Summer 1995

In vitro Expression of Human Zona Pellucida Protein 3

Chih-Wei Chen
Old Dominion University

In vitro expression of human zona pellucida protein 3

by

Chih-Wei Chen

B.S. June 1987, Cheng-Kung University

M.S. June 1989, Tunghai University

A Dissertation Submitted to the Faculty of Old Dominion University and Eastern Virginia Medical School in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

OLD DOMINION UNIVERSITY
and
EASTERN VIRGINIA MEDICAL SCHOOL

Aug 1995

Approved by:

_____
Dr. Sergio Oehninger

_____
Dr. Frank J. Castora

_____
Dr. James H. Yuan

_____
Dr. Ke-Wen Dong

_____
Dr. Mark S. Elliott

ABSTRACT

In vitro expression of human zona pellucida protein 3

Chih-Wei Chen
Old Dominion University
and
Eastern Virginia Medical School
1995

Human zona pellucida protein 3 (hZP3) is the putative receptor on the zona pellucida of the mature oocyte that recognizes and binds sperm, and therefore plays a critical role in fertilization.

A full length cDNA of hZP3 (1278 bp) was amplified from the human ovary mRNA by reverse transcription-polymerase chain reaction (RT-PCR). The hZP3 cDNA was subcloned into PSK and pREP4 expression vectors. The cDNA of hZP3 was further characterized by restriction mapping, PCR, auto-sequencing and Southern blot analysis by using an internal oligonucleotide probe, and found to be identical to the one reported by J. Dean. Using autosequencing, 289 bases were identified and more than 98% homology was found at the 3'-end with the reported sequence, whereas, 224 bases were identified and more than 91% homology was found at the 5'-end. *In vitro* translation was performed to ensure the capacity of the cloned cDNA for producing a full length,

non-glycosylated hZP3 protein (47 kDa). Stable transfection of a human ovarian tumor cell line, PA-1, was produced by introduction of the hZP3/pREP4 expression vector construct using the calcium phosphate precipitation method. Approximately 200 single colonies of the stable-transfected cells were isolated by the cloning-ring method. Direct PCR, RT-PCR, and ELISA were used to identify and select clones; ten of the isolated clones were found to be positive.

Two of the ten selected clones (SKC-P3-12 and SKC-P3-19) were used for further studies. Culture medium was collected from transfected and non-transfected cells (negative control), and the samples were purified by wheat germ agglutinin (WGA)-lectin chromatography and anti-WGA immunoaffinity chromatography; the final eluted fraction was concentrated by ultrafiltration with a limiting molecular weight cut off at 30 kDa. The secreted glycoproteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining, Western blot analysis using a specific anti-ZP3 antibody, and by a metabolic labelling study using ^{3}H-galactose. Several bands including a band at 65 kDa, were observed in both non-transfected and transfected medium by silver staining. Distinct bands were identified by Western blot analysis at 65 kDa and 100 kDa for the recombinant product; solubilized human zonae pellucidae demonstrated a molecular mass of native hZP3 at approximately 65 kDa, and was at the same position identified for the recombinant product. In the

metabolic study, several distinct bands including the band at 65 kDa, were identified as biosynthesized glycosylated recombinant proteins.

In the hemizona assay (a test for sperm-zona pellucida binding), a significant inhibition (>40%) of binding was observed in competition studies using a 1:4 dilution of the WGA-purified recombinant glycoprotein solution. This material did not affect sperm viability or motility, and induced a more than 50% increase in the acrosome reaction of sperm after overnight incubation.

ACKNOWLEDGEMENTS

I wish to especially thank Dr. Sergio Oehninger, my dissertation advisor, for his direction and encouragement throughout the entire course of this study.

I would also like to thank the other members of my dissertation committee, Dr. Ke-Wen Dong, Dr. Mark Elliott, Dr. Frank Castora, and Dr. James Yuan, for their support, suggestion, comments and advice on my research and dissertation.

Also, my appreciation goes to Dr. William Gibbons, Dr. Howard White, Dr. Christopher Osgood, Dr. Gary Clark, Dr. Mary Mahony, Dr. Klaus Hinsch, Ms Linda Adamson, Ms Suzanne Cartwright, and Mr. Simon Chi for their support, help and assistance on this study.

Lastly, I want to express my gratitude to my family and my other friends here in the Jones Institute. It has been a privilege to work here for the past years.

TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

I. INTRODUCTION

A. Human fertilization

Fertilization is one of the most important and interesting subjects in the study of human reproduction because it concerns the first contact between the two gametes and the very beginning of human life. It occurs as a series of orchestrated and regulated events that culminate in the fusion of the sperm and oocyte to form a one-cell zygote, the obligatory precursor of all cells in the embryo.

Fertilization can be divided into the following sequence of events: (1) penetration of the cumulus mass by the sperm cells, (2) attachment and primary binding of sperm to the egg's zona pellucida, (3) induction of the acrosome reaction, (4) secondary binding and penetration of sperm through the zona pellucida, and (5) fusion of the ovum and spermatozoon and the activation of egg [1-8].

Gametogenesis is the process of formation and development of gametes or germ cells. During spermato-genesis, the chromosome number is reduced by half (haploid), and the shape of the cells is altered. A mature sperm cell must undergo two meiotic divisions and a process of differentiation which takes about sixty-four days to be

completed. The mature sperm cell is a free-swimming, actively motile cell consisting of three regions, namely head, neck, and tail. The head contains the haploid nucleus, and the anterior two thirds of the nucleus is covered by a head cap known as acrosome which contains a large array of powerful hydrolyzing enzymes and structural proteins [7,9, 10]. Among these enzymes, hyaluronidase and acrosin are the two acrosomal enzymes that have been most extensively studied and well characterized. It seems likely that a high degree of acrosomal organization may be required to assure the release of acrosomal components in a precise order as the spermatozoa penetrate through the egg's zona pellucida [7,9,11]. Interestingly, glycoprotein is a major component of the acrosomal matrix [11-13] and therefore is supposed to be important in many physiological functions of sperm during fertilization, including the conversion of proacrosin into enzymatically active acrosin [14-17]. Mitochondria are abundant in the proximal portion of the sperm tail, supplying the energy for the vigorous sperm motility [1,2,7, 18].

Spermatozoa that have matured in the epididymis are capable of moving actively, yet they still have to undergo the process known as capacitation to gain the capacity for fertilization after residing in the female tract for some period of time [19,20]. Even though the molecular basis is not fully understood, capacitation is believed to involve a

variety of metabolic/biochemical changes in preparation for fertilization [1,21].

Under *in vitro* conditions, and after about two hours of contact with capacitation medium, the spermatozoa begin to move much more actively than ever before. They swim in a characteristic movement seen as a very vigorous whiplashlike beating of the tail, often with the sperm head tracing an erratic figure-eight pattern (also known as nonprogressive or dancing movement) interspersed with brief episodes of linear movement. This is known as hyperactivity motility. The strong thrusting power generated by the vigorous tail movements may facilitate spermatozoa in swimming through the viscous fluid of the oviduct [18,22-24] as well as the relatively thick zona pellucida [1,7,15,19,20,22,23,25,26].

Acrosome reaction as well as hyperactivity motility are the outcome of the capacitation process. The healthy sperm have to go through this modification step (capacitation), especially the modification of the composition of the cytoplasmic membrane, in order to make themselves suitable for a successful physiological response during acrosomal exocytosis and fertilization events [3,7, 8,21].

Many compounds have been found to demonstrate the ability for activating the acrosome reaction, for example, follicular fluid, hyaluronidase, calcium ionophores, and zona pellucida proteins. In fact, a large fraction of sperm lose their acrosomes before they reach the surface of zona

pellucida during their penetration through the cumulus matrix. Although both of the acrosome-intact and acrosome-reacted sperm cells are able to attach to the zona pellucida in the mouse, the species best characterized so far, only the acrosome-intact ones are found to be capable of tight binding to the zona pellucida [7,17,27-36].

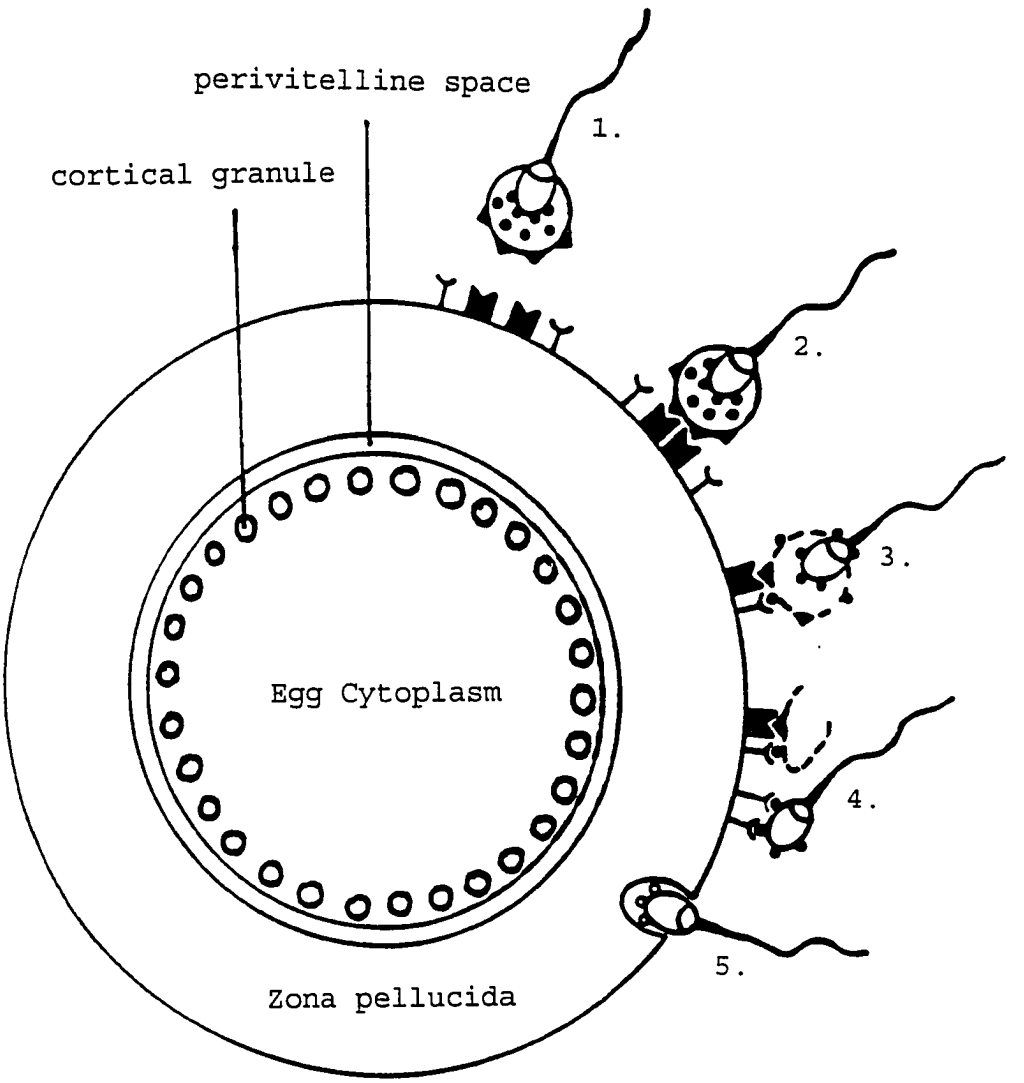
Zona pellucida protein 3 (ZP3) and zona pellucida protein 2 (ZP2) are, respectively, thought to be the first receptor and the second receptor for the specific recognition and binding between the zona pellucida and the sperm cells, but the detailed mechanism of their interaction is still unknown [4,6,37-39]. Generally speaking, the mechanism of acrosome reaction and even the individual physiological functions of the activators of the reaction, are still not clear.

B. Sperm-egg interaction and hZP3

In the mouse, sperm-egg interaction during fertilization consists of a sequence of events that take place in a compulsory order as follows (Fig 1) [2,5,7,8, 15,18,39]:

1) First of all, the sperm cells pass through the corona radiata and contact the oocyte. At this step, the attachment is random, loosely associated and not species-specific. Therefore the attached sperm can be usually washed away by simple agitation, and this is one of the most

Fig 1. Sperm-egg interaction during fertilization: The mouse model. The picture is adapted and modified from reference (54). According to this model, a spermatozoon recognizes and binds with ZP3 (primary receptor in the zona) ; this is followed by induction of the acrosome reaction by ZP3 in the zona-bound sperm cell; after this, a secondary sperm receptor is exposed and interacts with ZP2. This is followed by zona pellucida penetration.



important steps of the hemizona assay (HZA) method which will be discussed in a later chapter.

2) Subsequently, some spermatozoa bind tightly and specifically with the oocyte via the interaction between sperm and oocyte receptors, i.e. ZP3 and its corresponding sperm receptor. This process is defined as the tight binding event. Unlike the previous attachment event, tight binding is thought to be irreversible, and it is generally agreed that tight binding is required to allow penetration of the specialized covering of the ovum, the zona pellucida [29].

3) The acrosome reaction is the next event that follows the tight binding event. During fertilization, this organelle has to break to release its contained enzymes to enhance the penetration of the sperm into the zona pellucida. As we mentioned before, some sperm cells' acrosomes are broken during their penetration process through the cumulus matrix and follicular fluid, but only the acrosome-intact ones have the potential to penetrate the extremely thick extracellular matrix surrounding the ovum and fertilize the ovum. The acrosome reaction occurring between the intact sperm and the zona pellucida can be divided into two steps: (1) fusion of the outer acrosomal membrane and sperm plasma membrane, and (2) releasing of the associated enzymes and small membrane vesicles from the sperm head and inner acrosomal membrane. It is believed that the acrosome reaction is a signal transduced exocytosis

process involving at least two of the protein kinase systems (protein kinase A and protein kinase C) [4,36,40,41], but the detailed mechanism is still to be clarified [16,30,31].

4) The acrosome-reacted sperm cells are the only ones that have the chance to penetrate the zona pellucida and reach the perivitelline space. Normally only one of them is able to be the first and the unique one to fuse with the plasma membrane of the ovum, and then get into the ovum to form a zygote [29,32,42].

5) Then the development of the zygote starts. Meanwhile, the electrical potential of the egg plasma membrane changes almost immediately after the spermatozoon reaches the ovum. The plasma membrane of the egg becomes "hardened" in seconds due to these effects, and therefore blocks additional sperm binding and penetration.

6) The zona pellucida is modified and becomes "hardened" by the releasing of enzymes from the cortical granules which are membrane bound, lysosome-like organelles, occupying a region of the egg cytoplasm just beneath the plasma membrane. The cortical granules which accumulated during oocyte growth as a product of the Golgi body, fuse with the egg plasma membrane and release their contents into the perivitelline space. This so called "zona reaction" happens in seconds after fertilization.

Among all these events, the event that influences the sperm binding to the zona pellucida is an initial, crucial

recognition step leading to fertilization. Recent investigations into the molecular composition and genetics of the zona pellucida and sperm membrane receptors have provided much more detailed information about the molecular basis of this critical event during fertilization, especially in the murine species. It is well accepted that ZP3 has a major role on the specific, primary sperm binding to the zona, and has the potential to induce the sperm's acrosome reaction. This follows studies using both crude solubilized mixture of zonae pellucidae as well as intact eggs in the mouse model, which is the best characterized species so far in ZP3 research [4,9,30,37,38,40,43].

C. Zona pellucida

Beginning at puberty, as the primary oocyte ensues development, the single layer of flattened, follicular epithelial cells surrounding the egg become cuboidal and then columnar, forming a primary follicle. The primary oocyte soon becomes surrounded by a covering of amorphous intercellular material known as the zona pellucida. In fact, the zona pellucida is a very thick and unique extracellular matrix surrounding the female gamete, constituted by zona pellucida fibers in a specific manner [28,44-46]. Compared to the oocyte itself, the thickness of the zona pellucida is more than half of the radius of the ovum inside depending on the species and the stages of the growing follicle [5,7].

The organization of the human zona pellucida is not yet clear, whereas the evidence obtained from immunohistochemistry and autoradiographic visualization demonstrate that at least two layers of organization exist in the human zona pellucida [28,44,45]. Each fiber is composed of several individual filaments in the mouse model and it may be similar in the human [44].

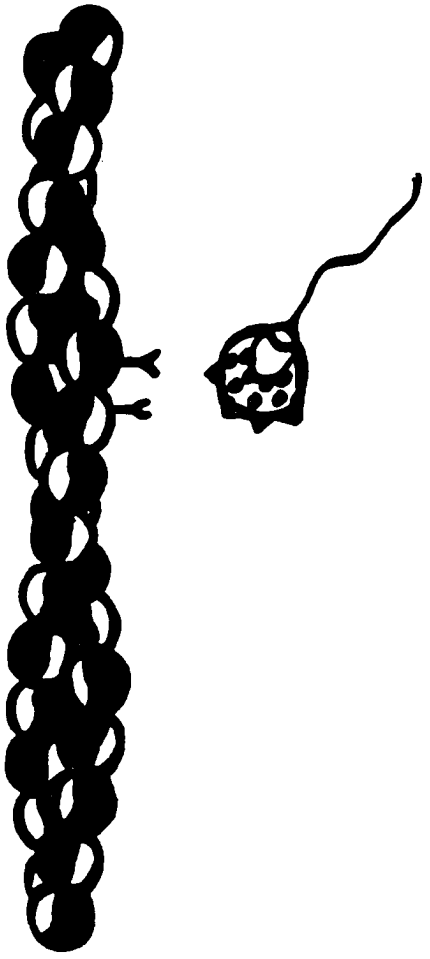
It is believed that ZP3 and ZP2, which are two of the major components of the zona pellucida, bind together to form a dimer and the dimer acts as the building subunit of the zona filament (Fig 2). Another glycoprotein, ZP1 which is also abundant in the zona pellucida serves as linker between zona filaments and ZP3-ZP2 dimer subunits [7].

D. Zona pellucida proteins

The zona pellucida proteins (ZP1, ZP2, and ZP3) are distinguished from each other by differences of their characteristic migration positions on denaturing gel electrophoresis (SDS-PAGE). ZP3 is believed to be the primary sperm receptor, and ZP2 the secondary sperm receptor, whereas the function of the ZP1 is structure-related [18,44,47].

Both the mouse and human ZP3 proteins are composed of 424 amino acids, and are 67% identical in their amino acids composition with the greatest similarity in the center of the protein. The human ZP3 protein (12% acidic, 8% basic,

Fig 2. Structure of the zona pellucida filaments: ZP3 (in black) and ZP2 (in white). In addition, ZP1 protein is another major component in the zona pellucida filament, but it is structure related, and is not shown in the figure. The arrow signs represent different oligosaccharides which are responsible for the recognition and binding with the sperm cell.



7% aromatic, and 32% hydrophobic residues) has a calculated molecular mass of 47,032 Da (nonglycosylated) [40].

ZP3 has a very hydrophobic region consisting of 26 amino acids near the carboxyl terminus which may play an important role in the intracellular trafficking of these secreted proteins or in their interactions in the extracellular matrix [39,40].

In the mouse, the ZP3 is believed to have 3-6 complex-type N-linked carbohydrate side chains, and an undetermined number of O-linked sugars [5,39]. The O-linked oligosaccharides on ZP3 have been identified as the primary sperm-binding moiety [48], and galactose and N-acetylglucosamine are two carbohydrates that have been implicated as essential for the sperm receptor activity of the zona in mice [49]. ZP3 and ZP2 share one common structure motif with a very hydrophobic region consisting of 23 and 26 amino acids respectively, near the carboxyl terminus. In the mouse, the ZP3 polypeptide chain has a 22 amino acids signal sequence for the glycosylation of the protein at its N-terminus [5,39,40,50-52].

Once the zona pellucida proteins are incorporated into the extracellular matrix, they are found to be very stable and have a long half-life, and it is estimated that the zona pellucida proteins in the natural conditions maintain their biological activities for days (>100 hr) [39].

Compared to the mouse zona pellucida (mZP), less information is available for the human zona pellucida (hZP). In the human, the acrosome-initiating activity of intact and solubilized human ZP has been reported [30,32,34,42]. However, these experiments are all performed by using very crude mixture samples with almost all of the zona pellucida components in it. Whether hZP3 alone or multi-components are necessary for the induction of the acrosome reaction of human sperm is still unclear. However, the intact and tertiary structure of ZP3 is suggested to be critical for the induction effect of the acrosome reaction [6,9].

Similar to the mouse, the human zona pellucida is also composed of three major glycoproteins: ZP1, ZP2, and ZP3. Besides, two isoforms of human ZP3 have been reported, named $ZP3_H$ and $ZP3_L$, but whether they represent different post-transcriptional modifications of a single ZP3 gene product, the presence of an additional gene, or they simply just demonstrate the heterogeneous glycosylation products remain to be clarified [39,40,42,53].

The molecular weights of human zona pellucida proteins are species-related and demonstrate heterogeneity due to various degrees of glycosylation. According to J. Dean, the molecular mass of human zona pellucida proteins are: ZP3, from 57 to 73 kDa; ZP2, from 64 to 76 kDa; and ZP1, from 90 to 110 kDa [39]. The human ZP3 has a polypeptide backbone of 424 amino acids with complex type N-linked and

O-linked oligosaccharide side chains [39]. The human polypeptide chain contains four potential N-linked glycosylation sites, and sixty-six potential O-linked glycosylation sites [40].

E. Developmental expression of hZP3 and the biosynthesis of glycoprotein

The synthesis of zona pellucida proteins is first detected when oocytes enter their two weeks growth phase before ovulation [39]. Investigation in the mouse model has concluded that the zona pellucida proteins are synthesized in only growing oocytes and secreted into the extracellular matrix [54-61].

In a mouse experiment, various sizes of oocyte follicles in the ovary were isolated and the expression of ZP3 was monitored by the ratio of its mRNA among total RNA. At its peak in 30-35 μm diameter oocyte, zona pellucida synthesis represented 7-8% of total protein synthesis. Zona production declined in the fully grown oocyte and was absent or not detectable in ovulated eggs and the embryo [39]. In the ovulated eggs, the mRNAs of ZP2 and ZP3 are found to be 200 nucleotides shorter than those found in the growing oocyte, implying the possibility of developmental regulation of the expression of the ZP gene between these two phases of the oocyte [56]. The mechanism for the regulation of the oocyte-specific and developmental expression of the ZP3 gene

is still not clear, although it is believed the hormones and cytokines may play important roles on these functions [7].

Little is known on the human zona pellucida due to the difficulty in obtaining human oocytes and zona pellucida protein. Therefore, it was believed to be necessary to apply molecular biology techniques to supply enough hZP3 for investigation. Recent work reported an established Chinese Hamster Ovarian cell line (CHO-K1) transfected with a hZP3-reconstructed mini-gene which was capable of expressing a recombinant hZP3 glycoprotein. However, the reported molecular mass was at least 5 kDa lower than the native hZP3 protein. The biological activity of the recombinant hZP3 protein was only reported in acrosome reaction assays and sperm-egg fusion assays. The most important biological activity, that is the sperm ligand capacity, was not reported [62].

