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Recombinant human oviductin regulates protein tyrosine phosphorylation and acrosome reaction

Yuewen Zhao[1,*], Xiaojing Yang[1,*], Zongchao Jia[1], Robert L Reid[2], Pierre Leclerc[3] and Frederick W K Kan[1]

[1]*Department of Biomedical and Molecular Sciences,* [2]*Department of Obstetrics and Gynecology, Faculty of Health Sciences, Queen's University, Kingston, Ontario, Canada, and* [3]*Center for Research on Reproductive Biology, Laval University, Quebec City, Quebec, Canada*

Correspondence should be addressed to F W K Kan; Email: kanfwk@queensu.ca

*(Y Zhao and X Yang contributed equally to this work)

Abstract

The mammalian oviduct synthesizes and secretes a major glycoprotein known as oviductin (OVGP1), which has been shown to interact with gametes and early embryos. Here we report the use of recombinant DNA technology to produce, for the first time, the secretory form of human OVGP1 in HEK293 cells. HEK293 colonies stably expressing recombinant human OVGP1 (rHuOVGP1) were established by transfecting cells with an expression vector pCMV6-Entry constructed with *OVGP1* cDNA. Large quantities of rHuOVGP1 were obtained from the stably transfected cells using the CELLSPIN cell cultivation system. A two-step purification system was carried out to yield rHuOVGP1 with a purity of >95%. Upon gel electrophoresis, purified rHuOVGP1 showed a single band corresponding to the 120–150 kDa size range of human OVGP1. Mass spectrometric analysis of the purified rHuOVGP1 revealed its identity as human oviductin. Immunofluorescence showed the binding of rHuOVGP1 to different regions of human sperm cell surfaces in various degrees of intensity. Prior treatment of sperm with 1% Triton X-100 altered the immunostaining pattern of rHuOVGP1 with an intense immunostaining over the equatorial segment and post-acrosomal region as well as along the length of the tail. Addition of rHuOVGP1 in the capacitating medium further enhanced tyrosine phosphorylation of sperm proteins in a time-dependent manner. After 4-h incubation in the presence of rHuOVGP1, the number of acrosome-reacted sperm induced by calcium ionophore significantly increased. The successful production of rHuOVGP1 can now facilitate the study of the role of human OVGP1 in fertilization and early embryo development.

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Introduction

The mammalian oviduct undergoes endocrine-induced morphological, biochemical and physiological changes during the estrous or menstrual cycle. These changes establish an essential microenvironment within the lumen of the oviduct for final maturation of gametes, capacitation of sperm, transport of gametes and embryos, fertilization, and early cleavage-stage embryonic development (Hunter 2003). Early investigations on many mammalian species, including mouse (Kapur & Johnson 1985, 1986), hamster (Leveille *et al.* 1987), rabbit (Oliphant & Ross 1982, Oliphant *et al.* 1984), sheep (Sutton *et al.* 1984, 1986, Gandolfi *et al.* 1989), pig (Buhi *et al.* 1990), cow (Malayer *et al.* 1988, Boice *et al.* 1990*a*), baboon (Fazleabas & Verhage 1986, Verhage & Fazleabas 1988, Verhage *et al.* 1989) and human (Verhage *et al.* 1988) identified and characterized a high-molecular-weight glycoprotein secreted by secretory cells of the oviduct collectively known as oviductin or oviduct-specific glycoprotein (OVGP1). Subsequent *in vitro* studies carried out in different animal models and in humans showed specific association of OVGP1 with zona pellucida (ZP) of ovulated ova and early embryos (Kapur & Johnson 1988, Kan *et al.* 1989, 1993, Boice *et al.* 1990*b*, Gandolfi *et al.* 1991, Wegner & Killian 1991, Buhi *et al.* 1993, Abe *et al.* 1995, McBride *et al.* 2004*a*) and correlated the secretion of OVGP1 with the estrogen-dominated stage of estrous (King *et al.* 1994, Boatman & Magnoni 1995) and menstrual cycles (Verhage & Fazleabas 1988, Verhage *et al.* 1989). The nucleotide and amino acid sequence of OVGP1 is highly conserved among mammalian species. Expression of OVGP1 appears to be dependent on estrogen or associated with estrous cycle in most species, but differences have been noted between species (Buhi *et al.* 1991, Murray 1993, Arias *et al.* 1994, Malette *et al.* 1995, Briton-Jones *et al.* 2001, 2004, McBride *et al.* 2004*b*, 2005).

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Previous results obtained from *in vitro* studies suggested several beneficial effects of OVGP1 for gametes and embryos. Bovine OVGP1 was shown to increase the motility and viability of homologous sperm (Abe *et al.* 1995) and enhance sperm capacitation and fertilizing capabilities *in vitro* (King *et al.* 1994). The presence of OVGP1 in the incubation medium has been shown to increase sperm–egg binding and penetration rates (Boatman & Magnoni 1995, O'Day-Bowman *et al.* 1996) and decrease polyspermy (Aviles *et al.* 2010). OVGP1 from different mammalian species has been shown to increase cleavage rates of embryos and the number of embryos that reach the blastocyst stage (Hill *et al.* 1997, Kouba *et al.* 2000). In our laboratory, we have recently shown that hamster OVGP1 prepared from the estrus stage of the estrous cycle can regulate the expression of tyrosine-phosphorylated proteins in hamster sperm during capacitation *in vitro* (Saccary *et al.* 2013). We also successfully produced a biologically active recombinant hamster oviductin that can exert positive effects on sperm functions and sperm–oocyte binding (Yang *et al.* 2015). Taken together, these results indicate the beneficial effects of OVGP1 and the importance of its presence in the luminal milieu of the oviduct during the process of fertilization and early embryo development.

Despite the findings from animal models supporting a role for OVGP1 in fertilization and early embryo development, the molecular mechanisms underlying the mode of action of OVGP1 are not fully understood. Information concerning the biological role of human OVGP1 is lacking behind its counterpart in other mammalian species. This is mainly due to the ethical issue and technical difficulty in obtaining sufficient amount of human oviductal fluid to prepare purified human OVGP1 for functional studies. In this study, our objective is to produce a secretory form of recombinant human OVGP1 (rHuOVGP1) in human embryonic kidney 293 (HEK293) cells. We also aimed at evaluating the effects of adding rHuOVGP1 in the capacitating medium on tyrosine phosphorylation of sperm proteins and acrosome reaction. Here we report, for the first time, the successful production of rHuOVGP1 of high purity. Experiments were carried out to show binding of rHuOVGP1 to sperm cell surfaces and its association with structural components that are resistant to Triton. In this study, *in vitro* functional studies demonstrated that the presence of rHuOVGP1 in the capacitating medium could further enhance tyrosine phosphorylation of human sperm proteins, a hallmark of sperm capacitation, in a time-dependent manner, and significantly increase the number of acrosome-reacted sperm induced by ionophore A23187. Taken together, the present findings confirm the biologically active nature of our newly produced rHuOVGP1, creating opportunities for further exploration of the role of human OVGP1 in reproductive functions.

