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·Original Article·

Inhibition of mouse acrosome reaction and sperm-zona pellucida binding by anti-human sperm membrane protein 1 antibody

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Abstract

Aim: To investigate the possible functions of human sperm membrane protein (hSMP-1) in the process of fertilization. **Methods:** A 576-bp cDNA fragment of *HSD-1* gene coding for the extracellular domain of hSMP-1 was cloned and expressed. The localization of this protein on human and mouse sperm was determined by indirect immunofluorescent staining by using anti-recombinant hSMP-1 (anti-rhSMP-1) antibodies. Sperm acrosome reaction and sperm-zona pellucida (ZP) binding assay were carried out in 10-week-old BALB/c mice. **Results:** Recombinant hSMP-1 was successfully cloned and expressed. The expression of the native protein was limited on the acrosome of human and mouse sperm. Treatment of anti-rhSMP-1 antibodies significantly decreased the average number of sperms bound to each egg. Meanwhile, the percentage of acrosome reaction was decreased in comparison to pre-immune control after treatment with anti-rhSMP-1 ($P < 0.05$). **Conclusion:** The results suggest that anti-rhSMP-1 antibody inhibited mouse acrosome reaction and sperm-ZP binding. *(Asian J Androl 2007 Jan; 9: 23–29)*

Keywords: human sperm membrane protein-1; SPAG8 protein; gene expression; acrosome reaction; sperm-oocyte interactions; zona pellucida; fertilization

1 Introduction

The sperm surface is covered by a continuous plasma membrane where functional molecules are distributed. The antigens on sperm plasma membrane provide potential immunogens for antifertility vaccine development and antibodies will interact with antigens accessible at the cell surface to cause cytolysis or immobilization [1]. It has been reported that some infertile couples may be so because of the presence of anti-sperm antibodies in the male and/or female partner [2]. Though the causal relationship between infertility and antibodies against sperm surface proteins has not been completely proved, there have been reports that show a significant correlation [3].

Previously, a serum containing antisperm antibodies

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that agglutinated human sperm was obtained from an infertile female patient. This serum was used to screen a human testis cDNA expression library, resulting in the identification of the *HSD-1* gene (PubMed Locus HSU12978) coding for a human sperm membrane protein (hSMP-1). Subsequent studies showed that hSMP-1 (PubMed Locus U12978) is a testis specific human sperm membrane protein expressed during human development [4]. However, until now the effect of hSMP-1 on the process of fertilization, and the mechanism of infertility induced by the appearance of anti-hSMP-1 antibodies remains uncertain.

In this study, a 576-bp cDNA fragment of *HSD-1* gene coding for the extracellular domain of hSMP-1 was successfully cloned and expressed. Bioinformatic analysis shows that the fragment appears to be highly immunogenic and displays a high probability of surface expression. Moreover, specific anti-hSMP-1 antibodies raised against this fragment significantly inhibited sperm acrosome reaction and binding of spermatozoa to eggs.

2 Materials and methods

2.1 Subjects and animals

This study was approved by the Ethical Review Board at Shanghai Jiao Tong University School of Medicine. For human spermatozoa collection, healthy donors were recruited from RenJi Hospital, which is affiliated with Shanghai Jiao Tong University School of Medicine. All subjects signed informed consent before participation in the study. Adult (10-week-old) BALB/c mice were obtained from the Animal Center of the Chinese Academy of Sciences (Shanghai, China), and housed in specific pathogen-free conditions at Shanghai Jiao Tong University School of Medicine. All animal experiment was conducted in accordance with Shanghai Jiao Tong University School of Medicine Animal Studies Committee.

2.2 RT-PCR

Using DNAssist and Prosis Analytical software, we found that nucleotides 750-1325 of the HSD-1 cDNA contained antigenic determinants of hSMP-1. Therefore, this 576 fragment was cloned by RT-PCR. Total RNA was extracted from pathologically normal human testis (obtained from a patient with prostate cancer from the Department of Urology of Renji Hospital, Shanghai, China) using Qiagen RNAeasy kit (Qiagen, Hilden, Germany). Reverse transcription was performed according to the manual of TaKaRa AMV RT-PCR kit (TaKaRa, Da Lian, Japan). Two primers were designed by using the Primer Premier 5.0 software. The forward primer was 5'-GCG GAT CCT GTA TTC CTC CAG GGT TCA-3' (containing a *BamH*I site) and the backward primer was 5'-GCC TCG AGG TCG TGA GGC TTT GTT GG-3' (containing an *Xho*I site). A touchdown PCR reaction was carried out in a final volume of 25 μL with annealing temperature stepped down gradually from 52ºC to 48ºC.

