light/dark cycle. The animals were provided water and standard mouse lab chow (Oriental yeast, Tokyo, Japan) *ad libitum*. Animal experiments were carried out with the regulation and permission of the Institutional Animal Care and Use Committee protocol at National Taiwan University (NTU-107-EL-00102, Taiwan). To evaluate QSOX1 expression at different postnatal developmental stages, epididymal tissues were harvested from 20-, 30-, 40-, 50-, 60-, and 80-postpartum day mice. To investigate the effects of sex hormones on QSOX1 protein expression and secretion, loss- and restoration-of-function animal models were established. Briefly, 10 adult ICR mice were randomly allocated into the sham operation (n = 3) and castration (n = 7) groups. Castration was performed by means of bilateral removal of the testes, following which the remaining epididymis was placed back into the abdominal cavity. Animals in the sham operation group underwent the same surgical procedures, except for the removal of testes. Fourteen days post-surgery, four castrated animals received only 100 µl corn oil (catalog no. C8267, Sigma-Aldrich) as vehicle controls (castration + corn oil), while three other castrated mice were subcutaneously administered testosterone propionate (catalog no. T1875, Sigma-Aldrich, 5 mg/kg body weight, dissolved in 100 µl corn oil) for 10 consecutive days (castration + testosterone).

Collection of epididymal sperm cells and epididymal fluid

After the mice were euthanized, the epididymes were carefully dissected from their fat and connective tissue, and mouse epididymes were punched using an 18 G syringe needle in 50 µl PBS, on a temperature-controlled dissection stage (Tokai Hit, Fujinomiya, Japan). To release sperm cells from either the caput or cauda epididymal lumen, a further 5 min incubation at 37°C was allowed. Epididymal fluid and sperm cells were separated by means of centrifugation at 600 × *g* for 10 min, following which the epididymal sperm cells were washed twice with pre-warmed PBS before further experiments. To obtain a sufficient amount of epididymal fluid, the epididymal fluid from three male mice at the same postnatal time point was pooled. The sperm cells and epididymal fluid were used without further storage.

Immuno-blotting

For protein sample preparation, freshly obtained epididymes were homogenized on ice with tissue homogenization buffer (250 mM sucrose, 1 mM ethylenediaminetetraacetic acid [EDTA], 20 mM Tris/HEPES, 1% Triton™ X-100, pH 7.4) supplemented with protease inhibitor cocktail tablet (EDTA-free, Roche, Mannheim, Germany).

Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. Equivalent amounts of total protein were resuspended in lithium dodecyl sulfate loading buffer (NuPAGE™; catalog no. NP0007, Thermo Fisher Scientific, Waltham, MA, USA) in the presence of a reducing agent (50 mM dithiothreitol, catalog no. NP0009, Thermo Fisher Scientific). The samples were heated at 95°C for 10 min and cooled on ice before loading on the gels. The Bio-Rad Mini-PROTEIN® electrophoresis system was used (Bio-Rad Laboratories, Hertfordshire, DX, USA) according to the manufacturer's protocol, as previously described [18, 23]. Briefly, proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (gradient T-Pro EZ Gel Solution, catalog no. JB02-B010M, T-Pro Biotechnology, NTC, Taiwan) and wet-blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA). After blocking for 1 h with blocking buffer (Tris-buffered saline/Triton™ X-100 [TBST; 5 mM Tris, 250 mM sucrose, pH 7.4, 0.05% v/v Tween-20] supplemented with 5% milk powder) at room temperature (RT), the blots were incubated with primary antibody (1:1000 dilution for QSOX1 and 1:5000 dilution for QSOX1c), overnight at 4°C. After washing in TBST, anti-mouse or anti-rabbit secondary antibodies were added to the blots at a 1:10000 dilution and incubated at RT for an additional 1 h. Protein signals were visualized using chemiluminescence (Merck, Kenilworth, NJ, USA) and detected using the ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA). The relative intensity of each band was semi-quantified using the ImageJ (NIH) software. When necessary, the blots were stripped with stripping buffer (Restore™ Western Blot Stripping Buffer, catalog no. 21059, Thermo Fisher Scientific) and re-probed for other proteins of interest.

Cell culture

Mouse caput epididymal epithelial cells (meCap18), which lack an endogenous androgen receptor [24], were kindly provided by Dr. Petra Sipilä (University of Turku, Finland) and used as a control cell line for steroid-induced QSOX1 secretion experiments. The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco), at 37.5°C, in a humidified atmosphere with 5% CO_2. Immortalized mouse distal caput epididymal epithelial cells (DC2) obtained from Applied Biological Materials (catalog no. T0599 Richmond, BC, Canada) were cultured in Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% FBS and 1% antibiotic-antimycotic medium (Gibco), at 33°C, in a humidified atmosphere with 5% CO_2. For whole cell lysates, after the designed treatments, the cells were rinsed three times with ice-cold Dulbecco's phosphate-buffered saline (DPBS, Gibco) and subsequently scraped into radioimmunoprecipitation assay buffer lysis buffer (catalog no. BP115, Boston BioProducts, Boston, MA, USA) supplemented with EDTA-free protease inhibitors (Roche). The cells were lysed on ice for 15 min and sonicated for 1 min in an ice-cold water bath. Lysates were centrifuged at 10,000 × *g* for 10 min at 4°C, to remove the cellular debris. Protein quantification was performed using a BCA protein assay kit (Pierce Biotechnology), and cell lysates were stored at –20°C until use.

Mouse QSOX1-eGFP plasmid construction and transient transfection

The plasmid mRNA containing mouse QSOX1 (mQSOX1, variant 1, clone ID no. OMu01097, accession no. nm_001024945. 1) nucleotide sequence was purchased from GenScript (Piscataway, NJ, USA). The 2263 bp QSOX1 sequence was cloned into the pcDNA3.1(+)-eGFP vector using *BamHI/NotI* restriction enzymes from the original pcDNA3.1-C-(k)DYK vector. To amplify the plasmid, the mQSOX1-eGFP plasmid was transformed into One Shot™ TOP10 competent *E. coli* (catalog no. C404010, Invitrogen), and the bacteria were spread onto ampicillin-containing lysogeny broth (LB) agar plates and incubated at 37°C for 12–16 h. Isolated single colonies were selected and amplified in LB broth containing ampicillin, at 37°C for 12–16 h, on a constant rotating shaker (225 rpm). The plasmid was extracted using the QIAprep® Spin Miniprep Kit (catalog no. 27106X4, Qiagen, Hilden, Germany), according to the manufacturer's protocol. The concentration and quality of the plasmid were determined using a Picodrop Microliter UV/Vis spectrophotometer (Pico p100, Hinxton, UK). To validate the plasmid structure, it was cleaved using *BamHI/NotI* restriction enzymes. The cleaved vector and DNA fragments were separated using 1% agarose gel electrophoresis and visualized using ultraviolet illumination, after ethidium bromide staining.

Mouse QSOX1-eGFP plasmid was chemically transfected into meCap18 and DC2 cells using Lipofectamine™ 3000 Transfection Reagent (catalog no. L3000001, Invitrogen). In brief, 2 × 10^6 cells were cultured in 24-well plates for 24 h, until 90% confluence. Thirty minutes before transfection, the medium was refreshed and the standard transfection protocol was followed, according to the manufacturer's instructions. Twenty-four hours after transfection, the transfection efficiency was evaluated using an IX83 epifluorescent microscope (Olympus, Tokyo, Japan). To further validate the expression and secretion of mQSOX1-eGFP in transfected cells, western blot analysis and indirect immunofluorescence staining were performed using antibodies against QSOX1, secretory QSOX1c, and eGFP.

