Biology of Reproduction, 2020, 102(1), 92–101
doi:10.1093/biolre/ioz171
Research Article
Advance Access Publication Date: 28 August 2019

Research Article

Beta-defensin126 is correlated with sperm motility in fertile and infertile men†

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† **Grant Support:** This work was supported by a Canadian Institutes for Health Research Operating grant (84576) (DGC and PTKC). RA received partial support from the Armand-Frappier Foundation and an a Natural Sciences and Engineering Research Council Create grant to the Fonds de recherches nature et technologies-Réseau Québécois en Reproduction Network.

Received 04 February 2019; Revised 14 August 2019; Accepted 23 August 2019

Abstract

A crucial function of the epididymis is providing a surface glycocalyx that is important for sperm maturation and capacitation. Defensins are antimicrobial peptides expressed in the epididymis. In the macaque epididymis, defensin beta 126 (DEFB126) is important for sperm motility, however, it is not known whether this is the case in humans. The objectives were to determine: (1) if DEFB126 on human ejaculated sperm was correlated with sperm motility in fertile and infertile men, (2) that recombinant DEFB126 could induce immature sperm motility in vitro. Immunofluorescence staining indicated that the proportion of DEFB126-positive sperm was significantly higher in motile sperm. Furthermore, the proportion of DEFB126-labeled sperm was positively correlated with sperm motility and normal morphology. Additional studies indicated that the proportion of DEFB126-positive spermatozoa in fertile volunteers was significantly higher than in volunteers with varicocele, and in infertile volunteers with semen deficiencies. To determine the role of DEFB126 on sperm motility, the DEFB126 gene was cloned and used to generate recombinant DEFB126 in H9C2 cells (rat embryonic heart myoblast cells). Deletion mutations were created into two regions of the protein, which have been linked to male infertility. Immotile testicular spermatozoa were incubated with cells expressing the different forms of DEFB126. Full-length DEFB126 significantly increased motility of co-cultured spermatozoa. However, no increase in sperm motility was observed with the mutated forms of DEFB126. In conclusion, these results support the notion that DEFB126 is important in human sperm maturation and the potential use of DEFB126 for in vitro sperm maturation.

Summary sentence

DEFB126 is correlated with sperm motility and fertility in human.

Key words: DEFB126, epididymis, spermatozoa, sperm motility, infertility, human

Introduction

The epididymis is essential for male fertility [1, 2]. Mammalian spermatozoa leave the testis as immature cells that lack the ability to swim, synthesize proteins, or fertilize an oocyte. They must complete the maturation process during their transit through the epididymis. The epididymis plays a role in the reabsorption of testicular fluid, synthesis, and secretion of specific proteins in order to create an

appropriate milieu for sperm maturation [3]. Secreted glycoproteins and glycolipids from epididymal principal cells bind to maturing spermatozoa, creating a surface glycocalyx that encapsulates them. These modifications of the sperm surface are considered essential for motility and maturation [4–6].

Beta-defensins (DEFBs) are a family of cationic antimicrobial peptides, which contribute to host immune responses [7]. They are characterized by the presence of a conserved six-cysteine residue, which form three disulfide bonds (1–5, 2–4, and 5–6) [8]. Studies have shown that the epididymis is the main site of DEFB expression in mammals and that these proteins are secreted into the lumen and bind to the sperm plasma membrane, thereby contributing to the formation of the glycocalyx [6, 9–11]. DEFBs have been shown to play a critical role in sperm maturation and capacitation in various species [11–13]. Rat DEFB, or Bin1b, is important for the acquisition of sperm maturation and the initiation of sperm motility by inducing Ca^{2+} uptake in immature rat spermatozoa [6]. Defensin beta 126 (DEFB126) coats the entire surface of macaque spermatozoa and has a role in sperm capacitation, penetration through the cervical mucus, and sperm protection [10, 14]. A recent study on bovine DEFB126 (BBD126), which is expressed in the cauda epididymidis, indicated that it has a role in inducing motility in immature bovine spermatozoa [4]. Moreover, the presence of two common mutations in the coding sequence of human DEFB126 has been associated with patient sub-fertility and infertility [15, 16]. Previous studies from our lab have shown that DEFB126 mRNA is expressed primarily in the caput epididymidis and that its levels are downregulated in the caput epididymidis of infertile non-obstructive azoospermic patients [17, 18].

The objective of this study was to determine whether or not there is an association between DEFB126 and both sperm motility and fertility in humans. Furthermore, we investigated whether or not recombinant DEFB126 and mutated forms of the protein generally associated with sub-fertility or infertility could induce sperm motility.

Materials and methods

Ethics statement

All subjects provided informed consent and the study was approved by the McGill University Research Ethics Board (protocols A06-M61-04B and A03-M27-10B).

Preparation of human ejaculated semen samples

Men ($n = 99$, 20–54 years of age) were recruited from a university-based reproductive center. Fertile subjects ($n = 42$) had a history of natural fecundity with semen parameter values above the [42] fertile reference values. Infertile subjects ($n = 19$) included: (1) men with idiopathic infertility with a history of clinical infertility, and in whom at least one of the semen parameters among sperm concentration, motility, or morphology, was consistently below the lower WHO reference limit; (2) infertile men with varicoceles ($n = 14$) and a history of clinical infertility with at least one of the semen parameters consistently below the WHO reference values and a diagnosis of clinical varicoceles.

In this study, subjects with severe oligozoospermia (sperm concentration below 2×10^6/ml) were excluded to ensure that an adequate number of spermatozoa was available for the experiments. For each experiment described in this study, the number of subjects used was a reflection of the status of recruitment at the time of the experiment, and varied for different experiments.

Semen collection and evaluation were performed according to [42] guidelines. Ejaculated semen samples were collected from each subject by masturbation following a 3- to 5-day period of abstinence. Sperm concentration and motility were analyzed using an IVOS semen analyzer (Hamilton Thorne, Beverly, MA, USA). The morphology of 100 spermatozoa per sample was evaluated manually. Briefly, sperm smears were prepared, dried at room temperature and stained with Diff-Quick (Medion Diagnostics AG, Duedingen, Switzerland), and analyzed by microscopy (1009; Nikon Instruments Inc., Melville, NY, USA). Following semen analysis, samples were transferred (<1 h) to INRS, and washed in phosphate-buffered saline (PBS) by centrifugation at 300*g* for 7 min. The supernatant was removed and the cell pellets were prepared for either immunofluorescence staining or western blot analyses.