Since hZP3 is a glycoprotein, and the oligosaccharides have been proven to be critical for the biological functions of sperm-egg interaction [29,39,48-50,52,63-65], it is believed that the biological activity of the hZP3 is dependent on the secondary modification of the polypeptide backbone, that is the glycosylation. The glycosylation of a protein is thought to be species-dependent due to the different "glycosylation machinery" in various cell types, therefore, it is of highly interest to study the production of recombinant hZP3 glycoprotein in human ovarian cell

lines. In addition, the "glycosylation machinery" is organized by the Golgi body and rough endoplasmic reticulum (RER), including their constituted glycosylation-related enzymes, and peptidyl-receptors. In addition, oligosaccharide-carriers, and the sugar transportation systems of the cell itself are also important for the biosynthesis of a glycoprotein.

It is believed that hZP3 has three complex type N-linked (asparagine-linked) oligosaccharide side chains and an unknown number of complex type O-linked oligosaccharides [39]. Typically, these two types of oligosaccharides in hZP3 glycoprotein are both rich in galactose. The complex type asparagine-linked oligosaccharides contain a variable number of outer chains linked to a core of N-acetylglucosamine and mannose. The outer chains are consist mostly of galactose and sialic acid. In the complex type O-linked oligosaccharides, a similar core exists which is also rich in galactose residues (typically, >50%) [66-68].

One obvious difference between the biosynthetic pathways of these two types of oligosaccharides lies in the involvement of the oligosaccharide-pyrophosphate-dolichol (oligosaccharide-P-P-Dol) which is necessary for the biosynthesis of N-linked oligosaccharide but not O-linked oligosaccharides.

The pyrophosphate-dolichol (P-P-Dol) is important for the transportation of N-linked oligosaccharide, and

therefore the biosynthesis of N-linked oligosaccharide is dependent on the concentration of intracellular P-P-Dol. It is expected that P-P-Dol plays a crucial role for the regulation of glycosylation and biosynthesis of a glycoprotein. The assembling, transferring, and processing of the oligosaccharide-P-P-Dol is finished in the RER and the product is localized on the inner membranes of the RER. Actually the glycosylation of all glycoproteins is started at the inner membrane of RER. And generally speaking, most of the modification, addition and deletion processes of the oligosaccharides of the glycoproteins is completed in the Golgi body.

F. hZP3 gene and cDNA

The human ZP3 gene is comprised of eight exons and the overall structure of the zona genes and their products is thought to be conserved among mammals. Using cross-hybridization with mouse nucleic acid sequences as a criterion, the conservation of ZP3 genes of human is more related to mouse than that of pig and rabbit [39,40].

Similar to mouse and hamster, the human ZP3 mRNA has short 5′ and 3′ untranslated regions which are very unusual and characteristic among all species found. The polypeptide of human ZP3 deduced from the open reading frame of its mRNA has a molecular mass of approximately 47 kDa and consists of 424 amino acids [1,28,69-71].

The characterization of the cDNA encoding mouse ZP3 has made the expression of recombinant ZP3 (rZP3) in tissue culture lines possible and represents a potential option for obtaining large amounts of murine ZP3. However, since glycosylation appears mandatory for ZP3-ligand function, it has been unclear whether rZP3 obtained from cells other than oocytes would possess the necessary post-translational modifications required for biological activity [37].

Recently, a full length cDNA encoding mouse ZP3 was cloned into expression vectors [37]. Mouse L-929 cells and green monkey cells were transfected with the mouse ZP3-expression vector constructs, and the recombinant mouse ZP3 protein (rmZP3) was purified by immunoaffinity chromatography from the culture medium and detected on Western blots using a mouse monoclonal antibody against an oligosaccharide side chain of hZP3. Although this rmZP3 differed slightly from native mZP3 in its molecular mass, it demonstrated biological activity in homologous murine sperm-zona pellucida competition assays and in triggering mouse acrosomal exocytosis in capacitated mouse sperm. Thus, according to these authors, rmZP3 isolated from both rodent and primate cells appears to contain those carbohydrate and protein structures necessary for ZP3's dual role in fertilization in the mouse.

Another approach has been tried by introducing a hZP3-minigene construct into the Chinese Hamster cell line (CHO-

K1), but the recombinant hZP3 produced was at least 5 kDa lower in molecular mass even though the transfection process seemed to be successful. As discussed previously, the biological activity was not satisfactory [62]. These facts imply that the glycosylation process of hZP3 in the transfected host cell may be critical for the expression of biologically active hZP3.

II. SPECIFIC AIMS

The specific aims of this study are: (1) To produce a full-length cDNA of hZP3 and to express human ZP3 in a human ovarian cell lines (PA-1) ; and (2) To characterize the biological activity of the recombinant product as sperm ligand and acrosome reaction inducer.

A. Significance

Many couples suffer from infertility, but in some of the cases the pathophysiological causes are still unclear. This mainly applies to male-factor infertility, where many cases are still considered "idiopathic", or, in other words, the cause is unknown. Even though *in vitro* fertilization (IVF) and other assisted reproductive techniques have been widely and successfully applied to these problems, little is known about their pathological origin, and the efficiency of the therapy is actually low and depends on an uncertain principle of "getting the gametes closer together". Because of our lack of complete understanding in the molecular details of fertilization, there is not an accurate and satisfactory tool or assay to assess all of these sperm dysfunctions. Recently, cellular and molecular advances in gamete biology have elucidated the structure and interaction

of the binding receptors on the surfaces of both sperm and egg, principally in the mouse model. It is very interesting for us to study the binding receptors in human species by using molecular biology techniques.

Among several molecules involved in the event of fertilization, ZP3 protein is believed to be the first receptor in the zona pellucida that specifically recognizes and binds with the sperm. Therefore, ZP3 may play a very important role in fertilization, as determined during the pathophysiological study of some potential causes of male infertility and in the clinical diagnosis of fertility. In addition, ZP3 has been reported to be highly immunogenic [53,72-74] and therefore ZP3 may also be critical for the design of long-term immunocontraceptive methods.

The difficulty in obtaining enough human oocytes and preserving solubilized hZP3 protein has made further studies difficult due to the scant natural source of human ZP3. Therefore we sought to produce a stable transfected cell line to supply enough recombinant human ZP3 with biological activity for further studies by using molecular biology techniques.

The information provided by this study could have significant impact in many areas within the Reproductive Biology/Medicine field: (1) the physiology of human sperm-zona pellucida interaction and pathophysiology of the clinical disorders affecting human fertilization may be

critically reviewed and expanded (i.e., temporal relationship between zona binding and penetration, acrosome reaction and motility patterns, isolation of the putative ZP3 receptor from human sperm, etc.); (2) a solid or liquid phase assay may be developed using the artificial (recombinant) ZP3 thus giving access to a much simpler and universally applicable clinical diagnostic method to assess human sperm-zona pellucida interaction; and (3) the generation of a "naturally" glycosylated recombinant ZP3 may be exploited for engineering a contraceptive vaccine against the human zona pellucida and therefore advance fertility regulation research.

B. Experimental design

Our initial approach consisted of the isolation of mRNA from the human ovary, followed by the use of RT-PCR for generation of a full length cDNA for human ZP3. The cDNA was then purified by the geneclean glass-milk method and was respectively subcloned into PSK and pREP4 expression vectors. Autosequencing, Southern blot analysis, restriction mapping, PCR and in vitro translation were performed to identify the cDNA of hZP3.

The hZP3/pREP4 DNA construct was stably transfected into human ovarian PA-1 cell line by the calcium phosphate precipitation method. Single colonies were isolated by the cloning-ring technique [75]. PCR was performed to confirm

the integration of the inserted cDNA into the genomic DNA of the host cell. Because that the mRNA of zona pellucida proteins are expressed in the oocytes at their two-weeks growth phase before ovulation, and there is little or no mRNA of ZP3 found in ovulated oocytes in the study of the mouse model [39,57], there will be no mRNA expected in the non-transfected PA-1 cells. RT-PCR was performed to identify the expression of the inserted cDNA in transfected cells. Identification of the production of hZP3 protein was performed by ELISA and Western blot analysis. A metabolic labelling study by substitution of ^{3}H-galactose in the culture medium was performed to identify the glycosylated product of hZP3 expression. Solubilized human zonae pellucidae were used as positive controls. Lectin-affinity chromatography, immunoaffinity chromatography and ultra-filtration were used for protein isolation and purification.

The biological activity of the recombinant ZP3 was assessed in two ways: (1) The capacity of the recombinant protein to compete (competitive inhibition) with the natural zona pellucida for sperm binding was examined by the hemizona assay. Dose-response studies were performed to determine the efficiency of the recombinant ZP3 as a sperm ligand, and sperm motility was also monitored to further characterize the biological activity. (2) The capacity of the recombinant protein to induce a "physiologic" acrosome reaction was assessed by using lectin staining (Pisum

sativum agglutinin) staining combined with immunofluorescent techniques. Consequently, the criteria used for the identification of the secreted recombinant protein were immunogenicity, molecular mass, and biological activity.

III. MATERIALS AND METHODS

A. Materials

1. Oligonucleotide primers:

Oligonucleotide primers (CH-1, CH-2, and S1 primers) were designed for this study as follows: CH-1 primer (5' ACCATGGAGCTGAGCTATAGG 3') and CH-2 primer (5'TTATTCGGAAGCAGACACAGG 3') were purchased from Oligos, Etc. (Wilsonville, OR). S1 primer (5' GTCAGCCGGGCCTTCCACTGG 3') was synthesized at the Microbiology Department, EVMS. (Dr. Stenberg)

2. Cells, vectors and culture supplies:

A human ovarian tumor cell line, PA-1, was purchased from American Type Culture Collection (ATCC CRL 1572, Rockville, MD). XL1-Blue Ecoli cells were from Strategene (La Jolla, CA). pBluescript SK (+) vector (PSK) was purchased from Stratagene. A mammalian cell expression vector, pREP4, was purchased from Invitrogen (San Diego, CA). Trypsin-EDTA solution and minimum essential medium eagle (MEM) were purchased from Sigma (St. Louis, Mo). Fetal bovine serum (FBS) was from Hyclone (Logan, Utah), Ham's F-10 was from Gibco Lab (Grand Island, NY).

3. DNA markers:

DNA molecular weight marker III was purchased from Boehringer Mannhein (Indianapolis, IN), and 123 DNA ladder was from Gibco BRL technologies (Grand Island, NY).

4. Radioactive materials:

[γ-^{32}P]-Deoxyadenosine 5'-triphosphate for radioactive labelling of oligonucleotide probe and ^{35}S-methinine (translation grade) for *in vitro* translation were purchased from Du-Pont NEN (Boston, MA). ^{13}C-protein rainbow marker (14.3-200 kDa) for fluorography was purchased from Amersham (Arlington Heights, IL).

5. Enzymes and other chemicals:

Restriction enzymes used in recombinant DNA procedures were purchased from New England BioLabs (Beverly, MA), Promega (Madison, WI) and Boehringer Mannheim.

Most of the chemical materials purchased from Sigma Chem. Co. (St. Louis, MO) except the following materials:

TNT T3 Couple reticulocyte lysate (for *in vitro* translation), polytract mRNA isolation kit, Magic Maxiprep kit (for plasmid DNA isolation), T4 polynucleotide kinase, alkaline phosphatase (Calf intestine), Tag polymerase (for "direct PCR" and "RT-PCR diagnosis", see Methods), lysozyme, RNasin and dNTPs were purchased from Promega. Taq polymerase (for production of full length cDNA of hZP3), Proteinase K,

IPTG (isopropyl-beta-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) were purchased from Stratagene. M-MLV reverse transcriptase, DTT and oligo$(dT)_{12-18}$ were from Gibco BRL. (FITC)-labeled Pisum Sativum agglutinin (PSA), Vectastain Alkaline Phosphatase ABC kit, BCIP/NBT kit, Wheat germ agglutinin (WGA) lectin and anti-WGA antibody were from Vector (Burlingame, CA). The non-radioactive protein rainbow marker (14.3-200 kDa) was purchased from Amersham, Geneclean glassmilk (for DNA isolation) was purchased from Biol 101 (La Jolla, CA), BCA protein assay kit was from Pierce, sheared salmon sperm DNA was from 5 prime -> 3 prime Inc. (Boulder, CO), TMB (for ELISA) was purchased from Pierce (Rockford, IL), peroxidase-Goat-anti-rabbit-IgG (H+L) was from Zymed (San Francisco, CA) and fluorographic amplification reagent was purchased from Amersham.

6. Non-chemical materials:

Most of the non-chemical supplies were purchased from Fisher (Pittsburgh, PA), or Costar Corning (Cambridge, MA) except the following materials.

PCR reaction tube was purchased from Perkin Elmer (Norwalk, CT), Whatman 3 MM paper was from Schleicher & Schuell (Keen, NH), dialysis tubing (3500 Da MW cut off) was from Spectra (Houston, TX), X-ray film (Fuji RX) was from Fisher, nitrocellulose membrane (0.45 μm) was from Costar

and Centricon and Centriprep (with 30 kDa cut-off) were purchased from Amicon (Beverly, MA).

B. Methods

1. Cell culture

PA-1 (ATCC CRL) and the transfected cells were cultured with MEM containing 10% FBS [77,78]. Typically, confluent cells were split at 1:6 dilution in MEM containing 10% FBS. The stable transfected cells were maintained in MEM with 150 μg/ml of hygromycin (antibiotic) for at least three weeks to establish the stable transfection cells, and then the culture was continued in regular medium for further application [76]. FBS with 9% Dimethylsulfoxide (DMSO) was used as cryogenic medium for the storage of cells. The cells were spun down, washed by PBS buffer, resuspended in the cryogenic medium, and then frozen in liquid nitrogen following standard procedures.

2. Medium collection from the culture cells

For ELISA, the culture medium was collected when the cells reached 70% confluence to get a detectable amount of the recombinant glycoprotein secreted. The collected medium was centrifuged promptly to separate the supernatant, and protease inhibitors (1.0 mM PMSF, 0.7 mg/ml Pepstatin A, 0.5 mg/ml Leupeptin, and 1.0 mM EDTA) were added. The samples

were stored at -80 °C until used [79].

For the isolation of the recombinant glycoprotein by the chromatography method (described later), culture medium was collected in short periods of culture by using the following procedure: Cells were adjusted to 15% confluence in medium (MEM) containing 10% FBS, cultured for 4 hr to allow cells to attach to the plate, washed once with PBS to remove the suspended cell bodies. Then culture medium was changed by 5.0 ml of fresh MEM with 10% FBS. Culture medium was collected every 12 hr until the cells reached 70% confluence. The collected medium was treated as above description (protease inhibitors added and centrifuged promptly) and stored at -20°C until used.

3. Isolation of RNA

Glassware used for the RNA isolation was all baked at 200°C overnight. Plastic centrifuge tubes were thoroughly rinsed before usage with washing solution (0.1 N NaOH, 1.0 mM EDTA), and then rinsed with RNase-free water. Other plasticware was soaked in 0.05% diethyl pyrocarbonate (DEPC) overnight to remove the organic impurity and then autoclaved for 30 min to degraded residual DEPC. All solutions were treated with 0.05% diethyl pyrocarbonate (DEPC) overnight at room temperature and then autoclaved for 30 min to remove any trace of DEPC.

Isolation of total RNA from the human ovary

Total RNA was isolated by modifying Chirwin's guanidine thiocyanate method [80]. "Denaturing solution" was prepared by adding 132.0 ml of CSB buffer (0.042 M sodium citrate, 0.028 M N-lauroylsarcosine, 0.0014% mercapto-ethanol, pH 7.0) to 100.0 g of guanidine thiocyanate. An aliquot of 12.0 ml of "denaturing solution" was dispensed into a sterile 50 ml centrifuge tube and chilled on ice for 5 min. Approximate 1.0 g of human ovary was chopped into small pieces by sterile surgical knife, transferred into the denaturing solution, and homogenized by a polytron. After 1.2 ml of sodium acetate solution (2.0 M, pH 4.0) was added to the lysed cell solution, the RNA was extracted with 12.0 ml of phenol:chloroform:isoamyl alcohol (25:24:1). The RNA was precipitated at -20°C overnight by adding 1 volume of isopropanol. The RNA was pelleted by centrifugation at 10,000 x g for 15 min at 4°C, washed with 12.0 ml of 75% ethanol, dried in a vacuum desiccator for 20 min, and then dissolved in RNase-free water for storage at -20°C. Finally, the concentration of RNA was determined by measuring the absorbance at 260 nm [81].

Isolation of mRNA

The mRNA was further purified by the method of polytract mRNA isolation system following manufacturer's description (Promega). A biotinylated oligo(dT) primer was

used in the system to hybridize to the 3'poly(A) region present in eukaryotic mRNA species. The mRNA-biotinylated oligo(dT) hybrids were captured by paramagnetic particles which were pre-coupled with streptavidin at high stringency, washed thoroughly and then isolated by using a magnetic separation stand. The mRNA was eluted from the solid phase with RNase-free water.

One milligram of total RNA isolated as described above was brought to a final volume of 500 μl in RNase-free water. For the purpose of annealing of biotinylated-oligo-$(dT)_{12-18}$ primer with the mRNA, the total RNA solution (500 μl) was incubated at room temperature for 10 min by adding 3.0 μl of the biotinylated-oligo$(dT)_{12-18}$ primer and 13.0 μl of 20 x SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0).

Streptavidin-coupled magnetic sphere particles (SA-PMPs) were prepared by gently flicking the bottom of the tube until completely dispersed, and captured by placing the tube in the magnetic stand. The SA-PMPs were collected at the side of the tube, washed three times with 0.5 x SSC (0.3 ml each) and then resuspended in 0.1 ml of 0.5 x SSC.

In order to isolate the mRNA, the entire content of the annealing reaction above was added into the tube which contained the washed SA-PMPs, and the reaction was incubated at room temperature for 10 min. Then the mRNA-oligo(dT) hybrids were captured by SA-PMPs, washed four times with 0.1 x SSC (0.3 ml per wash) , and then eluted from SA-PMPs by

two fractions of 0.1 ml of RNase-free water. The eluted mRNA solution was centrifuged again at 10,000 x g at 4°C in order to remove the magnetic particles.

Isolation of cytoplasmic RNA (sucrose gradient method)

For the purpose of clonal selection of the stable transfected cells by RT-PCR, the total cytoplasmic RNA was isolated by sucrose gradient method as follows: A plate of 70% confluent cells was washed with sterile PBS, and then the cells were scrapped out from the petri dish by a rubber policeman with 500 μl of lysis buffer (1.5 mM $MgCl_2$, 0.5% NP-40, 0.25% Sodium deoxycholate, 0.3 M sucrose, 10.0 mM Tris, pH 7.4) on ice. The cell lysate was carefully laid onto a 350 μl of Cushion buffer (1.5 mM $MgCl_2$, 10.0 mM Tris, 0.4 M sucrose, pH 7.4) and spun at 5000 x g for 10 min at 4°C. A 500 μl fraction of the upper aqueous solution was transferred to a fresh tube. Then 58.0 μl of 10 x STE buffer (0.1 N NaCl, 10.0 mM Tris, 1.0 mM EDTA, pH 8.0), 12.0 μl of 10.0 mg/ml of proteinase K, and 17.0 μl of 5.0 M NaCl were added. The mixture was incubated at 45°C for 45 min, extracted with equal volume of phenol:chloroform (1:1). The RNA was precipitated overnight at -20°C with equal volume of isopropanol, spun down and washed once with 1.0 ml of 70% ethanol. The RNA pellet was dried and dissolved in RNase-free water. The quality of RNA was assessed spectrophotometrically (A_{260}/A_{280}) and by agarose gel electrophoresis [81].

4. RT-PCR

The purified mRNA was reversed transcribed to cDNA, and then the hZP3 cDNA was specifically amplified by PCR. The RT reaction was performed as follows: One microgram of mRNA, 20.0 units of RNasin, 100.0 pmol oligo$(dT)_{12-18}$, 1.0 mM of each dNTPs (dATP, dTTP, dCTP, and dGTP), 10.0 mM DTT and 100 U of M-MLV reverse-transcriptase were added in 5 x first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15.0 mM $MgCl_2$), and the final volume was added up to 20.0 μl by DEPC-treated water. The reaction was performed at 37°C for 30 min, then at 42°C for 15 min, stopped by incubation at 95°C for 5 min and chilled on ice at once [83].

The RT reaction above was directly applied to a PCR. The PCR was accomplished by using a pair of 21 base-pairs (bp) oligonucleotide primers (CH-1 primer and CH-2 primer, see Materials) designed based on the published sequence of the cDNA of human ZP3 [40]. The synthesized first-strand cDNA (20.0 μl) described above was denatured at 100°C for 3 min and then amplified by PCR with 2.5 U Taq polymerase by adding 15 pmole of each CH-1 and CH-2 primers and 10.0 μl of 10 x PCR reaction buffer (100 mM Tris-HCl (pH 8.8), 500 mM KCl, 15.0 mM $MgCl_2$, 0.1% (w/v) gelatin) in a final reaction volme of 100 μl. The thermocycler was programmed as follows: 94°C denaturing for 1 min, 55°C annealing for 1 min, and 72°C extension for 30 sec with a 2 sec extension per cycle, for a total of 35 cycles of amplification, followed by final

extension at 72°C for 3 min [83].

5. Subcloning of the cDNA of hZP3

The RT-PCR product was blunt-end ligated with EcoRI linkers, and then inserted into the PSK vector. In order to create blunt-ends, the RT-PCR product was subjected to a fill-in reaction at room temperature for 30 min by adding 2.5 mM of each dNTPs and 2.5 U the Klenow (large fragment of DNA polymerase I). For blunt-end ligation, EcoRI linkers were phosphorylated by T4 polynucleotide kinase. The fill-in reaction was heat-inactivated at 75°C for 20 min and then combined with the linker reaction prepared above. This was followed by addition of T4 DNA ligase and 10 x ligase buffer (500 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, 10.0 mM ATP and 500 μg/ml BSA), and incubated for 5 hr at 15°C for the purpose of blunt-end ligation. The reaction was again heat-inactivated at 75°C for 20 min, and EcoRI was then added to produce EcoRI sticky ends on the blunt-end ligated DNA in order to be inserted into the EcoRI site of the vector (PSK) [81,82].

Meanwhile, the PSK vector was digested by EcoRI, purified by phenol extraction. To prevent recirculation of the sticky-ends vector, the DNA solution was 5'-end dephosphorylated with alkaline phosphatase (CIAP, Promega).

Fine-ground glassmilk (Geneclean protocol, Biol 101) was utilized to purify the DNAs prepared above. The purified

hZP3 DNA prepared above, was ligated with the prepared PSK vector at 4-16°C for 18 hr. The DNA construct (hZP3/PSK) was transformed into XL1-Blue competent cells, using the Mandel and Higa method [71] and plated onto X-Gal ampicillin-agarose. The white clones were selected and grown in LB medium with 75 μg/ml ampicillin at 37°C.