Materials and methods

Materials

The following materials were purchased from the sources indicated: Dulbecco's Modified Eagle Medium (DMEM), CD293, fetal bovine serum (FBS), penicillin/streptomycin, Lipofectamine 2000, Geneticin and L-glutamine (Invitrogen); open reading frame (ORF) clone of human oviductal glycoprotein 1 (OVGP1, oviductin) (ORIGENE, Rockville, MD, USA); goat anti-oviductin antibody (P-20), oviductin immunizing peptide (P-20)P, horseradish peroxidase (HRP)-linked donkey anti-goat IgG, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, fluorescein isothiocyanate (FITC)-linked goat anti-mouse IgG and donkey anti-goat IgG-FITC (Santa Cruz Biotechnology); rabbit anti-human oviductal glycoprotein polyclonal antibody was a gift from Dr Patricia Mavrogianis, University of Illinois, Chicago, IL, USA (O'Day-Bowman *et al.* 1996); mouse anti-phosphotyrosine antibody (clone 4G10) (EMD Millipore); mouse anti-α-tubulin antibody (clone B-5-1-2) (Sigma-Aldrich); Western Lighting-enhanced Chemiluminescence Substrate, Micromass MassPREP Robotic Protein Handling System (PerkinElmer); Amicon Ultra-4 and -15 centrifugal filter units with a 30 kDa molecular weight cutoff (EMD Millipore); Superdex 200 (XK 16/60) and GammaBind plus Sepharose (GE Healthcare Bio-Sciences AB). All other chemicals were obtained from Sigma-Aldrich.

Production of rHuOVGP1 in HEK293 cells

HEK293 cells were a gift from Dr Leda Raptis, Queen's University, Ontario, Canada. The cells were cultured in DMEM supplemented with 10% FBS, penicillin (50 U/mL) and streptomycin (50 µg/mL) (growth medium). To establish a stable cell line producing rHuOVGP1, HEK293 cells were transfected with 4 µg human *OVGP1* cDNA ORF clone using Lipofectamine 2000 as described by the manufacturer. Briefly, 0.25×10^6 cells were passaged into a 35 mm dish and grown until 70–80% confluence. The cells were incubated in DMEM supplemented with 10% FBS at 37°C in 5% CO_2 for 6 h. Human *OVGP1* cDNA and Lipofectamine 2000 reagent were diluted, respectively, with DMEM serum-free medium without antibiotics. Diluted cDNA was added to diluted Lipofectamine 2000 reagent at 1:2.5 ratio (µg/µL). The transfection mixture was incubated at room temperature for 20 min and then added dropwise to the cells. The cells were incubated at 37°C in 5% CO_2. After 48 h of transfection, the medium was replaced with fresh growth medium containing 1 mg/mL Geneticin (G418) (selective medium). The selective medium was changed every 4–5 days. After 2-week incubation in the selective medium, the individual clones were transferred using cloning cylinders to 96-well plates and grown in selection medium until confluence. The cells were then passaged into 24-well plates and continually grown in the selective medium for 1–2 additional passages. Clones were screened for expression level by Western blot analysis of culture supernatants using the anti-oviductin antibody (P-20). HEK293 cells stably expressing rHuOVGP1 were maintained in the above medium supplemented with 200 µg/mL Geneticin. In order to produce large amount of rHuOVGP1, the stable rHuOVGP1-expressing

HEK293 cell clone was grown to confluence in 100 mm dishes in the above medium in the presence of 200 µg/mL Geneticin and passaged into 500 mL spinner flasks on a stirring platform (CELLSPIN CELL CULTIVATION SYSTEM, INTEGRA Biosciences AG, Zizers, Switzerland) at a density of 2.5×10^5 cells/mL. During production of rHuOVGP1, cells were grown in suspension in CD293 serum-free medium supplemented with 4 mM L-glutamine, penicillin (50 U/mL), streptomycin (50 µg/mL) and 200 µg/mL Geneticin at 37°C and 8% CO_2. The cell count and viability were determined daily by trypan blue staining. When the viable cell count reached ~1.5×10^6 cells/mL, the cells were diluted to $2.5–3.0 \times 10^5$ cells/mL with warm (37°C) CD293 medium. After 7–10 days, serum-free culture medium containing the recombinant glycoprotein was collected and centrifuged at 1000 ***g*** for 5 min at 4°C. The supernatant was collected and stored at −70°C for further purification. Production of rHuOVGP1 was monitored weekly by Western blot analysis of culture supernatants using the anti-human OVGP1 antibody (P-20).

Purification of secreted rHuOVGP1 collected from serum-free cell culture medium

The culture supernatant was concentrated by dialysis against poly(ethylene glycol) 20 K at 4°C. The first purification step was carried out by ammonium sulfate ($(NH_4)_2SO_4$) precipitation (Duong-Ly & Gabelli 2014). Briefly, $(NH_4)_2SO_4$ was added slowly to the concentrated culture supernatant at 4°C with constant stirring until 30% $(NH_4)_2SO_4$ saturation. After 1 h of incubation at 4°C, the sample was centrifuged for 30 min at 10,000 ***g*** at 4°C. The supernatant fraction was collected and subjected to a second saturation step until the sample reached 70% $(NH_4)_2SO_4$ saturation. After 3 h of incubation in the cold room, the precipitate was spun down by centrifugation for 30 min at 10,000 ***g*** at 4°C. The pellet obtained from 70% $(NH_4)_2SO_4$ saturation was dissolved in PBS and subsequently dialyzed overnight against a dialyzing buffer containing 0.05 M sodium phosphate and 0.15 M NaCl, pH 7.2, in the cold room. The buffer-exchanged solution was then concentrated by filtration using Amicon Ultra-15 centrifugal filter with a 30 kDa cutoff membrane. The concentrated samples were further purified using gel filtration chromatography. Gel filtration was performed on a fast protein liquid chromatography (FPLC) system (Pharmacia) using a Superdex 200 (XK16/60) column (120–124 mL bed volume) equilibrated with the dialyzing buffer. All solutions were de-gassed and filtered through 0.22 µm filter. The sample was centrifuged at 10,000 ***g*** for 10 min at 4°C before applying to the chromatography column. Absorbance at 280 nm was monitored and recorded. The sample was eluted isocratically from the column using the above buffer at the flow rate of 1.0 mL/min. The eluate was collected in 1 mL fractions and the peak fractions containing rHuOVGP1 were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified rHuOVGP1 was desalted in PBS and filtered under sterile conditions. The protein concentration of the samples at each purification step was determined by the Bradford protein assay using bovine serum albumin (BSA) as the standard. To assess the purity level of rHuOVGP1, 3 µg of the recombinant glycoprotein were used for SDS-PAGE followed by Coomassie staining.

SDS-PAGE, Western blotting and immunoprecipitation

The culture supernatants were collected as described above. The protein samples (20 µL each) were size-fractionated by SDS-PAGE on a 7.5% gel. The proteins were visualized with Coomassie Brilliant Blue R-250. For Western blot analysis, the protein samples were mixed with reducing SDS sample buffer (63 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1% β-mercaptoethanol, 0.0025% bromophenol blue), boiled for 5 min, separated on the gel and electrophoretically transferred to polyvinylidene fluoride (PVDF). After blocking with blocking buffer (5% nonfat milk Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.5) with 0.05% Tween 20 (TBST)) for 1 h, blots were probed sequentially with goat anti-oviductin antibody (P-20) at a dilution of 1:1000 and then with donkey anti-goat IgG-HRP at a 1:2000 dilution. Alternatively, the blots were probed sequentially with a polyclonal antibody against human oviductin (a gift from Dr P Mavrogianis) at a dilution of 1:50 and goat anti-rabbit IgG-HRP at a dilution of 1:10,000. Labeling was visualized by enhanced chemiluminescence.

Immunoprecipitation was performed on culture supernatants from the stable rHuOVGP1-expressing HEK293 cells. Samples (100 µL) that contained high concentrations of rHuOVGP1 were pre-cleared with 20 µL GammaBind Plus Sepharose for 1 h at 4°C. Samples were then immunoprecipitated overnight at 4°C with 30 µL GammaBind Plus Sepharose containing 0.2 µg/µL polyclonal anti-human oviductin antibody (Oviductin (P-20)) and subsequently analyzed by SDS-PAGE and Western blotting as described above.