2.3 Cloning and expression of recombinant hSMP-1

The amplified DNA was purified according to the procedure of TaKaRa Agarose Gel DNA Purification kit (TaKaRa, Da Lian, Japan), and then cloned into pBS-T vector. The recombinant plasmid DNA was confirmed by *BamH*I-*Xho*I digestion and also by sequencing. The *BamH*I-*Xho*I fragment was introduced into the *BamH*I-*Xho*I site of the Histidine-tagged pET-28a (+) expression vector (Novagen, Darmstadt, Germany) [5], and was subsequently propagated in *E. coli* BL21 (DE3) host cells. Recombinant protein expression was induced, and the IPTG-induced $(Histidine)_6$-tagged fusion proteins were analyzed on a discontinuous polyacrylamide slab gel (5% stacking and 15% separating gel, Bio-Rad, USA).

2.4 Purification of expressed proteins and production of polyclonal antibodies

Half a liter cell culture of *E. coli* expressing recombinant hSMP-1 (rhSMP-1) was centrifuged and washed with phosphate-buffered saline (PBS) containing 0.2 mmol/L PMSF (PBS 0.01 mol/L, pH 7 .4), which was then separated on 15% SDS-PAGE gels. Finally, the target protein was excised from the gels according to its molecular weight, purified by dialysis and concentrated by freeze-drying. Its purity was checked on an SDS-PAGE gel. The quantification of rhSMP-1 was determined by BCA Protein Assay kit (Pierce, Rockford, USA).

Purified rhSMP-1 was used to immunize healthy 6-month-old male New Zealand white rabbits with bodyweights of approximately 2.5 kg (purchased from Animal Center of the Chinese Academy of Sciences, Shanghai, China). The immunization was carried out as described in a previous study [6]. On the 35th day, the titer of the antiserum was checked with ELISA [7]. The antibodies were purified to a final concentration reached 0.5 mg/mL using Montage Antibody Purification PROSEP-A kit (Millipore, Billerica, USA). The specificity of the antiserum was judged by: (a) the reactivity of the antise-

rum to rhSMP-1; (b) the inability of the antiserum to cross-react with any other proteins from total sperm extracts on Western blot; (c) the ability of antigen pre-adsorption to abolish immunorecognition; and (d) reproducibility of the results with antiserum from different animals.

2.5 Western blot analysis

Western blot analysis was processed as described in a previous study [8]. Human sperm membrane proteins were extracted as described in a previous study [4], transferred to nitrocellulose membrane, and incubated with anti-rhSMP-1 antibodies (1:1 000). Control blots were incubated with pre-immune serum (1:1 000). After incubation, the membranes were rinsed and incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG. Reactive protein was visualized by chemiluminescence (ECL).

2.6 Indirect immunofluorescent staining of hSMP-1 on human and mouse sperm

Healthy donor sperm were collected and air-dried onto poly-L-lysine-coated slides. For mouse studies, swimming out mouse sperm [9] were washed by a two-step Percoll gradient (80%:40%), centrifuged at 400 × *g* for 20 min, and air-dried onto poly-L-lysine-coated slides. All slides were immersed in 4% paraformaldehyde for 30 min, each slide was respectively incubated with 1:100 dilution of anti-rhSMP-1 antibodies or pre-immune serum overnight. After being treated with 1:200 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) at room temperature for 2 h, digital images of fluorescent slides were viewed under a laser scanning confocal microscope (Carl Zeiss LSM-510, Jena, Germany).

