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Characterization of the Biological Activities of Recombinant Fusion Protein Green Fluorescent Protein/Human Zona Pellucida Protein 3 (GFP/HZP3)

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CHARACTERIZATION OF THE BIOLOGICAL ACTIVITIES OF RECOMBINANT FUSION PROTEIN GREEN FLUORESCENT PROTEIN/HUMAN ZONA PELLUCIDA PROTEIN 3 (GFP/HZP3)

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ABSTRACT

CHARACTERIZATION OF THE BIOLOGICAL ACTIVITIES OF RECOMBINANT FUSION PROTEIN GREEN FLUORESCENT PROTEIN/HUMAN ZONA PELLUCIDA PROTEIN 3 (GFP/HZP3)

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Despite numerous reports indicating the successful production of bioactive recombinant ZP3, no report has shown the rhZP3 having direct binding activity with human sperm. Recombinant ZP3 generated from our previous study displayed binding activity with human sperm through indirect evidence from hemizona assay (HZA).

This present study focused on the production of recombinant ZP3 with direct binding activity with human sperm. Through the application of a pEGFP expression vector, fusion protein GFP/ZP3 was successfully generated and expressed. The expression of GFP/ZP3 was evidenced by RT-PCR and western blot. The fusion protein was partially purified by Ni-NTA affinity column from cell culture medium of stably transfected cells generated from a single cell clone. Immuno-blotting analysis of the fusion protein indicated that the GFP/ZP3 was about 90-92 kD in molecular mass which is close to the sum of the size of GFP and ZP3. Flow cytometry was conducted to evaluate the direct binding activity of fusion protein with human sperm. The GFP/ZP3 showed dose-dependence in the binding assay. GFP tag removal resulted in almost no detectable binding as assayed by flow cytometry, thereby indicating that ZP3 binding was specific. Furthermore, immunofluorescence demonstrated that the fusion protein

interacted with human sperm on the acrosome region. In the acrosome reaction assay, fusion protein was proved to be able to induce the sperm acrosome reaction (19.2% ± 3.4% as compared to basal line 10.8% ± 2.3%) at the concentration of 1ug/ml. Therefore, the fusion protein displayed full spectrum biological activity of ZP3, and, most importantly unlike prior studies, this binding activity was elucidated in a direct way.

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Finally, I would like to dedicate this dissertation to my parents, my dear wife for their love and support.

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INTRODUCTION

Fertilization is defined as the process of the union of two germ cells, the egg and sperm, whereby the somatic chromosome number is restored and the development of a new individual exhibiting characteristics of the species is initiated. If fertilization fails to take place, both egg and sperm degenerate relatively rapidly in the female reproductive tract, since the two highly differentiated cells cannot survive long on their own. Among mammals, the process of union of germ cells includes several ordered steps (Wassarman, 1987; Yanagimachi, 1994; Snell and White, 1996). It begins in the oviduct with binding of free-swimming sperm to the ovulated egg extracellular coat, the zona pellucida (ZP), and ends a short time later with fusion of egg and sperm plasma membranes to form a single "activated" cell, the zygote. Along the way, several recognizable events take place, including the sperm acrosome reaction (a form of cellular exocytosis), penetration of the egg ZP by sperm, and the egg cortical reaction and zona reaction. The latter results in alteration of the ZP such that free-swimming sperm are unable to penetrate the egg.

A. Formation and structure of spermatozoa

Throughout postpubertal male reproductive life, spermatozoa are formed from spermatogonial stem cells by a highly orchestrated process referred to as spermatogenesis. The entire process consists of three sequential phases of cell proliferation and differentiation. First, there is an extensive multiplication and

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Model journal used in this dissertation was Molecular and Cellular Endocrinology.

proliferation of spermatogonial stem cells to produce an optimal number of spermatogonia which give rise to primary spermatocytes and also to maintain a pool of stem cells. Second, the primary spermatocytes undergo a lengthy meiotic prophase, followed by the first meiotic division resulting in the formation of two secondary spermatocytes, each undergoing the second meiotic phase to produce four haploid round spermatids. Finally, there is a gradual remodeling of the nuclear and cellular components of round spermatids during transformation into sperm cells by a process referred to as spermiogenesis (Eddy et al., 1994). Sperm development takes place within the germinal epithelium of the seminiferous tubules where germ cells are arranged along the wall of tubules in a well-defined combination of various developmental stages. Due to continuous maturation of the testicular germ cells from each generation of cells, the combination of cells varies along the length of the tubule. The well programmed multiple changes occurring between the appearance of one pattern and its reappearance in a different segment of a given tubule is defined as the "cycle of the seminiferous epithelium" (Clermont, 1972).