Trypsin digestion of samples and sequencing of peptides by mass spectrometry

Samples of purified rHuOVGP1 were electrophoretically separated by SDS-PAGE. After Coomassie staining of the gels, protein bands were cut from the gels and digested using the Micromass MassPREP Robotic Protein Handling System. The tryptically digested sample was subjected to SCIEX Voyager DE Pro Matrix-Assisted Laser-Desorption (MALDI) mass spectrometer at the Protein Function and Discovery Facility of Queen's University, Ontario, Canada. Data from peptide mass fingerprinting were acquired over the mass range between m/z 700 and 4000. Mass spectrometry (MS) data were processed using Applied Biosystems Data Explorer version 5.1 and submitted to the Genebio Aldente search engine for comparison against the Swiss-Prot database.

Immunofluorescence and confocal microscopy

HEK293 cells transfected with *HuOVGP1* cDNA and mock-transfected cells were grown, respectively, on glass coverslips in culture dishes in DMEM growth medium. Cells grown to 80% confluence on the coverslips were washed three times with PBS and then fixed in 2% paraformaldehyde in PBS for 10 min at room temperature. After fixation, cells were washed with PBS once for 5 min and permeabilized with

0.5% Triton X-100 in PBS for 20 min at room temperature. Nonspecific binding was blocked with 1% BSA in PBS for 45 min at room temperature. The cells were probed with goat polyclonal anti-human oviductin antibody (P-20) and subsequently stained with donkey anti-goat IgG-FITC and DAPI (nuclei staining). Cells were washed and mounted in Mowiol 4-88/glycerol solution before imaging using a Leica TCS-SP2 Multiphoton Confocal Laser Scanning Microscope (TCS-MP, Heidelberg, Germany).

Preparation of sperm from fresh semen samples

Studies with human semen were approved by the ethics committee for research on human subjects from the Laval University Medical Center and by Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board. Human semen samples were obtained from healthy donors by masturbation with consent after 3 days of sexual abstinence. Upon receiving the samples, they were liquefied in a 37°C water bath. Semen viscosity and volume, sperm motility, and sperm concentration were assessed according to the WHO laboratory manual for the examination and processing of human semen (WHO 2010). Only normozoospermic samples were selected for this study. Liquefied semen was layered on the top of a gradient composed of 2 mL fractions each of 20, 40 and 65% and 0.1 mL of 95% Percoll, and then centrifuged at 1000***g*** for 30 min to isolate sperm from the seminal plasma. Percoll was made isotonic in HEPES-buffered saline (HBS; 25 mM HEPES, 130 mM NaCl, 4 mM KCL, 0.4 mM $MgCl_2$, 14 mM fructose, pH 7.6). Following centrifugation, sperm from the 65–95% interface and within the 95% Percoll fraction representing the highly motile population were pooled and their concentration was determined by a hematocytometer.

Sperm incubation

Sperm were incubated at a concentration of 2×10^7 cells/mL in the presence or absence of 10, 25, 50 or 75 µg/mL of rHuOVGP1 for 0, 1, 2, 3 or 4 h, respectively, at 37°C in 5% CO_2 with 100% humidity in modified Biggers–Whitten–Whittingham medium (BWW; 10 mM HEPES, 94.6 mM NaCl, 4.8 mM KCl. 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.1 mM $NaHCO_3$, 5.6 mM D-glucose, 21.6 mM Na lactate, 0.25 mM Na pyruvate and 0.1 mg/mL phenol red, pH 7.4) supplemented with 0.3% fatty acid-free BSA.

Binding of rHuOVGP1 to human sperm

A dose-dependent experiment was first carried out to determine the effect of various concentrations of rHuOVGP1 on enhancement of protein tyrosine phosphorylation and acrosome reaction of sperm after 4-h capacitation (see 'Materials and methods' section for evaluation of protein tyrosine phosphorylation and acrosome reaction). A concentration of 50 µg/mL was found to be optimal for significantly enhancing the level of protein tyrosine phosphorylation and chemical-induced acrosome reaction. Based on these initial results, rHuOVGP1 at a concentration of 50 µg/mL was used for the binding study. Sperm incubated in the presence of 50 µg/mL rHuOVGP1 for 1 and 4 h, respectively, were washed with HBS twice and resuspended in phosphate-buffered 1% Triton X-100 solution with protease inhibitors for 1 h at 4°C. After Triton treatment, the samples were centrifuged at 10,000***g*** for 10 min. The cell pellets were washed in PBS twice and fixed in phosphate-buffered 1% paraformaldehyde solution for 1 h at room temperature. Samples were then transferred onto poly-L-lysine pre-coated slides allowing sperm to attach. Excess of fixative solution on the slides was washed off with PBS. The samples were blocked with 5% donkey serum in PBS for 1 h at room temperature and incubated overnight at 4°C with goat anti-oviductin (P-20) primary antibody at a dilution of 1:100 in 1% donkey serum in PBS. Following incubation, the slides were washed three times each for 10 min with PBS. The samples were then stained for 1 h at room temperature with donkey anti-goat IgG-FITC at a dilution of 1:200 in 1% donkey serum in PBS. After washing with PBS, coverslips were mounted on slides with 90% glycerol containing 1% (w/v) 1,4-diazabicyclo-(2,2,2)-octane (DABCO) as an anti-bleaching agent. Fluorescent signals were detected by confocal microscopy.

Evaluation of protein tyrosine phosphorylation

At the end of each time interval, sperm were washed with 1ml Hepes-buffered saline (HBS) and centrifuged at 1000***g*** for 5 min. Sperm proteins were solubilized in electrophoresis sample buffer (2% SDS, 10% glycerol, 50 mM dithiothreitol, 62.5 mM Tris–HCl, pH 6.8) and denatured at 100°C for 5 min. Sperm proteins were resolved by SDS-PAGE and transferred onto PVDF. Nonspecific binding on the PVDF membrane was blocked with 5% (w/v) skim milk in TBST. The PVDF membrane was then incubated with a mouse anti-phosphotyrosine antibody diluted 1:10,000 in TBST for 1 h at room temperature. Following several washes with TBST, the PVDF membrane was incubated for 1 h with goat anti-mouse IgG-HRP diluted 1:10,000 with 5% milk in TBST. After five washes in TBST, positive immunoreactive bands were detected using the ECL kit according to the manufacturer's instructions.

To ensure that an equal amount of protein was loaded in each well of the gel apparatus, the same membrane was reprobed with a mouse anti-α-tubulin antibody diluted 1:50,000 with 5% milk in TBST. After incubation, the membrane was labeled with goat anti-mouse-HRP diluted 1:10,000 with 5% milk in TBST. Immunoreactive bands were detected as outlined above. The band intensities were quantified by densitometry using ImageJ software. The band intensities of tyrosine-phosphorylated proteins were normalized to the ones of α-tubulin. The level of tyrosine phosphorylation at each time point was further compared with the 0-h time point and expressed as fold increase.

Acrosome reaction assays

After 4-h incubation in the capacitating medium in the presence or absence of rHuOVGP1, the sperm were divided into two aliquots – both were washed with 1 ml HBS and then centrifuged at 1000***g*** for 5 min before being resuspended

in BWW. Calcium ionophore A23187 was added (10 µM final concentration) to one of the two aliquots to induce the acrosome reaction, while dimethyl sulfoxide (DMSO) was added to the other aliquot that served as a control for spontaneous acrosome reaction. Subsequently, both samples were incubated for 1 h at 37°C in 5% CO_2 with 100% humidity after which sperm were washed with 1 mL HBS and centrifuged at 1000 ***g*** for 5 min. Sperm pellet was resuspended in 100% methanol for 30 min on ice, spread on slides and dried at 37°C on a heated stage. The acrosome of sperm was stained for 30 min with *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (*psa*-FITC) (75 µg/mL in PBS) in a humid chamber. The slides were washed with water and air-dried. Coverslips were mounted on slides with 25 µL DABCO. The acrosomal status was determined using fluorescence microscopy and evaluated based on the immunostaining pattern of *psa*-FITC as described previously (Cross *et al.* 1986). For each sample, more than 200 sperm were counted to obtain the percentage of acrosome-reacted sperm.