The DNA plasmid (hZP3/PSK) was isolated from the selected clones by the alkaline-detergent method (mini-prep method) [81] and identified by restriction mapping (using BstXI, MvnI, and ScaI restriction enzymes)[40], Southern blot analysis (using a probe designed to identify an internal sequence of the cDNA), and PCR (using the same 5' terminal primer used in previous RT-PCR and the internal probe used in Southern blot analysis). In addition, *in vitro* translation (TNT T3 coupled reticulocyte lysate system, Promega) using radioactive methionine was carried out to ensure that a full length protein (non-glycosylated ZP3) is synthesized by this cDNA of hZP3 as determined by SDS-PAGE and autoradiography.

In order to express the cDNA of hZP3 in mammalian cells (human ovarian tumor cell), the cloned cDNA was subcloned into a mammalian cell expression vector (pREP4). The plasmid DNA (hZP3/PSK) isolated from the selected clones was digested with BamHI and XhoI restriction enzymes to produce sticky ends and then ligated with the episomal expression vector pREP4 (containing the Rous sarcoma virus

promotor and hygromycin-resistant gene) with T4 DNA ligase at 4-16°C for 18 hr. Restriction mapping, Southern blot analysis and PCR were again performed to select the correct clones. Approximately, 300 clones were analyzed. Spin column (Maxiprep, Promega) was used to produce high quality and large amounts of supercoiled DNA construct for the further application. Further purification of the cDNA construct was achieved by alcohol precipitation with the addition of 0.3 M sodium acetate at -80°C for 4 hr.

6. 5'-end radioactive labelling of oligonucleotide probe

The reaction for the radioactive labelling of the oligonucleotide probe S1 (see Materials) for Southern blot analysis was performed at 37°C for 10 min by adding 200 ng of the internal probe (S1), 70 μCi of [γ-^{32}P]ATP and 10 U of T4 polynucleotide kinase in 1 x forward exchange buffer (50 mM Tris-HCl, 10.0 mM $MgCl_2$, 0.1 mM spermidineand and 5.0 mM DTT, pH 7.5) with a final volume of 10.0 μl. The reaction was stopped by adding 5.0 μl of 0.1 M EDTA. Right before use, the radioactively labelled S1 probe solution was boiled for 10 min and then chilled on ice.

7. Southern blot analysis

Southern blot analysis was performed to identify the DNA constructs of the cDNA of hZP3 by using an internal probe S1 [81]. DNAs were run on an agarose gel, then the gel

was soaked in denaturing solution (1.5 M NaCl, and 0.5 M NaOH) for 45 min, and then in neutralization solution (1.5 M NaCl, 1.0 M Tris, pH 7.4) for 30 min. Blot transfer of DNA fragments to nitrocellulose membrane was performed by capillary action (simple diffusion method) in 10 x SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for 16 hr [81]. The blot membrane was air-dried on a 3 MM paper for 30 min, UV cross-linked at 0.12 joules per cm^2 of membrane for 1 min, and then prehybridized in a sealable bag with pre-hybridization solution (25.0 mM KH_2PO_4, 5 x Denhardt's solution, 50 μg/ml salmon sperm DNA, and 50% formamide in 5 x SSC buffer) at 42°C for 3 hr by gentle shaking [81].

The prehybridization solution was removed from the sealable bag and the blot membrane was transferred to a new bag containing the prepared oligonucleotide probe in hybri-dization solution (the same as prehybridization solution but without the Denhardt's solution). The bag was placed in water bath at 42°C with gentle shaking for 10 hr. The membrane was removed and washed in 1 x SSC with 0.1% SDS twice for 15 min each at room temperature, and then in 0.25 x SSC with 0.1% SDS twice for 15 min each at 42°C. The membrane was then air-dried on a piece of 3 MM paper, wrapped in saran wrap and put in a Kodak X-ray holder with Kodak XAR-5 X-ray film and intensifying screen for an appropriate duration of time at -70°C.

8. *In vitro* transcription/translation

TNT Couple Reticulocyte Lysate system was used to test the capacity of the cloned hZP3 cDNA to produce a full length nonglycosylated protein following manufacturer's instruction. DNA template (2.0 μg) was added into 25.0 μl of TNT rabbit reticulocyte lysate. Then 2.0 μl of TNT reaction buffer (Promega), 1.0 μl of T3 RNA polymerase, 1.0 μl of amino acid mixture without methionine, 4.0 μl of ^{35}S-methionine (1000 Ci/mmol at 10 mCi/ml), and 40 U of RNasin (RNase inhibitor) were added into the DNA solution above, and the final volume was adjusted to 50.0 μl by RNase-free water. The reaction was performed at 30°C for 2 hr. Luciferase DNA was used as a positive control, and the plasmid with opposite orientation insertion of hZP3 cDNA into PSK vector was used as a negative control. In order to analyze the result of translation, a discontinuous SDS-PAGE was run (with 10% separating gel and 4% stacking gel), gel was dried by gel-drier, and autoradiography was performed as described before.

9. DNA purification by CsCl ultracentrifugation

For the purpose of cell transfection, DNA construct (hZP3/pREP4) was further purified by ultracentrifugation in CsCl as follows: One liter of cells incubated overnight was pelleted at 5000 x g for 5 min and then was resuspended in 30.0 ml of alkaline lysis solution I (50.0 mM of glucose,

10.0 mM EDTA and 25.0 mM Tris, pH 8.0) by shaking and vortexing. A 30.0 ml of lysis solution buffer II (0.2 M NaCl and 1% of SDS) was added, and the mixture was incubated on ice for exactly 5 min. Then 22.5 ml of 5.0 M potassium acetate (pH 5.5) was added, and the lysate solution was incubated on ice for exactly 5 min. Centrifugation was performed at 10,000 x g for 20 min at 4°C and the supernatant containing DNA was carefully transferred to another tube. DNA was precipitated at room temperature for 20 min by adding 1 volume of isopropanol, spun down, air-dried, and then dissolved in 15.0 ml of TE buffer (1.0 mM EDTA, 10.0 mM Tris.Cl, pH 7.4).

Purification was achieved by sequential extraction one time with 1 volume of phenol, once with one volume of phenol: chloroform: isoamyl alcohol (25:24:1), and one time with one volume of chloroform: isoamyl alcohol (24:1). DNA was precipitated at -20°C overnight by adding 2.5 volumes of 100% ethanol and 0.3 M sodium acetate (pH 5.2), spun down, air-dried, and dissolved in TE buffer again to a final volume of 17.0 ml. Then 1.9 ml of 5% lauroylsarcosine solution and 19.6 g CsCl were added and mixed well until CsCl dissolved completely. After 1.2 ml of 10.0 mg/ml of ethidium bromide was added, the solution was separated to two "heat sealable" centrifuge tubes. Balance was corrected up to 0.1 g. The tubes were capped by heat, and then centrifugation was performed at 55,000 rpm (26,000 x g) with a

VTi65 rotor for 16 hr. The supercoiled DNA (the lower band) was collected through the side of the tube by syringe (with 18 gauge needle) under UV lamp, and was extracted with water-saturated n-butanol to remove the ethidium bromide until clear by checking under UV light.

The solution was dialyzed overnight with 2 l TE buffer to remove CsCl, and then DNA was precipitated at -20°C overnight by adding 1/10 volume 3.0 M sodium acetate (pH 5.2) and 2.5 volume 100% ethanol. The DNA was spun down, washed with 70% ethanol, air-dried, and then dissolved in 0.5 ml of TE buffer (pH 7.0). The concentration and quality was measured with A_{260} and A_{280} spectrophotometrically [81].

10. Stable transfection

PA-1 cell was stably transfected with the hZP3/pREP4 DNA construct by using calcium phosphate-mediated transfection method [81,82]. A 50 μg/ml of DNA solution was prepared by adding 62 μl of $CaCl_2$ and sterile water to a final volume of 0.5 ml. The DNA solution was added dropwise into 0.5 ml of sterile 2 x HBS (280 mM NaCl, 10.0 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 12.0 mM dextrose, and 50.0 mM HEPES, pH 7.05) under a stream of air, and then incubated at room temperature for 20 min to precipitate DNA. Right before use, the DNA precipitate was dispersed by vortex gently (speed 4 with a Vortex-Genie (Scientific Industries Inc, NY)) for 10 sec, and the medium was removed from the cells. The calcium

phosphate-precipitated DNA suspension was transferred carefully onto the cell monolayer and incubated at room temperature for 15 min.

Culture medium (MEM with 10% FBS) was then laid gently onto the cells. The cells were cultured for 18 hr to allow endocytosis happen, washed twice by PBS, and cultured for another 24 hr in regular medium.

Selection medium (regular medium with 150 μg/ml of hygromycin) was then used for three weeks to established the stably transfected cells [76,81,84,85], and the selection medium was changed every 2-3 days.

Cloning ring with 3 mm internal diameter was used to isolate the viable single colonies [75], and trypsin-EDTA was used to release cells. Approximately, 150 single colonies were isolated.

The integration of hZP3/pREP4 DNA into the host genomic DNA was identified by PCR, the expression of the cDNA of human ZP3 in the transfected cells was assessed by RT-PCR and the secretion of recombinant protein was assessed by ELISA (described later).

When the selected single colonies of transfected cells reached about 70% confluence in six-wells clusters, the cells were harvested, resuspended in cryogenic medium (9% DMSO/FBS), and stored in liquid nitrogen by following standard protocol.

11. RT-PCR for the clonal selection of stable-transfected cells

RT-PCR was performed for the identification of hZP3 cDNA expression in the transfected cells. The cytoplasmic total RNA was isolated by the method described before, dissolved in RNase-free water, and stored at -20°C if not used immediately [83].

In order to select the stably transfected clones from a large number of candidate clones, total RNA (10 μg) was used instead of mRNA in this RT-PCR. The RT reaction was performed as described before except that 1.5 mM of each dNTPs (dATP, dTTP, dCTP and dGTP) and 0.5 μg BSA were used to optimize the reaction. In addition, reaction was performed at 37°C for 1.5 hr, stopped by adding 30 μl of sterile water, incubated at 95°C for 5 min and then cooled on ice immediately.

The RT reaction was followed directly by PCR reaction. Only 7.5 μl of the RT final solution (prepared above) was used to perform a PCR, the remaining material was stored at -20°C. The sample used was equivalent to the RT product of 1.5 μg of total RNA.

The PCR was optimized by the following conditions: Each CH-1 and S1 primer (see Materials) was added in the amount of 0.15 μM into 7.5 μl of the RT solution. Then 2.0 mM $MgCl_2$, 2 U Taq polymerase, and 0.1 mM of each dNTPs were added in 5.0 μl PCR reaction buffer and the final volume was

brought to 50.0 μl with sterile water. The thermocycler was programmed as follows: 94°C denaturing for 45 sec, 55°C annealing for 1 min, and then 72°C primer extension for 1 min, for a total 35 cycles of amplification, followed by a final extension at 72°C for 5 min.

12. Isolation of genomic DNA

The genomic DNA was isolated by Higuchi's method [86]. The cells were lysed in a alkaline buffer containing non-ionic detergents and Proteinase K. In this method, the RNA was destroyed by alkaline hydrolysis, and the DNA remained intact and was used directly in PCR.

A "PCR buffer" (50.0 mM KCl, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, 2.5 mM $MgCl_2$ and 10.0 mM Tris, pH 8.3) was prepared, autoclaved and stored frozen. When ready to use, the buffer was thawed, and 0.6 μl of 10.0 mg/ml Proteinase K was added per 100 μl of the solution.

A 40 mm dish of cells were washed with PBS, scrapped free by rubber policeman, and resuspended in 150 μl of the prepared buffer (to make a solution of about 6 x 10^6 cells per ml). The cell suspension was incubated at 55°C for 1 hr to lyse the cells, release chromosomal DNA from histone, and hydrolyze the RNA. Then the proteinase K was inactivated at 95°C for 10 min. The lysate was either stored frozen at -20°C or used directly as sample for PCR. Typically, 25 μl of the lysate thus prepared was about 1 μg of genomic DNA according

to Higuchi's estimation [86].

13. PCR for the clonal selection of the stable-transfected cells

A PCR was performed to verify genomic integration of the hZP3 cDNA construct in transfected cells.

The conditions for the PCR were optimized as follows: Two microliters of the lysate mentioned above (which was about 80 ng of the genomic DNA), 1.0 mM of $MgCl_2$, 0.4 mM of each dNTPs, 2.5 U of Taq polymerase, and 0.075 μM of each primer (CH-1 and S1 primers, see materials) were added in 5.0 μl of 10 x Thermophilic Buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100) to a final reaction volume of 50.0 μl. The other reaction conditions were the same as that in the "RT-PCR for clonal selection of the transfected cells" described previously.

14. Protein purification

Purification by WGA-Lectin chromatography

The WGA-coupled particles were equilibrated with PBS buffer, and then packed into a column (5 cm long with a diameter of 1.5 cm). The glycoproteins were initially isolated by WGA-Sepharose chromatography at 4°C. Typically, 150.0 ml of culture medium was collected, and then concentrated to a final volume of 40.0 ml by centrifugation 4 times at 1500 x g with Centriprep-30 following manu-

facturer's instruction. To protect the protein, proteinase inhibitors (as mentioned in Medium Collection) and sodium azide (0.1%) were added before the sample was applied to a WGA-Sepharose column equilibrated with PBS with 0.1% sodium azide. After washing with PBS for 4 hr at a flow rate about 5 ml/hr, the absorbed fraction was eluted with 0.5 M N-acetylglucosamine in PBS [87]. In order to remove the suspended WGA lectin molecules, the eluted solution was passed through an anti-WGA immunoaffinity column (2 cm long and 1.5 cm in diameter) packed with anti-WGA immobilized Sepharose 6B particles. The preparation of the anti-WGA immunoaffinity chromatography was described as follows.

Preparation of anti-WGA immunoaffinity chromatography

One milligram of antibody was dissolved in 1.5 ml of coupling buffer (50.0 mM sodium acetate, pH 5.5), purified and washed three times with coupling buffer by ultra-filtration (Centricone, Amicon). To oxidize the carbohydrate moieties on IgG, the antibody solution was incubated at room temperature in the dark for 1 hr by adding One-tenth volume of $NaIO_4$ solution (25.0 mg/1.2 ml).

The oxidized IgG was purified by Sepharose 10 column (Econo-Pac 10 DG column, Bio-Rad, 2.0 cm diameter, 5.0 cm long) as manufacturer's instruction. Right before use, the column was washed with 20.0 ml of coupling buffer, and then exactly 3.0 ml of IgG solution was added to the column. The

column was eluted with coupling buffer. The first 3.0 ml of eluent was discarded and then 0.5 ml fractions were collected for 5 ml total elution. The optical absorbance of every fraction was measured at 280 nm. The first peak fractions were collected. The Sepharose 10 column was re-generated by at least 20 ml of coupling buffer and stored in coupling buffer with 0.02% of sodium azide.

After 1.5 ml of "affini-hydrazide gel" (affi-gel Hz, Bio-Rad) was washed twice with 10 ml of coupling buffer in a polypropylene tube, the prepared IgG was added directly into the washed gel and incubation was performed for 10-24 hr at room temperature by shaking gently.

The IgG-coupled gel particles were packed into a prepared column (1.0 cm diameter, 10.0 cm long), washed with 1 volume of 0.5 M NaCl/PBS to remove the unbound IgG, and washed with ten bed-volumes of PBS before usage. For long term storage, the column was stored in PBS buffer with 0.02% of sodium azide.

Protein concentration

Centriprep and centricone (with a 30 kDa cut off range) were used for concentrating the protein solution before being applied onto the columns. The protein was concentrated by Centriprep-30 and Centricone-30 to suitable volume depending on the initial amount of sample used following manufacturer's instruction. In order to increase

the efficiency of protein recovery, 3% BSA solution was used to pre-coat the membrane of the Centriprep-30 by centrifugation at 1500 x g for 15 min. The pre-coated Centriprep-30 was washed thoroughly with PBS twice by centrifugation at 1500 x g for 15 min. For Western blot analysis, the recombinant glyprotein was isolated by the Centricone-30 without the pre-treatment of BSA to prevent the cross-reaction of suspended BSA with the antibody used. The final product of the recombinant glycoprotein was approximately concentrated to 1.0 mg/ml, aliquoted and stored by frozen at -80°C by adding 6.1% glycerol.

15. Protein assay

BCA protein assay was used to measure the quantity of the protein content as manufacturer's instruction (Pierce, Rockford, IL) [88].

BSA were prepared in PBS buffer from 100 μg/ml to 1200 μg/ml and used as standards of the protein assay. The working buffer was prepared as manufacturer's instruction by mixing fifty parts of reagent A (bicinchoninic acid (BCA) solution) with one part of reagent B (4% copper sulfate solution). Each standard or unknown sample was pipetted in the amount of 25.0 μl into the appropriately labelled test tube, and PBS was used as a blank control. Then 500 μl of working buffer prepared above was added into each tube, mixed well, covered with Saran wrap, and incubated at 37°C

for 20 min. After incubation, all tubes were set on the bench to allow the solution cool down to room temperature. Exactly 200 μl of solution was transferred from each test tube to microtiter plate. The absorbances at 570 nm were measured by microtiter plate reader. The concentration of protein was obtained by the absorbance and the linear equation of the standard curve.

16. ELISA

For the detection of hZP3 secretion from stably transfected cells, A rabbit polyclonal antibody (Ab5a) against a conserved oligopeptide sequence of ZP3 protein (PIECRYPRQGNVSS) which was amino acids 137-150 deduced from the DNA sequence among several species (human, mouse, and hamster) was used in ELISA. This antibody was a kind gift from Dr. Hinsch and was previously characterized by positive immunohistochemical staining in human oocytes and by Western blotting *in vitro* [45,46].

The production of the antibody was as follows: Five milligrams of peptide was dissolved in 500 μl of distilled water, added to 10.0 mg of KLH (keyhole limpet hemocyanin), dissolved in 1.5 ml sodium phosphate buffer, pH 7.0, and mixed with 0.5 ml of 10.0 mg/ml EDC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride). The pH was maintained at about 5.1 for 1 hr. Then an additional 0.5 ml of 10.0 mg/ml EDC was added, and the mixture was incubated

for 24 hr at room temperature. The conjugate was dialyzed against 15 l of distilled water for 24 hr, lyophilized, and stored desiccated at -20°C. Female New Zealand rabbits were inoculated intradermally with 1.0 mg of the conjugate and antisera were collected. For affinity purification of antibody, peptide used for immunization was coupled to an AminoLink affinity column, and performed according to manufacturer's instruction [45].

Samples and controls were coated to a microtiter plate (EIA grade, Costar) in 100 μl aliquots at room temperature for 9 hr in a humid atmosphere. The microtiter plate was washed six times with PBS by a 12-channel plate washer (Nunc well washer, Fisher) to remove the unbound antigen. The blocking buffer (0.2% Tween 20 in PBS buffer) was added and incubated for two hours in a humid atmosphere at room temperature. The plate was washed with PBS buffer, and then 100 μl of Ab5a (1:100 dilution in 0.2% Tween 20/PBS) was added and incubated for 1.5 hr. Horseradish peroxidase conjugated goat anti-rabbit IgG was used as second Ab (1:200 dilution in 0.2% Tween 20/PBS), and was incubated for one hour. A 100 μl of tetramethyl-benzidine (TMB, Pierce) was added to each well, and incubated at room temperature for 5 min for color development. The positive wells appeared pale blue. The reaction was stopped by adding 120 μl of 1.0 M H_2SO_4, and the absorbance at 450 nm was measured.

17. Metabolic labelling experiment

In order to distinguish the biosynthesized glycoproteins from the endogenous glycoproteins, a ^{3}H-galactose metabolic labelling experiment was performed [89]. Transfected cells and non-transfected cells were split to 15% confluence in MEM containing 10% FBS. The cells were cultured for 4 hr until attached to the plate surface, and then washed by PBS to remove the suspended cell-debris. The "radioactive" medium containing 250 μCi of ^{3}H-galactose was added, and cells was cultured for additional 16 hr. The medium was collected as described in previous section. Meanwhile, another portion of 5.0 ml of ^{3}H-galactose medium was added into the cells for another 12 hr incubation.

The unincorporated ^{3}H-galactose was removed from the collected medium by Centriprep with a 30 kDa cut-off range (1500 x g for 30 min). The retentate was washed three more time with PBS buffer (approximately, a total 40.0 ml of buffer was used for the washing). Both the retentate and the residual (washing) solutions were collected for further study. Another Spin was made to concentrate the retentate. The final volume was measured, and sodium azide with protease inhibitors were added immediately. The final retentate was adjusted to 2.0 ml by PBS. An aliquot of 5.0 μl solution in every samples (the retentate and washing solutions) was used to measure the radioactivity.

One million cpm of metabolic solution (retentate) was passed through a wheat germ agglutinin (WGA) lectin column prepared as described previously, washed by ten bed volumes of PBS solution, and eluted by elution buffer (0.5 M N-acetylgalactosamine/PBS solution). Both of the WGA bound, and non-bound fractions were collected respectively. A suitable amount (10,000 cpm) of samples were loaded in SDS-PAGE gel with 4% stacking and 8% separation layers [81]. The SDS-PAGE was run with Tris-glycine buffer at 13 mA. The gel was fixed in 500 ml of methanol:acetic acid:water (25:10:65) for 30 min, soaked in 200 ml of "enhancer" solution (Amersham) for 30 min, and then dried by heating under vacuum for 1 hr. Autoradiography was performed as described before. In general, it took one week for the gel exposure to X-ray film at -80°C before development.

18. Silver-staining of SDS-PAGE

SDS-PAGE was prepared with a 10% separating gel and a 4% stacking gel [81]. The protein samples was added by 1 volume of a loading buffer containing 2% SDS, 10% glycerol, 10% beta-mercaptoethanol, 0.001% bromophenol blue and 100 mM dithiothreitol in 50.0 mM Tris, pH 6.8, was used. After heating the samples at 100°C for 2 min, electrophoresis was run with Tris-Glycine buffer (pH 8.3) at 100 volts until the dye reached the bottom of the gel [81]. The gel was incubated at 4°C overnight in fixing solution (40% methanol/

10% acetic acid), and then in 10% ethanol/5% acetic acid twice for 30 min each time.

The oxidizer reagent and silver reagent are prepared according to manufacturer's instruction (Bio-Rad) by diluting the concentrate solution ten times with deionized distilled water before use. The Development solution was also prepared according to manufacturer's instruction.

The gel was oxidized by prepared oxidizer reagent for 10 min with gentle shaking, washed three times with deionized distilled water for 10 min each time, and then incubated at room temperature for 30 min with prepared silver reagent by shaking gently. The gel was washed again with deionized distilled water for 2 min with gentle shaking, and then developed by development solution until the solution turned yellow or until brown smokey precipitate appeared. The development solution was exchanged for another fraction of the same solution and incubated at room temperature by gentle shaking until the desired intensity was observed. The reaction was stopped by 5% acetic acid solution. The gel was washed in deionized distilled water for 10 min before photography.