The normal human spermatozoon is morphologically divided into two parts, head and tail. The sperm head has a flattened, pyriform shape, it condensed nuclear chromatin, and acrosomal cap has a length of approximately 5 to 6 μm and a width of 2.5 to 3.5 μm. The head is divided into the acrosomal and postacrosomal regions. The acrosomal region is covered by the plasma, outer acrosomal, inner acrosomal, and nuclear membranes. The postacrosomal region and neck connects the head to the middle piece. The tail or flagellum

allows motility in a vibratory, circular, or linear manner. The tail has three regions: a middle piece, a principle piece, and an end piece of variable length. The middle piece has a core of flagellum, surrounded by mitochondria. In cross section the flagellum has an axoneme, a central pair of single microtubules surrounded by nine doublets. The end piece is a short segment that lacks any components other than the axoneme and the plasma membrane.

B. Oogenesis and oocyte structure

The task of oogenesis is to construct a large cell containing a large and complex dowry of resources for construction of the embryo before it can either make them on its own or obtain them from its environment. Oogenesis begins with primordial germ cell formation and encompasses a series of cellular transformations, from primordial germ cells to oogonia, from oogonia to oocytes and from oocytes to egg. Oogonia can undergo mitotic divisions, but then stop dividing. In the human female, millions of oogonia are produced during the second and seventh months of fetal life. Most of these primary oocytes die, others enter the first meiotic prophase – chromosomes replicate and pair. There is a continuing atresia during human development, by puberty, only about 300,000 follicles remain, they are in a state of arrested Prophase I and will not begin maturing until puberty. In the human female primary oocytes remain in meiotic prophase until puberty. Only one of these primary oocytes per menstrual cycle normally undergoes further changes and mature each month. The primary oocytes (1° oocyte) (4n) are within a follicle. Cortical granules form as does the

egg coat after completion of meiosis I. There are two different "cells," the secondary oocyte (2° oocyte) (2n) and the first polar body. This is due to uneven cytokinesis at the end of meiosis I. The first polar body can go on to undergo meiosis II to form two more polar bodies. The secondary oocyte undergoes meiosis II only after fertilization, converting the 2° oocyte into an egg (and a second polar body). The oocyte measures about 120μm in diameter in human, it is a highly specialized cell. The oocyte is surrounded by a transparent non-cellular membrane, the zona pellucida, and a layer of follicular cells, the corona radiata and cumulus cells, immediately underneath the zona pellucida is the perivitelline space followed by the plasma membrane. The yolk made up of glycogen, lipids and proteins provides nutrients for the oocyte. Some are produced by the oocyte, others are synthesized elsewhere (liver) and transported to the oocyte. Oocytes accumulate components, in the form of mRNAs and proteins for translational machinery, DNA replication machinery, energy systems, cytoskeleton, metabolism etc. As a consequence, in most species, enucleated fertilized egg can proceed through some cell cycles. Transcription is initially not necessary during early development. Translation is, on the other hand, one of the first processes to be activated after fertilization.

C. The sperm acrosome

The sperm acrosome is a Golgi-derived structure forming a cap over the anterior region of the nucleus, which contains many hydrolytic enzymes and consists of an anterior cap and a posterior region called the equatorial segment.