Establishment of an in vitro two-dimensional sperm-epididymal epithelium co-culture system

To establish an *in vitro* polarized co-culture system, mQSOX1-eGFP-transfected cells were cultured in Transwell® (catalog no. 3470, Corning, New York, USA) with a 0.4 μm pore size membrane. The cells were washed twice with DPBS before incubation with Phenol Red and FBS-free FluoroBrite™ DMEM medium (Gibco). The cell culture medium was collected at different time-points (12, 24, 36, 48, and 60 h post-transfection) and centrifuged at 3000 × *g*, 4°C for 10 min, to remove the cellular debris. The cultured supernatant was further concentrated using a Vivaspin™ protein concentrator spin column (GE Healthcare, Chicago, IL, USA) with a molecular weight cut-off of 3 kDa (Vivaspin™ 2 MWCO 3000; catalog no. 28932240). The fluorescence intensity, which correlated with the concentration of mQSOX1-eGFP, was measured using a SpectraMax® M5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

To standardize the measurement with minimal spontaneous secretion, all stimulation experiments were performed after spontaneous QSOX1-eGFP protein secretion reached a plateau, in the absence of exogenous stimulation. To examine the effects of sex steroids on QSOX1 secretion, 5–40 nM testosterone or 5α-dihydrotestosterone (DHT, Sigma-Aldrich) was added to transfected epididymal epithelial cells, following which the accumulated fluorescence intensity was measured, at intervals of 12 h, until 60 h post-transfection. To investigate the effects of sperm cells on QSOX1 secretion, sperm cells from caput or cadua epididymis were retrieved as described above and incubated further in pre-warmed Whitten's HEPES medium (WH medium, 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 1 mM pyruvic acid, 4.8 mM calcium

L-lactate hydrate, 20 mM HEPES, pH 7.4). The sperm suspension was collected and further filtered through a 40 µm pore size Falcon™ Cell Strainer (Fisher Scientific, Hampton, NH, USA). Direct and indirect interaction assays were performed using Transwell® (Corning) with a 0.4 µm pore size membrane.

Proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

To identify candidate proteins responsible for sperm-epithelium communication, media from the lower chamber of the above-mentioned indirect co-culture system were collected and analyzed. Media in the presence and absence of 2×10^6 caput sperm cells were used as the stimulation and control groups, respectively. To achieve a sufficient protein concentration, the collected medium was centrifuged at 3300 × *g*, 4°C until a 15- to 30-fold concentrated volume was achieved. Soluble protein samples were denatured in reduction buffer (2 mM dithioerythritol/8 M urea) for 1 h at 37°C. Lys-C (1 h, 37°C) and trypsin (16 h, 37°C) were subsequently used as the digestion enzymes, and the digested peptides were suspended in 0.1% formic acid and desalted using Ziptips. Liquid chromatography-tandem mass spectrometry was performed using an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific). The mass spectra data files were analyzed using Mascot Daemon (version 2.6.0, Matrix Science, London, UK) searched against the SwissProt *Mus musculus* protein database (dated: 5/17/2019). Only two missed cleavages were allowed, and a MASCOT score ≥ 35 was used to identify proteins of interest (score: E-value < 0.05). These protein IDs were converted into UniProt gene IDs for future categorization and molecular function annotation studies. Protein IDs that reached the above-mentioned requirements were first categorized into three major subgroups, (1) sperm-origin, (2) epithelium/luminal fluid-origin, and (3) uncategorized, based on published literature [2, 4, 7, 25–28].

Gene Ontology (GO) analysis and network analysis

Protein network analysis was conducted using Cytoscape (version 3.7.1), with the addition of the ClueGo plugin (version 2.5.4) [29]. GO Biological Process Annotation (downloaded 27.02.2019) for caput sperm protein, epididymal lumen protein, and uncharacterized protein were compared for gene enrichment (right-sided hypergeometric test) using Benjamini–Hochberg multiple test correction. Network parameters were set as follows: GO tree levels (min = 3, max = 8), GO term restriction (minimum number of genes = 5, minimum % = 4), and GO term connection restriction (kappa score threshold = 0.4). Only terms with $P \leq 0.05$ are shown. Following GO analysis, PANTHER (protein analysis through evolutionary relationships; version 14.1) was used to classify proteins based on their biological process and molecular function.

Statistical analyses

Results are expressed as mean ± standard deviation. Comparative studies of means were performed using one-way analysis of variance, followed by Kruskal-Wallis test. Statistical significance was set at $P < 0.05$.

Results

Postnatal epididymal QSOX1 protein expression coincided with the presence of spermatozoa and testosterone surge

To investigate potential stimuli for postnatal mouse epididymal QSOX1 protein expression and secretion, whole epididymal tissue and epididymal fluid from 20-, 30-, 40-, 50-, 60-, and 80-day-old ICR mice were collected and subjected to western blot analysis. The QSOX1 antibody, which detected both membrane and secretory forms of QSOX1 at 75 kDa and 63 kDa, respectively, was used for whole tissue analysis. As shown in Fig. 1A, while a weak signal was detected in the postnatal tissue on day 20, at the molecular size of 75 kDa, which corresponds to the membrane form of QSOX1, an apparent increase in total QSOX1 was detected after day 30 in postnatal samples, which coincided with the first appearance of sperm cells and testosterone surge in the epididymis. In agreement with our earlier finding [18], the dominant form of epididymal QSOX1 at the later postnatal stages was the secretory form, QSOX1c, present at ~65 kDa (Fig. 1A). The secretory activity of QSOX1c was evidenced as we also detected a significant increase (2.4-fold increase) of QSOX1c protein in postnatal epididymal fluid using anti-QSOX1c antibody, after day 30 (Fig. 1B), while the luminal QSOX1c signal became relatively stable 30 days after birth (Fig. 1B).

In most cases, epididymal protein expression and secretion are regulated by luminal factors [19], and to demonstrate whether sperm cells or other stimuli (*e.g.*, steroid hormones) affect QSOX1 protein expression and secretion, we applied the castration model, as described earlier [19]. As shown in Fig. 1C, removal of the testes significantly decreased epididymal QSOX1 protein expression (62.5% decrease when compared to the sham operation group), and supplementation with exogenous testosterone rescued QSOX1 protein expression (238.9% increase when compared to the castration group, Fig. 1C). When we further dissected QSOX1 signal into membrane or secretory form, we observed no significant changes in the percentage of either membrane or secretory QSOX1 in castrated animals; however, upon testosterone supplementation, an increase in secretory QSOX1 was apparent (74.7% and 89.5% in the castration and testosterone addition groups, respectively), suggesting that testosterone affected not only total QSOX1 protein expression but also altered QSOX1 secretion activity (Fig. 1C). Interestingly, when we examined the effect of sperm cells on secretory QSOX1 protein expression in azoospermic mice (Vasa-Cre:*Elp1*$^{-/-}$), despite no apparent changes in the size or the morphology of the epididymis, a sharp decline (59%) in QSOX1c was detected at 65 kDa (Fig. 1D).