Statistical analysis

The band intensities of tyrosine-phosphorylated proteins and the number of acrosome-reacted sperm between groups were compared using Student's paired or unpaired *t*-test. The statistical analysis was performed using GraphPad software PRISM. Data are expressed as mean ± S.E.M. *P* values equal to or less than 0.05 with 95% confidence intervals were considered statistically significant.

Results

Establishment of a stable rHuOVGP1-expressing HEK293 cell clone and purification of rHuOVGP1

To produce rHuOVGP1, we used the HEK293 cells transfected with the ORF clone of human OVGP1. Cell clone stably expressing human OVGP1 was selected using Geneticin. The clone that stably produced the recombinant glycoprotein was isolated and cultured. Immunofluorescent staining was used in conjunction with confocal microscopy to confirm protein expression and subcellular localization of rHuOVGP1 in the HEK293 cells. Confocal microscopy revealed a strong immunoreaction in the perinuclear region and the cytoplasmic region (Fig. 1A).

As OVGP1 is a secretory glycoprotein, HEK293 cells are expected to secrete the mature form of rHuOVGP1 into the culture medium. We probed the presence of rHuOVGP1 in the culture medium by Western blot analysis using a polyclonal anti-human oviductin antibody (oviductin (P-20)). As expected, the antibody detected a single band (120–150 kDa) in the supernatant fraction (Fig. 1B: lanes 2 and 3)

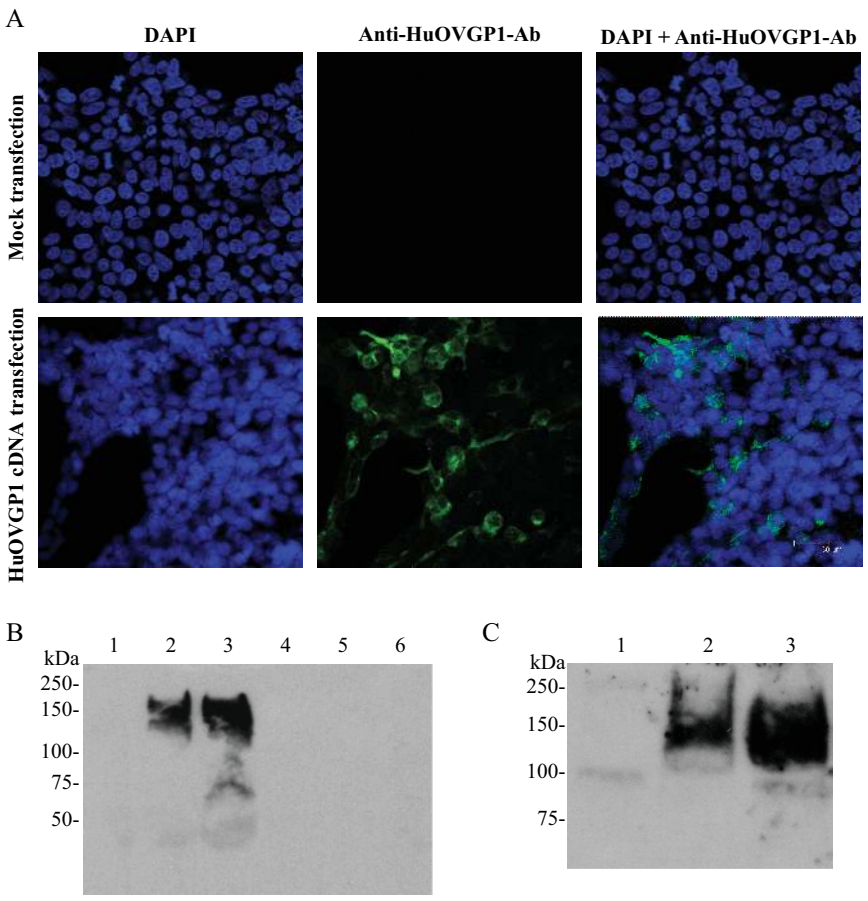

Figure 1 Detection of rHuOVGP1 expression in *HuOVGP1* cDNA-transfected HEK293 cells. (A) Immunofluorescent staining results showing transfection of HEK293 cells with *HuOVGP1* cDNA ORF clone (lower panel). Mock transfection (upper panel) was used as control. Cells were probed with a goat anti-HuOVGP1 polyclonal antibody (Oviductin (P-20)) followed by a donkey anti-goat IgG-FITC secondary antibody (green) and DAPI (blue nuclei staining). Scale bar = 30 µm. (B) Western blot results showing the expression of rHuOVGP1 in HEK293 cell culture supernatant. Lanes 1 and 4 represent results obtained from mock transfection and lanes 2, 3, 5 and 6 represent results obtained from OVGP1 human cDNA ORF clone transfection. The blot was probed with a goat anti-HuOVGP1 polyclonal antibody (oviductin (P-20)) (lanes 1–3) or with the same antibody in the presence of the antibody blocking peptide (lanes 4–6). (C) A similar blot was probed with a rabbit anti-HuOVGP1 polyclonal antibody and similar results were obtained. Mock transfection (lane 1), OVGP1 human cDNA ORF clone transfection (lanes 2 and 3).

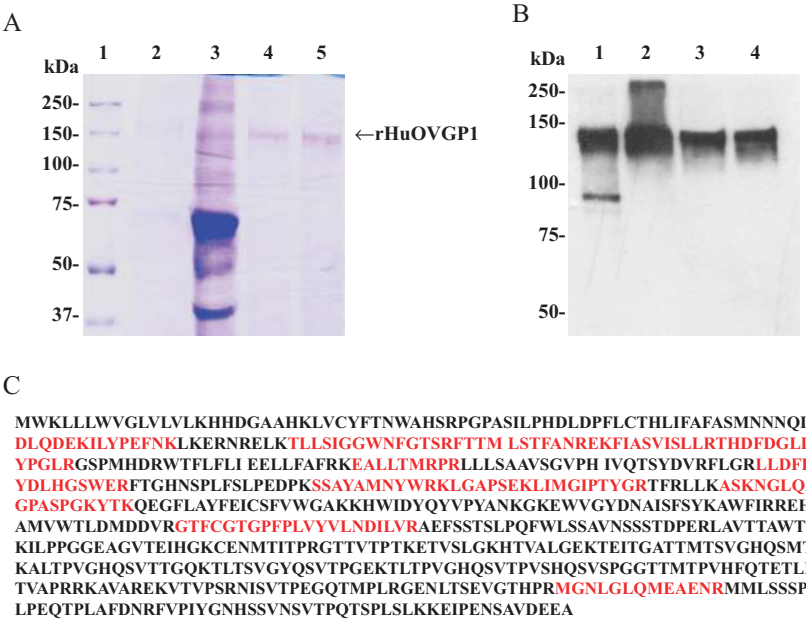

Figure 2 Purification of rHuOVGP1 and identity confirmation of the purified protein. (A) Coomassie-stained SDS-PAGE analysis: lane 1 is a molecular mass ladder; lane 2 represents culture supernatant; lane 3 is the concentrated culture supernatant; lanes 4–5 are purified rHuOVGP1 from different fractions of the gel filtration chromatography. (B) Western blot analysis showing rHuOVGP1 expression levels in main fractions from the separation process: culture supernatant (lane 1); proteins captured by 70% ammonium sulfate saturation (lane 2); purified rHuOVGP1 from different fractions of the gel filtration chromatography (lanes 3 and 4). The extra band with lower molecular weight in lane 1 is considered as nonspecific found in the culture medium as this band is absent in lane 2 and in the purified fractions (lanes 3 and 4). (C) Mass spectrometry analysis showing the entire sequence of HuOVGP1 with the coverage of peptides (bold red) identified from the trypsin digest of rHuOVGP1.