The acrosome plays an important role at the site of sperm–zona (egg) binding during the fertilization process. Clinical studies have identified a distinct group of men whose infertility is associated with abnormal acrosome reaction (Benoff, et al., 1997). The organelle is a Golgi-derived secretory granule, which is formed during an early stage of spermiogenesis. It resembles the cellular lysosome, a bag-like structure that normally functions in intracellular digestive and defensive mechanisms. The sperm acrosome is a sac-like structure surrounded by inner and outer acrosomal membranes. Immediately after sperm (receptor)–zona (ligand) binding, at least in the mouse, the outer acrosomal membrane fuses with the overlying plasma membrane, releasing the acrosomal contents (glycohydrolases, proteases, etc.) at the site of sperm–egg binding (acrosomal exocytosis). The acrosome is formed during spermiogenesis and represents one of the defining features of sperm development in the testes. At the beginning of spermiogenesis, meiosis is complete and the nondividing haploid cells begin to transform into a uniquely shaped spermatozoon which takes 12–14 days for completion. The Golgi complex at an early stage of spermiogenesis (round spermatid) consists of a prominent system of closely packed tubules and vesicles localized close to one of the poles of the nucleus. Some of these vesicles enlarge and become filled with dense, fine granular components that migrate and empty their glycoprotein- rich contents into the forming acrosome system (head cap and acrosome). The vesicles that contribute to the acrosome formation are derived from the trans-Golgi stacks. The shape of the acrosome is quite variable between species, ranging from skull-cap in man to sickle-shape in rodents (Eddy et al.,

1994; Clermont et al., 1993). Modifications, such as condensation of the acrosome and intra-acrosomal modifications of several antigens are completed as the spermatozoa transit through the epididymis. The sperm acrosome contains a variety of acid glycohydrolases, proteinases, phosphatases, esterases, and aryl sulfatases.

D. Mammalian fertilization

The final steps of spermatogenesis and oogenesis produce sperm and eggs respectively, that will be available for fertilization. Mammalian fertilization is the net result of a complex set of molecular events in which capacitated spermatozoa bind to the homologous egg and undergo several programmed events before penetration of the zona pellucida and fusion with the egg. Fertilization is defined as the process of union of two germ cells, egg and sperm; complementary molecules on the surface of sperm and eggs are responsible for interactions between gametes during the course of fertilization (Farley, 1982). There are highly orchestrated events involved in the interaction between mammalian gametes. The major events include gamete maturation, regulated sperm transport, sperm-oocyte recognition, sperm-zona penetration, and sperm-oocyte fusion.

Sperm maturation involves capacitation and acquisition of a hyperactive state, and expression of cell-cell adhesive ligands. Upon ejaculation and residence in the female reproductive tract, mammalian spermatozoa bind to oviduct cells; after undergoing capacitation, sperm pass through the enveloping

cumulus oophorus, which is composed of a glycosylaminoglycan matrix and cumulus cells, then the primary binding occur between the extracellular matrix called zona pellucida on the egg and the sperm, which is a species-specific event, results in sperm activation and induction of acrosome reaction, the latter facilitates secondary binding to the zona. Only acrosome-intact sperm can bind to the ovulated eggs in mouse and possibly in human. Proteolytic and hydrolytic enzymes released during the course of the acrosome reaction then facilitate the penetration of the fertilizing sperm through the ZP and into the perivitelline space. Then spermatozoa enter into the perivitelline space, bind to the egg plasma membrane and fuse with it. Following fusion of the sperm head and oolemma at discrete sites, sperm and egg pronuclei are formed, and fusion of both pronuclei results in the completion of fertilization. This is followed by nuclear decondensation and activating zygote development. Immediately after fertilization, two important changes contribute to prevent polyspermy: the zona pellucida is biochemically modified, and also a rapid electrical depolarization of the egg plasma membrane blocks additional sperm in the perivitelline space from fusing with the egg.

E. Sperm capacitation and acrosome reaction

Although the spermatozoa are "mature" when they leave the epididymis, nevertheless, their activity is still held in check by multiple inhibitory factors secreted by the genital duct epithelia. Therefore, when they are first expelled in the semen, they are still unable to perform their duties in fertilizing the ovum.

However, on coming in contact with the substances/fluids of the female genital tract, multiple biochemical and functional modifications occur, which are collectively called capacitation of the spermatozoa. This process will then enable the binding of sperm to zona pellucida of the ovum more feasible which subsequently leads to fertilization. The net change during capacitation is a combined effect of multiple molecular modifications in sperm plasma membrane proteins/glycoproteins and lipid components that modify the ion channels in the plasmlemma of spermatozoa. The preparatory modifications include removal of seminal plasma proteins adsorbed to the surface of ejaculated spermatozoa and reorganizations/modifications of sperm surface molecules (Yanagimachi, 1994). The molecular mechanisms underlying the functional changes are not fully understood. As capacitation proceeds, a number of biochemical changes allow the transmembrane flux of ions that are believed to be important in initiating the events of capacitation, hyperactivation, and acrosome reaction. Only capacitated spermatozoa are able to recognize and bind to the ZP (Yanagimachi, 1994).