Swim-up technique

The separation of motile sperm-enriched and immotile sperm-enriched portions from semen samples was done by using the standard swim-up method [19]. A 0.75 ml aliquot of semen from consecutive fertile men ($n = 18$) was washed by centrifugation in pre-warmed M199 medium (Life Technologies, Burlington, ON, Canada) containing 0.3% BSA, at 300*g* for 7 min. The supernatant was removed by aspiration, and the pellet gently layered with M199 medium (containing 0.3% BSA). Samples were then incubated at 37 °C, in a humid chamber with 5% CO_2, for 45 min. The supernatant enriched with selected motile spermatozoa was then gently separated from the pellet enriched with unselected spermatozoa.

Western blots

Semen samples from each of three fertile men were washed with PBS containing a protease inhibitor cocktail (Active Motif, Carlsbad, CA, USA). Samples were centrifuged at 500*g* for 7 min. Following centrifugation, the upper layer was removed, and the pellet was resuspended in 100 µl of cold lysis buffer (1% Igepal CA-630, 154 mM NaCl, 0.4 M Tris, protease inhibitor cocktail, pH 8.0). Samples were then placed on a shaker at 4 °C for 30 min, and centrifuged at 10 000*g* for 10 min at 4 °C [20]. The protein concentration of the samples was determined with the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Ottawa, ON, Canada) and the supernatant was aliquoted and stored at −80 °C.

A 50 µg aliquot of total protein extracted from the spermatozoa was denatured in Laemmli buffer (Tris-HCl 60 mM, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.01% bromophenol blue) containing 100 mM dithiothreitol (Sigma-Aldrich, Oakville, ON, Canada) for 2 min at 95 °C and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Proteins were electroblotted onto polyvinylidene difluoride membranes via a semi-dry transfer apparatus (1.3A, 25 V for 10 min, Trans-Blot Turbo Transfer System; Bio-Rad Laboratories, Mississauga, ON, Canada). The efficiency of the transfer was determined by staining the blots with Ponceau red (0.6% w/v in 1% acid acetic) for 15 min. The membranes were then rinsed and blocked in Tris-buffered saline-Tween20 buffer (TBST; 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween20, pH 7.4) containing 5% non-fat dry milk (TBST-M) for 90 min. The membranes were then incubated with either anti-DEFB126 antibody or anti-His-antisera, diluted in TBST-M, overnight at 4 °C (Supplementary Table S1). Blots were then washed three times with TBST at room temperature and incubated with goat anti-rabbit IgG linked to horseradish peroxidase (HRP) or rabbit anti-mouse IgG (HRP) in TBST-M for 1 h. The blots were then washed three times

with TBST, and the signal of the immune complexes was detected with eClarity Western enhanced chemiluminescence substrate (Bio-Rad Laboratories). Data were analyzed in a ChemiDoc MP imaging system (Bio-Rad Laboratories).

Immunofluorescence

Spermatozoa were washed with phosphate buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS at room temperature for 20 min. They were then washed in PBS, collected by centrifugation and blocked in blocking solution (2% gelatin and 0.1% NaN_3/PBS; 20 min). The cells were subsequently incubated with anti-DEFB126 antibody (Supplementary Table S1) in blocking solution overnight at 4 °C. Spermatozoa were then washed three times with PBS, and incubated with goat anti-rabbit Alexa Fluor 488 (green)-conjugated secondary antibody (Life Technologies) in blocking solution for 1 h. Finally, cells were washed three times with buffer and resuspended in a solution containing propidium iodide (Vector Laboratories, Burlingame, CA, USA) and mounted on a glass slide [21]. Slides were visualized using a Leica DMRE microscope (Leica Microsystems, Inc., Concord, ON, Canada), and the percentage of DEFB126-positive spermatozoa was determined on a minimum of 100 spermatozoa in each semen sample.

Recombinant DEFB126

Primers (Supplementary Table S2) were designed to amplify the coding sequences of native DEFB126 and mutated forms of the gene that contained either a two-nucleotide deletion (DEFB126-2del) or a four-nucleotide deletion (DEFB126-4del) in the open reading frame (Supplementary Figure S1A). These mutations have been associated with male subfertility [16]. Total RNA was extracted from the caput epididymidis of two normal volunteers, and from a volunteer with secondary infertility using a commercial kit (NucleoSpin, Macherey-Nagel, Bethlehem, PA, USA). Sequence analysis had previously revealed the presence of the 2-nucleotide deletion and 4-nucleotide deletion in the *DEFB126* gene from two of the individuals. The coding region of human DEFB126, DEFB126-2 nucleotide deletion, and DEFB126-4 nucleotide deletion cDNAs were generated by real-time polymerase chain reaction (RT-PCR). RNA (500 ng) was reverse transcribed using an oligo $(dT)_{16}$ primer (R&D Systems, Minneapolis, MN, USA) and the coding sequences of DEFB126 and mutated forms of the gene were amplified by PCR with Mango-Taq DNA polymerase (Bioline, Toronto, ON, Canada) using an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s and 70 °C for 1 min. A Kozak sequence was embedded adjacent to the ATG start codon in the forward primer to increase the efficiency of translation and overall expression of the gene product [22]. A 6X Histidine-tag was inserted upstream of the stop codon at the C-terminus of the protein (Supplementary Figure S2). Amplicons obtained from agarose gel electrophoresis were purified using the NucleoSpin DNA purification Kit (Macherey-Nagel). DNA concentrations were determined using a NanoDrop 1000 (ThermoFisher, Ottawa, ON, Canada). The pcDNA3.1(+) plasmid (ThermoFisher), which contains the hygromycin resistance gene, was digested with BamHI and NotI (Invitrogen, Burlington, ON, Canada) restriction enzymes and the amplicons for DEFB126 and DEFB126-4del were cloned into the vector. For DEFB126-2del, the plasmid was digested with EcoRV and NotI (Invitrogen). The orientation of cloned DNA was verified by sequencing (Genome Québec Innovation Center, Montreal, QC, Canada).

Chemically competent Top10 *Escherichia coli* were transfected using the $CaCl_2$ method [23]. Competent cells were transformed with the pcDNA3.1-DEFB126, -DEFB126-2del, and -DEFB126-4del plasmids by heat shock at 42 °C, for 90 s. The presence of the DNA insert in ampicillin-selected transformed bacteria was verified by digestion of subclones using HindIII and NotI restriction enzymes (Invitrogen; Supplementary Figure S2).