corresponding to the molecular weight of human OVGP1. These immunoreactive protein bands (Fig. 1B: lanes 2 and 3) were abrogated in the presence of the blocking peptide (oviductin(P-20)P) (Fig. 1B: lanes 5 and 6), confirming the specificity of the antibody. Supernatant fractions from the mock transfection were not reactive to the antibody (Fig. 1B: lanes 1 and 4). To further confirm the identity of the glycoprotein detected by the aforementioned antibody, another primary antibody known to recognize human OVGP1 (a gift from Dr P Mavrogianis) was used to probe the presence of rHuOVGP1 in the culture medium prepared from HEK293 cells. Again, a single protein band of approximately 110–150 kDa was detected (Fig. 1C). Supernatant fractions of the culture medium collected from duplicated experiments of *HuOVGP1* cDNA transfection (Fig. 1B: lane 2 and 3, 5 and 6; Fig. 1C: lane 2 and 3) were assessed by Western blot analysis for the reproducibility of the transfection procedure. Subsequently, individual rHuOVGP1-transfected cell clones were selected for establishing stable transfected cell clone. Expression of rHuOVGP1 in stable cell clone was also confirmed by Western blotting using the oviductin (P-20) antibody (data not shown).

In order to obtain purified rHuOVGP1, CELLSPIN, a large-scale cell cultivation system, was used to collect secreted rHuOVGP1 stably produced by HEK293 cells in the culture medium. After ammonium sulfate precipitation followed by size exclusion chromatography, rHuOVGP1 was obtained with a purity of over 95% based on a conservative estimation using densitometric analysis of the SDS-PAGE resolved purified glycoprotein. Using this approach, a considerable amount of rHuOVGP1 protein was produced from HEK293 cells stably expressing rHuOVGP1. In our laboratory, a typical yield for 1L of the conditioned medium varied between 1.8 and 2.7 mg rHuOVGP1. The purity of OVGP1 was assessed by SDS-PAGE in association with Coomassie Blue staining (Fig. 2A) and Western blot analyses (Fig. 2B). As shown in Fig. 2A and B, purified OVGP1 predominantly revealed a single band of the expected molecular mass of 120–150 kDa under reducing conditions. The identity of the purified recombinant protein was verified by mass spectrometric analysis (Fig. 2C). The peptide mass fragment spectra of the 120–150 kDa protein demonstrated that 19 of 30 peptides at 50 ppm mass accuracy were matched to human OVGP1 protein with 23% sequence coverage, thus confirming the identity of rHuOVGP1 as human OVGP1.

Binding of rHuOVGP1 to human sperm

Having obtained the purified rHuOVGP1, we then investigated the binding of this glycoprotein to sperm. First, Western blot analysis was performed to confirm the presence of rHuOVGP1 in sperm protein extractions after incubation with rHuOVGP1 in capacitating medium. After 4-h incubation, a fair amount of rHuOVGP1 was present in the Triton X-100-soluble fraction, indicating binding of the glycoprotein to sperm (Fig. 3A). Noticeably, an evident amount of rHuOVGP1 was still present in the Triton-insoluble fraction even after extensive washing (Fig. 3A). As a positive control, the supernatant recovered from the medium showed the presence of rHuOVGP1 in the capacitating medium after 4-h of incubation. The HBS wash following incubation was found to be negative in immunoreaction for rHuOVGP1, and the PBS wash after Triton extraction was also found to be negative in

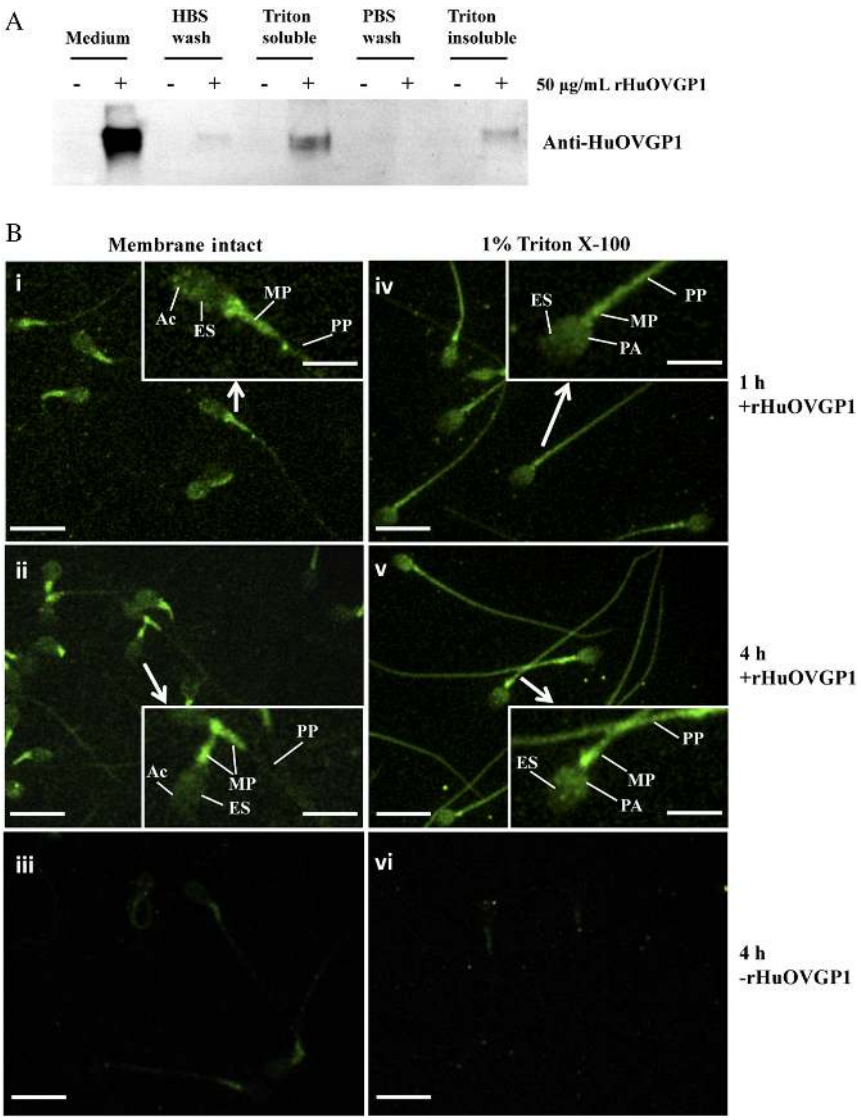

Figure 3 Binding of rHuOVGP1 to human sperm during *in vitro* capacitation. (A) Western blot showing the presence of rHuOVGP1 in 1% Triton X-100-soluble and -insoluble fractions of sperm after 4-h capacitation. Lanes from left to right: supernatant from medium, HBS wash following incubating with capacitating medium, Triton-soluble fractions, PBS wash after Triton solubilization and Triton-insoluble cell pellets. Minus "–" and plus "+" indicate incubation in the absence or presence of rHuOVGP1, respectively. (B) Confocal microscopy imaging of human sperm incubated in the presence of rHuOVGP1 shows binding of the recombinant glycoprotein to membrane-intact sperm (i and ii) and 1% Triton X-100-treated sperm (iv and v) following 1 h (i and iv) and 4 h (ii and v) of capacitation. Controls show negative immunostaining when sperm were incubated in the absence of rHuOVGP1 for the membrane-intact sperm (iii) and Triton-treated sperm (vi). Scale bar = 10 µm. Inserts: high magnifications of sperm cells revealing various immunolabeled structures. Ac, acrosomal region; ES, equatorial segment; MP, mid-piece; PA, post-acrosomal region; PP, principal piece. Scale bar = 5 µm.

immunoreaction for rHuOVGP1. Samples from sperm incubated in the absence of rHuOVGP1 were used as negative controls. These results provide evidence for the binding of rHuOVGP1 to both Triton-soluble and -insoluble fractions of sperm protein extractions.