As a result of these modifications, spermatozoa become acrosomally responsive by an unknown mechanism. Evidence from several studies suggests that the sperm plasma membrane loses cholesterol before becoming acrosomally responsive (Cross, 1998). Although the importance of capacitation has been known for nearly 40 years (Yanagimachi, 1994), the molecular mechanisms underlying the functional changes are not fully understood.

Capacitation and hyperactivation take place before sperm–zona (egg) interaction, and the latter event, at least in mouse and several other species

including man, is initiated after sperm–egg interaction. It is, therefore, reasonable to assume that these events are independent and involve regional-specific changes in sperm plasma membrane. All mammalian spermatozoa undergo capacitation after residing in the female genital tract. Similarly, spermatozoa recovered from the female genital tract after mating, or ejaculated spermatozoa following incubation with the cumulus masses and oviductal secretions, are able to fertilize an egg *in vitro,* a result consistent with the suggestion that these sperm have undergone capacitation (King et al., 1994). Mammalian spermatozoa can also be capacitated in vitro in a chemically defined medium containing bovine serum albumin and energy substances, such as glucose and pyruvate, as well as components used in Krebs–Ringer bicarbonate medium (Dow et al., 1989). It is noteworthy that albumin is the major protein in female genital tract secretions and is an important component during *in vivo* and *in vitro* capacitation (Dow et al., 1989). Kopf's lab has showed the essential role of bicarbonate ion, albumin and cAMP in the sperm capacitation in mouse, hamster, bovine and human (Galantino-Homer et al, 1997; Visconti, et al., 1995, 1998, 1999; Osheroff et al., 1999). The protein facilitates capacitation by efflux of fatty acids/cholesterol from sperm plasma membrane. The loss of fatty acids/cholesterol increases fluidity and permeability of the sperm plasma membrane, initiating the events of capacitation and the acrosome reaction (Cross, 1998).

Acrosome reaction (AR) is an exocytotic process physiologically induced by ligand (ZP3)-receptor interaction, consisting in multiple fusions between outer

acrosomal membrane and the overlaying plasma membrane leading to the release of acrosomal enzymes and exposure of the molecules present on the inner acrosomal membrane surface that mediate fusion with oolemma. As mentioned above, only capacitated sperm are physiologically able to undergo AR in response to physiological stimuli. It is thus conceivable that the two process, capacitation and AR, are sequentially and functionally linked such that several of the effectors involved in mediating intracellular signaling activated by AR start to be tuned during capacitation. For instance, the increase of intracellular calcium levels and tyrosine phosphorylation of proteins accompanying capacitation is also essential for the subsequent exocytosis in response to P4. The process of AR consists of development of multiple fenestrations between the outer acrosomal membrane and the plasma membrane of the spermatozoa leading to the release of the enzymatic content of acrosome and to the exposure of the enzymes bound to the inner membrane adjacent to the nuclear envelope. In the absence of any specific stimuli only a low percentage of human spermatozoa can undergo AR. It has been suggested that self-aggregation of the sperm receptor for ZP may account for this spontaneous acrosome reaction. A wide variety of molecules present on sperm surface have been proposed as putative candidate for ZP3 receptor. This rather long list of possible candidates may be justified by the involvement of different receptors in different species or different binding affinities, or even multiple receptors that may cooperate in sequence to induce AR: however none of these molecules has been definitively recognized as "the sperm-egg receptor". AR can also be physiologically induced by P4, present at

high levels in the cumulus matrix surrounding the oocyte that must be crossed by sperm to reach the zona pellucida. The steroid has been described to affect several other sperm functions, including capacitation, motility and priming effect on ZP3-induced AR through stimulation of a rapid nongenomic signaling pathway mediated by recently characterized P4 receptors present on sperm surface (Meizel, et al., 1991; Blackmore et al., 1991). P4-induced AR is inhibited by 17 - estradiol through interaction with a specific nongenomic estrogen receptor on sperm plasma membrane, suggesting that estradiol, which is present at micromolar levels in the follicular fluid, may act as a physiological modulator of P4 action on sperm assuring the appropriate timing of activation in the fertilization process.