19. Western blot analysis

SDS-PAGE with a 10% separating gel and a 4% stacking gel was performed as described previously [81], using a minigel system (Hoefer Scientific, San Francisco). Wet

transfer of protein was performed at 63 volts for 14 hr in transfer buffer prepared by adding 5.8 g Tris, 1.0 g SDS, 29.0 g Glycine, and 200 ml methanol in 1 l of water [79]. After transfer, the membrane was blocked with blocking buffer (0.2% Tween 20 in PBS buffer) for 2 hr by gentle shaking. The membrane was aligned onto a Deca-probe incubation manifold (Hoefer).

Then 1.5 ml of the Ab5a (1:50 dilution in 0.2% Tween 20/PBS) was added and incubated at room temperature for one hour by shaking gently. The membrane was washed three times with washing solution (0.2% Tween 20/ PBS) for 5 min each to remove unbound antibody. A second antibody solution (biotinylated goat anti-rabbit IgG) and "ABC solution" (Avidin DH and biotinylated alkaline phosphatase H) were prepared in washing solution as manufacturer's instruction (Vector). The prepared second antibody solution was poured over membrane and incubation was continued for 30 min at room temperature with gentle shaking. The membrane was washed three times with washing solution for 5 min each to remove unbound second antibody. The previously prepared "ABC" solution was then poured over membrane, and incubation was performed with gentle shaking for 30 min at room temperature.

After another three washes with washing solution, the membrane was removed from manifold, and rinsed once in washing solution 2 (0.2% Tween 20, 150 mM NaCl, and 50.0 mM Tris, pH 7.5), and then soaked in another aliquot of the

same solution with agitation for 5 min. The membrane was washed twice with washing solution 3 (150 mM NaCl, 50.0 mM Tris, pH 7.5) for 2 min. Meanwhile, BCIP/NBT solution was prepared as manufacturer's instruction (Vector). The immune complexes were visualized with BCIP/NBT in the dark for 5-30 min until the satisfactory staining intensity depending on the amount of antigen loaded on the gel. Another Wash for 5 min in water was made before photography. The stained membrane was dried and stored folded between two pieces of 3 MM filter paper.

20. Hemizona assay

Human immature (prophase I) oocytes were stored in a hyperosmotic solution consisting of 1.5 M $MgCl_2$ supplemented with 0.1 % polyvinylpyrrolidone (PVP) in 40 mM Hepes buffer (pH 7.3). Oocytes prepared in this manner can be stored at 4°C for up to 90 days without loss of activity in the HZA. Before the assay, the oocytes were removed from storage and rinsed in Ham's F-10 medium. Narishige micromanipulators (Tokyo, Japan) mounted on a phase-contrast inverted microscope (Nikon Diaphot, Garden City, NY) are used for cutting the oocytes into matching hemizonae. A detailed protocol for the microbisection procedure and oocyte handling has been described.

Semen samples were provided by fertile men (donors) by masturbation after 3-5 days of sexual abstinence. Sperm were

washed twice by Ham's F10 medium with 3.5% of human serum albumin (swim-up solution) and centrifuged at 400 x g for 7 min and 5 min respectively. The sperm pellet was overlaid with 0.5ml of swim-up solution as described above for one hour at 37°C, and the upper fraction of motile swim-up sperm was collected for further experiment. The sperm numbers before and after swim-up procedure were counted for the preparation of sperm concentration.

For the HZA, two sperm suspensions were prepared: one was utilized as the control sperm (incubated at 37°C in 5% CO_2 in air), while the other one was incubated under identical conditions but in the presence of the test substance (hZP3). Different given concentrations of hZP3 were examined (60 min incubation time). After this incubation period, the sperm was subjected to a wash and centrifugation step to remove excess protein (control sperm are treated identically). A 100 μl sperm droplet from the control sperm was placed under previously equilibrated mineral oil, and incubated with a hemizona for 4 hr at 37°C in 5% CO_2 in air. A 100 μl droplet of the test sperm suspension was incubated with the matching hemizona (from the same pair) under identical conditions. Following the 4 hr incubation period, the hemizonae were rinsed in culture medium using a finely drawn glass pipette in order to dislodge loosely attached sperm. Thereafter, the number of spermatozoa tightly bound to the outer (convex) surface of

each hemizona was counted employing phase-contrast microscopy (x 200). For each assay, the HZA results were expressed as hemizona index (HZI) calculated as follows: numbers of sperm bound for test/control x 100.

Typically, the control sperm suspension was incubated in culture medium while the test sperm suspension was incubated with hZP3. Experiments were also carried out assaying control sperm (in Ham's F-10) versus test sperm exposed to the different cell transfection and protein separation conditions including: culture medium of non-transfected cells and WGA non-bound fraction from the protein separation procedures. For each given condition tested in the HZA, 5-8 oocytes were used and binding results statistically analyzed by a paired t-test [90-107].

21. Acrosome reaction assay

The acrosomal status of spermatozoa was evaluated using the fluorescent probe fluorescein isothiocyanate-labeled Pisum Sativum agglutinin (FITC-PSA) following previously described techniques. Assay slides were read using an epifluorescent microscope at 400 x magnification; duplicate slides were evaluated for each treatment and time analyzed, assessed blindly by two different experienced observers and results averaged. At least 100 cells were evaluated per slide. Acrosome reacted sperm were diagnosed when a total loss of the acrosomal cap was observed (bar

pattern), or no immunofluorescence was seen at all. Acrosome-intact sperm typically show staining of the entire acrosomal cap [29-35,108].

The acrosomal status of control and test (rZP3-treated) sperm was initially examined under basal conditions (spontaneous acrosome reaction). Different concentrations of rZP3 were assessed. In addition, sperm motility was evaluated by the aid of a semen computer analyzer (Hamilton-Thorn, Beverly, MA).

22) Statistical analysis

The results of the Hemizona assay (test versus control) were analyzed by paired t-test by the aid of computer (CSS, Statistica). The formula for paired t-test is as follows:

$$t = \frac{(\bar{X}_1 - \bar{X}_2) - (\mu_1 - \mu_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2} - 2r\left(\frac{s_1}{\sqrt{n_1}}\right)\left(\frac{s_2}{\sqrt{n_2}}\right)}}$$

Where s_1 and s_2 are the standard deviations of the test and control groups respectively, and n_1 and n_2 are the number of samples in test and control groups respectively. (X_1-X_2) represents the difference between the means of the two groups. The term $(\mu_1-\mu_2)$ is based upon the null hypothesis, which assumes that the two populations are not different and therefore the difference is equal to zero. So $(\mu_1-\mu_2) = 0$.

The term r is the correlation coefficient, and the formula is as follows:

$$r = \frac{\Sigma XY - \frac{(\Sigma X)(\Sigma Y)}{n}}{\sqrt{\left(\Sigma X^2 - \frac{(\Sigma X)^2}{n}\right)\left(\Sigma Y^2 - \frac{(\Sigma Y)^2}{n}\right)}}$$

Where X and Y represents the score in test and control group respectively, and n is the number of pairs of scores.

The results of the paired t-test were scored by the t and p values. The larger the t score is and the smaller the p score is, the more significant the results are [103,109].

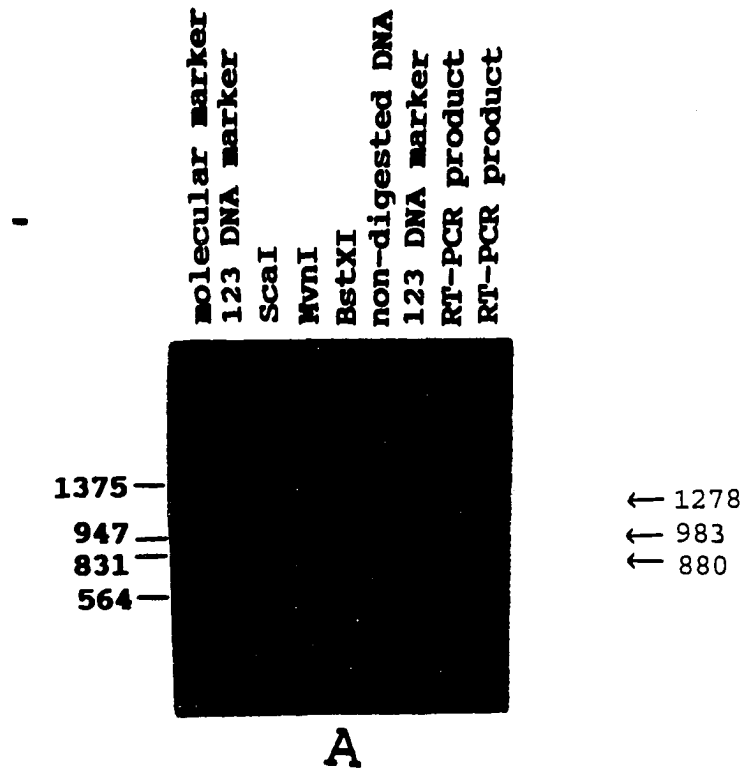
IV. Results

A. Subcloning of the full-length cDNA of human ZP3

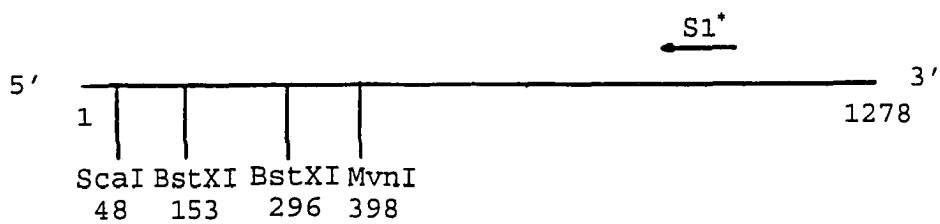
Dean reported that in the mouse, the expression of hZP3 protein is highly restricted to the growing oocyte [39,40]. We obtained total RNA from fresh, surgically removed human ovary using Chirwin's guanidinium thiocyanate extraction method [80]. The result of the extraction was confirmed by the observation of the sharp bands of the 28 S and 18 S rRNAs that appeared on agarose gel. This was followed by isolation of polyadenylated mRNA by using the biotin-labeled oligo(dT) and avidin-bound polymagnetic particle method.

A pair of primers (CH-1 and CH-2 primers) was designed based on the reported sequence of hZP3 [40]. Results of the restriction digestion and the length of produced cDNA from the RT-PCR are shown in Fig 3. The full length and the restriction digestion fragments of the RT-PCR product are exactly as expected [40], suggesting that the RT-PCR product may be a full length cDNA of hZP3 and that the amplification is completely specific because there was only one single band found. In order to further identify the RT-PCR product, a 21 bases internal probe (S1 primer) was designed to

Fig 3. Restriction digestion analysis of the RT/PCR product.
(A) After mRNA was isolated from the ovary, RT-PCR was performed and the product (cDNA) was identified by restriction mapping. The produced RT/PCR product is probably the full length cDNA of hZP3 (1278 bp) estimated according to the 123 DNA marker(ladder) based on that difference between every band in the marker is 123 base-pairs. In addition, three restriction enzymes were used: ScaI, BstXI, and MvnI. The restriction mapping was matched with hZP3 as expected. The 401 bp band in MvnI digestion; 156 bp and 142 bp bands in BstXI; 51 bp band in ScaI can not be seen in this figure due to low intensity.
(B) Scheme for the restriction digestion.



A



ScaI : 1229, 51
MvnI : 880, 401
BstXI: 983, 156, 142

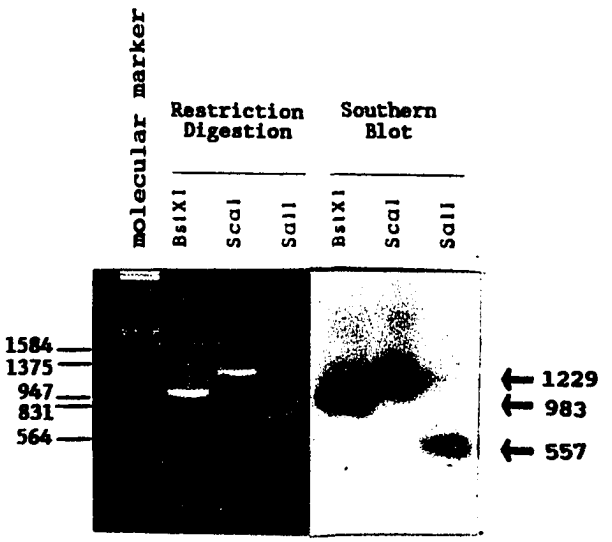
B

specifically recognize the cDNA of human ZP3 [40,65]. This probe was labelled with ^{32}P-ATP and hybridized to the intact cDNA and its restriction digested fragments, and visualized by autoradiography (Fig 4). The results of Southern blot analysis were exactly as expected. The RT-PCR product (cDNA solution) was directly purified by phenol extraction and stored at 4°C.

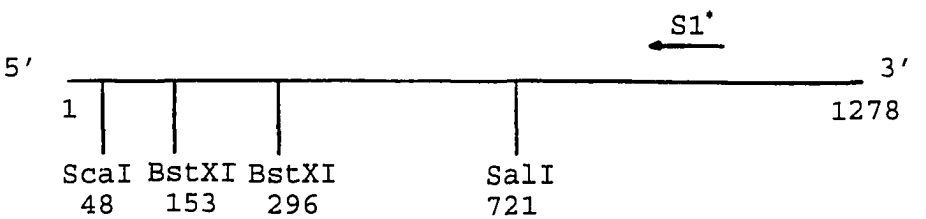
For further application, the purified RT-PCR product (hZP3 cDNA) was inserted into the PSK (pBluescript SK) vector, and then the hZP3/PSK construct was introduced into XL-1 Ecoli [7,71]. Approximately 100 clones were selected by color selection as described in the Methods. DNA was extracted from each individual clone of the positive ones, and further selection and identification were fulfilled by restriction mapping, Southern blot analysis and PCR using an internal probe (S1 primer)(Fig 5 and Fig 6). Seven clones demonstrated expected pattern of restriction digestion and Southern blot analysis (Fig 5).

Polymerase chain reaction (PCR) was performed to further identify the selected clones by using two pairs of primers (CH-1/CH-2 and CH-1/S1) as shown in Fig 6. The results demonstrated single band amplification at the expected sites by CH-1/CH-2 and CH-1/S1 primers (Fig 6). Both linear and circular DNAs from selected transformed clones (hZP3/PSK DNA construct) were used to identify the correct insertion of the cDNA of hZP3 into the PSK vector.

Fig 4. Southern blot analysis of the RT-PCR product.
(A) Restriction digestion analysis of the RT-PCR product (left), and Southern blot analysis by an internal probe S1 (see materials) (right). The same three restriction enzymes were used: BstXI, ScaI, and SalI. The restriction mapping was matched with the cDNA of hZP3 as expected, and the Southern blot analysis demonstrated the hybridizations at the right sites further identified the RT-PCR product as cDNA of hZP3.
(B) Scheme and results for the restriction digestion and Southern blot analysis.



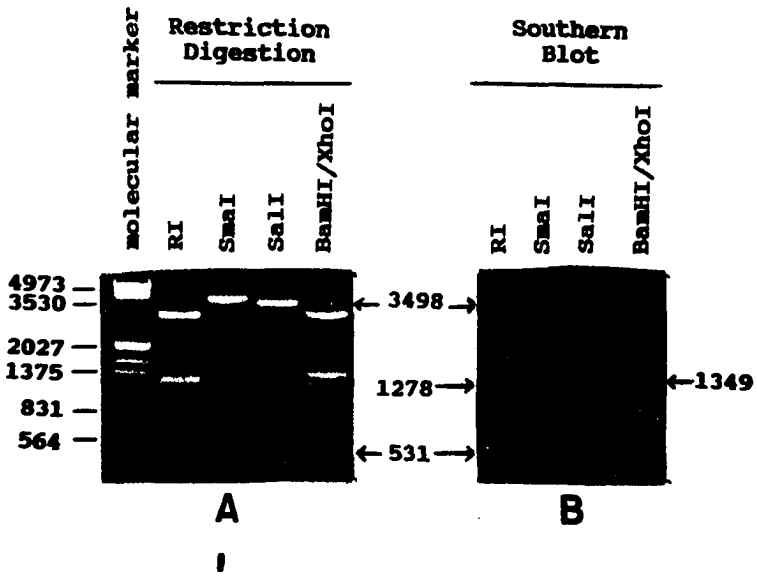
A

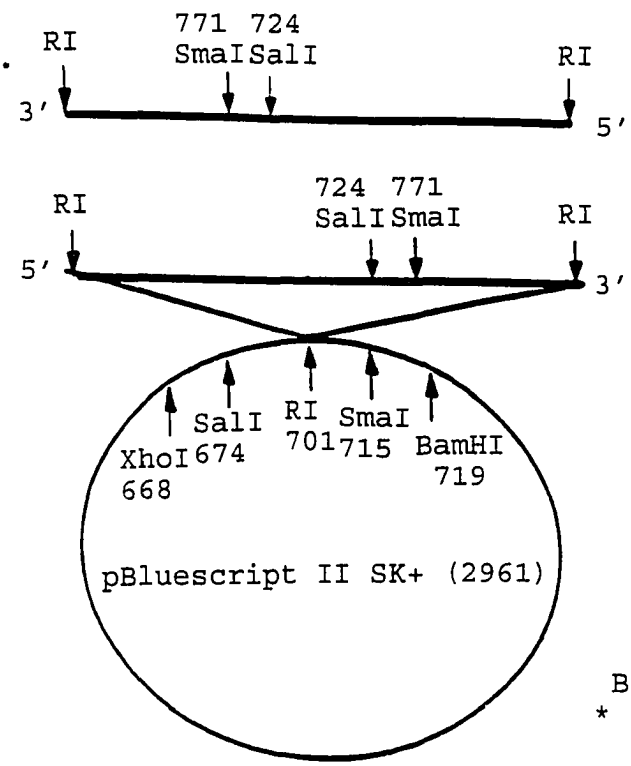


BstXI: 983*, 156, 142
ScaI : 1229*, 51
SalI : 724, 557*
* represents radioactive labelled

B

Fig 5. Identification of the hZP3/PSK construct by restriction digestion and Southern blot analysis.
(A): Restriction digestion analysis of the hZP3/PSK DNA construct. EcoRI, SmaI, SalI, and BamHI/XhoI were used for the restriction digestion.
(B): Southern blot analysis of the same gel by using an internal probe (S1).
(C): Scheme for the restriction digestion of hZP3/PSK construct.
The cDNA of hZP3 (RT-PCR product) which was blunt-end ligated with EcoRI linkers, was inserted into PSK vector for the purposes of clone selection, amplification, long-time storage, and further identification. The ligated product of the hZP3/PSK construct was transformed into XL-1 bacteria; clone selection was made on the agarose plate by color selection and restriction mapping analysis of the isolated plasmid DNA (hZP3/PSK construct) was performed.
The scheme for the restriction digestion analysis of the isolated plasmid DNA of hZP3/PSK construct and the expected results of the restriction digestion and Southern blot analysis were shown.

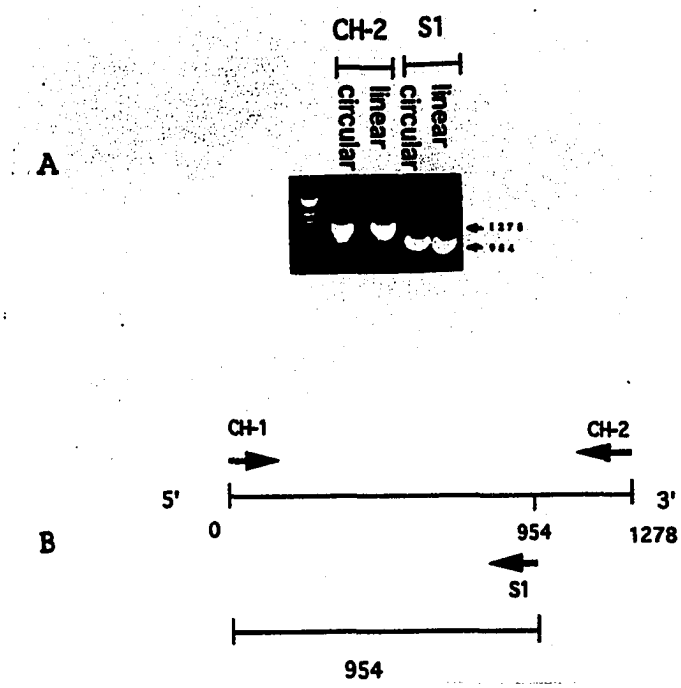




RI: 2961, 1278*
SmaI: 3728, 531*
SalI: 3498*, 761
BamHI/XhoI: 2910, 1349*
* represents radioactive labelled

C

Fig 6. PCR of the cDNA of hZP3 by an internal probe S1.
(A) DNAs from selected transformed clones (hZP3/PSK DNA construct) were used to identify the correct insertion of the cDNA of hZP3 into the PSK vector. Two pairs of primers were used for this identification : CH-1/CH-2, and CH-1/S1. A specific amplification of a 1278 base-pairs product by using CH-1 and CH-2 primers, and a 954 base-pairs product by using CH-1 and S1 primers were observed.
(B) Schemes for the PCR method: the identification of the insertion of the hZP3 cDNA into the PSK expression vector.



The linear DNA was produced by restriction digestion and purified by the gene-clean glassmilk method.

To further verify the cloned cDNA as hZP3 cDNA, autosequencing was performed by taking advantage of the T3 and T7 promoters inherent in the PSK vector [110] and the sequence obtained was compared with the sequence published by Dean (Appendix I).

Based on the computer print-out, a total of 289 bases was identified from the 3'-end of hZP3 cDNA by taking advantage of the T3 promoter and a more than 98% homology was found with the sequence of hZP3 cDNA reported by Dean (Fig 7 and Appendix III). A gap was found (base 196-186) and mis-matched bases were located at the following positions: 331, 314, 310, 284, 289 and 262. However, the sequence under the big T peak of the gap was found identical to the published sequence except base 186 and base 196. An extra T base (base 196) was read mistakenly by the sequencer due to the large T signal (Appendix III). The mis-matched bases at position 331, 284, 289 and 262 were found to be machine error. The sequencer did not recognize the right bases because the peaks fell just outside the window of detection set by the technician. The other extra bases obtained (base 363, 353, 349, 345, 298 and 290) were found to be read mistakenly due to low intensity of signals. The deletion at base 84 was definitely a machine error because it was located right inside the sequence of CH-2 primer used for

Fig 7. Comparison of the sequence of hZP3 cDNA from the 3'-end sequencing.