To determine the specific sites of binding of rHuOVGP1 to human sperm, indirect immunofluorescence was performed. At 1-h capacitation in the presence of rHuOVGP1, immunoreaction of rHuOVGP1 was detected over the head, mid-piece and, and to a lesser extent, over the principal piece of the tail (Fig. 3Bi). In the sperm head, immunoreaction exhibited as a punctated staining pattern and was mainly found over the acrosomal region and the equatorial segment, but the immunostaining extended to the post-acrosomal region in some cells. The connecting piece in the sperm neck and the mid-piece were found to be intensely stained, whereas the principal piece was weakly stained in all cells examined. Sperm incubated for 4 h in the presence of rHuOVGP1 displayed a similar immunostaining pattern with a persistent staining intensity (Fig. 3Bii). The aforementioned immunostaining was not seen at both time intervals when sperm were incubated in the absence of rHuOVGP1 (Fig. 3Biii). Sperm cells also showed a negative immunoreaction when the primary antibody was omitted in the staining process of the sperm incubated in the presence of rHuOVGP1 (result not shown).

Interestingly, when sperm were treated with 1% Triton X-100 before immunostaining for rHuOVGP1, the signal was more intense over the equatorial segment and post-acrosomal region (Fig. 3Biv), which was not seen in sperm without Triton treatment. Also after treatment with 1% Triton, while the mid-piece remained strongly stained, the fluorescent signal was found relatively uniform throughout the sperm tail including the mid-piece and the principal piece (Fig. 3Biv). This altered pattern of immunostaining after Triton treatment

persisted after 4-h incubation of sperm in the presence of rHuOVGP1 (Fig. 3Bv). The immunostaining was also not seen at both time intervals when sperm were incubated in the absence of rHuOVGP1 (Fig. 3Bvi).

Effect of rHuOVGP1 on the expression of phosphotyrosine-containing proteins in human sperm

As one of the characteristic features of sperm capacitation is the increase of protein tyrosine phosphorylation, the effect of rHuOVGP1 on enhancing sperm protein tyrosine phosphorylation following capacitation was evaluated. A dose-dependent experiment was first carried out where sperm samples were incubated for 4 h in BWW medium supplemented with different concentrations of rHuOVGP1 (0, 10, 25, 50 and 75 µg/ml). Western blot analysis was performed to detect the level of protein tyrosine phosphorylation expression by probing with an anti-phosphotyrosine antibody. Our result indicated that two proteins migrated at 105 kDa (p105) and 81 kDa (p81) exhibited an increase in tyrosine phosphorylation in a dose-dependent manner in the presence of rHuOVGP1 at 4-h incubation (Fig. 4A). The immunolabeling intensity of protein tyrosine phosphorylation was almost undetectable at 0 h for uncapacitated sperm. The strongest labeling intensity of the two proteins was detected when rHuOVGP1 was used at a concentration of 50 µg/mL. Statistical analysis was also performed, confirming that the levels of tyrosine phosphorylation of p105 and p81 were significantly increased in the presence of rHuOVGP1 at a concentration of 50 µg/mL compared with incubation in the absence of the protein (Supplementary Figure, see section on supplementary data given at the end of this article). The levels of tyrosine phosphorylation of p105 and p81 were also found to be the highest at a concentration of 50 µg/mL compared with other concentrations (10, 25 and 75 µg/mL). Based on these results, rHuOVGP1 at 50 µg/mL was considered the optimal concentration to be used for enhancing protein tyrosine phosphorylation during sperm capacitation.

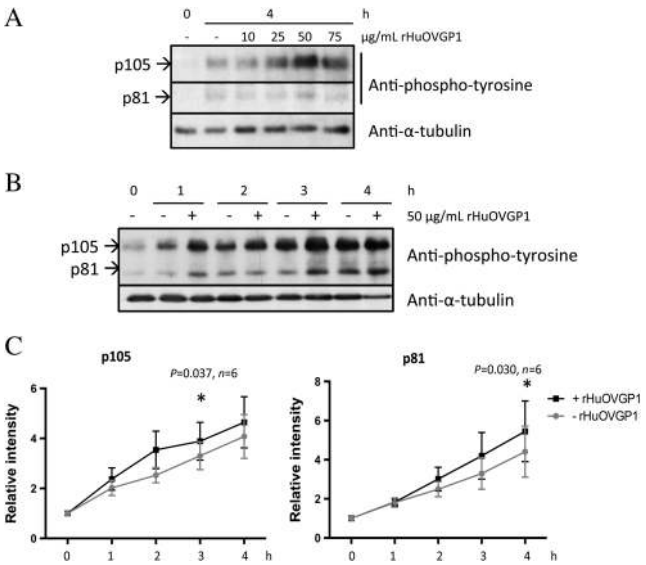

Figure 4 Effect of rHuOVGP1 on tyrosine phosphorylation of proteins prepared from fresh human sperm. (A) Western blot showing dose-dependent effect of rHuOVGP1 on tyrosine phosphorylation of proteins from sperm incubated for 4 h in BWW medium alone or in BWW medium supplemented with different concentrations of rHuOVGP1 (10, 25, 50 and 75 µg/mL) (upper panels: proteins migrated at 105 and 81 kDa that detected by Western blot using anti-phosphotyrosine antibody; lower panel: Western blot using anti-α-tubulin antibody), (B) Western blot showing protein tyrosine phosphorylation in the absence (–) or presence (+) of 50 µg/mL rHuOVGP1 following 0-, 1-, 2, 3- and 4-h capacitation, (C) statistical analysis of the levels of tyrosine phosphorylation of 105 and 81 kDa proteins obtained with Western blot ($n = 6$ samples each from individual donors for each time point, $^*P < 0.05$).

A time-dependent experiment was subsequently carried out, where the sperm samples were incubated for 0–4 h in BWW medium alone or supplemented with 50 µg/mL rHuOVGP1. Results obtained with Western blot analysis demonstrated a time-dependent increase in tyrosine phosphorylation of p105 and p81 during 4-h capacitation that was further potentiated in the presence of rHuOVGP1 (Fig. 4B). Variability in the amplitude of increased levels of tyrosine phosphorylation during capacitation is commonly found among sperm samples from different donors, as observed in Fig. 4A (lane 2 from left) compared with Fig. 4B (lane 8 from left). Nonetheless, densitometric image analysis of labeling intensities of p105 and p81 indicated that the level of tyrosine phosphorylation of p105 and p81 was significantly increased at 3 and 4 h, respectively, when incubated in the presence of rHuOVGP1 compared with their corresponding counterparts obtained from sperm without rHuOVGP1 treatment (Fig. 4C).