2.7 Assessment of mouse spermatozoa acrosome reaction

Mouse spermatozoa were obtained and washed as before. For *in vitro* capacitation, they were adjusted to 1×10^6/mL in BWW (Sigma, St. Louis, MO, USA) with 3% BSA (Sigma, St. Louis, MO, USA) and cultured at 37ºC in 5% CO_2 for 1 h. Then they were incubated for 30 min in BWW-BSA containing pre-immune serum (1:200 dilution) as control or anti-rhSMP-1 antibodies (1:200, 1:800 dilutions, respectively). Ca^{2+} ionophore A23187 (final concentration 2.5 μmol/L; Sigma, St. Louis, MO, USA) was added simultaneously to induce acrosome reaction. The percentage of acrosome reaction of living spermatozoa was evaluated by staining with 0.1 mg/mL of fluorescein isothiocyanate (FITC)-labeled pisum sativum agglutinin (PSA, Sigma, St. Louis, MO, USA) [10]. Sperm were scored as acrosome intact when a bright staining was observed on the acrosome, or as acrosome reacted when either fluorescent staining was restricted to the equatorial segment or no labeling was observed, and diffuse fluorescence over the entire heads was deemed as dead sperm. For analysis of the FITC-PSA staining, a laser scanning confocal microscope was used to assess at least 200 spermatozoa for each slide. The experiment was repeated three times.

2.8 Sperm-ZP binding assay

BALB/c female mice were induced to superovulate by i.p. injection of 15 IU of hCG (Sigma, St. Louis, MO, USA), 48 h after 15 IU pregnant mare's serum gonadotropin (PMSG, Sigma, St. Louis, MO, USA) injection. Animals were killed 16h after hCG injection. The cumulus oocyte clumps were obtained by puncturing the distended ampulla as described in a previous study [11]. To disperse cumulus cells, cumulus/oocyte clumps were transferred to M16 medium (Gibco, Los Angeles, USA) containing 0.1% hyaluronidase (Sigma, St. Louis, MO, USA) and 3% BSA (Sigma, St. Louis, MO, USA). Completely denuded eggs were washed three times and placed in BWW.

For sperm collection, the cauda epididymides were minced and the swimming out sperms were collected after 15 min. Sperm concentration was adjusted to 1×10^6/mL in BWW. Capacitated spermatozoa were pretreated with 1:200, 1:800, 1:1 600 diluted anti-rhSMP-1 antibodies or 1:200 diluted pre-immune serum for 30 min. They were then incubated with eggs in a 5% CO_2 incubator at 37ºC. One hour later, eggs were washed three times with fresh medium. 25–30 eggs were taken and mounted on glass slides for analysis of sperm-ZP binding.

2.9 Statistical analysis

Results from the experimental and the control group were reported as mean ± SD. Difference of means was analyzed by the unpaired *t*-test, with $P < 0.05$ considered significantly different.

3 Results

3.1 Homology between hSMP-1 and mouse SPAG8

hSMP-1, also called SPAG8 (PubMed Locus Q99932),

is homologous to mouse SPAG8 (PubMed Locus NP_001007464). Within the target fragment region, the identity between human and mouse SPAG8 is 66.67%, and the similarity is 81.25%. The high homology between the two proteins suggests that the potential function of hSMP-1 during human fertilization may be deduced from experiments carried out in BALB/c mice.

3.2 Cloning, expression and purification of recombinant hSMP-1

The 576 bp fragment of hSMP-1 was successfully cloned. The IPTG-induced $(Histidine)_6$-tagged protein is approximately 26 kDa, which was mainly expressed in the insoluble inclusion bodies instead of being released in the supernatant of the *E. coli* BL21 (DE3) medium. The purified protein ran as a 26-kDa band in 15% SDS-PAGE gel by Coomassie Blue R-250 staining (Figure 1).

3.3 Generation of polyclonal antibody against recombinant hSMP-1

Data showed decreasing absorbance values of anti-rhSMP-1 antibodies or pre-immune serum with increasing dilutions of up to 1:64 000, and the absorbance value of the former was higher than that of the latter. In addition, Western blot analysis showed that purified rhSMP-1 and extracted human sperm proteins both reacted with anti-