H9C2 cells (Rat Myocardial Myoblasts; American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/Ham F-12 culture medium containing 10% FBS and 2 mM L-glutamine. One day before transfection, H9C2 cells were seeded in 12-well plate at 70% confluency. The next day, the medium was changed 1 h prior to transfection with pcDNA3.1, pcDNA3.1-DEFB126, -DEFB126-2del, or -DEFB126-4del plasmids using Lipofectamine 3000 (Invitrogen). P3000 reagent (Invitrogen) was used as a transfection enhancer. A total of 1 µg of each plasmid was added to each well of a 12-well culture plate and the medium was changed 12 h after transfection. H9C2 cells transfected with an empty pcDNA3.1 plasmid were used as a negative control.

Transfected H9C2 cells were trypsinized 48 h after transfection and seeded at low density (25% confluence). The next day, the medium was replaced with fresh medium containing 100 µg/ml hygromycin. The concentration of hygromycin was determined experimentally to represent the lowest concentration causing 100% mortality over 7 days in untransfected cells. Non-transfected H9C2 cells were treated with hygromycin and used as a positive control. Cells were grown in selection medium for 10 days, until all of the cells in the positive control died. The selection medium was changed every 48 h.

To isolate monoclonal stable cell lines that expressed either recombinant DEFB126 (rDEFB126) or one of the two recombinant mutant forms of the protein, single-cell isolations of selected cells were used. Serial dilutions were made and lines were derived from a single cell in a 96-well plate. The cells were allowed to reach confluency over a 14-day period. The mRNA levels of the transcribed DEFB126 gene and the two mutated forms of the gene in transfected cells were first assessed by RT-PCR with specific primers for each construct (Supplementary Figure S1B). Total RNA was extracted from the transfected and selected H2C9 cells as described above and mRNA transcripts were reverse transcribed using oligo $d(T)_{16}$ primers (R&D Systems) and MMLV-reverse transcriptase (Sigma-Aldrich). PCR amplifications were done with 35 cycles of denaturation (95 °C for 30 s), annealing and elongation (70 °C for 1 min). PCR was also done on RNA transcripts before reverse transcription in order to detect any genomic DNA contamination. PCR products were visualized on ethidium bromide-stained 1.6% agarose gels using a Gel Doc EZ Imager system (Bio-Rad Laboratories). The presence of recombinant proteins was assessed in the stably transfected cell lines by immunofluorescence and western blot analysis (Supplementary Figure S1C and D).

The secretion of recombinant proteins by monoclonal cell lines was assessed by western blot analysis of the culture medium (Supplementary Figure S1D). The protein concentration of the culture medium following 48 h of exposure to the transfected cells was assessed with the BCA protein assay kit (Thermo Scientific). Samples were stored at −80 °C until electrophoresis. Western blots were done as described above and the recombinant protein bands were revealed by an anti-6XHis Tag antibody (Supplementary Table S1) and a rabbit anti-mouse IgG (HRP) secondary antibody (Supplementary Table S1).

Transfected H9C2 cells were cultured in Dulbecco's modified Eagle medium (DMEM)/Ham F-12 medium containing nutrients, in 8-well chamber slides (Nalge Nunc International, Ottawa, ON,

Canada) for 48 h. The cells were fixed in ice-cold methanol for 10 min. They were then washed with PBS and incubated in blocking solution (5% BSA, 0.1% Tween 20 in PBS), for 30 min at room temperature. Subsequently, the cells were incubated with an anti-His Tag antibody (Supplementary Table S1) in blocking solution, for 90 min at room temperature. Cells were then washed three times in PBST and incubated with blocking solution containing the secondary antibody Goat anti-Mouse IgG Alexa Fluor 594 (Supplementary Table S1) and Hoechst dye (1 µg/ml; Biotium, Scarborough, ON, Canada) for 45 min, at room temperature. Finally, slides were washed three times in PBST and mounted in Fluoromount (Southern Biotech, Birmingham, AL, USA) [24].

Induction of sperm motility

To establish a protocol to induce sperm motility using rDEFB126, initial experiments were done using rat sperm. Male Sprague-Dawley rats ($n = 4$; 60–80 days of age) were purchased from Charles-River Laboratories (St-Constant, QC, Canada). Rats were maintained under a constant photoperiod of 12 h light/12 h dark cycle, and received food and water ad libitum. All animals were acclimated for at least 7 days before use. Animal protocols used in this study were approved by the University Animal Care Committee.

The initial segment of the rat epididymis was removed under sterile conditions and placed in cell culture medium (DMEM/Ham F-12 medium containing 2 mM L-glutamine and 10% FBS). Immature rat spermatozoa were isolated from the tissue and the culture medium containing immature spermatozoa was collected with a pipette and placed in a 1.5 ml microcentrifuge tube. After washing, sperm concentration was determined with a hemocytometer, and the suspension was diluted to a final concentration of 2×10^6 cells/ml. Co-incubation was performed using 24-well transwell culture inserts (0.4-µm polyester membrane cell culture inserts; Corning Life Sciences, Tewksbury, MA, USA). The inserts were placed in culture dishes containing H9C2 cells that had been in culture for 24 h. An aliquot of 0.1 ml of diluted immature spermatozoa was added to the apical chamber. Immature spermatozoa were co-cultured in the presence of either H9C2-pcDNA3.1 (sham-transfected), H9C2-DEFB126, H9C2-DEFB126-2del, H9C2-DEFB126-4del (experimental groups), and culture medium alone (negative control) for 24 h. The motility of co-cultured spermatozoa was analyzed at various time points (0, 2, 4, 6, and 24 h) on a 2X-CEL Chamber slide (Hamilton-Thorne), and verified manually according to WHO guidelines [42].

Binding of secreted recombinant proteins (rDEFB126, rDEFB126-2del, and rDEFB126-4del expressed from transfected-H9C2 cells) to the surface of co-cultured immature rat spermatozoa was assessed by immunofluorescence. Spermatozoa were collected 24 h after co-incubation with transfected cells and fixed in 1% paraformaldehyde in PBS. Immunofluorescence staining to detect recombinant fusion proteins was done using anti-6XHis Tag antibody according to the procedures described above.

Human immature testicular spermatozoa were collected from the seminiferous tubules obtained from three men with obstructive azoospermia undergoing testicular biopsies. Samples were placed in DMEM/Ham F-12 culture medium and transported to INRS within 1 h of surgery. Under sterile conditions, a 30G needle was used to perforate the tissue and release immature spermatozoa. The culture medium containing immature spermatozoa was collected with a pipette and placed in a 15 ml centrifuge tube. The tissue was incubated for 15 min to allow sedimentation of the seminiferous tubules. Subsequently, the supernatant containing released spermatozoa were collected. Co-incubation of immature testicular human spermatozoa with transfected-H9C2 cells was performed as described for rat spermatozoa. The motility of co-cultured spermatozoa was analyzed at various time points (0, 2, 4, 6, and 24 h) as described above.