Effect of rHuOVGP1 on sperm acrosome reaction

The ability of sperm to undergo acrosome reaction is essential for the subsequent sperm–oocyte binding and successful fertilization. In order to examine if rHuOVGP1 exerts any positive effects on acrosome reaction, fresh sperm were incubated in BWW medium alone or in the presence of different concentrations of rHuOVGP1 (10, 25, 50 and 75 µg/mL). The percentage of acrosome-reacted sperm was measured and compared between spontaneous (DMSO) and chemically induced (A23187) acrosome reaction (Fig. 5). Spontaneous acrosome reaction of sperm remained low and was not significantly different among samples incubated with various concentrations of rHuOVGP1 or with the medium alone. Sperm treated with A23187 showed an increase in acrosome reaction in the presence of rHuOVGP1 in a dose-dependent manner. The percentage of acrosome-reacted sperm was significantly increased after incubation with rHuOVGP1 at the concentration of 50 µg/mL compared

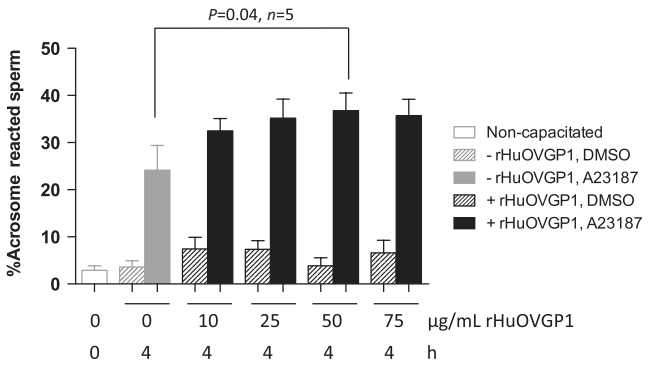

Figure 5 Effects of rHuOVGP1 on calcium ionophore-induced acrosome reaction of fresh sperm. Percentage of acrosome-reacted sperm (mean ± S.E.M., unpaired *t*-test) obtained under different experimental conditions was indicated by the blank or shaded symbols as shown in the histogram. Sperm were assessed for acrosome reaction after incubation in capacitating medium for 0 or 4 h in the absence or presence of different concentrations of rHuOVGP1 ($n = 5$ samples each from individual donors, $^*P < 0.05$).

with sperm incubated without rHuOVGP1. The increase in the percentage of acrosome-reacted sperm after incubation with 50 µg/mL rHuOVGP1 was at the highest level compared with those obtained with other concentrations (10, 25 and 75 µg/mL).

Discussion

Accumulating evidence indicates that mammalian OVGP1 exerts positive effects during early events of fertilization and early embryo development, but the molecular mechanisms of the physiological function of OVGP1 involved in these events are not clearly understood, especially in humans. In order to carry out functional and mechanistic studies, two approaches can be envisaged for obtaining purified OVGP1. One approach is to purify sufficient amount of the native glycoprotein from oviductal fluid and an alternative is to utilize recombinant DNA technology to produce a recombinant oviductin. In our laboratory, we have previously isolated and purified hamster oviductin (HamOVGP1) from oviducts at different stages of the estrous cycle and showed binding of the estrus stage-associated HamOVGP1 to the cell surfaces of homologous sperm (Kan & Esperanzate 2006). The estrus stage-specific HamOVGP1 has also been found to enhance, during *in vitro* capacitation, tyrosine phosphorylation of a subset of sperm proteins known to play important functions in fertilization (Saccary *et al.* 2013). We have recently produced a biologically active recombinant hamster OVGP1 (rHamOVGP1) and demonstrated that this recombinant glycoprotein can enhance tyrosine phosphorylation of hamster sperm proteins, acrosome reaction and sperm–egg binding (Yang *et al.* 2015). However, isolating and purifying large amounts of native OVGP1 from human Fallopian tubes is not feasible mainly due to ethical reasons and limited accessibility to human tissue samples. In this study, we have successfully produced a biologically active rHuOVGP1 protein using the recombinant DNA approach and a two-step protein purification procedure. We have found that rHuOVGP1 can enhance tyrosine phosphorylation of two human sperm proteins with a molecular weight of 105 and 81 kDa, respectively, during capacitation. In addition, rHuOVGP1 has been found to increase the number of acrosome-reacted sperm after *in vitro* capacitation.

In the beginning of our study, we attempted to produce rHuOVGP1 in the immortalized human oviductal cell line OE6/E7 (Lee *et al.* 2001). We have previously demonstrated that this cell line expresses endogenous human *OVGP1* mRNA and protein (Ling *et al.* 2005). Although we were able to overexpress human *OVGP1* mRNA in the transfected OE6/E7 cells, only small amounts of secreted rHuOVGP1 were obtained from the culture medium. In order to increase the secretion levels of rHuOVGP1, HEK293 cells were used instead. In the past, HEK293 cells have been successfully used for the production of several recombinant human glycoproteins such as cathepsin E (Cappiello *et al.* 2004) and IFNα2b (Loignon *et al.* 2008). The use of HEK293 cells is also compatible with the large-scale cell cultivating system CELLSPIN as these cells can be grown in suspension. Our present results showed the establishment of a HEK293 cell clone stably expressing and secreting glycosylated rHuOVGP1 and that the cells can be cultured to produce large amounts of rHuOVGP1 using the CELLSPIN system. rHuOVGP1 was further purified from serum-free culture medium by a two-step procedure starting with ammonium sulfate precipitation followed by gel filtration chromatography on Superdex 200. The identity of the glycoprotein was confirmed by Western blot analysis using both a commercially available antibody generated against a peptide mapping near the C-terminus of human OVGP1 and an antibody against the full sequence of partially purified native HuOVGP1. Mass spectrometric analysis further confirmed the purified glycoprotein as human OVGP1.

OVGP1 has been previously shown to bind to the acrosomal region of hamster sperm (Boatman & Magnoni 1995) and to the head and mid-tail regions of bovine sperm (King *et al.* 1994). Using the purified rHuOVGP1, results of this study demonstrated, for the first time, the binding of OVGP1 to human sperm. Our Western blot results showed that rHuOVGP1 was not only present in the Triton-soluble fraction but also detected in the insoluble fraction. These findings suggest the association of rHuOVGP1 not only with the plasma membrane of sperm but also with certain structural elements underlying the cell surfaces. Results obtained with immunofluorescence further revealed the sites of localization of rHuOVGP1 in human sperm during capacitation. On the membrane-intact

sperm cells, rHuOVGP1 was found to bind primarily to the connecting piece and mid-piece and, to a lesser extent, to the principal piece. Immunostaining of rHuOVGP1 with a punctated pattern was observed over the acrosomal and equatorial regions of the sperm head. However, when treated with 1% Triton X-100, a relatively intense immunostaining of rHuOVGP1 was found to be associated with the equatorial and post-acrosomal regions in the sperm head and throughout the mid-piece and principal piece of the tail. As Triton X-100 is a nonionic surfactant, which can be used to solubilize cell membranes and peripheral membrane-bound proteins, the present results indicate that rHuOVGP1 binds not only to the cell surfaces of human sperm but also to certain structural elements that are resistant to Triton. Our immunofluorescent results are supported by findings of previous studies showing binding of OVGP1 to capacitated sperm and that the binding sites can be visualized on membrane-permeabilized sperm from bovine and monkey (King & Killian 1994, Natraj *et al.* 2002). In monkey, the *N*-terminal conserved region of OVGP1 has been found to be associated with a cytoskeletal protein, non-muscle myosin IIA (MYH9) (Kadam *et al.* 2006). MYH9 is also expressed on the cell surface (Olden *et al.* 1976, Arii *et al.* 2010) and is enriched in detergent-resistant membrane skeleton and lipid rafts (Li *et al.* 1994, Nebl *et al.* 2002). Furthermore, MYH9 is thought to be a functional viral glycoprotein receptor for entry of viruses into cells (Arii *et al.* 2010, Xiong *et al.* 2015). Based on these premises, we speculate that once rHuOVGP1 binds to the sperm surfaces, it is incorporated into detergent-resistant lipid rafts that could possibly bind to MYH9. Future experiments are necessary to verify the presumptive association of OVGP1 with MYH9. Conversely, Reuter *et al.* were not able to detect binding of native human OVGP1 to human sperm using partially purified OVGP1 from hydrosalpinx fluid (Reuter *et al.* 1994). The discrepancy in OVGP1 binding to human sperm may be due to the use of partially purified protein by Reuter's group vs pure recombinant protein used in this study. The use of rHuOVGP1 with high purity in this study for examining its binding ability to the sperm surface is also considered more specific compared with the use of partially purified human OVGP1 reported previously.