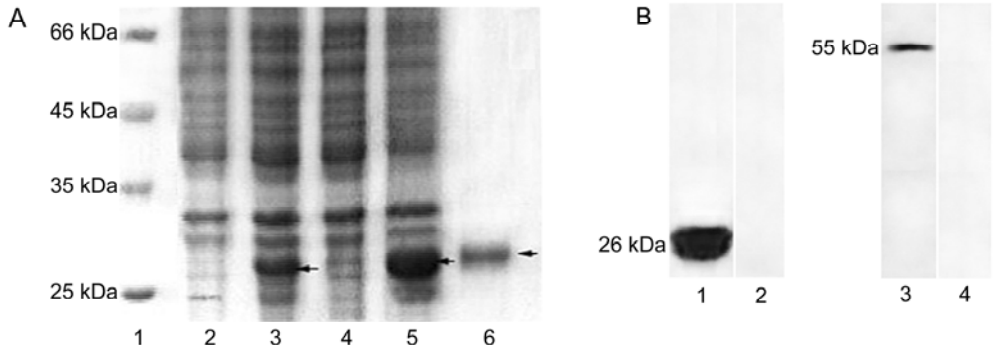

Figure 1. (A): Expression and purification of *IPTG*-induced rhSMP-1. Lane 1: protein molecular; Lane 2: bacteria lysate, IPTG (-); Lane 3: bacteria lysate, IPTG (+); Lane 4: supernatant, IPTG (+); Lane 5: insoluble inclusion bodies, IPTG (+); Lane 6: purified rhSMP-1. Arrows indicate the expressed rhSMP-1. (B): Western blot analysis. Lane 1: purified rhSMP-1 reacted with anti-rhSMP-1 antibodies (1:1 000); Lane 2: purified rhSMP-1 did not react with pre-immune serum (1:1 000); Lane 3: extracted human sperm proteins reacted with anti-rhSMP-1 antibodies (1:1 000); Lane 4: extracted human sperm proteins did not react with pre-immune serum (1:1 000).

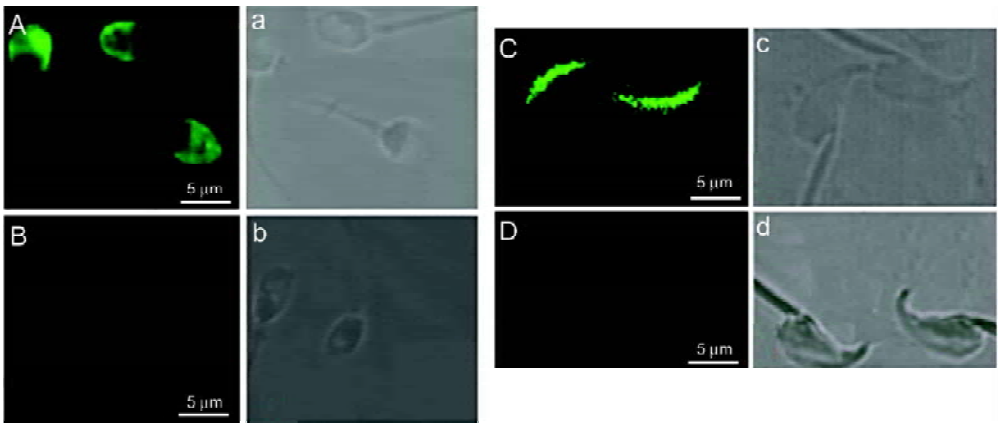

Figure 2. Immunolocalization of sperm membrane protein (*SMP-1*). Intense immunoreactivity was observed on human and mouse sperm acrosome (A, C). Control specimens were negative for immunofluorescent labeling (B, D). Corresponding phase-contrast were shown (a, b, c, d).

rhSMP-1 antibodies, while no reaction was observed with the addition of pre-immune serum (Figure 1).

3.4 Localization of SMP-1 on human sperm and mouse sperm

Indirect immunofluorescent technique was performed to determine the location of SMP-1 (Figure 2). Intense immunoreactivity was observed on the acrosome of human sperm and BALB/c mouse sperm. In controls incubated with pre-immune serum, no obvious reaction was observed.

3.5 Effects of anti-rhSMP-1 antibodies on mouse spermatozoa acrosome reaction

Under the laser scanning confocal microscope, three patterns of PSA-stained spermatozoa were observed. Nonfluorescent acrosomal region with or without a fluorescent postacrosomal region indicated capacitated acrosome-reacted spermatozoa. Sperm were acrosome intact when a bright staining was observed on the acrosome. Diffuse fluorescence over the entire heads indicated dead sperm.