Validation of the two-dimensional polarized culture system

To elucidate the potential stimulatory factors for QSOX1 secretion, we established an *in vitro* two-dimensional polarized culture system and an analysis platform. As shown in Fig. 2A, the mQSOX1-eGFP plasmid was transiently transfected into the epididymal epithelium after a polarized monolayer was observed. Culture supernatants were collected, centrifuged, measured, and analyzed at intervals of 12 h. To standardize the measurement, the transfection rate was calculated based on the % of cells expressing eGFP and used for signal intensity correction in each measurement. As shown in Fig. 2B, when the anti-eGFP antibody was used, the QSOX1-eGFP fusion protein was detected in the perinuclear region, similar to the known cellular localization of endogenous QSOX1 (Fig. 2B), and the signal of the QSOX1-eGFP fusion protein largely overlapped with the ER marker calreticulin, in both meCap18 and DC2 cells, suggesting that QSOX1-eGFP fusion proteins might be secreted *via* the ER-mediated secretory pathway (Fig. 2B). Overexpression of the QSOX1-eGFP fusion protein was validated using western blot. A single protein band corresponding to the molecular weight of the QSOX1-eGFP fusion protein, at ~97 kDa, was detected in the cell lysates of both meCap18 and DC2 (Fig. 2C). Moreover, to demonstrate the secretory activity of these transfected cells, culture supernatants from transfected meCap18 and DC2 cells were collected for ELISA

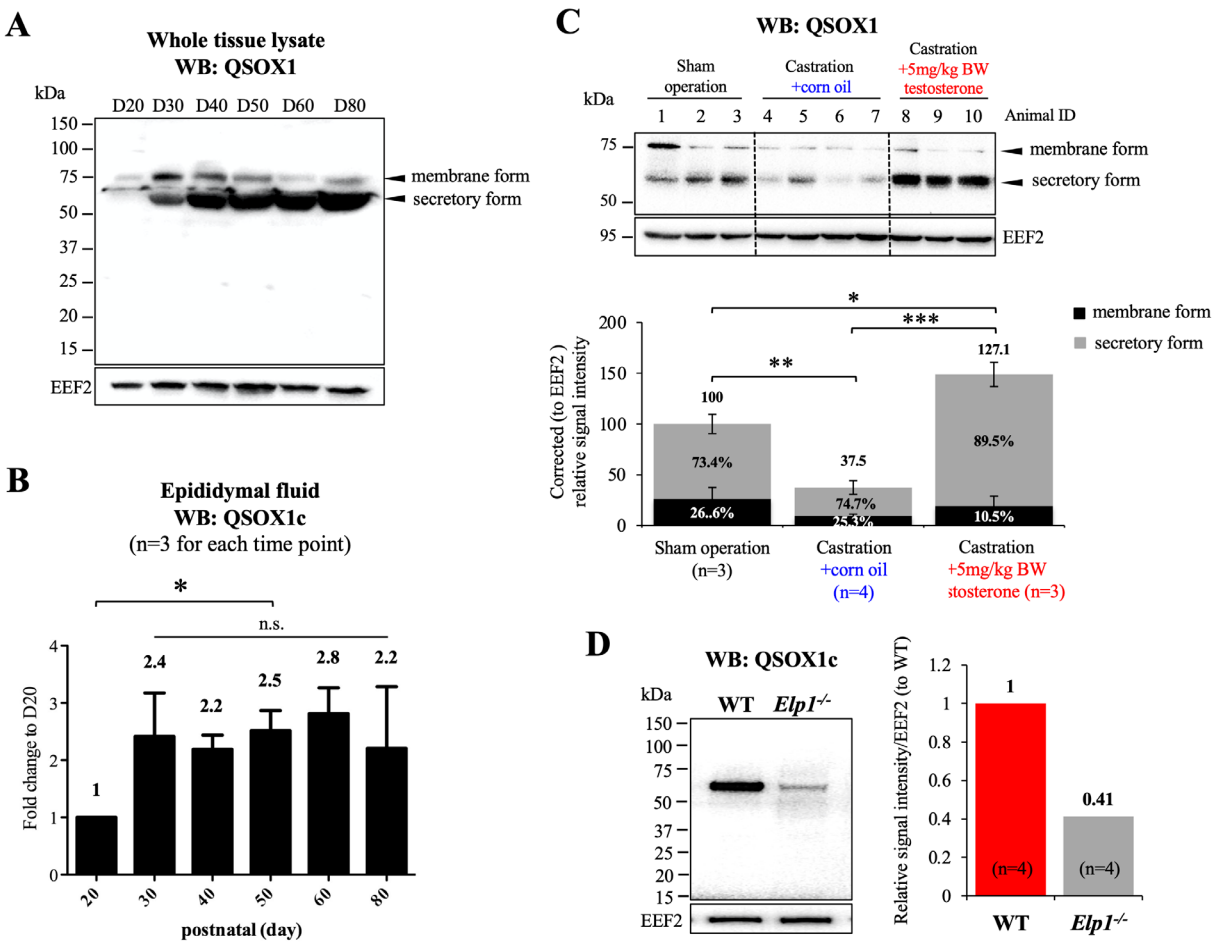

Fig. 1. Effects of testosterone and spermatozoa on QSOX1 protein expression and secretion in the mouse epididymis. (A) Epididymal tissue from 20- to 80-postnatal day mice were analyzed using western blot, and a representative blot has been presented. Postnatal epididymal QSOX1 expression coincided with the presence of spermatozoa and 1st testosterone surge at day 30. (B) Epididymal fluid collected from 20 to 80-postnatal day mice was subjected to western blot analysis for the detection of secretory QSOX1 (QSOX1c). A significant 2.4-fold increase in QSOX1c was detected at postnatal day 30, following which the signal became steady. For each postnatal time-point, epididymal fluid from 3 individual animals was collected, for quantitative analysis. (C) Epididymal tissues from loss- and gain-of-function mouse castration models were homogenized to examine the effect of testosterone on QSOX1 protein expression. Ten ICR mice were randomly allocated into sham operation (n = 3), castration+corn oil (n = 4, IP 100 µl corn oil), and castration+testosterone (n = 3, IP 5 mg/kg body weight in 100 µl dissolved in corn oil) groups. Significant deceases in both membrane and secretory forms of epididymal QSOX1 were detected after castration; addition of exogenous testosterone rescued epididymal QSOX1 protein expression. (D) Azoospermia mouse model supported a positive association between the presence of sperm and epididymal QSOX1 protein expression, as a pronounced decline (–59%) in QSOX1c was detected in the absence of sperm cells. Four animals for each phenotype (wild-type or Vasa-Cre:*Elp*$^{-/-}$) were used for western blot quantification analysis, and a representative blot has been presented. Statistical analysis was performed as described in the Materials and Methods section. Bars represent mean +/– standard deviation. Statistical significance was considered at $P < 0.05$ (*). N.S indicates a non-significant difference.

detection. Based on the data provided in Fig. 2D, no significant changes in the eGFP signal were detected in the meCap18 culture supernatant; in contrast, positive secretory activities were measured in the DC2 culture supernatant (Fig. 2D). To minimize the detection of spontaneous secretion, time-course eGFP detection from the collected culture supernatant was performed. We measured a gradual increase in eGFP signal, from 12 h to 36 h post-transfection, in DC2 cells; however, the accumulated eGFP signal became relatively stable between 36 h and 60 h post-transfection, indicating that without exogenous stimuli, spontaneous secretion of the QSOX1-eGFP fusion protein reached a plateau at 36 h. Thereafter, we applied 36 h post-transfection as the time-point to start our stimulatory experiments (Fig. 2D).