Statistical analysis

All statistical analyses were done with the Prism 6.0 software (GraphPad, San Diego, CA, USA). Data were assessed for normal distribution using both the D'Agostino-Pearson and the Shapiro-Wilk normality tests, and all data passed the normal distribution tests. The percentages data were transformed to arcsin values for analysis. Statistical significance between groups was established at $P \leq 0.05$. Two-tailed Student t-test was used for comparison analysis and two-way ANOVA was used to for multiple comparisons. Results are expressed as mean values ± SD for each series of experiments. Two-tailed Pearson correlation analysis was used to estimate the correlation between the proportion of DEFB126-positive spermatozoa and semen parameters (semen volume, sperm motility, morphology, and concentration) as well as with circulating hormonal levels.

RESULTS

DEFB126 immunostaining and western blotting

Western blot analysis of proteins isolated from ejaculated spermatozoa of three volunteers and probed with anti-DEFB126 antibody revealed the presence of a 30 kDa band (Figure 1). DEFB126-immunofluorescent staining of spermatozoa on the acrosome and mid-piece were seen in all of immunostained spermatozoa (Figure 1a, c). DEFB126 immunostaining of the equatorial segments, post-acrosomal region, and tail was punctate and not uniform, as observed under confocal microscopy (Figure 1c). Spermatozoa incubated with primary antisera did not show any immunopositive reaction (Figure 1b, d).

Correlation of DEFB126 and semen parameter values

From immunofluorescent staining for DEFB126, the proportion of DEFB126-positive spermatozoa was evaluated for each semen sample. Subsequently, the correlation between the percentage of DEFB126-positive spermatozoa and semen parameters, including semen volume, sperm concentration, sperm morphology, and sperm motility (grade A + B) was investigated. A significant correlation was observed between the proportion of DEFB126-positive spermatozoa and sperm motility (grade A + B; $P < 0.0001$), as well as with the percentage of normal sperm morphology ($P < 0.0001$; Figure 2). However, there was no correlation between the percentage of DEFB126-positive spermatozoa, semen volume, and the age of the volunteers ($P > 0.05$; Figure 2). The final cohort of subjects for this analysis ($n = 58$) had a mean sperm motility of 50.9 ± 15.5 (mean ± SD). There was a correlation between sperm concentration and DEFB126, but not with the age of the patients (Figure 2 and Supplementary Figure S2).

To evaluate the relationship between sperm motility and percentage of DEFB126-positive spermatozoa, using the swim-up technique, motile spermatozoa were separated from non-motile spermatozoa (the pellet) in semen samples from fertile volunteers ($n = 18$). The results indicated that the percentage of DEFB126-positive spermatozoa in motile spermatozoa was significantly higher than in unselected spermatozoa ($P < 0.0001$; Figure 3).

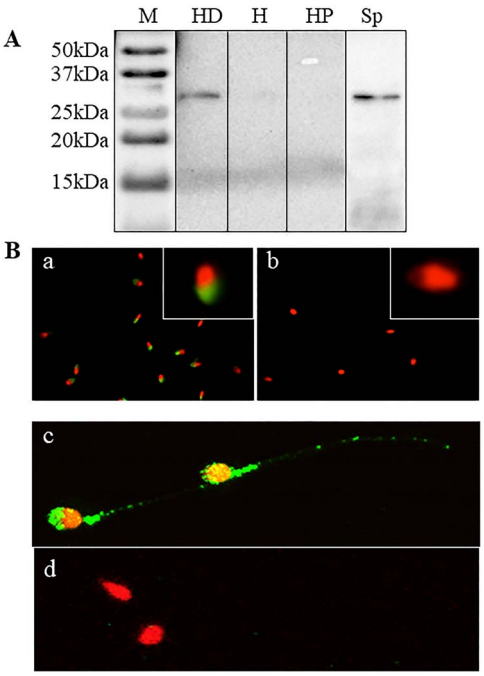

Figure 1. Detection of DEFB126 in human spermatozoa. (A) Western blot analysis of human spermatozoa protein lysate indicates a band of approximately 30 kDa (Sp). Transfected H9C2-DEFB126 (HD) is shown as a positive control, and H9C2 cells (H), transfected H9C2 with empty vector H9C2-pcDNA3.1 (HP) are shown as negative controls. (B) Immunofluorescence staining of human spermatozoa with anti-DEFB126 antibody (panel a and c, green) indicates that DEFB126 was present on the acrosome and mid-piece of all immunostained spermatozoa, as observed by fluorescent microscopy (a, insert). Beta-defensins 126 immunostaining of the equatorial segments, post-acrosomal region and tail was punctate and not uniform, as observed under confocal microscopy (c). Spermatozoa not treated with primary antibody (panel b and d; negative control) show no immunoreactivity. Nuclei are stained with propidium iodide (red).

Further investigations were done to determine if DEFB126 was associated with male fertility and sperm motility. According to the World Health Organization's [42] standard, the percentage of total motile spermatozoa (progressive motility + non-progressive motility) in a fertile man is greater than 40%, while volunteers with asthenozoospermia are defined as having less than 40% motility. The results showed that the percentage of DEFB126-positive spermatozoa in fertile men ($n = 39$) was significantly higher than levels in asthenospermic volunteers ($n = 20$; $P < 0.0001$; Figure 3).

DEFB126-positive spermatozoa in fertile and infertile volunteers

The present study indicated a strong positive correlation between DEFB126 and sperm motility (Figure 3). Based on these results, the proportion of DEFB126-positive spermatozoa between semen samples from fertile ($n = 42$) and infertile volunteers ($n = 19$), and between fertile and varicocele volunteers ($n = 14$), were compared. A significant difference was observed between the proportion of DEFB126-positive spermatozoa in semen samples obtained from fertile volunteers versus infertile ($P < 0.0001$; Figure 4A) and between fertile and varicocele volunteers ($P = 0.0012$; Figure 4B). The proportion of DEFB126 positive spermatozoa in samples from volunteers with leukocytospermia was also measured but did not show any significant difference from fertile volunteers (data not shown).