Acrosomal integrity was expressed as the percentage of living acrosome-reacted mouse spermatozoa over total living spermatozoa. Because there were no significant differences in the total number of living versus dead sperm among treatments with different dilutions of the antiserum. The addition of anti-rhSMP-1 antibodies at 1:200 dilution to mouse sperm resulted in a decrease of acrosome reaction. The percentage of living acrosome-reacted sperm was (23.21 ± 3.79)% in the experimental group *vs.* (37.96 ± 3.98)% in the control group ($P < 0.05$). No difference was found when the spermatozoa were treated with anti-rhSMP-1 antibodies at 1:800 dilution (38.32 ± 6.86)% (Figure 3).

3.6 Effects of anti-rhSMP-1 antibodies on sperm-ZP binding assay

To assess the possible role of anti-rhSMP-1 antibodies in sperm-egg interaction during fertilization, capacitated mouse spermatozoa were treated with anti-rhSMP-1 antibodies and pre-immune serum at different dilutions. Results showed that anti-rhSMP-1 antibodies significantly inhibited sperm-ZP binding. The average number of sperm bound to each egg was 2.15 ± 0.69, 3.94 ± 1.98, 13.25 ± 1.18 at 1:200, 1:800, 1:1 600 dilutions of anti-rhSMP-1 antibodies. In contrast, the average number of sperm bound to each egg was 13.38 ± 0.92 at 1:200 dilution of pre-immune serum. The inhibition of sperm binding to each egg was statistically significant ($P < 0.01$) at 1:200 (91.40%) and 1:800 (70.55%) dilutions of anti-rhSMP-1 antibodies. However, no inhibition was observed when the anti-rHSMP-1 antibodies were diluted to 1:1 600 (Figure 4).

4 Discussion

In this study, a 576-bp cDNA fragment of *HSD-1* was successfully cloned and expressed. Polyclonal antibodies against this fragment specifically resulted in in-

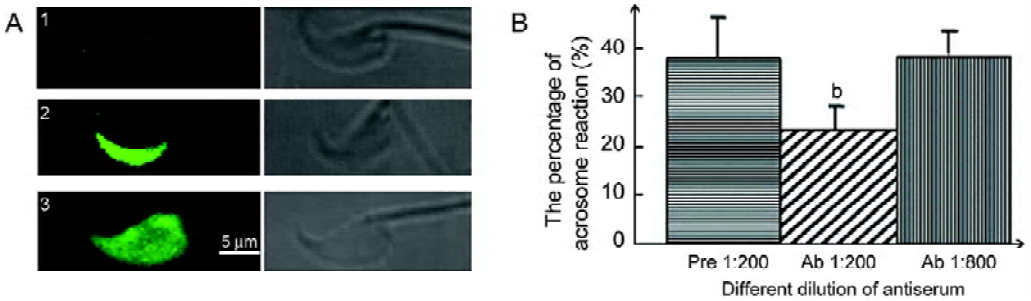

Figure 3. Acrosome reaction assay. (A): Three staining patterns observed from FITC-PSA-stained mouse spermatozoa: 1), the acrosomal region of the sperm head is nonfluorescent, with or without a fluorescent postacrosomal region. This indicates a capacitated, acrosome-reacted spermatozon; 2), the acrosomal region of the sperm head fluoresces brightly but the postacrosomal region does not. This indicates a capacitated, acrosome-intact spermatozon; 3), the whole sperm head shows diffuse fluorescence, which is indicative of dead sperm. (B): The percentage (mean ± SD) of acrosome-reacted mouse sperm with the treatment of 1:200 and 1:800 dilution of anti-rhSMP-1 antibodies (Ab 1:200 and Ab 1:800), and the percentage of that with 1:200 dilution of pre-immune serum (Pre 1:200). A bar chart demonstrates mean ± SD ($n = 200$). $^bP < 0.05$, compared with the pre-immune serum (Pre 1:200).