Testosterone and DHT stimulated epididymal QSOX1 secretion

To examine whether the most important male sex steroids, testosterone and DHT, stimulated QSOX1 secretion, two mouse epididymal epithelial cell lines, meCap18 and DC2, were used. When meCap18 was used, no significant elevation of the QSOX1 signal was detected in the culture medium, after addition of either testosterone or DHT (Fig. 3A). In contrast, when DC2 was used, a dose-dependent increase in QSOX1 signals was observed in both testosterone- and DHT-addition groups. Interestingly, as compared to the testosterone effect, a persistent stimulatory effect was observed from 36 h to 52 h, upon addition of DHT (Fig. 3B). Although many reasons could lead to the observed lack of changes in QSOX1 signal in meCap18 cells, such as low ligand-receptor binding efficiency, dull secretory activity, or responses to stimuli, one of the many

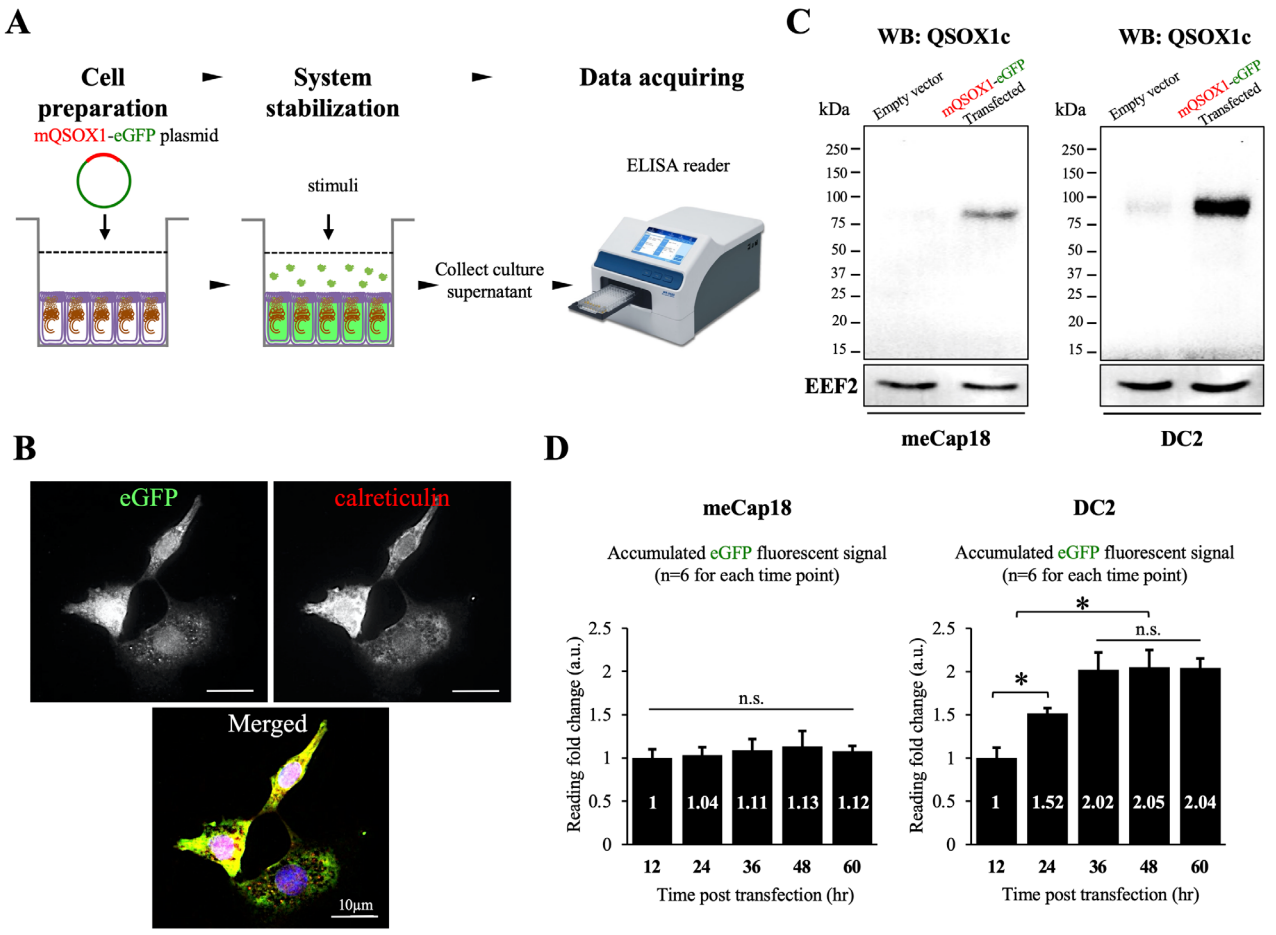

Fig. 2. Validation of mouse QSOX1-eGFP transfection and secretion in epididymal epithelial cell lines. (A) Mouse caput epididymal epithelium were transiently transfected with mouse QSOX1-eGFP plasmid. After the designed experimental procedures, the culture supernatant was collected for ELISA detection of the eGFP signal. (B) Indirect immunofluorescent staining against eGFP was used to validate the presence of transfected mQSOX1-eGFP fusion protein. The perinuclear eGFP staining was similar to the known cellular localization of endogenous QSOX1. The eGFP signal largely overlapped with calreticulin, an ER marker, indicating that eGFP-QSOX1 is involved in the ER-mediated secretory pathway. Representative images from DC2 cells have been presented. (C) Western blot analysis for QSOX1c confirmed the presence and overexpression of transfected mQSOX1-eGFP, at the size of ~97 kDa, both in meCap18 and DC2 cells. Representative western blot images have been presented. (D) Culture supernatants from transfected meCap18 and DC2 cells were collected at intervals of 12 h, following which the accumulated eGFP signals were measured. No apparent changes were detected from the meCap18 culture supernatants. An elevated signal was detected in the DC2 culture supernatants at 24 h and 36 h post-transfection; the fact that the signal became steady after 36 h indicated a minimal spontaneous secretion activity after this time-point. Statistical analysis was performed, as described in the Materials and Methods section. Bars represent mean +/– standard deviation. Statistical significance was considered at $P < 0.05$ (*). N.S. indicates a non-significant difference.

possibilities may be meCap18's natural lack of endogenous androgen receptors, that results in no or minimal response to either testosterone or DHT stimulation [26].