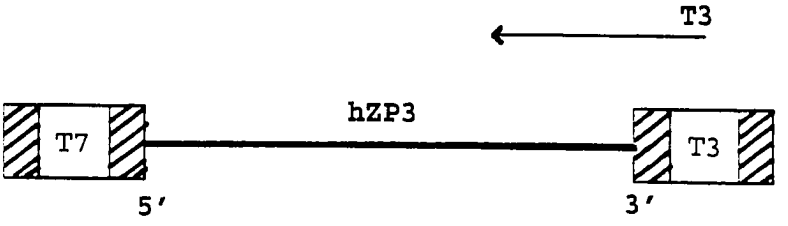
(A): 3'-end sequence of our cloned hZP3 cDNA. The numbers labelled are according to the print-out result of autosequencing for the convenience of comparison (see also Appendix III).

(B): 3'-end sequence of the hZP3 cDNA published by J.Dean. The numbers labelled are according to the published sequence (See also Appendix I).

(C): Scheme for the 3'-end autosequencing of cDNA of hZP3. The cDNA of hZP3 (RT-PCR product) inserted into the PSK vector was further identified by DNA sequencing. DNA sequencing was achieved from 3'-end of the cDNA of hZP3 by using T3 primer.

Two hundred eighty nine bases of hZP3 cDNA were identified, and a more than 98% homology with Dean's published sequence was found. A gap located from base 196-186 was underlined by dashed periods. The matched bases were represented by large letters, and the mis-matched ones were represented by small letters. The mis-matched bases were located at the following positions: 331,314,310,284 and 262 (according to the print-out result of autosequencing).

(A):	370	NNGTTCNCNT	NAGGNCCCTC	CTCTCTGNAN	341
(B):	990	AGGTTCG-GT	AAGGTCC-TC	C-CTC-GGAG	1015
	340	TACANTACTt	NNTCACCAGG	TCCNGAtGNN	311
	1016	TACAGTACTc	GGTCACCAGG	TCCAGAcGAA	1045
	310	cGGCANTGGC	GTCCNGTACA	TtTGTCaTCN	281
	1046	gGGCATTGGC	GTCC-GTACA	-cTGTCtTCT	1073
	280	TCNTCAACAG	NGGCACCCtG	GTGACTAGAA	251
	1074	TCGTCAACAG	TGGCACCCcG	GTGACTAGAA	1103
	250	GGACCTGTCC	TCCCCACTGG	TACTTCATCT	221
	1104	GGACCTGTCC	TCCCCACTGG	TACTTCATCT	1133
	220	CGTCACCCGN	AACGGAAGAC	TGTGTNTTTT	191
	1134	CGTCACCCGA	AACGGAAGAC	TGTG-GAGTC	1162
	190	TTTNTNACGA	CCCGCATCCG	GACCGACACC	161
	1163	ACCACGACGA	CCCGCATCCG	GACCGACACC	1192
	160	ACCACAGGGA	CTGAGACTGA	CGACAATAGG	131
	1193	ACCACAGGGA	CTGAGACTGA	CGACAATAGG	1222
	130	ACCAAGAGTG	GTCCTCCACA	GCGTGACGGA	101
	1223	ACCAAGAGTG	GTCCTCCACA	GCGTGACGGA	1252
	100	GGGTGGGACA	CAGACG-AGG	CTTATT	76
	1253	GGGTGGGACA	CAGACGAAGG	CTTATT	1278



(C)

PCR (see Appendix I and Materials).

By taking advantage of the T7 promoter, 224 bases were identified from the 5'-end of hZP3 cDNA and a more than 91% homology was found with the sequence published by Dean (Fig 8 and Appendix IV). A 15 bases gap was found (base 182-196), and the sequence under the big T peak of the gap was found identical to the published sequence except base 191, 192 and 193. In addition, two extra T bases were read mistakenly by the sequencer due to the large T signal (Appendix IV). Due to the low intensity of signals, the mis-matched bases after the position 228 (11 bases in a total of 15 bases) may be due to machine error as mentioned above. The other extra bases obtained (base 255, 261, 266, 279 and 286) were read mistakenly by the same reason.

In order to express the cloned hZP3 cDNA in PA-1 cell, the cDNA was subcloned into a mammalian expression vector (pREP4). The pREP4 vector is mainly composed of a Rous sarcoma virus long terminal repeat (RSV LTR) as upstream promoter, Epstein Barr virus OriP element (EBV OriP), EBNA-1 gene, SV40 poly A element and the hygromycin resistance gene [81]. Restriction mapping and Southern blot analysis were again performed to identify the correct clones of human ZP3 cDNA (Fig 9). Due to the non-specific digestion of EcoRI ("star" activity), an extra fragment of 400 bp appeared in the restriction mapping. By using an internal probe (S1), the Southern blot analysis demonstrated that this non-

Fig 8. Comparison of the sequence of hZP3 cDNA from the 5'-end sequencing.

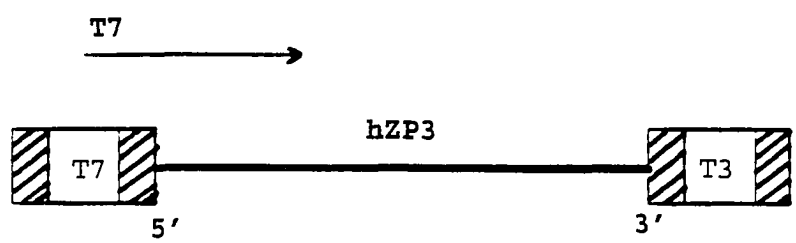
(A): 5'-end sequence of our cloned hZP3 cDNA. The numbers labelled are according to the print-out result of autosequencing for the convenience of comparison (see also Appendix IV).

(B): 5'-end sequence of the hZP3 cDNA published by Dean. The numbers labelled are according to the published sequence (see also Appendix I).

(C): Scheme for the 5'-end autosequencing of cDNA of hZP3. The cDNA of hZP3 (RT-PCR product) inserted into the PSK vector was further identified by DNA sequencing. DNA sequencing was achieved from 5'-end of the cDNA of hZP3 by using T7 primer.

Two hundred twenty four bases of hZP3 cDNA were identified and a more than 91% homology with Dean's published sequence was found. A gap located from base 182 to 196 was underlined by dashed periods. The matched bases were represented by large letters, and the mis-matched ones were represented by small letters. The different bases (mis-matched ones) were located at the following positions: 170, 173, 201, 208, 228, 231, 238, 246, 247, 249, 250, 259, 260, 270 and 293 (according to the print-out result of autosequencing).

(A):	66	ACCATGGAGC	TGAGCTATAG	GCTCTTCATC	95
(B):	1	ACCATGGAGC	TGAGCTATAG	GCTCTTCATC	30
	96	TGCCTCCTGC	TCTGGGGTAG	TACTGAGCTG	125
	31	TGCCTCCTGC	TCTGGGGTAG	TACTGAGCTG	60
	126	TGCTACCCCC	AACCCCTCTG	GCTCTTGCAG	155
	61	TGCTACCCCC	AACCCCTCTG	GCTCTTGCAG	90
	156	GGTGGAGCCA	GCCAcCCcGA	GACGNCTTTT	185
	91	GGTGGAGCCA	GCCAtCCtGA	GACGTCCGTA	120
	186	TTANTTTTTT	TCNGGaGGAG	NNcCAGGAGG	215
	121	-CAG-CCCGT	ACTGGtGGAG	TGtCAGGAGG	148
	216	CCACTCTGAN	GGcCAcNGNN	AGtANNGACC	245
	149	CCACTCTGAT	GGtCAtGGTC	AGcAAAGACC	178
	246	caNaaGNNAA	CCNccTAAGC	CNCAcCANGG	275
	179	ttTttGGCA-	CCGgg-AAGC	-TCAtCAGGG	205
	276	CTGNNTGNCC	CNCACCTcNG	N	296
	206	CTG-CTGACC	-TCACCTtGG	G	224



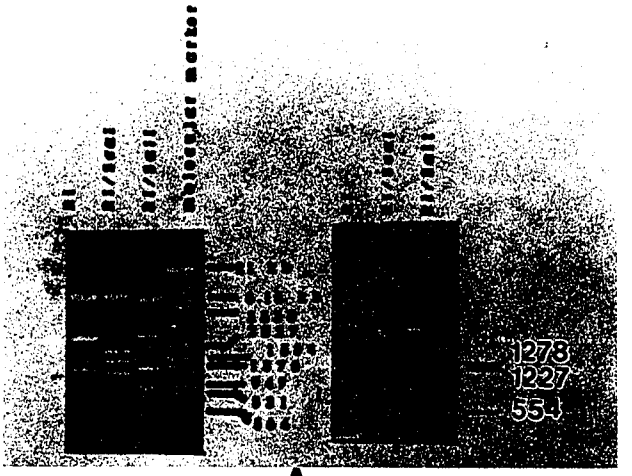
(C)

Fig 9. Restriction digestion and Southern blot analysis of the hZP3/pREP4 construct.

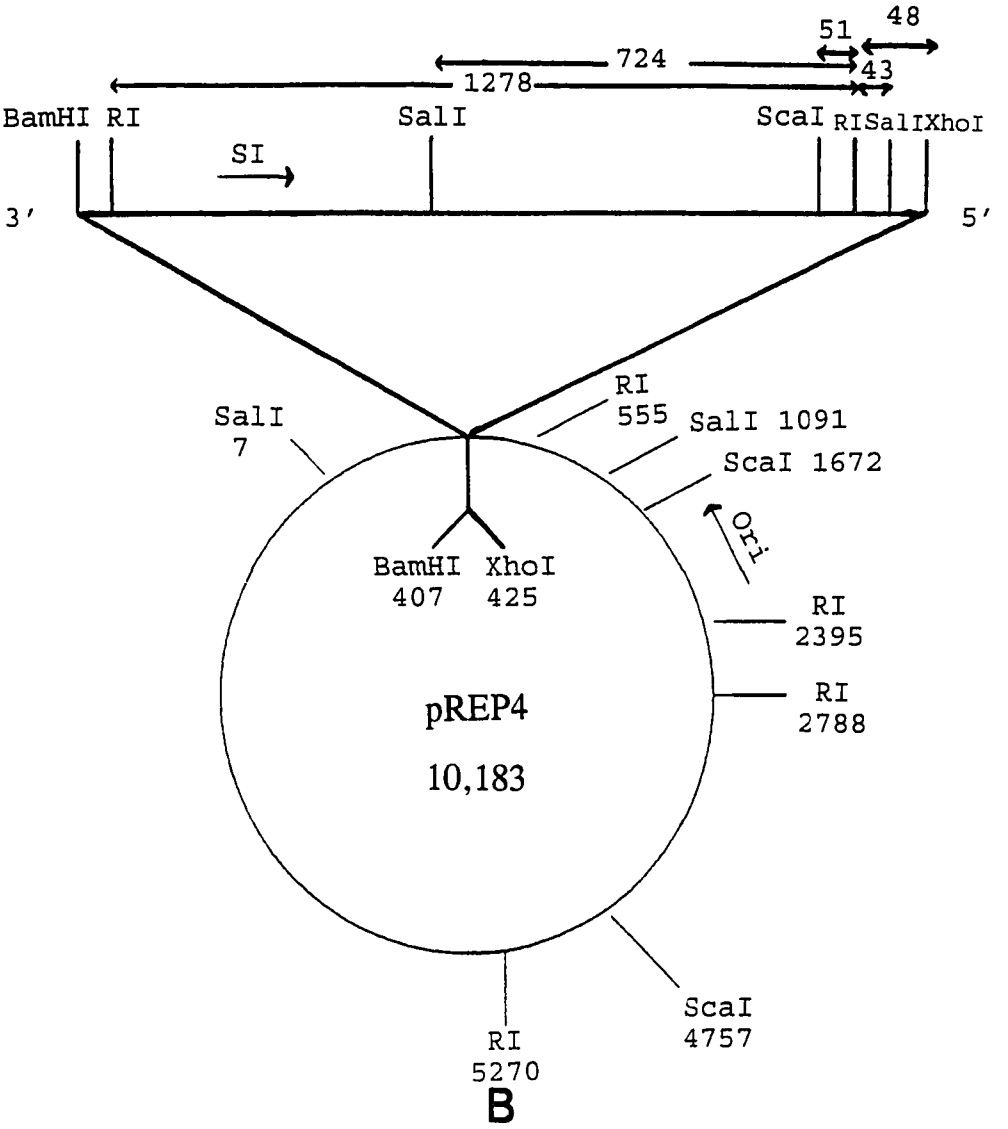
(A) Left: Restriction digestion analysis of the hZP3/pREP4 DNA construct. RI, RI/ScaI, and RI/SalI were used for the restriction digestion. RI/ScaI digestion was performed with two restriction enzymes: RI and ScaI ; RI/SalI digestion was performed with RI and SalI.

(A) Right: the Southern blot analysis of the same gel with an internal probe S1.

(B):Scheme for the restriction digestion analysis of the hZP3/ pREP4 construct.
The hZP3/PSK DNA construct was amplified in bacterial cells, isolated by guanidine thiocyanate method and purified by the maxi-prep method (spin column, Promega); then it was ligated into a mammalian expression vector (pREP4) at the restriction cutting sites BamHI and XhoI. $S1^{*}$ which represents the radioactive-labelled internal prob S1 is also shown in this figure.



A



B

specific cutting site was located inside the pREP4 vector, because a 1278 bp fragment resulted from the cDNA of hZP3 was the only one band hybridized.

Therefore, by using five different identification criteria based on length of the produced RT-PCR product, restriction mapping, Southern blot analysis, autosequencing and PCR, we have convincing evidence that the cDNA of human ZP3 was correctly amplified by our designed RT-PCR protocol and cloned into the PSK and pREP4 expression vectors.

B. *In vitro* transcription/translation

In vitro transcription/translation was performed to ensure the capacity of the cloned cDNA for producing a full length non-glycosylated hZP3 product. The TNT Coupled Reticulocyte Lysate System used bypass many steps in the standard rabbit reticulocyte lysate translation process by incorporating transcription directly in the translation mix.

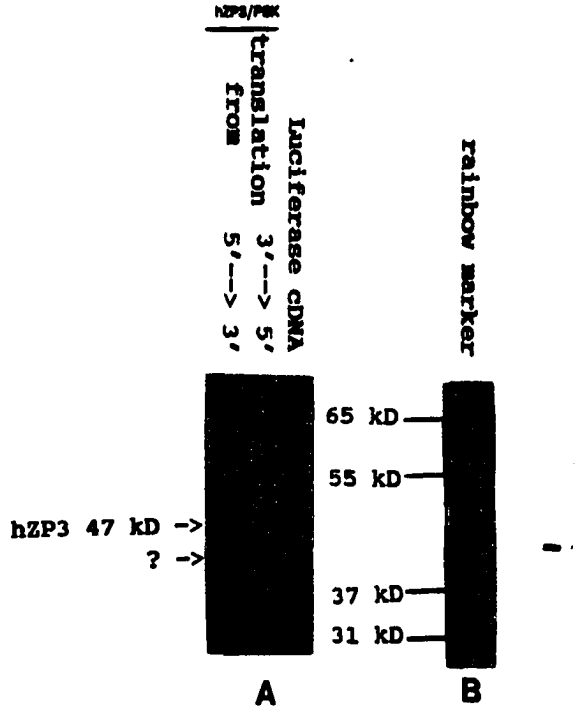
The sequence between the open reading frame of the cDNA of hZP3 and the T3 promoter of the hZP3/PSK DNA construct had been checked and no additional translation starting site (i.e. ATG) found.

A hZP3/PSK DNA construct with the correct orientation insertion of hZP3 (5'-> 3',see Fig 10) was used as the test sample for *in vitro* transcription/translation. According to the manufacturer's instruction, ^{35}S-methionine was used with the TNT Lysates for the purpose of autoradiography. A 47 kDa

Fig 10. *In vitro* transcription/translation of the hZP3 (nonglycosylated) protein.

(A) An 47 kDa protein was produced in the reticulocyte lysate system by taking advantage of the T3 promoter inherently present in the PSK vector. T3 RNA polymerase was added for the transcription of the inserted DNA. [^{35}S]-methionine was substituted in the reaction for the later autoradiography. In addition, Luciferase cDNA was used as a positive control, and the hZP3/PSK construct with the opposite orientation of insertion of the cDNA of hZP3 was used as a negative control.

(B) The protein marker was cut off from the gel and stained with Coomassie brilliant blue.



product was produced by the cloned cDNA of hZP3 with 5'-> 3' orientation insertion. However, the DNA construct with the opposite orientation insertion (3'-> 5') was used as a negative control and nothing was found by the same application. In addition, luciferase cDNA was used as a positive control according to manufacturer's instruction (Fig 10).

This result indicated that a full-length nonglycosylated hZP3 (47 kDa) was produced in the TNT Lysate reaction (transcription and translation) by using the cloned cDNA construct as template, and there was no reading frame shift because of the point mutation in the sequence of the cloned cDNA. Another protein which may be due to the non-completed transcription or translation reaction was also observed with the molecular mass at about 40 kDa (Fig 10).

C. Stable transfection

A stable transfected human ovarian cell line was established by the $CaPO_4$-mediated transfection method [77,78,81,111], followed by antibiotic selection (hygromycin) for three weeks [76,84,85]. Since the quality of the DNA used and the particle size of the DNA produced by $CaPO_4$ precipitation were critical for the efficiency of the introduction of the DNA construct into the host cells by phagocytosis [81], the supercoiled hZP3/pREP4 DNA construct was purified by CsCl gradient ultracentrifugation as described previously. Approximately, 200 single colonies

were isolated by cloning rings [75].

PCR was used to identify the integration of the inserted cDNA of hZP3 into the genomic DNA by using CH-1 primer and an internal primer (S1) (Fig 11). In order to quickly isolate genomic DNA from a large number of clones, Higuchi's method was used as described in the Methods [86]. The transfected cell clones demonstrated a very strong specifically amplified product at the expected position (954 bp) on the gel, but not in the non-transfected cells as well as the cells transfected by vector only (pREP4 only, without cDNA of hZP3). A total number of 12 clones were identified as transfected cells by this method. The high background resulted from (1) the complexity of genomic DNA in the host cell, (2) the inherent hZP3 gene in the PA-1 cell (Appendix VIII), and (3) the PCR conditions used. By Higuchi's method, the individual genomic DNA was isolated conveniently from a huge number of clones and the cell lysates with a lot of impurity were used directly as samples for PCR. Therefore, the conditions for PCR were not optimized and resulted in high background.

After knowing that the hZP3 cDNA was integrated into the genomic DNA in the transfected cells, the RT-PCR was performed to further identify the expression of the inserted cDNA by using the same pair of primers (CH-1/S1 primers). As can be seen in the Fig 12, only the transfected cells were found to show distinct specifically amplified product. The

Fig 11. Identification of the insertion of hZP3 cDNA into the transfected PA-1 cells by PCR.
Identification of the introduction of hZP3 cDNA into the genomic DNA of the host cell, PA-1, from the selected stable transfected cell clones, SKC-P3-15, SKC-P3-23, SKC-P3-19, and SKC-P3-12 by using CH-1 and an internal primer S1 as shown at the bottom of the figure. A 954 base-pairs of specific amplification was observed in the transfected cells. The non-transfected cell and transfected cell with vector only were used as negative controls. The background bands were thought to be due to the high complexity of the genomic DNA, the endogenous hZP3 gene, and mostly the non-optimized PCR conditions (see also Fig 15).

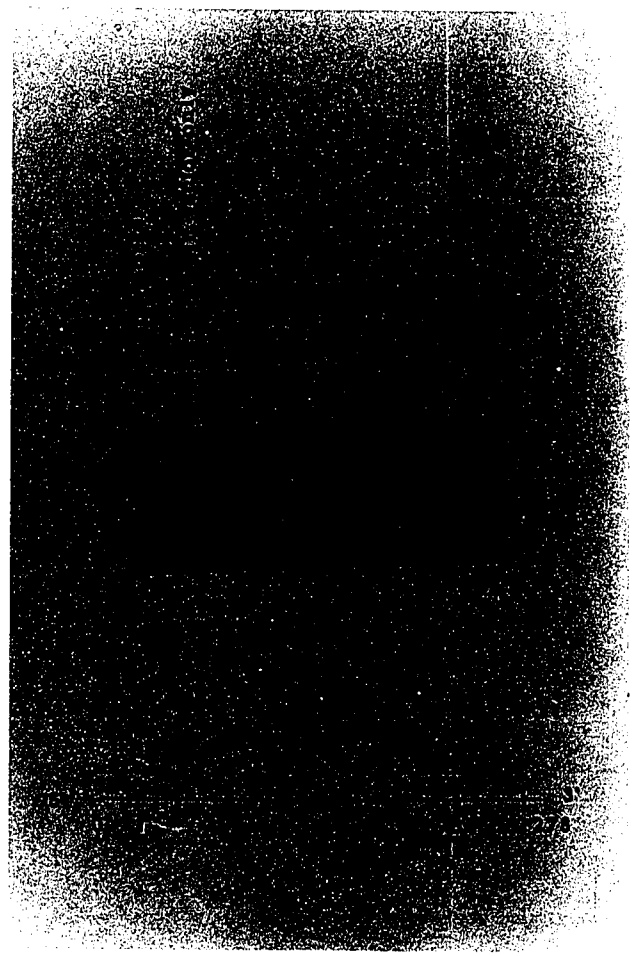
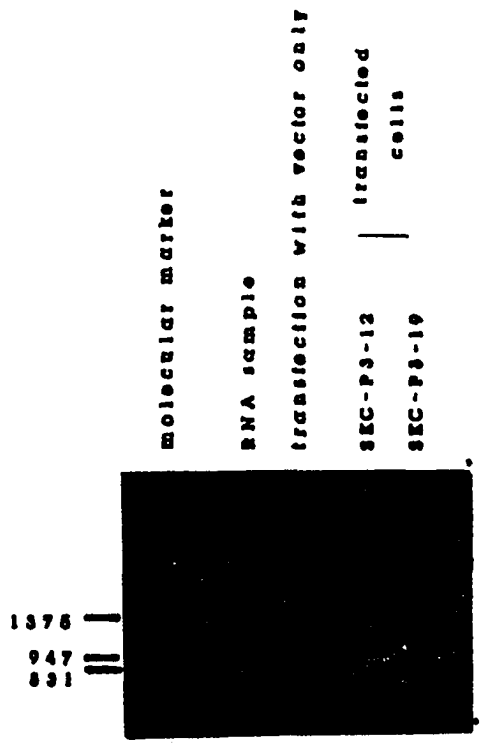


Fig 12. Identification of the expression of hZP3 mRNA from the stable-transfected cell clones by RT-PCR.
The RT-PCR results of the stable transfected cell clones, SKC-P3-12, and SKC-P3-19 by using the same pair of CH-1 primer and an internal primer S1 as shown at the bottom of the figure. A 954 base-pair product was observed from the transfected cells, but not in the cells transfected with vector only. In addition, RNA sample without being transcribed by RT reaction was also performed by PCR as another negative control.

-



cells transfected by vector only (pREP4, without insertion of cDNA of hZP3) did not show any RT-PCR product (Fig 12). This result indicated that the hZP3 cDNA was expressed in the transfected cells, and little or no hZP3 mRNA was detected in the cells transfected with vector only.