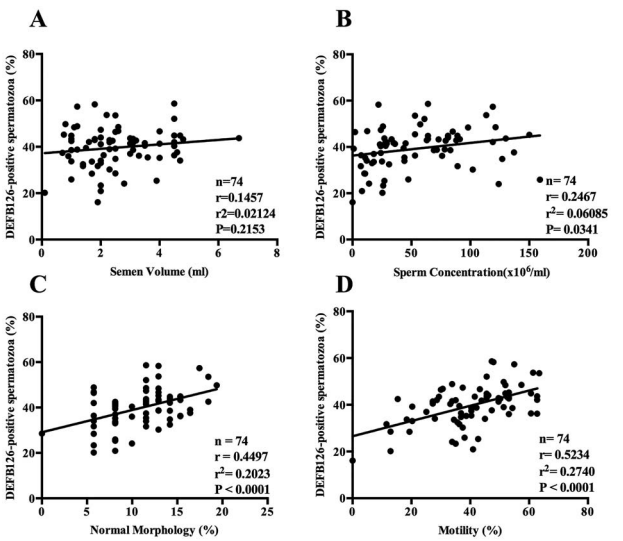

Figure 2. Correlation analysis of DEFB126 with semen parameters. Percentage data were arcsin transformed before statistical analysis. Pearson correlation analyses were used to determine the correlation between the percentage of (A) DEFB126-positive spermatozoa and semen volume (ml), (B) sperm concentration (10^6) in 1 ml of semen, (C) the percent with normal morphology, and (D) percent motility. $P \leq 0.05$ was considered significant.

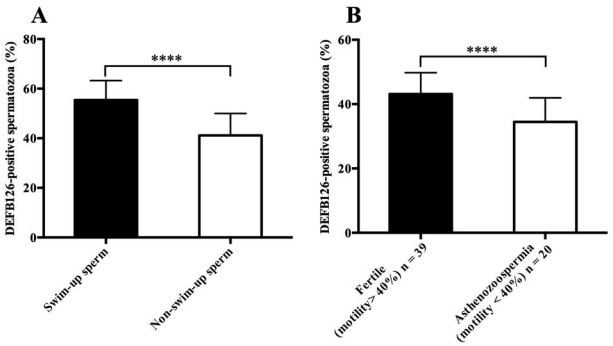

Figure 3. Assessing the proportion of DEFB126-positive spermatozoa in motile and non-motile spermatozoa. Motile spermatozoa were separated from non-motile spermatozoa by the swim-up method and were then immunostained for DEFB126. Percentage data, which refer to the percent of immunopositive sperm, were arcsin transformed before statistical analysis. (A) Paired student *t*-test analysis indicated that the proportion of DEFB126-positive spermatozoa in motile spermatozoa is significantly higher than in non-motile spermatozoa. (B) The proportion of DEFB126-positive spermatozoa was compared between fertile (sperm motility >40%) and asthenozoospermic volunteers (sperm motility <40%). There was a significant difference in the proportion of DEFB126-positive spermatozoa between fertile and asthenozoospermic volunteers. Results are expressed as mean ± SD. $^{****}P \leq 0.0001$ (Student *t*-test).

Sperm DEFB126 association with circulating levels of estradiol and testosterone

In the present study, correlations between serum levels of testosterone or estradiol with DEB126 were determined for a subset of volunteers. Statistical analysis indicated a positive correlation between the proportion of DEFB126-positive spermatozoa and serum testosterone levels in these men; however, additional volunteers will be needed to strengthen this observation ($P = 0.0185$; $n = 36$; Figure 5A). However, there was no association between the proportion of DEFB126-positive spermatozoa and serum estradiol levels ($P = 0.6475$; $n = 31$; Figure 5B).

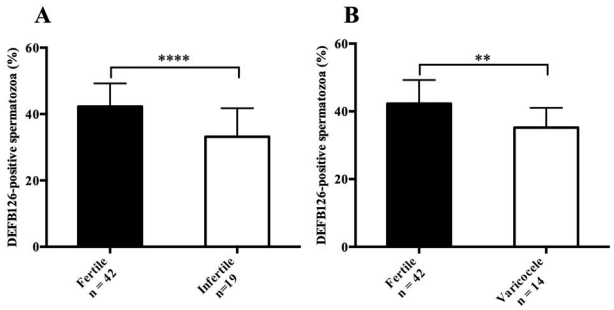

Figure 4. DEFB126-positive spermatozoa in fertile and infertile men. (A) DEFB126-positive spermatozoa in semen samples from fertile men were compared with men having a medical history of infertility. (B) DEFB126-positive spermatozoa in semen samples from fertile men were compared with spermatozoa from men with varicocele. Statistical analysis indicated that the proportion of DEFB126-positive spermatozoa in semen samples from fertile men was significantly higher than in either infertile (A) or men with varicoceles (B). Results were expressed as mean ± SD. $^{**}P \leq 0.01$; $^{****}P \leq 0.0001$ (Student *t*-test).

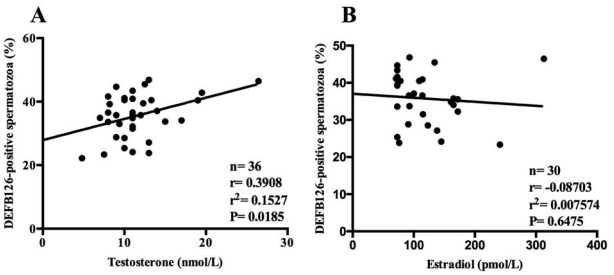

Figure 5. DEFB126 association with plasma sex hormones. Correlation between the proportion of DEFB126-positive spermatozoa in human ejaculated samples with circulating testosterone (nmol/l) (A) and estradiol levels (pmol/L) (B). Percentage data were arcsin transformed before statistical analysis. Statistical analysis indicates a significant ($P \leq 0.05$) positive association between testosterone levels and DEFB126. However, no significant relationship was found between estradiol levels and DEFB126.

Effects of DEFB126 on sperm motility

To investigate the effect of DEFB126 on sperm motility, immature rat spermatozoa from the initial segment were co-cultured with transfected-H9C2 cells, expressing rDEFB126, rDEFB126-2del, and rDEFB126-4del for 24 h ($n = 4$). In this experiment, two negative controls were used: immature rat spermatozoa were (1) co-cultured with transfected H9C2 cells with empty pcDNA3.1 vector; (2) co-incubated with culture medium without any cells. Evaluation of sperm motility of co-cultured spermatozoa in different time points displayed a significant 15% increase in the motility of spermatozoa co-cultured with H9C2-DEFB126 cells ($P < 0.001$; Figure 6) as compared with spermatozoa co-cultured with H9C2 cells transfected with an empty vector. In spermatozoa co-cultured with either of the two mutated forms of DEFB126, there was no increase in sperm motility. Experiments were repeated four times and similar results were observed. Likewise, immunostaining of spermatozoa incubated with rDEFB126 or one of the two mutated forms (rDEFB126-2del and rDEFB126-4del) indicated that more spermatozoa were immunopositive for those spermatozoa incubated with rDEFB126 than for those incubated with either of the two mutated forms (Figure 6).