F. *Zona Pellucida*

The Zona Pellucida is a porous and transparent extracellular glycoprotein matrix surrounding the female gamete, the thickness in the human is about 13 µm, it contains both primary and secondary sperm receptors involved in species-specific development and plays a pivotal role in sperm-egg interactions. The characterization of the protein components of the ZP has been conducted in a variety of mammalian species, including the murine, porcine species, as well as human. The reported number of discrete proteins comprising the ZP from different mammalian species varies (Dunbar et al., 1981; 1994; Timmons and Dunbar, 1987; Bleil and Wassarman, 1980). In the mouse, it is clear that is composed of three sulfated glycoproteins, namely, ZP1, ZP2 and ZP3. ZP2 and

ZP3 are present in about equimolar amounts in ZP, whereas ZP1 is present in much lower amount. These proteins play essential structural roles in assembling the extracellular coat during oogenesis. Each of these glycoproteins consists of a unique polypeptide that is heterogeneously glycosylated with both N-linked and O-linked oligosaccharides. Likewise, the human ZP is also composed of ZP1 (90-110 kDa), ZP2 (64-76kDa), and ZP3 (57-63 kDa) (Shabanowitz and O'Rand, 1988). The ZP is composed of very long, interconnected filaments of uniform width, ZP2 and ZP3 formed as a heterodimer filament construct, and ZP1 provides the structural integrity of the zona pellucida by cross-linking these ZP2/ZP3 heterodimer within the filaments (Wassarman, 1988). This structural model for ZP is supported by the fact that transgenic mice homozygous for an insertional mutation in the ZP3 gene are unable to form a zona matrix despite the presence of ZP2 and ZP1 (Liu et al., 1996; Rankin et al., 1996). Wassarman (1999) pointed out that the ZP of eggs from a wide variety of mammals, including humans, is composed of a small number of glycoproteins that are closely similar to that of mouse ZP1-ZP3.

The mouse and human zona proteins share certain protein motifs that are conserved in other vertebrates. Each protein has a signal peptide that directs it onto a secretory pathway and a transmembrane domain near its carboxyl terminus, upstream of which (20-30 amino acids) is a basic amino acid cleavage recognition sequence (absent in human ZP1) for furin, a protease located ubiquitously in the trans-Golgi. The most striking degree of conservation among zona proteins is the signature zona domain: a 260 amino acid motif defined by

eight conserved cysteine residues that is present in several extracellular proteins, some of which participate in fibrillar matrices (Bork and Sander, 1992; Legan et al., 1997)

The ZP matrix has multiple functions during fertilization and preimplantation development. The ZP3 is responsible for species-specific binding of spermatozoa to the zona pellucida and for induction of the acrosome reaction (Wassarman, 1990; Saling, 1991). After ZP3-mediated acrosomal exocytosis, the spermatozoon is postulated to bind to ZP2 (Bleil et al., 1988). This step is required for the second step of sperm-zona pellucida interaction by maintaining the association of the spermatozoon with the zona as it progresses through the oocyte glycocalix. After fertilization, the ZP2 protein is modified in the 'hardening reaction' to prevent polyspermy. This modification is caused by cortical granule exocytosis. In general, it plays at least the following important roles during development: 1) it mediates initial sperm-egg recognition and induces acrosome reaction; 2) regulation of normal endocrine profiles during folliculogenesis; 3) formation of a barrier to heterospecific fertilization; 4) formation of a block to polyspermic fertilization in some mammals; and 5) protection of the embryo during preimplantation development (Benoff, 1997).

Genes homologous to the ZP2 and/or ZP3 genes have been cloned for the mouse (ZP3 and ZP2), hamster (ZP3), human (ZP3), rabbit (rc75), and marmoset (Ringuette et al., 1986; Liang et al., 1990; Kinloch et al., 1990; Chamberlin & Dean, 1990; Liang & Dean, 1993; Lee et al., 1993; Thillai-Koothan et al., 1993). In addition, genes that represent a third class of zona protein have

been cloned from the rabbit (rc55) and pig (ZP3α) (Schwoebel et al., 1991; Yurewicz et al., 1993). Recently, full length ZP cDNAs from cat, dog and pig that are homologous to the ZP2/rc75 genes from mouse, human and rabbit, a full length zona pellucida cDNA from cat and a full length cDNA from human that are homologous to the rc55/ZP3α genes from rabbit and pig, and full length ZP cDNAs from cat, cow, dog, pig and rabbit that are homologous to the ZP3 genes from mouse, hamster, human and marmoset have been cloned and characterized (Harris et al., 1994). Genes encoding ZP2 and ZP3 are conserved among mammals and the DNA sequences of ZP3 cDNA coding regions show extensive homology between species studied so far.