Caput, but not cauda spermatozoa, stimulated epididymal QSOX1 secretion

As we showed a significant decrease in QSOX1 protein expression in azoopsermic mice (Fig. 1D), we further evaluated whether sperm cells of different maturation statuses resulted in different levels of stimulatory outcomes. Caput or cauda spermatozoa, representing immature or fully mature sperm cells, respectively, were used as exogenous stimuli. As sperm effects on epididymal proteins may be due to their direct physical contact or *via* intercellular communications by means of secretory or releasing factors, we established direct and indirect sperm-epididymal epithelium co-culture systems, as illustrated in Fig. 4. Because meCap18 did not respond to our earlier steroid stimulations and showed relatively low transfection and overexpression outcomes, to ensure successful detection of the signal, only DC2 cells were used in the following sets of experiments. To correct for the effects of residual luminal components on QSOX1 secretion, which may bias our measurements, we first established the baseline signal intensity using epididymal fluid as the stimulus. As shown in Fig. 4A, epididymal fluid significantly stimulated QSOX1 secretion, as compared to that in the control group (WH medium). This was likely due to the presence of luminal testosterone or DHT, which supports our earlier findings (Fig. 3). However, when caput sperm cells were used as a stimulus, a sharp increase (3.26-fold) was detected in the QSOX1 signal, indicating that sperm could serve as a stimulatory factor for the secretion of mouse epididymal QSOX1 (Fig. 4A). In both direct and indirect assays, caput sperm, but not cauda sperm, efficiently stimulated QSOX1 secretion, and a dose-dependent stimulation effect was observed when caput spermatozoa were used (Fig. 4B–C). Interestingly, we observed a better stimulatory effect when the indirect assay was applied (1.2- to

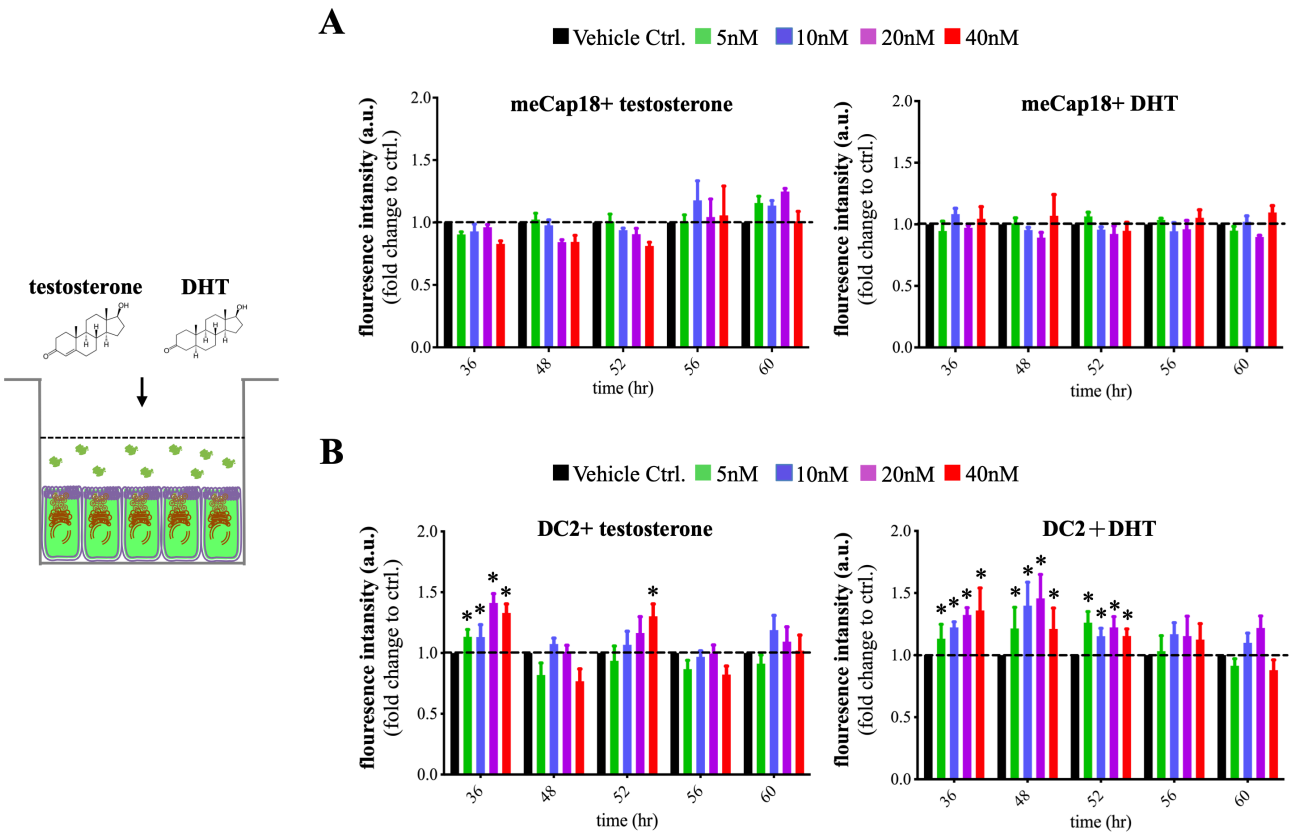

Fig. 3. Testosterone and 5α-dihydrotestosterone (DHT) stimulate QSOX1 secretion *in vitro*. (A) Upon transfection of meCap18 with mQSOX1-eGFP, neither testosterone nor DHT stimulated QSOX1 secretion, as no significant changes were detected in the eGFP signal, at all time-points measured. (B) When DC2 was used, testosterone exerted a dose-dependent stimulatory effect at 36 h. In comparison to testosterone, DHT showed persistent stimulatory effects on QSOX1 secretion, until 52 h. Statistical analysis was performed as described in the Materials and Methods section. Six experimental repeats were performed for each concentration tested, at each time-point. Bars represent mean +/– standard deviation. Statistical significance was considered at $P < 0.05$ (*).

2.6-fold *vs.* 2- to 6-fold increase for direct and indirect stimulations, respectively), indicating that non-physical communication between spermatozoa and epididymal epithelium *via* secretory and/or sperm releasing factors may exist and stimulate mouse epididymal QSOX1 secretion (Fig. 4B–C).

Proteomic identifications on sperm- and epididymal-derived secretomes

To identify potential secretory components that are responsible for inter-cellular communications, we collected supernatants from the lower chamber of an indirect co-culture system for proteomic identification, at 52 h of co-incubation of cauda sperm with DC2 (Fig. 5A). This time-point was chosen based on the detection of a significant increase in QSOX1-eGFP signals at this time-point in Fig. 4C. Among the 1871 proteins identified in the co-culture supernatant, 773 proteins were exclusively present in the sperm-containing group (stimulated), and 582 proteins were considered significant and reliable protein IDs, based on the criteria described in the Materials and Methods section (Fig. 5B, circle in purple). A literature search was performed to categorize the origins of these proteins. Among the 582 protein IDs, 258 were sperm-specific or sperm-origin proteins (Fig. 5C, Supplementary Table 1), 154 were epithelial-origin proteins (Fig. 5D, Supplementary Table 2), and 170 protein IDs could not be specified. Further analyses showed that the majority of the proteins released from sperm cells had known functions related to cellular catalytic activity (144 proteins, such as oxidoreductase and phospholipase) and cellular binding activity (70 proteins, such as hemoglobin and synaptotagmin) (Fig. 5C). Similar to sperm-origin proteins, a large proportion of epithelial-origin proteins were responsible for cellular catalytic activity (76 proteins) and cellular binding activity (50 proteins). It is worth noting that we also identified many proteins that are known to facilitate structural rearrangement or regulate molecular functions, and can be important to facilitate sperm maturation. Because we were interested in the sperm-derived secretome, which might be responsible for stimulating QSOX1 secretion, GO analysis of these 258 proteins was performed. As shown in Fig. 6, most of the identified sperm-origin proteins belonged to the following categories: metabolic process, oxidoreductase activity, antibiotic catabolic process, regulation of RNA process, and Golgi vesicle transportation. These proteins secreted or released from sperm cells may promote or activate various cellular activities, to promote the secretion or transportation of cytosolic proteins, such as QSOX1 (Fig. 6).