To assess the effect of DEFB126 on human sperm motility, co-culture of immature human testicular spermatozoa with transfected H9C2 cells expressing rDEFB126 resulted in about 15% increase in sperm motility, while testicular spermatozoa incubated with cells expressing either rDEFB126-2del, rDEFB126-4del showed no increase in sperm motility (Figure 7). Furthermore, approximately 20% of spermatozoa co-cultured with cells expressing rDEFB126 were stained positively for the HIS tag, while less than 5% of spermatozoa incubated with cells that expressed either rDEFB126-2del, rDEFB126-4del were immunopositive. Given the percentage of immunopositive cells, the observed increase in sperm motility represents a relatively high proportion of immunopositive sperm, suggesting an important association between motility and DEFB126 binding.

DISCUSSION

Epididymal DEFBs have been shown to be essential for sperm maturation and capacitation in different species [11, 21, 25–27]. DEFB126, or its species-specific homologue, is implicated in sperm maturation, initiation of sperm motility in bulls, as well as sperm capacitation and protection in macaques [4, 6, 10, 14]. Previous studies have reported that DEFB126 has a molecular weight of 35 kDa in macaque spermatozoa. While the predicted weight of macaque DEFB126 is approximately 13.2 kDa, the higher molecular weight was shown to be the result of O-linked glycosylation [13]. In the present study, western blot analysis of DEFB126 in human sperm lysates indicated a band of 30 kDa, which is consistent with a previous report by Xin et al., [28]. DEFB126 immunostaining of spermatozoa was localized to the acrosome and mid-piece of all spermatozoa while staining of the equatorial segments, post-acrosomal region and tail was punctate and not uniform. This is different from the macaque, where an intense DEFB126 immunostaining was present over the entire surface of the spermatozoon [10].

Our results indicate a significant correlation between sperm-associated DEFB126 and sperm motility. Furthermore, separation of motile spermatozoa by the swim-up method confirmed that DEFB126 is associated with sperm motility. The role of several epididymal DEFBs on sperm motility has been reported previously. Bin1b induces progressive sperm motility in immature rat spermatozoa in vitro by inducing sperm Ca^{2+} uptake [29]. Likewise, Bin1b-immunized rats display decreased sperm motility [6]. Furthermore, in vivo knockdown of DEFB15, which is normally bound to the acrosome of rat spermatozoa, results in decreased progressive motility and fertilization ability [11]. In another study, deletion of a cluster of nine beta-defensin genes caused a gross disruption in microtubule structure of spermatozoa, which subsequently led to a significant reduction in sperm motility and fertility. In fact, spermatozoa from mice in which nine DEFB genes were deleted displayed microtubule disruption, as well as a significant increase in intracellular calcium concentration, relative to spermatozoa from wild-type mice [27]. In bovine species, DEFB126 induces motility in immature spermatozoa in vitro [4]. Data from the present study support the notion that in humans, DEFB126 is also associated with the acquisition of sperm motility in the epididymis.

Sperm morphology was also correlated with DEFB126 immunostaining. Although the proportion of different abnormal forms of spermatozoa was not evaluated in the present study, the percentage of spermatozoa with normal morphology showed a strong positive correlation with the proportion of DEFB126-labelled spermatozoa. While morphology of spermatozoa is an important parameter in predicting fertility [30, 31], the degree of positive

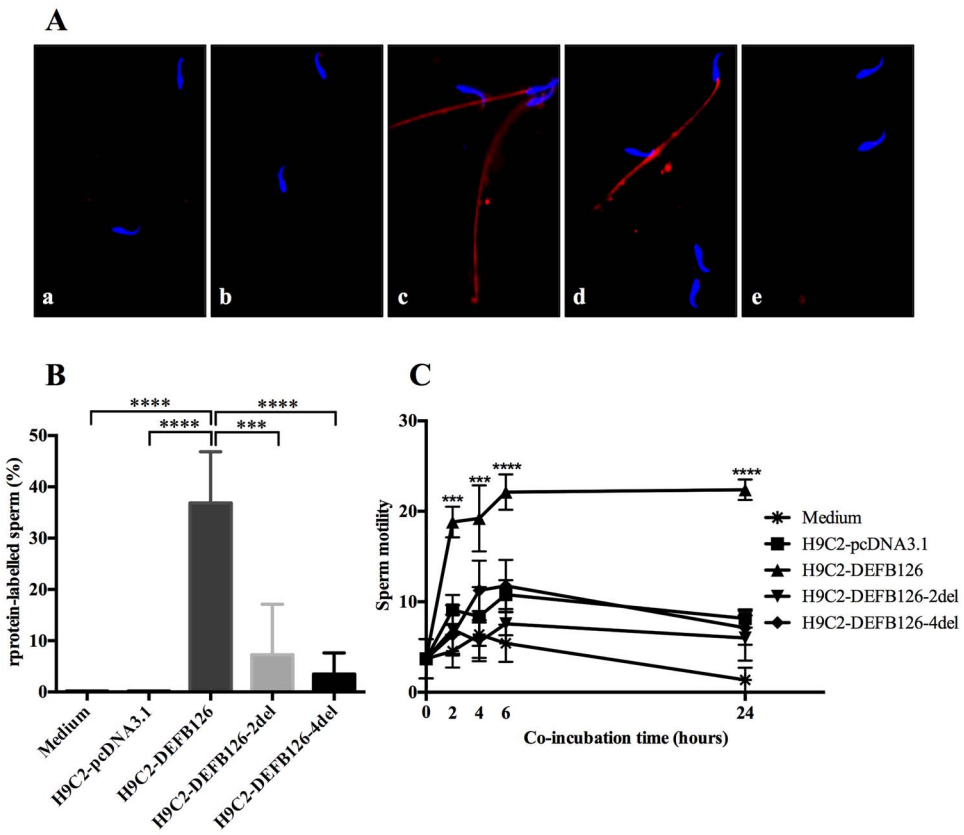

Figure 6. Influence of DEFB126 on sperm motility of immature rat spermatozoa collected from the initial segment of the epididymis. (A) Immunostaining of recombinant proteins following 24 h co-culture of immature rat spermatozoa with transfected H9C2 cells. Immature rat spermatozoa were incubated in (a) medium alone or with cells transfected with (b) an empty pcDNA3.1 plasmid; (c) H9C2-DEFB126, (d) H9C2-DEFB126-2del, or (e) H9C2-DEFB126-4del. Recombinant proteins were detected by anti-6XHIS Tag antibody (red). Nuclei were stained using Hoechst dye (blue). (B) The percentage of positive sperm for His-tag labeled recombinant proteins was determined for each group. The percentage of labeled spermatozoa, co-incubated with H9C2 cells, secreting rDEFB126, was significantly higher than in other groups ($P < 0.0001$). (C) Sperm motility of immature rat spermatozoa co-incubated with H9C2 cells, expressing rDEFB126, rDEFB126-2del, and rDEFB126-4del was manually assessed by microscopy according to the WHO guideline. Experiments were repeated four times. Data reported as mean ± SD, ($n = 4$). Percentage data were arcsin transformed before statistical analysis. Asterisks indicate a significant difference from other groups.$^{***}P \leq 0.001$; $^{****}P \leq 0.0001$ (Student *t*-test).

correlation between DEFB126 and sperm morphology suggests that spermatozoa with normal morphology are more likely to be coated by DEFB126 as compared to morphologically abnormal spermatozoa.