A previous *in vitro* study showed that incubation of bovine sperm in culture medium in the presence of bovine oviductal fluid enhanced sperm capacitation (King *et al.* 1994). A time-dependent increase in protein tyrosine phosphorylation of sperm proteins is associated with *in vitro* sperm capacitation and this is a common occurrence found in various mammalian species including humans (Visconti *et al.* 1995, Leclerc *et al.* 1996). As OVGP1 is present in the luminal milieu of mammalian oviducts with the highest expression level during the peri-ovulatory phase, it was of our interest to find out if addition of rHuOVGP1 in capacitating medium can further enhance tyrosine phosphorylation of sperm proteins as this glycoprotein is lacking in commercially available capacitating medium or in capacitating medium prepared in the laboratory setting. In this study, Western blot analysis revealed two proteins of 105 and 81 kDa both of which were found to be significantly increased in tyrosine phosphorylation level after 3 and 4 h, respectively, of incubation of sperm in capacitating medium in the presence of 50 µg/mL rHuOVGP1. The molecular weight of these two tyrosine-phosphorylated proteins correspond, respectively, to that of the A Kinase Anchor Protein (AKAP) 3 and 4, which have been shown to be tyrosine-phosphorylated during sperm capacitation (Luconi *et al.* 2011). AKAP 3 and 4 are mainly located in the principal piece of the sperm tail (Brown *et al.* 2003). Tyrosine phosphorylation of sperm proteins following capacitation occurs primarily in the principal piece, and to a lesser extent in the neck, equatorial and acrosome regions (Sati *et al.* 2014). As rHuOVGP1 binds to sperm cell surfaces, it is plausible that binding of rHuOVGP1 to the tail could affect the local signal transduction cascade leading to an increase in the level of tyrosine phosphorylation of a subset of sperm proteins, including the 105 and 81 kDa proteins that were found to be under the influence of rHuOVGP1. The time discrepancy between the increase in tyrosine phosphorylation levels of p105 (at 3 h) and p81 (at 4 h) could be due to mechanisms that regulate phosphorylation and de-phosphorylation of tyrosine residues at a precise time during sperm capacitation. For example, AKAP3 protein has been shown to undergo de-phosphorylation during capacitation that leads to degradation of the protein (Vizel *et al.* 2015). As capacitation can occur *in vitro*, we believe that capacitation and the events associated with this process are controlled intrinsically by sperm itself as long as specific requirements are met *in vitro*. This intrinsic control likely involves a series of phosphorylation and de-phosphorylation that occur at precise timing to prepare the sperm for successful sperm–egg binding, acrosome reaction and the subsequent fertilization. Future studies are warranted to examine if human OVGP1 is involved in regulating the signaling mechanism of tyrosine phosphorylation of sperm proteins.

Acrosome reaction is a critical event that follows capacitation and precedes fertilization. The percentage of acrosome-reacted sperm induced by A23187 correlates with the fertilizing capacity of the sperm sample (Yovich *et al.* 1994, Liu & Baker 1998). Concordantly, the present results showed that exposure of human sperm to rHuOVGP1 for 4 h before induction of acrosome reaction with calcium ionophore significantly increased the number of acrosome-reacted sperm. Therefore, rHuOVGP1 can likely increase the fertilizing capacity of the sperm following capacitation. Our results showed that the presence of rHuOVGP1 in the incubating medium

did not alter the percentage of spontaneous acrosome reaction, which is consistent with previous studies indicating that human sperm normally have very low percentage of spontaneous acrosome reaction following *in vitro* capacitation (Byrd & Wolf 1986, Fénichel *et al.* 1991). Our dose-dependent experiments showed that rHuOVGP1, at a concentration of 50 µg/mL, significantly increased the percentage of calcium ionophore-induced acrosome reaction. At a higher concentration of 75 µg/mL, the increase in acrosome reaction became insignificant compared with incubation without rHuOVGP1, suggesting that a moderate concentration of rHuOVGP1 is able to potentiate acrosome reaction. We also observed the similar effect on hamster sperm in a previous study carried out in our laboratory when hamster recombinant OVGP1 was used at a high concentration (Yang *et al.* 2015). Taken together, higher concentrations of OVGP1 seem to alleviate the effect of OVGP1 on sperm capacitation as shown in the human sperm in this study and in the hamster sperm as we reported previously. Our findings indicate that rHuOVGP1 significantly increased tyrosine phosphorylation of two sperm proteins during capacitation and significantly increased chemical-induced acrosome reaction. However, at this time, it is not clear whether the increase in acrosome reaction is directly linked to the increase in protein tyrosine phosphorylation during sperm capacitation under the influence of rHuOVGP1. It will be of interest to examine, in the future, the *in vitro* effects of rHuOVGP1 on sperm hyperactivated motility as hyperactivation is an important feature of sperm capacitation (Kulanand & Shivaji 2001).

In summary, we have successfully produced and purified, for the first time, a secreted form of recombinant human oviductin from HEK293 cells. Sufficient amount of this recombinant glycoprotein has been purified for *in vitro* functional studies as demonstrated in this study with respect to the effect of rHuOVGP1 on enhancement of tyrosine phosphorylation of sperm proteins and acrosome reaction. As OVGP1 is a secretory glycoprotein, the biological functions of OVGP1 may be attributable to its carbohydrate components. A direct comparison of *N*- and *O*-linked glycans between native human OVGP1 (HuOVGP1) and rHuOVGP1 by mass spectrometry is not feasible due to the unavailability of sufficient amount of purified native HuOVGP1. Based on the premise that the immortalized human oviductal cells display phenotype characteristics of primary human oviduct cells and that the former cells express both mRNA transcripts and protein of HuOVGP1 (Ling *et al.* 2005), we employed an alternative approach and compared the biosynthetic pathways of *N*- and *O*-glycosylation in HEK293 cells, where rHuOVGP1 is produced, with those of immortalized human oviductal cells. As expected, we found that the two cell lines are very similar in the pathways of *N*- and *O*-linked glycosylation (Yang *et al.* 2012). In view of these findings, it is reasonable to assume that the rHuOVGP1 described in this study is very similar to the native HuOVGP1.

In the past, despite the fact that OVGP1 has been identified and characterized in many mammalian species including humans, further understanding of the roles played by OVGP1 in human reproduction has been hampered by the unavailability of purified native HuOVGP1. The successful production and purification of rHuOVGP1 will now make it possible to determine the mechanisms that regulate its biological activity. For example, as human OVGP1 is a secretory glycoprotein, it will be of interest to examine if *N*- and/or *O*-glycans and specific carbohydrates of rHuOVGP1 are responsible for mediating the functions of rHuOVGP1. The fact that rHuOVGP1 binds to different regions of sperm during capacitation raises the question of the presence of potential rHuOVGP1 receptors on sperm cell surfaces. The rHuOVGP1 that we produced could also have clinical significance. Failed sperm binding and lack of fertilization is a major limitation of human *in vitro* fertilization mainly due to male factor indications. As rHuOVGP1 is able to enhance human sperm capacitation presumably through the increase in protein tyrosine phosphorylation, and potentiate the subsequent acrosome reaction, exploration of the use of rHuOVGP1 in enhancing sperm fertilizing competence in order to improve the rate of sperm–egg binding during *in vitro* fertilization is also warranted.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-16-0177.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

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