In order to identify the production and secretion of recombinant ZP3 protein in the transfected cells, ELISA was performed as described in the Methods. The polyclonal antibody (Ab5a) used for ELISA was produced against a conserved oligopeptide (epitope) of ZP3 protein among several species (human, mouse, and hamster) [45]. This antibody was previously proven to be effective for the recognition of human zona pellucida proteins (ZP3, specifically) in immunohistochemical studies and in Western blot analysis [45,46]. As shown in Table 1, the results of ELISA demonstrated the present of hZP3 protein secreted in the culture medium of transfected cells compared to that of the cells transfected with vector only (negative control). The same oligopeptide which was used to produced the antibody was coated on microtiter wells as a positive control. Two of the transfected cells (SKC-P3-12 and SKC-P3-19) that showed the highest absorbance at 540 nm by ELISA were chosen for further application.

After several experiments, the background of the ELISA by using the Ab5a was high (Table 1), implying there were cross-reaction of this antibody with proteins other than

Table 1. Identification of protein secretion of the stable-transfected cell clones by ELISA.
A polyclonal antibody (Ab5a) which was produced against a conserved oligopeptide epitope of hZP3 was used to monitor the secretion of hZP3 protein in the culture medium. The original oligopeptide (see Methods) was used as positive control; and the culture medium from cells transfected by vector only was used as a negative control. The average value of four absorbance reading at 540 nm in every sample was shown in the Table. The high background reading of the negative control implies the possibility of cross-reactivity of the polyclonal antibody with proteins other than hZP3.

	pREP4 vector only as negative control	oligopeptide as as positive control	stable transfected cell clones SKC-P3-12	SKC-P3-19
OD_{540}	0.293	0.465	0.396	0.371

N=4

hZP3. Therefore, various blocking buffers including BSA, Superblock (Pierce), Blotto, and Tween 20 in PBS buffer were tested (data not shown). The cross-reactivity of Ab5a was found to be strong when BSA and Superblock were used, and moderate when Blotto was used. The non-protein reagent, Tween 20 (0.2% in PBS buffer) was finally determined to be most useful for the blocking process in the Western blot analysis.

D. Isolation and identification of the recombinant hZP3

For the purpose of the recombinant hZP3 production, two of the stable transfected cell clones (SKC-P3-12 and SKC-P3-19) which demonstrated the largest amount of hZP3 secretion by ELISA were cultured and the culture medium was collected as described in Methods. Size-exclusion chromatography (Sepharose-6B) and Lectin chromatography were performed to isolate the recombinant hZP3 in the culture medium. After several experiments, WGA was found to be more specific to hZP3 than Con A (Data not shown), and then size-exclusion chromatography was performed and found not effective due to the close molecular weight of the eluted glycoproteins.

WGA-Sepharose chromatography was finally determined to be used to isolate the recombinant hZP3 glycoprotein for the further applications. After the sample was applied to the WGA-Sepharose column and incubated at 4°C overnight, PBS

buffer was used to wash the unbound molecules away and then N-acetylglucosamine was used as a competitor to the WGA-particle in the Lectin column to elute the bound fraction as shown in Fig 13. In addition, to prevent the influence of residual lectin molecules with the bio-assay, the anti-WGA immunoaffinity chromatography was followed to remove the suspended WGA molecules in the eluted fraction. The final product was concentrated and stored as described before. Typically, an approximate 3 mg of total glycoprotein was isolated from 150 ml of the culture medium. SDS-PAGE and Western blot analysis were performed to identify the re-combinant hZP3 protein in the WGA-purified glycoprotein solution. First of all, proteins were separated by SDS-PAGE and silver-stained as described in Methods and the results were shown in Fig 14. Five solubilized zonae pellucidae (from human oocytes) were also loaded on the gel and the silver staining demonstrated a strong wide band at about 65 kDa, suggesting the molecular mass of hZP3 may be 65 kDa. Both the cells which are not transfected and the transfected cells indicated the major band at the same molecular weight, suggesting that there were proteins other than hZP3 with the similar molecular weight in the non-transfected medium. In addition, the 65 kDa band in the non-transfected medium was stronger than that in the transfected medium because of the following two reasons. First, more bands were observed in the transfected medium (Fig 14), indicating more kinds of

Fig 13. Isolation of the recombinant hZP3 glycoprotein by WGA-Sepharose chromatography.
Culture medium was collected and concentrated by Centriprep-30 as described in Methods. The concentrated solution was applied to the WGA-Sepharose column, PBS was used to wash the WGA-absorbed glycoproteins and then 0.5 M N-acetyl-glucosamine was used to elute the WGA-absorbed glycoprotein.
(1): WGA-unbound fraction (washed out by PBS)
(2): Starting point for the N-acetylglucosamine elution
(3): Peak of the WGA-absorbed fraction (WGA-purified glycoproteins)
(4): Basal reading of the elution solution

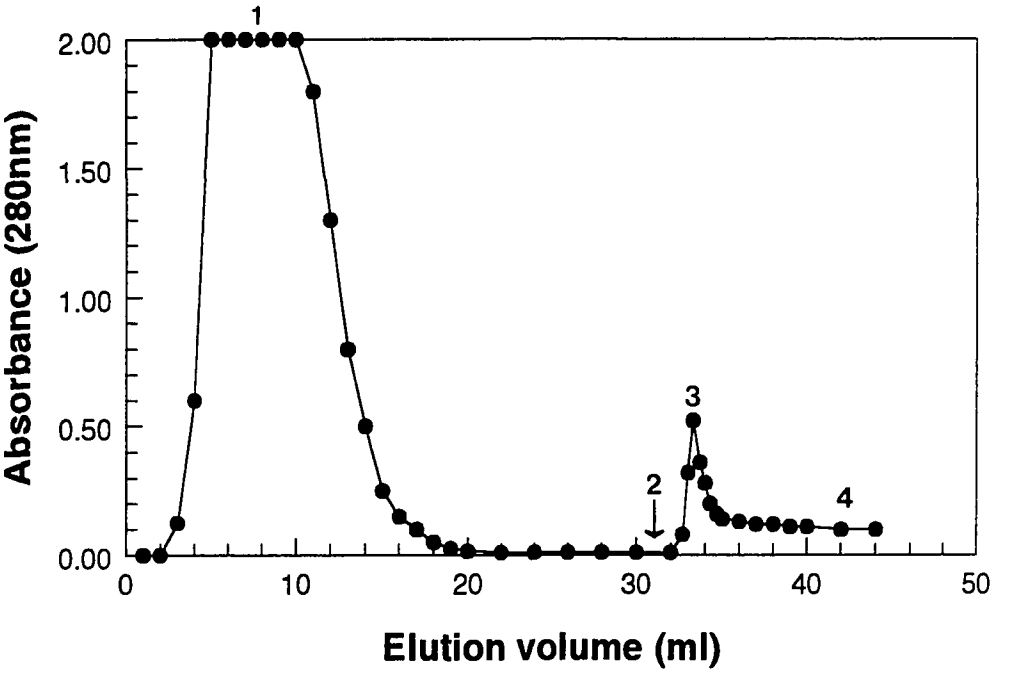
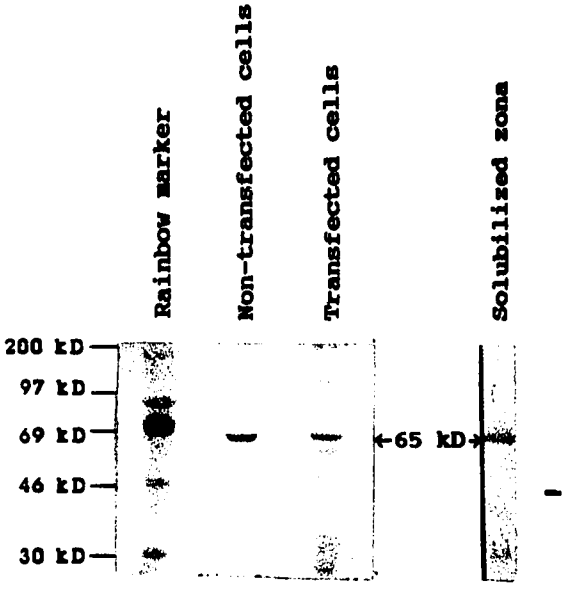


Fig 14. Separation of the recombinant hZP3 protein by SDS-PAGE.
Left: 50 μg of WGA-absorbed glycoproteins from non-transfected cells and transfected cells were loaded onto a denaturing SDS-PAGE gel.
Right: Solubilized proteins from five human zonae were used for the identification of the molecular weight of the hZP3. Silver stain was performed for the purpose of visualization.
Both the transfected cells and non-transfected cells have the stained band at 65 kDa. Distinct bands between WGA-purified glycoproteins of transfected and non-transfected cells were observed, implying distinct glycoproteins were produced and secreted by the transfected cells.

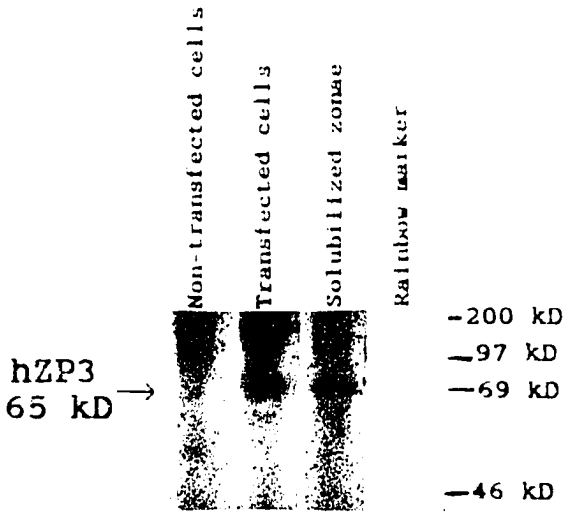


proteins were produced by transfected cells and therefore the intensity of each individual band was decreased. Second, in order to increase the recovery of glycoprotein, the Centriprep-30 used for the concentration was pre-coated with BSA solution. Therefore, there may be somehow more contamination of residual BSA in the non-transfected medium from this process.

Since the silver-staining can not specifically identify hZP3 from the other proteins with the similar molecular weight, Western blot analysis was performed. As shown in Fig 15, the Western blot analysis with polyclonal antibody Ab5a (as mentioned in Methods), verified the band at 65 kDa as the recombinant hZP3. In addition, a second band hybridized at 100 kDa (or heavier) was also found by the Western blot analysis in the transfected culture medium. This suggested the possibility of heterogenous glycosylation of hZP3 protein in the transfected tumor PA-1 cells [45,46, 77,78,111]. This will be discussed further in the later chapter. The solubilized zona pellucida was found hybridized at exactly the same position around 65 kDa (Fig 15).

In order to identify the newly biosynthesized and secreted glycoproteins, a metabolic labelling study was performed by substituting [6-^{3}H]-galactose in the culture medium of transfected cells and non-transfected cells as described in the Methods. The reasons for choosing this monosaccharide were: (1) Galactose is one of the major

Fig 15. Identification of the secretion of the recombinant hZP3 protein by Western blot analysis.
One microgram of WGA-purified glycoproteins from non-transfected cells and transfected cells, and solubilized proteins from five human zonae were loaded onto SDS-PAGE. And a polyclonal antiserum against a conserved oligopeptide of ZP3 protein was used for the identification of hZP3. Distinct bands were found at 65 kDa and 100 kDa from transfected cells, and the solubilized human zonae proteins demonstrated a major band around 65 kDa.



components (estimated at least more than 25%) of the complex type N-linked and O-linked oligosaccharides which are believed to be the major types of oligosaccharides in the hZP3 glycoprotein. [39,40,49,66-68,112-116]; and (2) there is less sugar conversion of galactose to other monosaccharides *in vivo* [89].

The culture medium was collected and ultrafiltration was performed to remove the suspended ^{3}H-galactose in the medium and PBS was used for the washing (totally, 40.0 ml were used). The final samples were concentrated to approximately 2.0 ml by Centricon 30 (Amicon). An aliquot of 5.0 μl solution was taken from the retentates and washing solutions of both transfected cells and non-transfected cells to be monitored for radioactivity. The results are shown in Table 2. According to these results, the glycoproteins produced and secreted in the transfected cells were estimated 3.29 times higher than that in the non-transfected cells from the total radioactivity shown in the retentate solutions (Table 2). Most of the ^{3}H-galactose was left inside the cells (estimated more than 90% of the total ^{3}H-Galactose used). Ten more million cpm of radioactivity was found in the washed solution (suspended ^{3}H-galactose) of the non-transfected cells than that in the transfected cells, implying that there was ten more million cpm of radioactivity left inside the transfected cells than that left inside the non-transfected cells. This also implied that

Table 2. Summary of metabolic labelling experiment.
^{3}H-galactose was substituted into the culture medium of the transfected and non-transfected cells to study the biosynthesized glycoprotein. The culture media from non-transfected cells and transfected cells were centrifuged to remove the suspending cells, concentrated by Centriprep ultra-filtration with a 30 kDa cut-off, and washed by 40 ml of sterile PBS (total volume) to remove the remaining ^{3}H-galactose in the medium. Five microliter of each sample was taken for the measurement of the radioactivity, and the results are shown in the table. The biosynthesized glycoprotein of the transfected cells was 3.29 times more than that of the non-transfected cells based on this calculation.
About 90% of the radioactivity was still left inside the cells. Less total radioactivity was found in retentate and washed solution of the transfected cells than that of the non-transfected cells implied that there were larger amount of processing glycoproteins insides the transfected cells than that in the non-transfected cells.

	variety of solutions	average cpm N=4	total volume	total cpm
transfected cell	retentate	8472.2	2 ml	3.39×10^6
	washed	3449.3	40 ml	27.59×10^6
non-transfected cell	retentate	2562.0	2 ml	1.03×10^6
	washed	4934.5	40 ml	39.47×10^6

* Glycoprotein in transfected cell = 3.29 x Glycoprotein in non-transfected cell (after WGA isolation)

there were still biosynthesized glycoproteins left inside the transfected cells (not secreted yet).

After the glycoproteins were purified by WGA-Sepharose chromatography from the culture medium of transfected cells and non-transfected cells, SDS-PAGE and fluorography were performed as described previously. As shown in Fig 16, distinct broad bands were found in the transfected medium at 55-70 kDa (at least three bandings), 80 kDa, and 100 kDa (Fig 16). Furthermore, on the same gel, the WGA-unbound fraction of protein solution (washed solution) from the culture medium of the transfected cells was also loaded, and most of the proteins were found at around 50 kDa and 80 kDa.

E. Biological activity of the recombinant hZP3

Sperm motility was examined by the assistance of a semen computer analyzer and no difference was found between test and control groups, implying that the WGA-purified recombinant protein solution did not have toxic effects on the sperm cells (Data see Appendix V).

The hemizona assay (HZA) was performed to characterize the biological activity of WGA-purified recombinant protein and the data were analyzed by a paired t-test (see also Appendix VI), and the results are shown in Fig 17. When WGA-purified culture medium of transfected cells (1:4 dilution, estimated 2.5 μg/ml of rZP3) was tested against culture medium (Ham's F-10), a 49% inhibition in binding was

Fig 16. Results of metabolic labelling experiment. ^{3}H-Galactose was substituted into the culture medium of the transfected and non-transfected cells to study the biosynthesized glycoproteins. The culture media from non-transfected cells and transfected cells were concentrated by Centriprep ultrafiltration with 30 kDa cut-off; the samples were purified by passing through WGA-Lectin affinity chromatography. Distinct bands were demonstrated at 55-70 kDa (three bands), 80 kDa, and 100 kDa. In addition, the WGA-unbound fraction (washed solution) of the transfected medium was also loaded on the gel as reference.

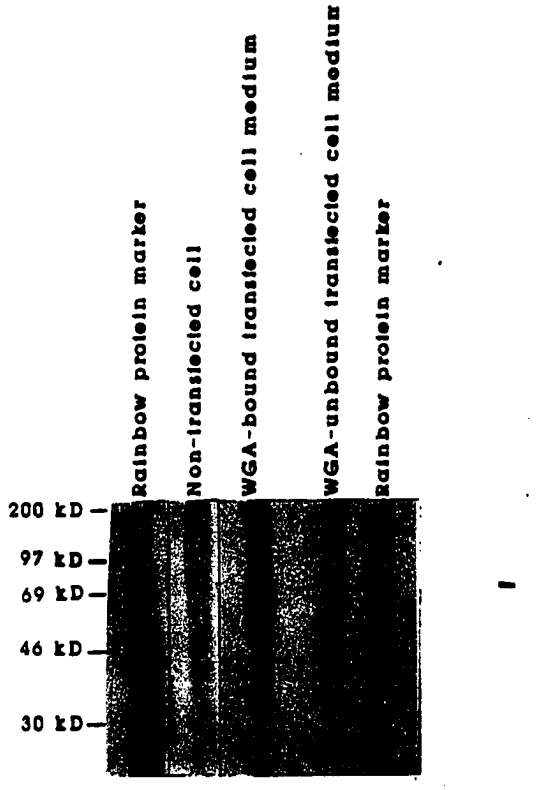
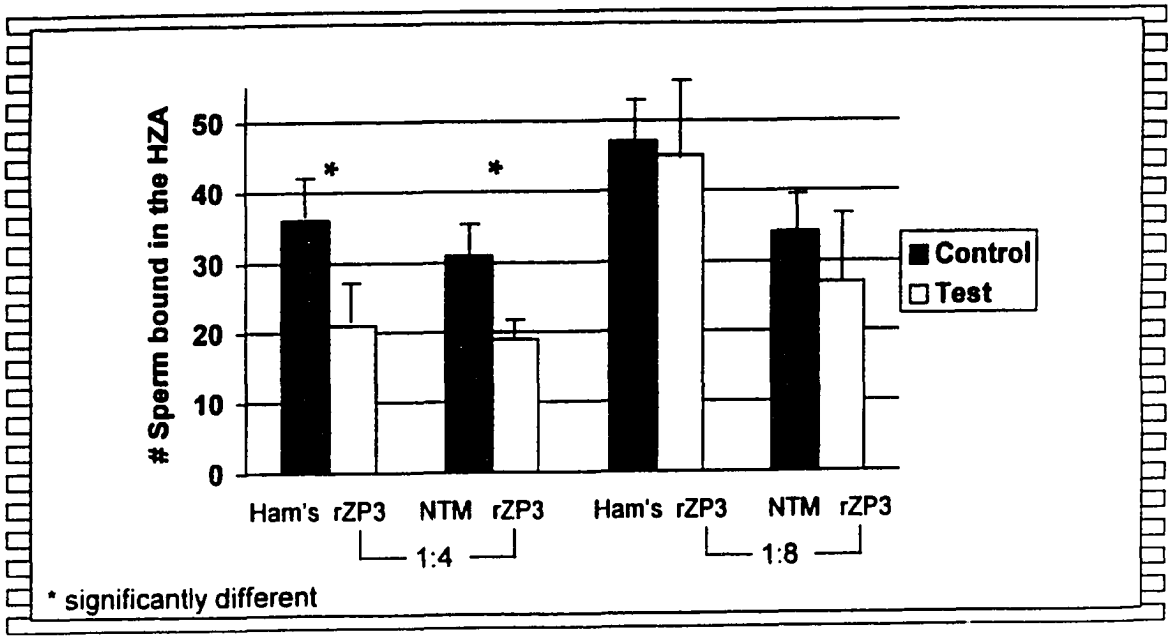


Fig 17. Characterization of the biological activity (sperm-binding) of the recombinant glycoprotein in the hemizona assay (HZA).
Paired t-test was applied to analyze the results of HZA. WGA-purified culture medium of transfected cells at (1:4 dilution) and (1:8 dilution) were tested by the hemizona assay, and showed 49% inhibition ($n=6$, $p<0.05$) in binding with (1:4 dilution) sample, but no significant difference was observed with (1:8 dilution) sample.
Ham's F-10 and WGA-purified culture medium of non-transfected cells were used as negative controls.
The concentrations of total glycoproteins in WGA-purified culture medium of transfected cells and non-transfected cells were controlled at 1 mg/ml.



observed and the results were statistically significant ($n=6$, $p<0.05$ by paired t-test) (Appendix VI). No significant differences were observed at a 1:8 dilution. This result demonstrated that there seems to be a concentration-dependent effect of the recombinant hZP3 in the HZA. In addition, the WGA-purified culture medium of non-transfected cells (both 1:4 and 1:8 dilution) did not show any inhibition (negative control) (Fig 17).

Furthermore, at a 1:4 dilution, the WGA-purified culture medium of transfected cells (rhZP3) produced a >50% induction of acrosome reaction (AR) after overnight incubation. Both Ham's F-10 and WGA-purified culture medium of non-transfected cells were used as negative controls (spontaneous AR). No difference was observed between the controls (20% basal AR were found). A 10.0 μM of calcium ionophore (A23187) was used as a positive control at the same conditions, and showed a similar effect to that achieved with a 1:4 dilution of WGA-purified transfected cells culture medium (about 70% AR were found).

V. DISCUSSION AND CONCLUSIONS

Recent studies report ZP3 to be critical for the initiation of fertilization in the mouse model [9,39,117-120]. More studies on the human ZP3 protein need to be performed to prove relevance in the human system. However, it is difficult to study hZP3 due to: (a) the difficulties in obtaining enough material for research, and (b) the heterogeneity of glycosylation of its polypeptide backbone, which results in difficulty isolating the protein [40,42, 53]. Here we produce the hZP3 cDNA by RT-PCR and express the hZP3 glycoprotein in stable-transfected PA-1 cells by using molecular biology techniques, and taking advantage of the published sequence for hZP3 cDNA [40].

A. Subcloning of hZP3 cDNA

In the mouse model, the expression of ZP3 is highly oocyte-specific and is restricted to the 2-weeks growth phase of oogenesis that occurs prior to ovulation [56,57, 60]. Therefore a fresh, surgically removed human ovary was used as source of the mRNA for the production of full-length hZP3 cDNA by RT-PCR. A single product was amplified by RT-PCR (Fig 3). The RT-PCR product was identified to be full length hZP3 cDNA by restriction mapping (Fig 3), Southern

blot analysis (Fig 4), and PCR (Data not shown). Further studies indicated that it is indeed hZP3 cDNA and there is no reading frame shift, although there may be some point mutations in the sequence (Figs 7, 8 and 10).

The RT-PCR product (hZP3 cDNA) was first inserted into pBluescript SK (PSK) vector and then subcloned into an mammalian expression vector (pREP4). A subcloning strategy and methodology was developed based on blunt-end ligation with an oligonucleotide linker, and has been proven to be effective for the introduction of the RT-PCR products into vectors. There were many advantages to insert the cDNA into PSK vector as follows: (1) An *in vitro* transcription/ translation (by taking advantage of the T3 promoter inherent in the PSK vector) was performed to identify the capacity of the cloned cDNA to produce a full-length non-glycosylated hZP3 protein (47 kDa, Fig 10). (2) Autosequening was performed by taking advantage of the T3 and T7 promoters inherent in the PSK vector (Fig 7, 8; Appendices III, IV). (3) "Color selection" using the PSK vector and XL1-Blue Ecoli bacterial system enhanced the efficiency of clone selection. Restriction mapping, Southern blot analysis and PCR were performed to identify the right clone (Figs 5, 6).