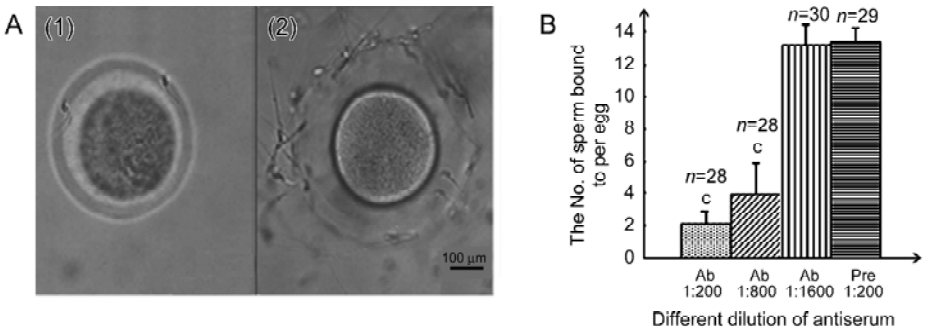

Figure 4. Sperm-zona pellucida (ZP) binding assay. (A): (1), The sperm binding to ZP at 1:200 dilution of anti-rhSMP-1 antibodies. (2), The sperm binding to ZP at 1:200 dilution of pre-immune serum (Pre 1:200). (B): The average number of bound sperm to per egg at 1:200, 1:800, 1:1 600 dilution of anti-rhSMP-1 antibodies (Ab 1:200, Ab 1:800, Ab 1:1 600) and 1:200 dilution of pre-immune serum (Pre 1:200). A bar chart demonstrates mean ± SD. *n*, total number of oocytes per group. $^{c}P < 0.01$, compared with pre-immune serum (Pre 1:200).

tense hSMP-1 immunoreactivity on the acrosome of human sperm. The staining results were the same when living sperm were probed by the antibody against recombinant hSMP-1 in comparison to dead sperm. Strong immunofluorescence was also observed on the acrosome of mouse sperm because of the strong homology between hSMP-1 and mouse SPAG8. In the control group, where pre-immune sera and natural rabbit IgG were used as primary antibody, immunofluorescence was not shown. The reproducibility in the sera of three rabbits making the antibody is good. The results suggested the quality and specificity of the antibody against recombinant hSMP-1. We infer that SMP-1/SPAG8 is probably related to acrosome reaction, an essential prerequisite for successful fertilization.

Our data showed a significant inhibition of acrosome reaction of living mouse sperm *in vitro* by the addition of anti-rhSMP-1 antibodies. However, the molecular mechanism of this inhibition still needs further study. In addition, anti-rhSMP-1 antiserum treatment also resulted in a significant decrease in sperm-ZP binding. Because sperm plasma membranes play a critical role in sperm-oocyte recognition, adhesion and fertilization, we propose that the specific anti-rhSMP-1 antiserum reacted with its corresponding antigen on the sperm membrane, and resulted in decreased ability of sperm-ZP binding.

Studies carried out in the late 1950s [12] demonstrated that a significant number of infertile men showed autoimmunity to spermatozoa and suggested that antisperm antibodies could interfere with the fertilizing ability of the spermatozoa. A subset of infertile subjects has been found to possess anti-sperm antibodies in blood, semen, ovarian follicular fluid and/or vaginal and cervical fluids [13]. Anti-sperm antibodies, which were bound to antigens of the gametes, can act negatively both on the motility to pass through female genital secretions and on the fusion of gametes and perhaps also on the first step of embryonic development [14]. Analyses of male fertility have revealed that several sperm surface proteins are associated with sperm function [15–18].

hSMP-1, a specific human sperm membrane protein, was originally identified in the serum from an infertile woman who showed the presence of antisperm antibodies. This fact is of great interest because one aspect of the modern male contraception research focuses on the immunological suppression of fertility through identification of an anti-sperm antibody. Studies on immunocontraceptive vaccines on the basis of sperm specific antigen have been performed throughout the world. However, until now no studies have examined the functional significance of acrosome-specific SMP-1 in terms of fertilization or its relevance to such vaccines.

To our knowledge, inhibited acrosome reaction and sperm-ZP binding by anti-SMP-1 antibodies may be a mechanism for infertility seen in patients positive for such antibodies. Moreover, recognition of antigens relevant to infertility may also be important for potential immunocontraception [19]. With the serious global issue of overpopulation, especially in developing countries, it becomes a necessity for us to find an effective contracep-

tive method to control the rapid growth of population. Immunocontraception deserves to be an important and potential means of birth control [20–22]. The fact that anti-hSMP-1 antibodies exist in the serum of infertile patients and its antifertility function suggest that the testis specific membrane protein, hSMP-1, may be used as an immunocontraceptive antigen. Our work implies that this kind of protein has a promising future in the research of human immunocontraception vaccine.

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