Discussion

Spermatozoa are not functionally mature after being released from testes. They acquire the capacity for progressive motility and fertilization ability through PTMs that occur in different segments of the epididymis [1, 4, 27, 28]. Among these PTMs, disulfide bond

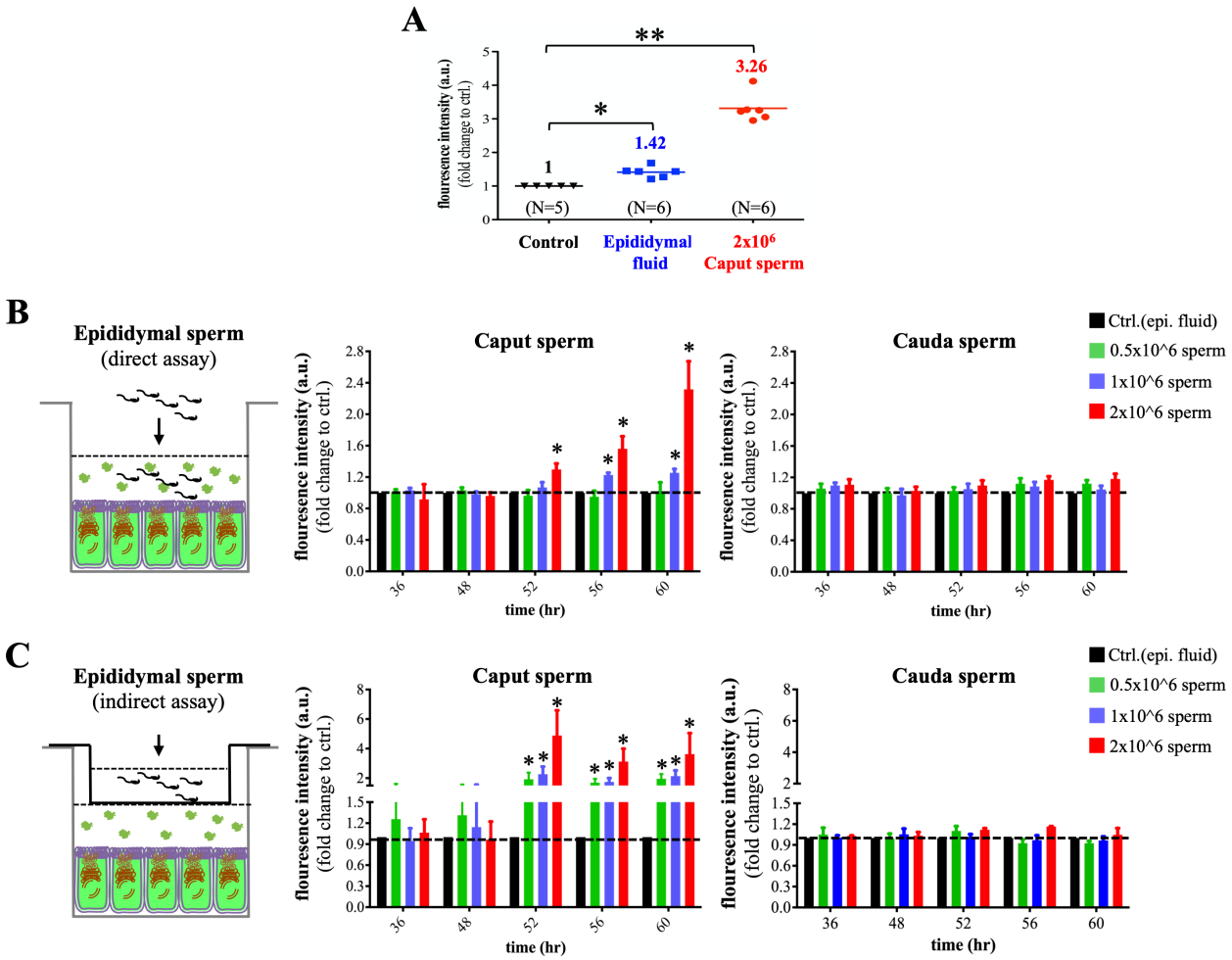

Fig. 4. *In vitro* evaluation of sperm as a factor for QSOX1 secretion. (A) Transfected DC2 was used to examine the effects of exogenous stimuli on the secretion of QSOX1. Epididymal fluid exerted significant stimulatory effect (1.42-fold) on QSOX1 secretion. A 3.26-fold increase in eGFP signal was detected when 2 × 10^6 caput sperm cells were co-cultured with DC2, which indicated that the caput sperm was more effective in stimulating epididymal QSOX1 secretion than luminal factors. (B) In the direct stimulation assay, we observed dose-dependent stimulatory effects on QSOX1 secretion from the caput sperm, but not the cauda sperm, at 52 h. (C) In the indirect stimulation assay, we observed dose-dependent effects from the caput sperm, but not the cauda sperm, on promoting QSOX1 secretion, at 52 h. Moreover, the level of stimulation was more apparent (2- to 6-fold), as compared to that upon direct stimulation (1.2- to 2.6-fold). For each concentration tested at each time-point, 6 experimental repeats were performed. Bars represent mean +/– standard deviation. Statistical significance was considered at $P < 0.05$. Asterisks indicate levels of significance, * $P < 0.05$; ** $P < 0.01$.

formation is essential for sperm proteins and structural stabilization. Quiescin sulfhydryl oxidases, which catalyze thiol oxidation and disulfide bond formation, have been identified in the epididymis [12, 18, 23]. In this study, we showed that mouse QSOX1 protein expression was upregulated from postnatal day 30, which coincided with the presence of sperm cells and the 1st surge in epididymal testosterone, suggesting the involvement of testosterone and sperm cells in the regulation and stimulation of QSOX1 protein expression/secretion. Moreover, we observed that secretory QSOX1 (QSOX1c) became more dominant at the later maturation stages in mice, which is consistent with our earlier finding that QSOX1 was mostly detected in the secretory form within the epididymal lumen in adult mice [18], which may also explain its function in eliminating defective sperm cells in adult animals [20]. To mimic the *in vivo* situation, we established a 2D-polarized sperm-epididymal epithelium co-culture system to investigate the effects of potential stimuli and measure the level of QSOX1 secretion *in vitro*. Our data showed that QSOX1 secretion can be stimulated *via* the addition of sex steroid hormones (testosterone and DHT) and sperm cells, and that the stimulatory effects were more apparent in the presence of caput sperm cells, suggesting that besides male sex steroids (such as testosterone and DHT), sperm cells *per se* and sperm maturation status are also the key regulators for mouse epididymal QSOX1 protein expression and secretion. LC-MS/MS and proteome analysis identified 582 secretory proteins that were exclusively present in the sperm-stimulated group. Further analysis showed that 258 sperm-origin proteins might be responsible for stimulating QSOX1 secretion in the epididymis.