The proportion of DEFB126-positive spermatozoa in infertile men was significantly lower than in fertile men. A possible explanation for this is the lower epididymal expression levels of the DEFB126, as previously reported for non-obstructive azoospermic volunteers [17]. A second possibility is that there is reduced binding affinity of DEFB126 to the surface of spermatozoa with abnormal morphology. Finally, two common frame-shift mutations to DEFB126 (nucleotide deletion at positions 313–314 and 158–162 bp from the start codon), associated with male sub-fertility and infertility [15, 16], have been shown in this study to result in reduced binding affinity of DEFB126 to the surface of spermatozoa, and therefore, lower DEFB126 immunostaining.

Varicoceles are a major cause of male infertility and have been shown to affect both testicular and epididymal functions [32, 33]. In the epididymis, varicoceles are associated with increased apoptosis of principal cells [32, 33]. Although there is limited information regarding the effects of varicocele on the epididymis and sperm maturation, it has been reported that varicocelectomy improves sperm motility [34]. In the present study, semen samples from varicocele volunteers displayed a lower percentage of DEFB126-positive spermatozoa than fertile men. From the relationship between varicocele and lower testosterone levels [35], and the positive correlation observed between DEFB126 and serum testosterone levels, the decrease in DEFB126-positive spermatozoa on volunteers with varicocele may result from the lower testosterone levels. It has been widely established that testosterone and its metabolites dihydrotestosterone and estradiol (E2) are the main regulators of epididymal structure and function [3, 36, 37]. Among 23 mouse caput epididymal DEFB, 6 genes are fully regulated by androgens and 10 genes are partially regulated by androgens [38, 39]. In the present study, a significant positive correlation between DEFB126-positive spermatozoa and serum testosterone level was observed. Interestingly, a previous study reported a positive relationship between serum testosterone levels and sperm motility [40]. Another possible explanation for the lower DEFB126-positive cells is the increased percentage of abnormal spermatozoa, which was observed in varicocele volunteers, and which we showed to be correlated with decreased binding of DEFB126 to spermatozoa.

To study the role of DEFB126 in sperm maturation, stable recombinant cell lines which express rDEFB126 were generated, as

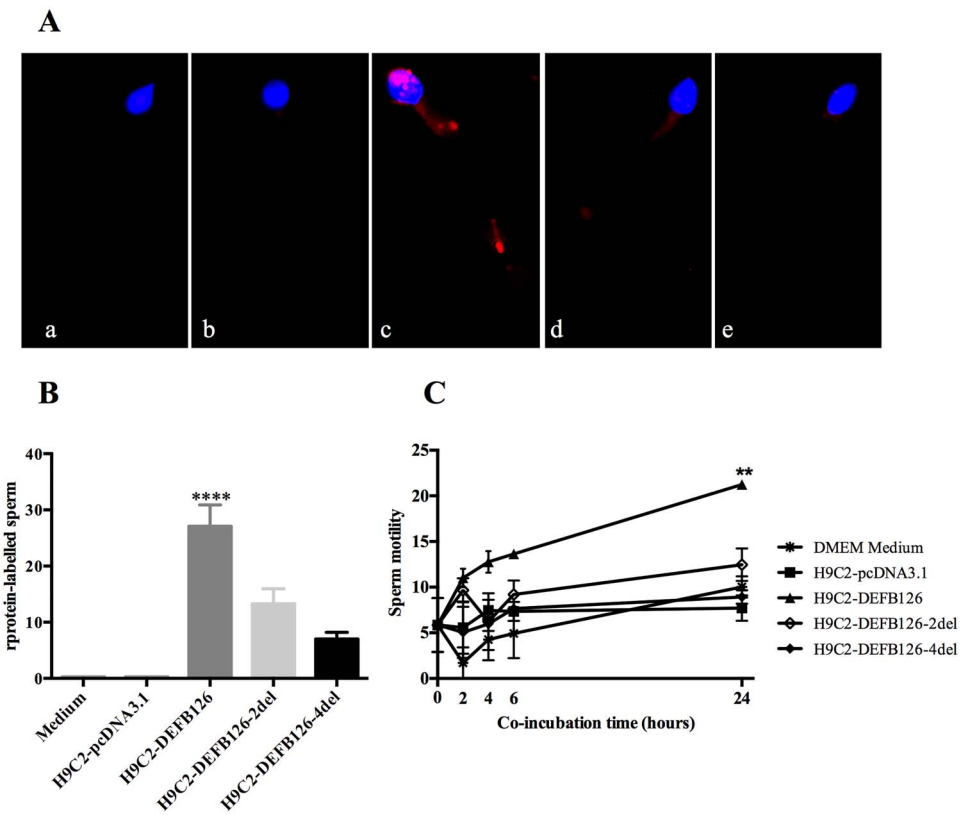

Figure 7. DEFB126 influence on sperm motility of immature human testicular spermatozoa collected from the seminiferous tubules. (A) Immunostaining of recombinant proteins following 24 h co-culture of immature human spermatozoa with transfected H9C2 cells. Immature spermatozoa were incubated in (a) medium alone or with cells transfected with (b) an empty pcDNA3.1 plasmid; (c) H9C2-DEFB126, (d) H9C2-DEFB126-2del, or (e) H9C2-DEFB126-4del. Recombinant proteins were detected by anti-6XHIS Tag antibody (red). Nuclei were stained using Hoechst dye (blue). (B) Sperm motility of immature spermatozoa co-incubated with H9C2 cells, expressing rDEFB126, rDEFB126-2del, and rDEFB126-4del was assessed manually according to the WHO guideline. Data are expressed as the mean ± SD, ($n = 3$). Percentage data were arcsin transformed before statistical analysis. Asterisks indicate a significant difference from other groups. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{****}P \leq 0.001$ (unpaired Student *t*-test).