In order to express the hZP3 in PA-1 cells, the cloned cDNA was subcloned into appropriate mammalian expression vectors. We have successfully inserted the hZP3 into an episomal vector, pREP4, which is mainly composed of a Rous

sarcoma virus long terminal repeat (RSV LTR), Epstein Barr virus OriP element (EBV OriP), EBNA-1 gene, SV40 poly A element and the Hygromycin resistance gene (Fig 9). There are two major advantages by using pREP4 as vector to express foreign cDNA in mammalian cells. First of all, the RSV LTR is active in a broad range of mammalian cell types; therefore, the foreign gene or cDNA inserted into the pREP4 vector is capable of being transfected into and expressed in a variety of mammalian cells. Second, the inherent EBV OriP element and EBNA-1 gene maintain and allow the introduced vector to be propagated for the expression of foreign genes in adherent mammalian cells [81].

B. Expression of hZP3

1. Stable transfection

The biological activity of the ZP3 (i.e. the receptor ligand ability) has been reported to be related to its carbohydrate side chains [28,40,48,49,98,100], therefore, the glycosylation of the protein is critical for the production of a recombinant hZP3 with biological function. Therefore, a human ovarian tumor cell line, PA-1, was selected as the host cell for the production of recombinant hZP3 glycoprotein based on three facts: (1) it is an ovarian cell line, (2) it is of human origin, and (3) it is known to produce glycosylated native proteins (lactosaminoglycan-carrier glycoprotein with 80-120 KDa molecular mass; and

heparin-binding protein which is about 44 kDa) [77,78], and recombinant proteins (recombinant fibronectin) [111].

After the cloned hZP3/pREP4 expression vector DNA construct was introduced into PA-1 cells by the calcium phosphate-mediated method and hygromycin selection, the single colonies of stable-transfected cells were identified and selected by three criteria: PCR (Fig 11), RT-PCR (Fig 12), and ELISA (Table 1).

Based on the results, the expression vector construct was integrated into the genomic DNA of PA-1 cell, and might be integrated into the chromosomal DNA. First of all, a specifically amplified product (954 bp) was identified by PCR using CH-1/S1 primers in the transfected cells, but was not found in the non-transfected cells, suggesting the integration of the hZP3 cDNA into the genomic DNA (Fig 11). Second, total cytoplasmic RNA (as well as small DNAs other than chromosomal DNA) was isolated by the sucrose gradient method as described previously, and a single specifically amplified product was identified by RT-PCR using CH-1/S1 primers in the transfected cells. However, nothing was found by the same application in the cells transfected with vector (Fig 12) or in the non-transfected cells (Data not shown). Therefore, no genomic DNA in the cytoplasm was amplified by PCR using CH-1/S1 primers, otherwise there ought to be some background observed in the RT-PCR identification (Fig 12). Third, it is believed that the introduced foreign DNA was

not stable in the cytoplasm and ought to be digested by DNase inside the cells in a few days.

The mechanism of the integration of the expression vector DNA construct (hZP3/pREP4) into the chromosomal DNA of the host cells (PA-1) is not clear, but it is believed to be a non-specific integration. Nevertheless, the Rous sarcoma virus long terminal repeat (LTR) inherent in the expression vector (episomal vector, pREP4) is able to be inserted into the chromosomal DNA by transposition [121]. Therefore, the vector-dependent mechanism (Rous sarcoma virus LTR) may be responsible for the integration of hZP3/pREP4 construct into the PA-1 genomic DNA [121].

2. Expression of hZP3

The *in vitro* transcription/translation experiment demonstrated that our cDNA of hZP3 was able to produce a nonglycosylated protein which exhibited full-length protein backbone of the hZP3 glycoprotein (47 kDa, Fig 10). In addition, a second product with a molecular mass about 40 kDa was also observed (Fig 10). We do not know whether this protein has any physiological meaning or just represents a non-completed product of the transcription or translation reaction.

In order to produce glycosylated hZP3, the cloned cDNA was introduced into and expressed in PA-1 cells. Culture medium was collected and the recombinant hZP3 was purified

by WGA-Sepharose chromatography and anti-WGA immunoaffinity chromatography as described in Methods (Fig 13). Centriprep-30 and Centricone-30 were used to concentrate protein following manufacturer's instruction.

Although there were still many different glycoproteins, the WGA-purified glycoprotein solution showed the banding at the expected position (65 kD) by SDS-PAGE and silver staining (Fig 14). The SDS-PAGE by silver staining could not be effectively analyzed for the identification of the secreted hZP3 glycoprotein because of the following reasons. First, the test and control solutions were still composed of many proteins including some proteins with the similar molecular mass (65 kDa). Second, the amount of recombinant glycoprotein produced may not be enough for the identification. Finally, silver staining is not able to specifically identify the hZP3 from other proteins (Fig 14).

Therefore, Western blot analysis with antibody (Ab5a) was performed and this further identified the hybridization at 65 kDa and 100 kDa (Fig 15). Moreover, solubilized human zonae demonstrated the exactly same size of hybridization at around 65 kDa by Western blot analysis, suggesting that hZP3 is 65 KDa (Fig 15). In fact, a very weak band at 100 kDa was also hybridized in the solubilized zonae, suggesting this high molecular weight protein has less immunogenicity than the 65 kDa protein and may be related to hZP3.

There are many possibilities for the 100 kDa (or heavier) glycoprotein which was produced by the transfected cells and also demonstrated immunogenicity in the Western blot analysis (Fig 15). According to Shabanowitz, a band heavier than 100 kDa and in the similar position as ours was also hybridized by Western blot analysis when solubilized human zonae proteins were used as sample [53]. Shabanowitz explained this band may be a dimer of ZP1 and ZP2 [53]. In fact, it does not seem to be accurate if Dean's estimation about the molecular mass of hZP proteins is correct (hZP2 about 76 kDa and hZP1 about 100 kDa) [40]. More importantly, it does not agree with our understanding of the hZP proteins that ZP2 and ZP3 are believed to bind together as dimer, but not ZP1 and ZP2 [7,39].

On the other hand, a transfected hamster ovarian cell line (CHO) was produced recently by the introduction of a hZP3 "minigene" construct [62]. The produced recombinant glycoprotein (55 kDa) was smaller than the native hZP3 and the most important biological activity of the hZP3, i.e. the sperm ligand activity, was not reported. Nevertheless, a band heavier than 100 kDa whose position was similar to our result, was also stained by western blot analysis shown in their figure [62]. This indicated the possibility that the protein (100 kDa or heavier) could be somehow related to ZP3 glycoprotein, especially since both of them (65 kDa and 100 kDa) demonstrated immunogenicity to ZP3 antibodies.

In addition, it had been shown that PA-1 tumor cell produced another recombinant glycoprotein (fibronectin) with heavier molecular mass than its native one [111]. It could be explained by the activation of the glycosylation system in the tumor cell itself. Therefore, it is still not clear whether the 100 kDa (or heavier) glycoprotein produced is really hZP3-related glycoprotein or a by-product due to overglycosylation of the hZP3 protein in tumor cells.

By using ^{3}H-galactose, a metabolic labelling study was performed as described in Methods to identify the newly biosynthesized and secreted glycoproteins. In the ^{3}H-galactose labelling metabolic study, the 100 kDa (or heavier) glycoprotein was distinct and obviously a product of the transfected cells (Fig 16) which implied that it is either a product of the introduced hZP3/pREP4 DNA construct or induced by the products of the introduced DNA construct in PA-1 cells. In addition, the total amount of glycoproteins produced in the transfected cells was about 3.29 times more than that in the non-transfected cells, and there may be a large amount of the biosynthesized recombinant glycoproteins (up to 10 millions cpm) which have not been secreted and are left inside the transfected cells (Table 2).

According to the results of SDS-PAGE with silver stain (Fig 14), ^{3}H-galactose labelling metabolic study (Fig 16), and especially Western blot analysis (Fig 15), the molecular mass of the recombinant hZP3 glycoproteins produced by the

transfected cells of human ovarian cell line (PA-1) was about 65 kDa. In addition, the non-glycosylated polypeptide backbone of hZP3 is 47 kDa as mentioned previously and a complex type N-linked or O-linked oligosaccharide is about 3-4 kDa. In this case, it implies that there ought to be four to six carbohydrate side chains (account for 18 kDa) in one molecule of hZP3 glycoprotein. And at least one or some of them must be O-linked carbohydrate side chains because it is believed that it is the O-linked carbohydrate side chains that are responsible for the biological activity of the hZP3 glycoprotein as receptor ligand of sperm cell [39,48].

Carbohydrate chains are believed to be able to stabilize the protein itself and a hZP3 molecule contained at least 18 kDa of carbohydrate side chains according to our results. This may be one of the reasons why the half-life of the zona pellucida proteins is so long (>100 hr and may be up to 10 days) [39,40]. On the other hand, zona pellucida proteins may polymerize and are organized as a filament structure [5,7,44]. This implies that there may be distinct molecular structure of the zona pellucida proteins in their polypeptide backbone that will enhance their protein-protein polymerization, or some specific metabolic pathway or intracellular signal transduction mechanism may exist to assemble this special organelle in the female germ cell during development.

C. Biological functions of hZP3

As mentioned before, hZP3 is expected to be the first receptor to bind and recognize sperm, and the hemizona assay (HZA) has been proven to be the best method to test the biological function of binding and recognition between sperm and egg [40,91]. Indeed, the biological activity of the recombinant hZP3 demonstrated significant results in the hemizona assay (Fig 17), and it was also demonstrated to be effective in the acrosome reaction assay (AR) in preliminary experiments. Ham's F-10 and the culture medium from non-transfected cells were used as negative controls to do a reasonable adjustment of the results in the bio-assays (HZA and AR). In addition, the paired t-test was applied to statistically analyze the results between test and control groups in the HZA. These results strongly suggest that hZP3 is really functioning as a receptor to bind sperm as well as inducer of the acrosome reaction.

To further investigate the molecular mechanisms of human sperm-zona pellucida interaction, future research will involve the following: (1) studies of signal transduction events after the recognition and binding of the receptors of zona pellucida and sperm cell at the cellular level (for example, Ca^{2+} influx), (2) dose-dependent and time-course studies of the recombinant hZP3 in the Hemizona assay (HZA) and Acrosome reaction assay (AR), and (3) further purification (i.e. immunoaffinity) of the recombinant hZP3

and amino acid sequencing.

D. Conclusion

In conclusion, this study has fulfilled our specific aims based on the following experiment results:

(1) The full-length cDNA of hZP3 has been successfully produced, identified and subcloned into expression vectors as proposed.

(2) A stable-transfected cell line has been produced by introduction of the hZP3/expression vector DNA construct into a human ovarian cell line, PA-1, and identified based on the following criteria: PCR, RT-PCR, and ELISA.

(3) The isolation of a secreted recombinant glycoprotein has been accomplished by WGA-Lectin chromatography and anti-WGA immunoaffinity chromatography.

(4) The secreted recombinant hZP3 has been identified by the following criteria: immunogenicity (ELISA and Western blot analysis), molecular mass (*in vitro* translation, and SDS-PAGE with silver stain), and biological characterization (HZA and AR). In addition, in the biological characterization, the recombinant glycoprotein demonstrated the dual functions of hZP3 (i.e.sperm-binding and induction of acrosome reaction).

VI. REFERENCES

1. Bedford JM, and Hoskins DD. (1990) The mammalian spermatozoa: morphology, biochemistry and physiology. In:(Lamming GE, ed.) Marshall's physiology of reproduction. Vol. 2. pp.379-568, Edinburgh: Churchhill Livingston.

2. Moore HDM (1990) J. Reprod. Fert., Suppl. **42**, 71-78.

3. Saling PM. (1989) Oxf. Rev. Reprod. Biol. **11**, 339-388.

4. Saling PM. (1991) Biol. Reprod. **44**, 246-251.

5. Wassarman PM. (1990) Development **108**, 1-17.

6. Wassarman PM. (1990) J. Reprod. Fertil. **42**, 79-87.

7. Yanagimachi R. (1988) Mammalian fertilization. In: (Knobil E, Neill J eds.) The physiology of reproduction. pp.135-185, Raven Press, New York.

8. Yanaqimachi R. (1981) Mechanisms of fertilization in mammals. In: (Mastroianni L, Biggers JD, eds.) Fertilization and embryonic development in vitro. pp.81-187, Plenum Press, New York.

9. Phillips DM. (1991) Sperm receptors of mammalian eggs; and Electron microscopy of mammalian fertilization. In: (Wassarman PM, ed.) Elements of mammalian fertilization. Vol.1. pp.133-151; pp.249-267, CRC Press, boca Raton, Florida.

10. Zaneveld LJD, and De Jonge CJ. (1991) Mammalian sperm acrosomal enzymes and the acrosome reaction. In: (Dunbar BS, O'Rand MG, eds.) A comparative overview of mammalian fertilization. pp.63-79, Plenum Press, New York.

11. Kopecny V, and Flechon JE. (1981) Biol. Reprod. **24**, 201-216.

12. Flechon JE. (1979) Gamete Res. **2**, 43-51.

13. Holt WV. (1979) J. Ultrastruct. Res. **68**, 58-71.

14. Goodpaster KL, Reddy JM, and Zaneveld JD. (1987) Biol. Reprod. **25**, 44-55.

15. Katz DF, Yanaqimachi R. (1981) Biol. Reprod. **25**, 785-791.

16. Leggio LL, Williams RM, and Jones R. (1994) J. Reprod. Fertil. **100**, 177-185.

17. Topfer-Petersen E, and Cechova D. (1990) Int. J. Androl. **13**, 190-196.

18. Jones R. (1990) J. Reprod. Fertil. **42**, 89-105.

19. Chang MC. (1951) Nature **168**, 697-698.

20. Chang MC. (1955) Nature **175**, 1036-1037.

21. Austin CR. (1967) Int. J. Fertil. **12**, 25-31.

22. Demott RP, and Suarez SS. (1992) Biol. Reprod. **46**, 779-785.

23. Suarez SS, Dai X. (1992) Biol. Reprod. **46**, 686-691.

24. Suarez SS, Katz DF, Owen Dh, Andrew JB, and Powell RL. (1991) Biol. Reprod. **44**, 375-381.

25. Leblond CP, and Clemmont Y. (1952) Am. J. Anat. **90**, 167-215.

26. Yanaqimachi R. (1989) J. Reprod. Fertil. **38**, 27-33.

27. Blackmore PF, Beebe SJ, Danforth DR, and Alexander NJ. (1990) J. Biol. Chem. **265**, 1376-1380.

28. Bleil JD, and Wassarman PM. (1986) J. Cell. Biol. April, **102**, 1363-1371.

29. Bleil JD, and Wassarman PM. (1986) J. Cell. Biol. **102**, 1363-1371.

30. Cross NL, Morales P, Overstreet JW, and Hanson FW. (1988) Biol. Reprod. **38**, 235-244.

31. Coddington CC, Fulgham DC, Alexander NJ, Johnson D, Herr JC, and Hodgen GD. (1990) Fertil. Steril. **54**,504-508.

32. Franken DR, Oosthuizen T, Cooper S, Kruger T, Burkman

LJ, Coddington CC, and Hodgen GD. (1991) Andrologia. **23**, 205-208.

33. Mahony M, Oehninger S, Clark GF, Acosta A, and Hodgen GD. (1991) Contraception **44**, 657-665.

34. Morales P, Cross NL, Overstreet JW, and Hanson FW. (1989) Devel. Biol. **133**, 385-392.

35. Oehninger S, Blackmore P, Morshedi M, Sueldo C, Acosta A, and Alexander N. (1994) Fertil. Steril. **61**, 349-354.

36. Tesarik J, Moos J, and Mendoza C. (1993) Endocrinology **133**, 328-335.

37. Beebe SJ, Leyton L, Burks D, Ishikawa M, Fuerst T, Dean J, and Saling P. (1992) Devel. Biol. **151**, 48-54.

38. Berger T, Davis A, Wardrip NJ, and Hedrick JL. (1989) J. Reprod. Fertil. **86**, 559-565.

39. Dean J. (1992) J. Clin. Invest. **89**, 1055-1059.

40. Chamberlin ME, and Dean J. (1990) Proc. Nat. Acad. Sci. USA. **87**, 6014-6018.

41. Kopf GS. (1990) J. Reprod. Fertil. **42**, 33-49.

42. Shabanowitz RB, and O'Rand MG. (1989) J. Reprod. Fertil. **82**, 151-161.

43. Dean J, Chamberlin ME, Miller SE, Ringuette MJ, Philpott CC, Baur AW, and Chamow SM. (1989) Developmental expression of ZP3, a mouse zona pellucida gene. Development of preimplantation, embryos and their enviroment. pp.21-32, Alan R. Liss Inc.

44. Bleil JD, and Wassarman PM. (1980) Devel. Biol. **76**, 185-202.

45. Hinsch KD, Hinsch E, Meinecke B, Topfer-Petersen E, Pfisterer S, and Schill WB. (1994) Biol. Reprod. **51**, 193-204.

46. Hinsch KD, Hinsch E, Pfisterer S, Schill WB, Gibbon WE, Hodgen GD, and Oehninger S. (1994) American J. Reprod. Immunology **32**, 146-151.

47. Bleil JD, Greve JM, and Wassarman PM. (1988) Devel. Biol. **178**, 376-385.

48. Florman HM, and Wassarman PM. (1985) Cell **41**, 313-324.

49. Bleil JD, and Wassarman PM. (1988) Proc. Natl. Acad. Sci. USA. **85**, 6778-6782.

50. Hokke CH, Damm JBL, Penninkhof B, Aitken RJ, Kamerling JP, and Vliegenthart JFG. (1994) Europ. J. Biochem. **221**, 491-512.

51. Kinloch RA, Roller RJ, Fimiani CM, Wassarman DA, and Wassarman PM. (1988) Devel. Biol. **85**, 6409-6413.

52. Noguchi S, Nakano M. (1993) Biochemica et Biophysica Acta **1158**, 217-226.

53. Shabanowitz RB. (1990) Biol. Reprod. **43**, 260-270.

54. Kimura J, Sato K, Okano M, and Tsukise A. (1994) Cell. Mol. Biol. **40**, 1097-1101.

55. Kinloch RA, Sakai Y, and Wassarman PM. (1995) Proc. Natl. Acad. Sci. USA. **92**, 263-267.

56. Liang LF, Chamou SM, and Dean J. (1992) Mol. Cell. Biol. **10**, 1507-1515.

57. Lira SA, Kinloch RA, Mortillo S, and Wassarman PM. (1990) Proc. Natl. Acad. Sci. USA **87**, 7215-7219.

58. Millar SM, Lader E, Liang LF, and Dean J. (1990) Mol. Cell. Biol. Dec, **11**(12), 6197-6204.

59. Murray RK. (1990) Harper's biochemistry. (22nd Ed.) pp.591-608, Appleton & Lange, Norwalk,Conn.

60. Ringuette MJ, Sobieski DA, Chomow SM, and Dean J. (1986) Proc. Nat. Acad. Sci. USA. **83**, 4341-4345.

61. Roller RJ, Kinloch PA, Hiraoka BY, L.Li SS, and Wassaman PM. (1989) Development **106**, 251-261.

62. Duin MV, Polman JEM, De Breet ITM, Ginneken KV, Bunschoten H, Grootenhuis A, Brindle J, and Aitken J. (1994) Biol. Reprod. **51**, 607-617.

63. Bagavant H, Yurewicz EC, Sacco AG, Talwar GP, and Gupta SK (1993) J. Reprod. Immunol. **25**, 277-283.

64. Oehninger S, Clark GF, Acosta A, and Hodgen GD. (1991) Fertil. Steril. **55**, 165-169.

65. Rosiere TK, and Wassarman PM. (1992) Devel. Biol. **154**, 309-317.

66. Chai W, Stoll MS, Cashmore GC, and Lawson AM. (1993) Carbohydr. Res. **239**, 107-115.

67. Hay ED. (1981) Cell biology of extracellular matrix. pp.20; pp.41, Plenum press, New York.

68. Lennarz WJ. (1980) The biochemistry of glycoproteins and proteoglycans. pp.1-34, Plenum Press, New York.

69. Kinloch RA, Ruiz-Seiler B, and Wassarman PM. (1990) Devel. Biol. **142**, 414-421.

70. Bagavant H, Thillaikoothan P, Sharma MG, Talwar GP, and Gupta SK. (1994) J. Reprod. Fertil. **102**, 17-25.

71. Berger SL, and Kimmel AR. (1987) Methods in enzymology. **152**,151-154, Academic Press.

72. Dietl J, and Mettler L. (1982) Experientia **38**, 502-503.

73. Sacco A, Yurewicz E, Subramaniah MG, and DeMayo F. (1981) Biol. Reprod. **25**, 997-1008.

74. Schwoebel ED, Vandevoort CA, Lee VH, Lo YK, and Dunbar BS. (1992) Biol. Reprod. **47**, 857-865.

75. Freshney RI. (1994) Culture of animal cell: A manual of basic techniques. pp.169-171, Wiley-Liss Inc.

76. Cabanas MJ, Vazquez D, and Modolell J. (1978) Eur. J. Biochem. **87**, 21-27.

77. Fukuda MN, Dell A, Oates JE, and Fukuda M. (1985) J. Biol. Chem. **260**, 6623-6631.

78. Furukawa T, Ozawa M, Huang RP, and Muramatsu T. (1990) J. Biochem. **108**, 297-302.

79. Harlow ED, and Lane D. (1990) Antibodies: A laboratory manual. pp.490-492; pp.496-497; pp.505; pp.574-576, Cold Spring Harbor Laboratory Press.

80. Chirwin JM, Przybyla AE, MacDonald RJ, and Rutter WJ. (1979) Biochem. **18**, 5294-5299.

81. Sambrook J, Fritsch EF, and Maniatis T. (1989) Molecular cloning: a laboratory manual. (2nd Ed) p1.22-1.37; p1.54-1.56; p1.74-1.84; p9.31-9.57; p16.6; p16.26; p16.32-16.37, Cold Spring Harbor Laboratory Press.

82. Zyskind JW, and Bernstein SI. (1989) Recombinant DNA laboratory manual. pp.36-38; pp.68; pp.179-180, Academic Press.

83. Mullis KB, Ferre F, and Gibbs RA. (1994) The polymerase chain reaction. pp.3-37; pp.97-109; pp.277-294, Birkhauser, Boston.

84. Gonzalez A, Jimenez A, Vazquez D, Davies JE, and Schindler D. (1978) Biochemica et Biophysica Acta. **521**, 459-469.