Interactions between epididymal epithelium and spermatozoa in the facilitation of sperm maturation have been studied intensively in the past decades [2, 30–33]. Since sperm cells are considered inactive in gene and *de novo* protein synthesis, essential surface modifications for competent sperm-egg interactions rely mostly on constant epididymal epithelium-sperm interactions. It is known that the region-specific composition of epididymal epithelial cells creates a suitable microenvironment for stage-wise sperm maturation. For example, luminal acidification established by cell-cell cross-

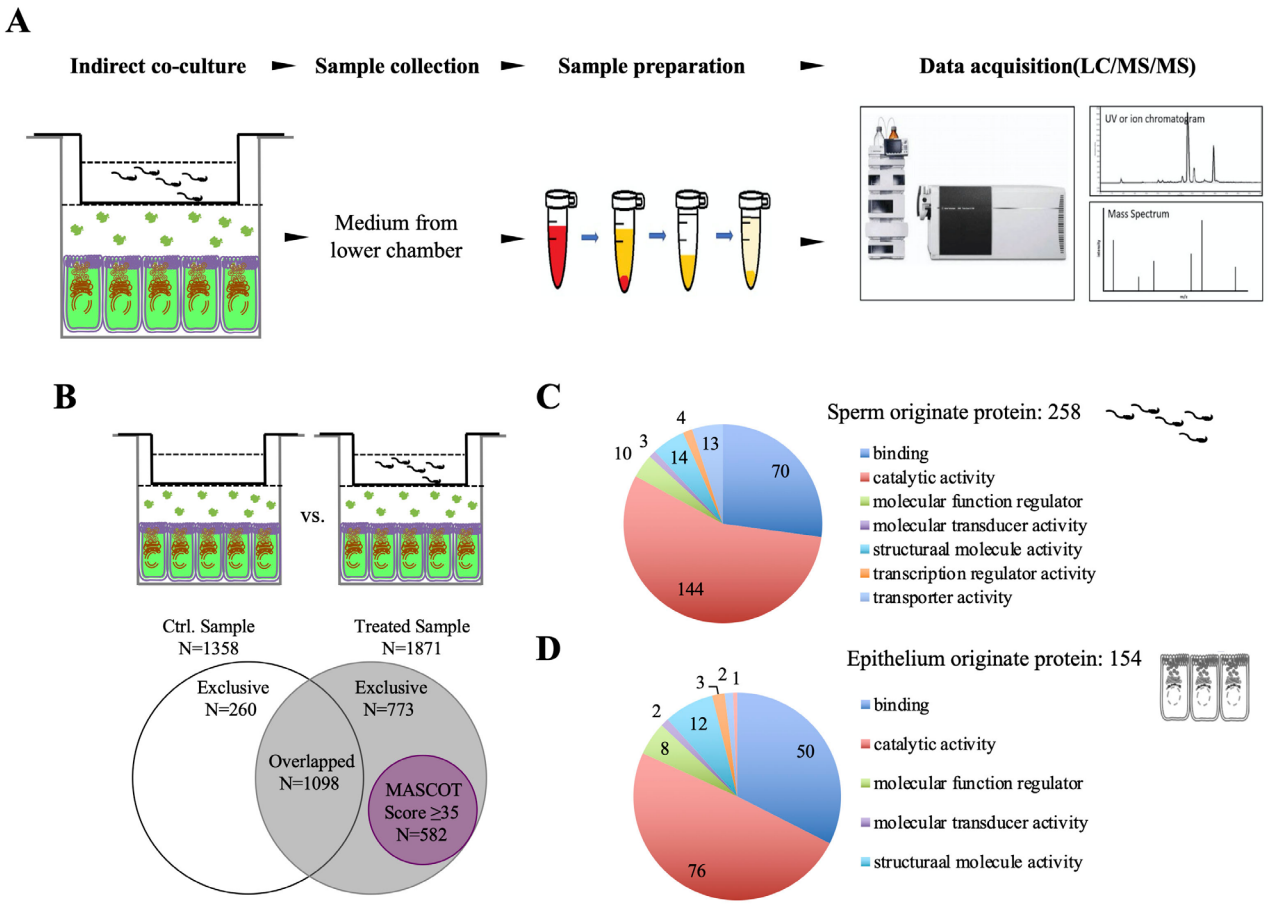

Fig. 5. Proteomic identification of sperm-epithelium interactomes responsible for QSOX1 secretion. (A) To identify sperm-releasing factors that might be responsible for stimulating QSOX1 secretion, supernatant from the lower chamber of the indirect co-culture (sperm-DC2) system was collected at 52 h, for proteomics analysis. (B) After subtracting protein IDs from the control group (without the presence of sperm cells), 773 proteins were found to be exclusively present in the stimulated group (in grey). Based on MASCOT proteomic criteria described in the Materials and Methods section, 582 proteins were considered reliable IDs (in purple circle). (C) Further analyses showed that among the 582 identified IDs, 258 were sperm-origin proteins. The majority of sperm-origin proteins (55.8%) had known function for catalytic activity, while around 27% were responsible for the cellular binding process. (D) Analysis of 154 epididymal epithelium proteins indicated that two major functions for epithelium secretomes were related to catalytic activity (49.4%) and cellular binding process (32.5%).

talk between epididymal clear cells and principal cells maintains epididymal sperm in a quiescent state [30, 33–35]. The complexity of the transcriptome network and endocrine, lumicrine, and paracrine factors tightly regulate epididymal protein expression, synthesis, and secretion [2–4, 26, 27]. Consistent with this, we observed that epididymal QSOX1 protein expression coincided with the postnatal testosterone surge, with peaks detected at days 30 and 60. Moreover, our *in vitro* data provided solid evidence that an essential male sex steroid, testosterone, and its catalytic metabolite, DHT, efficiently promoted QSOX1 secretion. Interestingly, although testosterone and DHT could both induce QSOX1 secretion at similar levels in DC2 cells, at 36 h, DHT showed a more persistent stimulatory effect until 52 h. One of the possibilities behind this observation could be that testosterone has to be converted into a more biologically active metabolite DHT by 5-alpha reductase; however, compared to testosterone, DHT is a primary and more dominant form of androgen in the male reproductive organ. Therefore, despite the fact that DC2 utilizes testosterone and converts testosterone into the active metabolite DHT, under our *in vitro* setup, DHT likely binds to the AR with higher affinity, so DC2 may utilize DHT more efficiently and directly, without additional conversion processes. Future cell uptake experiments with fluorescently labeled testosterone and DHT may confirm this hypothesis.

In addition to hormones, the maturation status of migrating sperm cells has been considered a critical factor that regulates epididymal activities. In agreement with this, we observed that compared to cauda sperm, caput sperm cells, which are considered “immature sperm cells”, were more effective in stimulating QSOX1 secretion *in vitro*. Upon post-testicular modification, disulfide bond formation is essential for the stabilization of sperm structure and sperm membrane proteins. The amount of disulfide bonds on the sperm membrane exhibits a progressive increase from the caput toward the cauda epididymis, and coincides with advances in sperm maturation status. This essential thiol-to-disulfide bond conversion supports the need for the QSOX protein in the maturation of spermatozoa [9]. In agreement with this concept, we also detected the presence of QSOX2 in the co-culture supernatant, and based on the known epididymal distribution of QSOX2 [18], it was detected in the co-culture supernatant, suggesting epithelium-initiated proactive secretion activity or a stimulated secretory mechanism initiated by immature sperm. Sperm-epididymal epithelium interactions have long been thought to be unidirectional, from the epithelium toward sperm cells,