were cell lines expressing two of the common human DEFB126 mutations. One of these expressed rDEFB126 containing a common 2-nucleotide deletion in the coding sequence of DEFB126 [16], while the second had a 4-nucleotide deletion in the reading frame of DEFB126 [15]. Although the 2-nucleotide deletion in the coding sequence of human DEFB126 generates a non-stop mRNA, the detection of the protein was highly variable in sperm of the volunteers with del/del genotype [15, 28]. Co-incubation of immature rat spermatozoa with transfected H9C2 cells expressing rDEFB126, showed that rDEFB126 bound to and initiated motility in both immature rat spermatozoa as well as in human testicular spermatozoa. In contrast, co-incubation of immature spermatozoa with cells expressing either of the two mutant forms of DEFB126 protein, rDEFB126-2del, and rDEFB126-4del, yielded DEFB126 proteins with lower binding affinity to both the rat and human immature spermatozoa. Deletion mutations to the DEFB126 open reading frame of the carboxyl terminal resulted in predicted changes to the peptide composition of DEFB126, which appears to result in the synthesis and secretion of non-functional proteins with either no binding or reduced binding to spermatozoa (Figure 8). These results can explain the variability in the detection of DEFB126-2-nucleotide deletion and undetectable presence of DEFB126-4nucleotide deletion in spermatozoa of the volunteers with del/del genotype in previous studies [15, 28]. Furthermore, sperm motility in these groups did not show any increase over those with rDEFB126 protein. These data confirm that DEFB126 can induce motility in immature spermatozoa. While there is no evidence in literature, or in the present study, that H9C2 cells influenced sperm motility in any way, nor that DEFB126 binds to other secretory proteins, the use of purified recombinant DEFB126 would be required to demonstrate that our observations are not influenced by an indirect effect by H9C2 cells.

Interestingly, rDEFB126 was effective in stimulating both human and rat immature spermatozoa. This is particularly intriguing since DEFB126 has very low homology (24%) with its rat DEFB126 homologue, DEFB22, which is also found on rat spermatozoa. While the mechanism by which DEFBs, including DEFB126, regulate sperm motility has not been established, defensins are cationic (polar) molecules with a hydrophobic, charged region that likely allows them to be inserted into the phospholipid moiety of plasma membranes and make holes or form ion-permeable channels [41]. This may enable the movement of essential ions for sperm motility, such as calcium, to enter the cells. Further studies will be needed to prove this.

In conclusion, DEFB126 appears to play an important role in the acquisition of human sperm motility. Furthermore, DEFB126 binding to spermatozoa is lower in infertile volunteers than in volunteers with proven fertility. From the use of recombinant proteins, our data support a role for DEFB126 in sperm motility and suggest that mutations to DEFB126 that have been identified in human

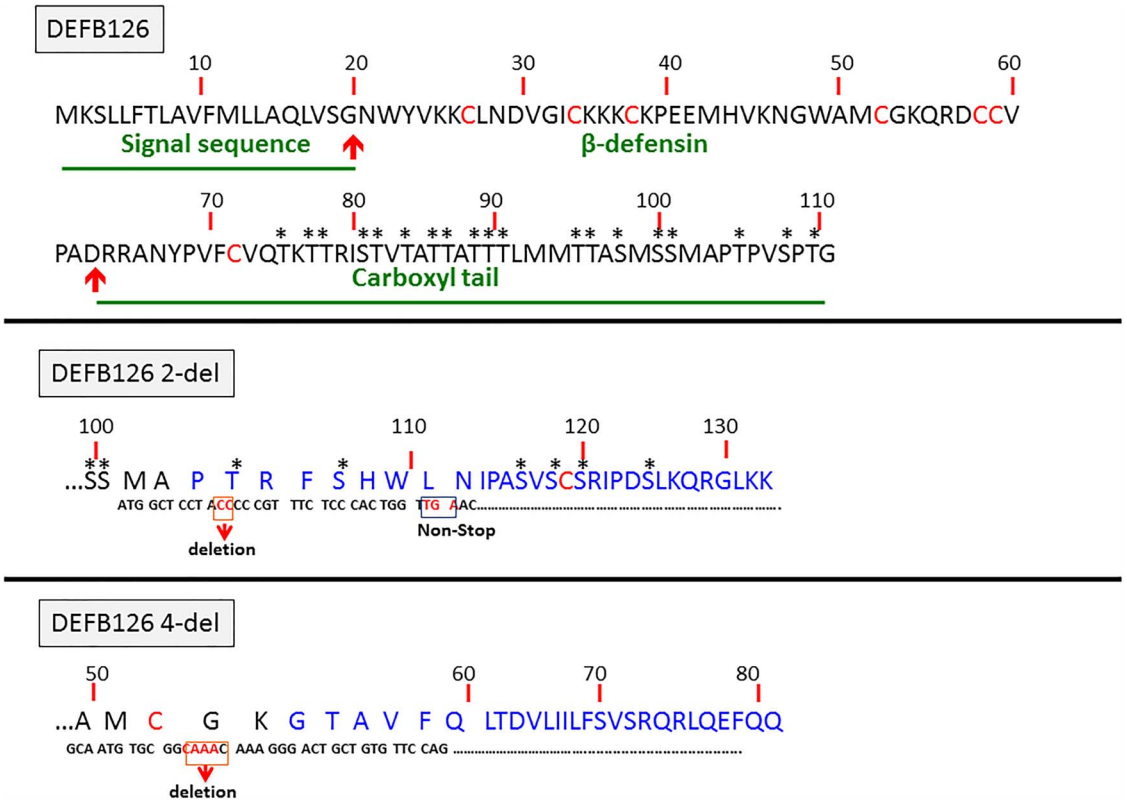

Figure 8. Schematic diagram of human DEFB126 peptide sequence showing the signal sequence, beta-defensin core, and carboxyl tail. The carboxyl terminal includes an unpaired cysteine and several potential O-glycosylation sites (*; serines and threonines). Deletion mutations (DEFB126 2-del; DEFB126 4-del) to the DEFB126 open reading frame of the carboxyl terminal resulted in predicted changes to the peptide composition of the protein. This yielded non-functional proteins, which showed decreased or lack of binding to the spermatozoa (arrows indicate cleavage sites; cysteine residues are displayed in red).

populations result in decreased binding of DEFB126 to spermatozoa and consequently, decreased motility.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Acknowledgements

The authors thank Mary Gregory and Julie Dufresne (INRS) for their assistance and scientific input throughout the study. The authors would further acknowledge the assistance of Lorraine Lavigne R.N. in the procedures of subject recruitment and enrolment. DGC holds the Canada Research Chair in Reproductive Toxicology.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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