85. Singh A, Ursic D, and Davies J. (1979) Nature **277**, 146-148.

86. Higuchi,R (1989) Perkin Elmer/cetus, Newsletter Amplification **2**, 1

87. Skutelsky E, Ranen E, Shalgi R. (1994) J. Reprod. Fertil. **100**, 35-41.

88. Redinbaugh MG, and Turley RB. (1986) Anal. Biochem. **153**, 267-271.

89. Lennarz WJ, Hart GW. (1994) Guide to techniques in glycobiology. Methods in enzymology **230**, 2, 16-32.

90. Burkman LJ, Coddington CC, Franken DR, Kruger T, Rosenwaks Z, and Hodgen GD. (1988) Fertil. Steril. **49**, 688-693.

91. Coddington CC, Franken DR, Burkman LJ, Oosthuizen WT, Kruger TF, and Hodgen GD. (1991) J. Androl. **12**, 1-18.

92. Franken DR, Burkman LJ, Oehninger SC, Coddington CC, Veeck LL, Kruger TF, Rosenwaks Z, and Hodgen GD. (1989) Gamete Res. **22**, 15-26.

93. Franken DR, Oehninger S, Burkman LJ, Coddington CC, Kruger TF, Rosenwaks Z, Acosta AA, and Hodgen GD. (1989) J. In Vitro Fertil. Embryo. Transf. **6**, 44-49.

94. Franken DR, Kruger TF, Menkveld R, Oehninger S, Coddington CC, and Hodgen GD. (1990) Fertil. Steril. **54**, 497-503.

95. Franken DR, Coddington CC, Burkman LJ, Oosthuizen WT, Oehninger S, Kruger T, and Hodgen GD. (1991) Fertil. Steril. **56**, 1156-1161.

96. Franken DR, Windt ML, Kruger T, Oehninger S, and Hodgen GD. (1991) Mol. Reprod. Devel. **30**, 56-61.

97. Kruger TF, Oehninger S, Franken DA, and Hodgen GD. (1991) J. In Vitro Fertil. Embryo. Transf. **8**, 154-156.

98. Oehninger S, Acosta R, Morshedi M, Philput C, Swanson RJ, and Acosta AA. (1990) J. Androl. **11**, 446-452.

99. Oehninger S, Veeck LL, Franken D, Kruger TF, Acosta AA, and Hodgen GD. (1991) Fertil. Steril. **55**, 1165-1170.

100. Oehninger S, Alexander NJ, Franken DR, Coddington CC, Burkman LJ, Kruger TF, Acosta A, and Hodgen GD. (1991) Antifertilization assays. In: (Alexander N, Griffin D, Spieler J, Waites G eds) Gamete Interaction: Prospects for Immunocontraception. pp.473-488, Wiley Liss, New York.

101. Oehninger S, Acosta AA, Veeck L, Brzyski R, Kruger T, Muasher S, and Hodgen GD. (1991) Am. J. Obstet. Gynecol. **164**, 1210-1215.

102. Oehninger S, Clark GF, Fulgham D, Blackmore P, Mahony M, Acosta A, and Hodgen GD. (1992) J. Androl. **13**, 519-525.

103. Oehninger S, Toner JP, Muasher SJ, Coddington CC, Acosta AA, and Hodgen GD. (1992) Am. J. Obstet. Gynecol. **167**, 1760-1767.

104. Oehninger S, Franken D, Alexander N, and Hodgen G. (1992) Andrologia **24**, 307-321.

105. Oehninger S. (1992) Diagnostic significance of sperm-zona interaction. In: (Hodden KS, and Stoughton K eds) Reeproductive Medicine Review. 1:pp.57-81, England.

106. Oehninger S, Mahony M, Swanson JR, and Hodgen GD. (1993) Mol. Reprod. Devel. **35**, 59-61.

107. Windt ML, Franken DR, deBeer PM, Bouic PJD, and Kruger TF. (1991) Andrologia. May-Jan, **23**(3), 209-212.

108. Cross NL, Morales P, Overstreet JW, and Hanson FW. (1986) Gamete Res. **15**, 213-217.

109. Munro BH, Visintainer MA, and Page EB. (1986) Statistical methods for health care research. pp.155-171; pp.63-84, J. B. Lippincott Co, Philadelphia.

110. Sanger F, Nicklen S, and Coulson AR. (1977) Proc. Nat. Acad. USA. **74**, 5463-5467.

111. McIlhinney PA, and Patel S. (1983) Cancer. Res. **43**, 1282-1288.

112. Mahony M, Clark G, Oehninger S, Acosta A, and Hodgen G. (1993) Contraception **48**, 277-283.

113. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, and Kobata A. (1979) J. Biol. Chem. **254**, 6419-6425.

114. Oehninger S, Acosta A, and Hodgen GD. (1990) Fertil. Steril. **53**, 143-149.

115. Stoll MS, Hounsell EF, Lawson AM, Chai W, and Feizi T. (1990) Eur. J. Biochem. **189**, 499-507.

116. Tong ZB, Nelson LM, and Dean J. (1995) J. Biol. Chem. **270**, 849-53.

117. Chamberlin ME, and Dean J. (1989) Devel. Biol. **131**, 207-214.

118. Cheng A, Le T, Palacios M, Bookbinder LH, Wassarman PM, Suzuki F, and Bleil JD. (1994) J. Cell. Biol. **125**, 867-878.

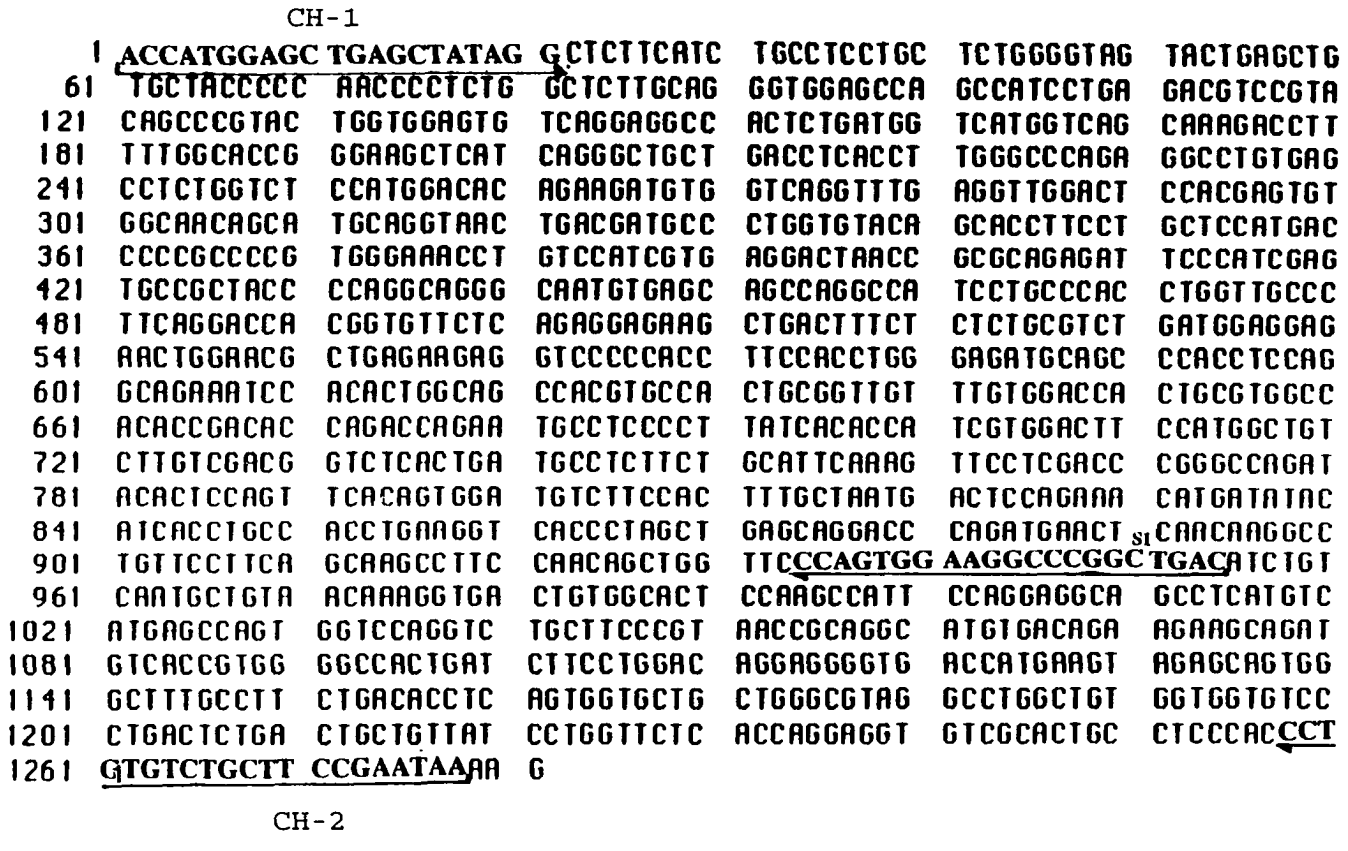
119. Bleil JD, and Wassarman PM. (1990) Proc. Natl. Acad. Sci. USA. Jul, **87**(14), 5563-5567.

120. Ringuette MJ, Chamberlin ME, Baur AW, Sobieski DA, and Dean J. (1988) Devel. Biol. **127**, 287-295.

121. Watson JD, Gilman M, Witkowsk J, and Zoller M. (1992) Recombinant DNA. (2nd Ed.) pp.175-90; pp.225-227, Scientific American, Inc.

APPENDIX I

Sequence of the cDNA of hZP3 (J. Dean) and the locations and orientations of the primers and probe designed for this study (attach behind)



APPENDIX II

Estimated cell number in various culture dishes

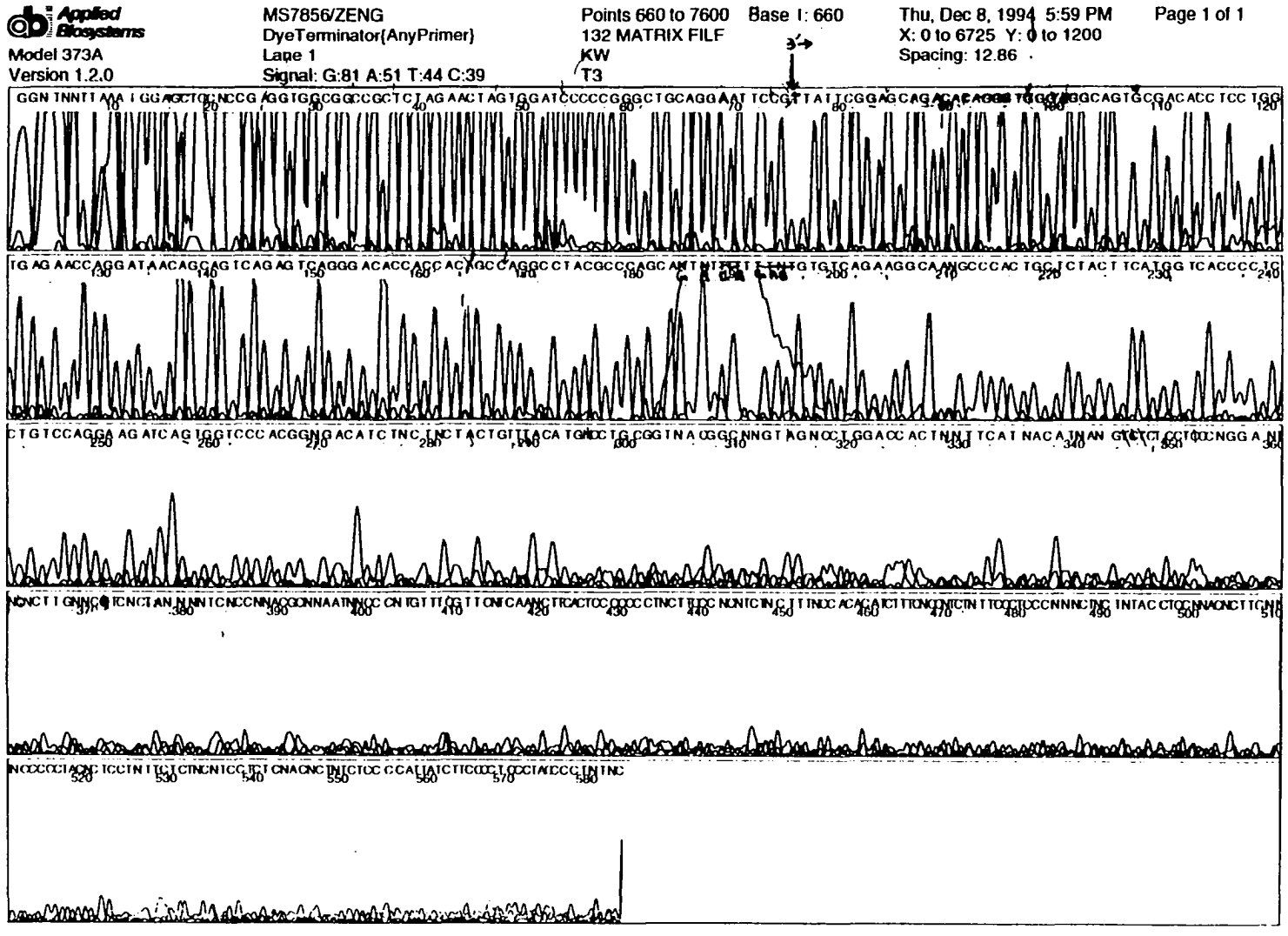
	Internal radius(cm)	Area(cm^2) (per well)	Cell Number(x million) (100% confluence)
24 well cluster (Costar)	0.75	1.77	0.5
6 well cluster (Costar)	1.70	9.08	2.57
Petri dish (100x20mm) (Corning)	4.00	50.26	14

APPENDIX III

Autosequencing of the RT-PCR product (cDNA of hZP3) by taking advantage of the T3 promoter inherent in the hZP3/PSK DNA construct

This sequence read was reversed and complementary to that of hZP3 cDNA published by J. Dean (see Appendix I).

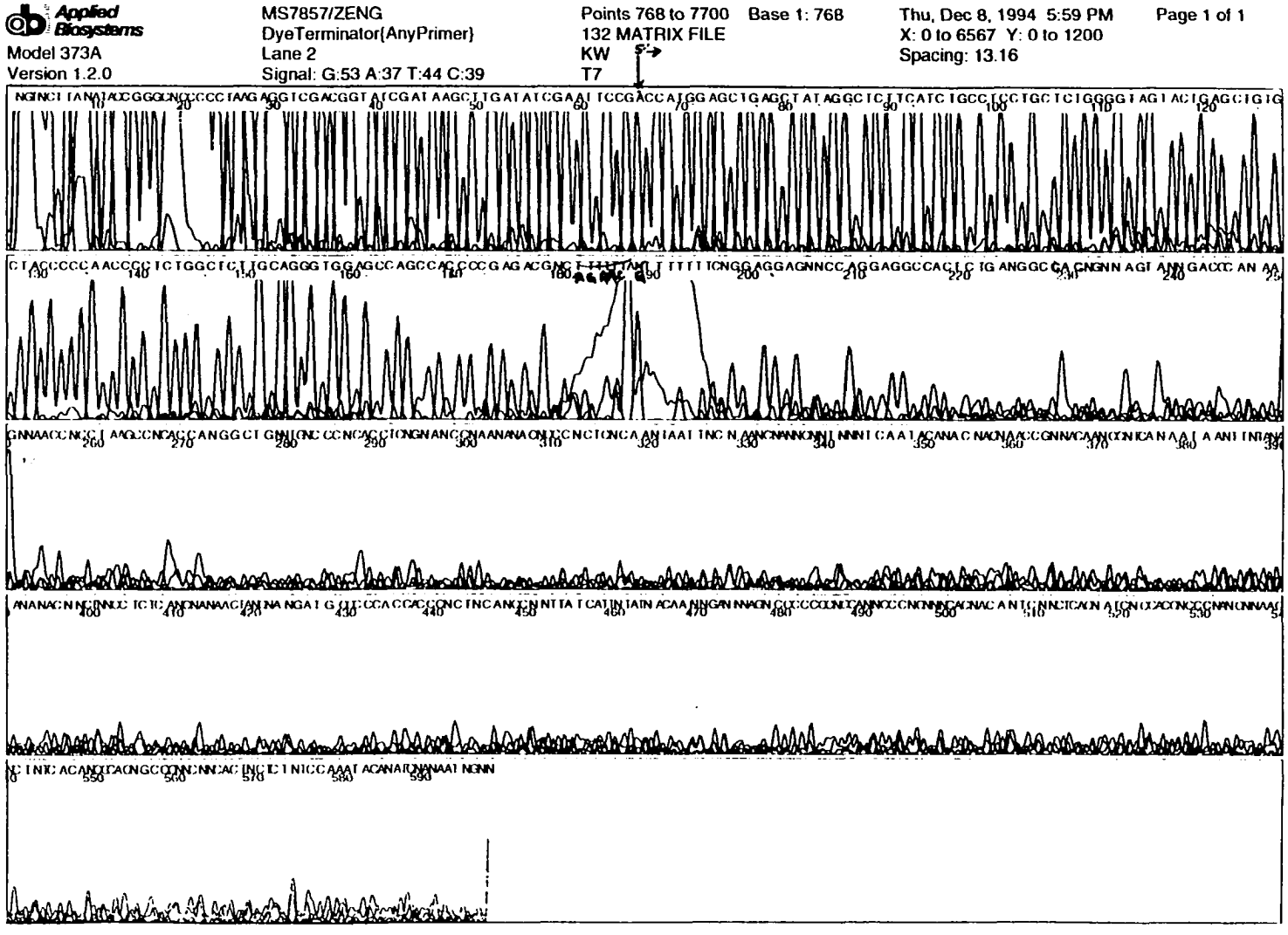
The position of the first base (base 1278) of hZP3 cDNA identified was marked in the figure attached behind.



APPENDIX IV

Autosequencing of the RT-PCR product (cDNA of hZP3) by taking advantage of the T7 promoter inherent in the hZP3/PSK DNA construct

The position of the first base (base 1) of the hZP3 cDNA identified was marked in the figure attached behind.



APPENDIX V

Sperm motility examination

Conclusion: There was no significant toxic effect of the recombinant hZP3 (rZP3) found on the sperm motility.

	% Motility
Ham's	79 ± 2
rZP3 (1:4)	72 ± 3.5
NTM	74 ± .5
rZP3 (1:4)	76 ± 1
Ham's	71 ± 2
rZP3 (1:8)	64 ± 4
NTM	77 ± 2
rZP3 (1:8)	70 ± 3.1

APPENDIX VI

Computer print-out of the statistic analysis of the hemizona assay results by single (paired) t-test.

The larger the |t| score and the smaller the p score (two-tailed test) are, the more significant the results demonstrate.

6 cases with 2 variables

	1 C	2 T
1	54.000	29.000
2	26.000	0.000
3	41.000	42.000
4	30.000	15.000
5	45.000	22.000
6	20.000	23.000

C: Control group (the non-transfected medium)
T: Test group (1:4 dilution of rZP3 solution)

<- Number of sperm cells bound to the identical pairs of hemizonae (6 pairs)

css/3: basic stats	Single t-Tests N. of Cases = 6 (MD casewise deleted)		
	t	p	n
T-C	-2.6446534129700	.04571945965290	6

css/3: basic stats	Single t-Tests N. of Cases = 6 (MD casewise deleted)	
	E(X-Y)	D(X-Y)
T-C	-14.166666666667	13.1212301093170

APPENDIX VII

Other informations

1). 1 pmol of 1.3 kb of double-strands DNA = 0.48 μg

2). 50 pmol of 21 bp primer = 0.5 μM in a 50 μl final reaction volume

3). 1 A260 unit of 10 bases linker = 50 μg

4). 1 μCi = 2.2 x 10^6 cpm

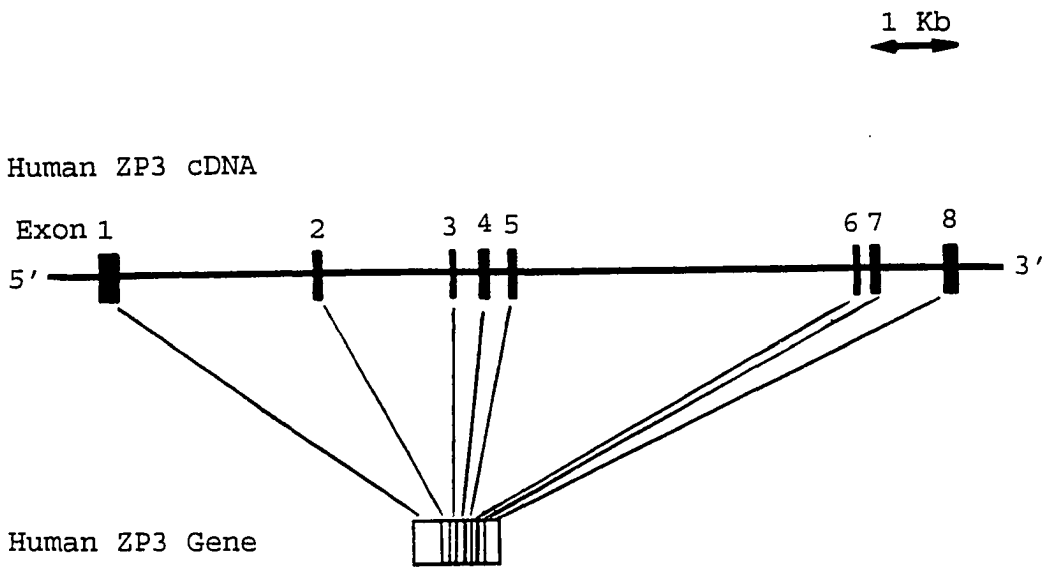
5). [RNA] = A_{260} x 40.0 μg/ml

6). [DNA] = A_{260} x 50.0 μg/ml

APPENDIX VIII

Comparison of the length of hZP3 gene and its cDNA.

The human ZP3 gene is composed of eight exons and corresponds to 18.3 kilobase-pairs of DNA, while the cDNA of hZP3 which is produced by RT-PCR is only 1.3 kilobase-pairs long.



AUTOBIOGRAPHICAL STATEMENT

Name: Chih-Wei Chen

Date of Birth: Aug 28, 1963

Place of Birth: Taiwan, Republic of China (R.O.C.)

Education:

PhD 1990-Aug,1995
Biomedical Science
Old Dominion University & Eastern Virginia Medical School, Norfolk, Va, USA.

MS 1987-1990
Applied Chemistry (Clinical Chemistry)
Tungphai University, Taichung, Taiwan, ROC.

BS 1983-1987
Chemistry
National Changkong University, Tainan, Taiwan, ROC.

Academic appointments:

Teaching Assistant, 1991-1992, Department of Chemistry, Old Dominion University, Norfolk, Virginia, USA.

Research Assistant, 1992-1995, Ob/Gyn Department, Eastern Virginia Medical School, Norfolk, Virginia, USA.

Fellowship: 1992-1995, Jones Institute,Ob/Gyn Department, Eastern Virginia Medical School, Norfolk, Virginia, USA.