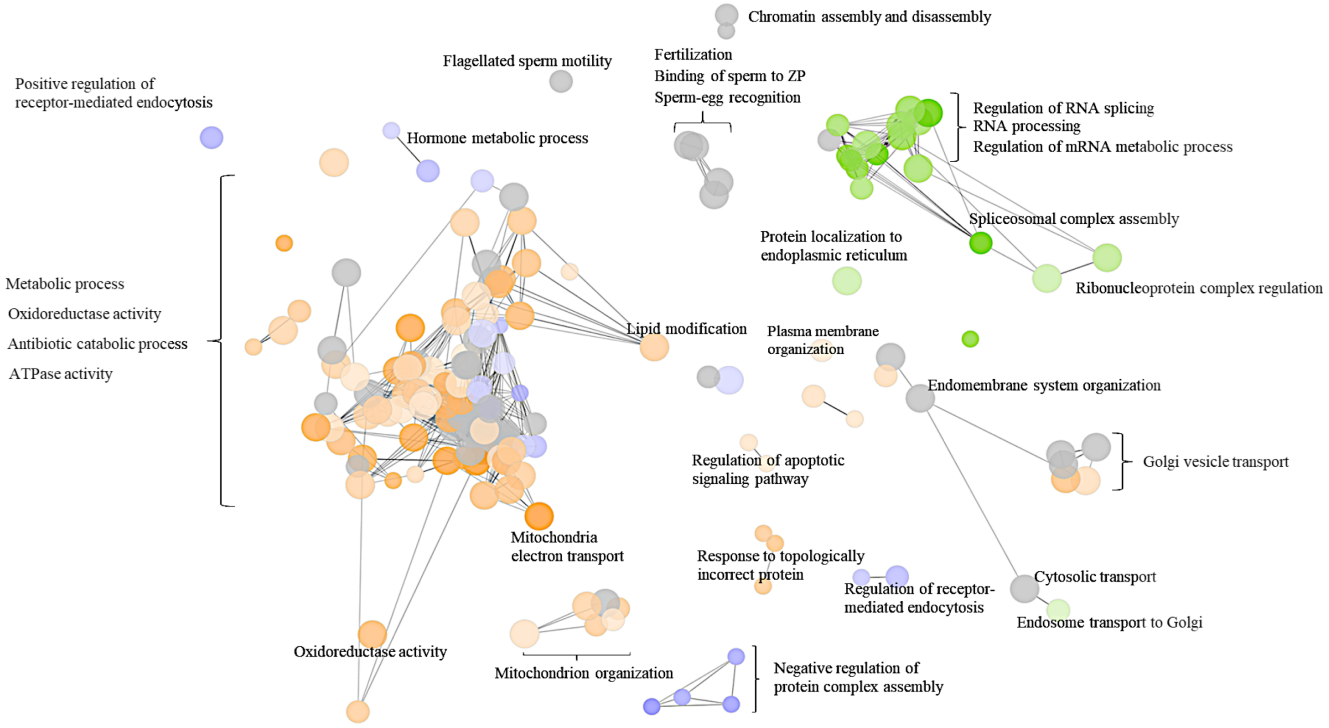

Fig. 6. Panther Gene Ontology (GO) analyses of proteomics data based on biological processes and molecular functions**.** Reliable protein IDs (n = 582) were processed for Panther GO analysis. Subcategories were created and are shown using different colors. Associated molecular functions have also been indicated accordingly. The majority of the identified proteins exhibited a known function related to metabolic process, oxidoreductase activity, and antibiotic catabolic process. Other proteins identified were also highly related to sperm maturation processes, such as sperm oocyte recognition, sperm-oocyte binding, chromatin assembly, plasma membrane organization, and lipid modification. Another group of proteins were found to have function in regulating Golgi vesicle transport, which might be responsible for the ER-mediated cellular secretory pathway of epididymal QSOX1.

and it is known that during sperm transit in the epididymis, sperm receives additional proteins, mRNA, sugars, and lipids from the epithelium, *via* specific tethering and cargo transfer mechanisms [2, 36–38]. However, a proactive signaling mechanism from the sperm towards the epididymal epithelium should also be considered. Most of the current studies have focused on unidirectional epithelium-to-sperm interactions [2, 4, 7, 28, 39–41], and there is little information available regarding sperm proactive participation in the stimulation of epididymal protein secretion. Using the azoospermia mouse model, we observed a sharp decrease in epididymal QSOX1c levels. In combination with an *in vitro* assay, we demonstrated that epididymal QSOX1 secretion was highly correlated with sperm maturation status, which was evidenced by the fact that caput spermatozoa elicited significantly 3–6-fold more QSOX1 protein secretion into the culture medium than epididymal fluid or cauda sperm cells alone. Since one of the intriguing observations was that indirect assay showed a better stimulatory effect on QSOX1 secretion from caput sperm, we hypothesize that sperm transit within the epididymal does not necessary require physical contact with the epithelium as it does in the oviduct. Interestingly, there is evidence from literature or our observation that the binding of sperm to epididymal epithelium is hardly observed. However, we do not believe that contact of immature sperm with epithelial cells inhibits QSOX1 expression, as both direct and indirect assays showed pronounced stimulatory effects on QSOX1 secretion. Thus, one of the reasons could be that when immature sperm cells migrate through the epididymal lumen, the secretion or release of "communication molecules" serves better as stimulation signals to the epithelium for QSOX1 secretion, which could also be a combined effect of various molecules (those we identified from the proteomics data of the indirect assay) that were being secreted or released by sperm during epididymal transition. Taken together, our data demonstrated that not only epididymal luminal factors but also immature sperm cells actively stimulate the epididymal epithelium to secrete maturation-essential proteins (such as QSOX1), thus facilitating sperm membrane stabilization.

Based on proteomics identification, the majority (55.8%) of sperm-releasing factors belong to catalytic activity; these sperm-origin proteins are likely to serve as signals or promote cellular activity of the epithelium to release or secrete molecules that facilitate sperm maturation and surface modifications, which was also evidenced by the fact that many identified epithelium releasing factors are known to have the functions related to cellular adhesion or binding (Supplementary Table 2). Of particular interest is that we identified dynamin-2 (DNM2), a large GTPase with a region-specific epididymal distribution present in our co-culture supernatant. DNM2 is highly expressed in both caput epididymal principal cells and in the sperm acrosome region [7, 28, 42], DNM2 is known to regulate bi-directional membrane trafficking events [43], which supports our idea of bi-directional sperm-epithelium communication that is essential for sperm maturation. The epididymis exhibits distinct gene and protein expression patterns, ensuring segmental-specific functions essential for different steps of sperm maturation [4, 5, 44, 45]. In line with this, we also identified the presence of beta-defensin 20, 47 and lipocalin 5, 8 (Lcn) in the co-culture supernatant, and these proteins have been shown to be located at the proximal region of the epididymis and have been postulated to facilitate sperm maturation and motility acquisition upon sperm epididymal transition [44, 46, 47].

In conclusion, we showed that epididymal QSOX1 secretion

can be stimulated by both steroids (testosterone and DHT) and immature sperm cells. Epididymal sperm with different maturation statuses exhibited different stimulatory effects on QSOX1 secretion. Moreover, using a combination of a co-culture system and proteomics analysis, we demonstrated that sperm cells could actively participate in signaling the epididymal epithelium to secrete proteins that are essential for sperm membrane surface modification.

Conflict of interests: The authors declare no conflicts of interest that could prejudice the impartiality of the research reported.

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