The expression of the zona genes is restricted to oocytes. The coordinate transcription of zona genes is restricted to the two-week growth phase of oogenesis and serves both as a molecular marker for oocyte growth and differentiation. Virtually nothing is known about the mechanisms that restrict gene expression to the female germline, and although previously characterized transcription factors are present in oocytes, their target genes have yet to be determined. In the mouse, the promoters for these three zona genes contain potential binding sites for a variety of known transcription factors. Some binding sites are present upstream of one gene but not the others, or at significantly different distances from the transcription start sites.

G. ZP3 and its biological functions

The genetic and molecular information of the zona pellucida proteins have been documented including the cDNA clone of human ZP3 that has been isolated and characterized from the loci of the human genome (Chamberlin, and Dean, 1990). The human ZP3 gene encodes a polypeptide of 424 amino acids (Chamberlin and Dean, 1990; Harris, 1994). The first 22 amino acids forms the putative signal peptide for the secretion of ZP3; there is a hinge region which spans amino acids 220 to 260; and near the end of the ZP3 polypeptide, there is a hydrophobic region (387-409). Among the three ZP proteins, ZP3 is the putative species-specific sperm-binding protein, is the most conserved between human and mouse. A single open reading frame encodes a protein that is identical in length with the mouse ZP3 protein and has a calculated molecular mass of 47,032 Da. The positions of all the 13 cysteine residues, as well as all of the recognizable domains of the mouse ZP3 polypeptide are conserved in the human ZP3 polypeptide. The human polypeptide chain contains four potential N-linked glycosylation sites, three of which are conserved in the mouse; and sixty-six potential O-linked sites, 71% of which are conserved in the mouse (Chamberlin and Dean, 1990).

During fertilization in mammals, zona pellucida protein 3 (ZP3) is thought to be the primary zona protein that contributes to the initial sperm-egg recognition and induces sperm acrosome reaction. (Bleil and Wassarman, 1980; Wassarman, 1990; Wassarman, 1995). Only acrosome intact sperm binds to the ovulated egg in mouse, initial binding of spermatozoa to the zona pellucida is

supported by ZP3 through) O-linked oligosaccharide side chains and complementary sperm-binding proteins present on the sperm plasma membrane. ZP-3 functions as the primary sperm receptor, experimental evidence strongly supports this conclusion in the mouse. Of the three glycoproteins that constitutes the ZP, only purified mZP3 binds exclusively to heads of acrosome-intact sperm, thus prevents sperm from binding to ovulated eggs in vitro (Bleil and Wassarman, 1980, 1986; Wassarman 1990). Even at nanomolar concentrations, purified, unfertilized egg mZP3 is very effective inhibitor of sperm binding in this competition assay. In a competition assay, the binding of a fixed amount of radioiodonated-ZP3 to acrosome-intact sperm was measured in the presence of unlabeled solubilized egg zonae pellucida (ZP1, ZP2, and ZP3). Increasing concentrations of the unlabeled zonae inhibited ZP3 binding. Bleil and Wassarman were thus able to conclude that there are a limited number of binding sites on the sperm head, for which the radiolabeled and unlabeled ZP3 compete. On the other hand, at similar concentrations, mZP3 from fertilized eggs or early embryos has no effect on binding of sperm to eggs in vitro. This is consistent with the failure of free-swimming sperm to bind to the ZP of fertilized eggs and preimplantation embryos. Thus it can be concluded that mZP3 is altered due to zona reaction such that free-swimming sperm no longer can recognize and bind to the glycoprotein.

It has been postulated that ZP3 binding and ZP3-induced acrosomal reaction can be dissociated from each other because sperm binding and the acrosome reaction seem to represent two independent process (Kopf, 1990). A

number of physiological and nonphysiological inducers of the acrosome reaction have been reported. These include progesterone, follicular fluid, and cumulus cell secretions containing prostaglandins, sterol sulfate, and glycosaminoglycans, and neoglycoproteins. However, it is now generally accepted that ZP3 is the natural agonist that initiates the acrosome reaction upon binding of acrosome-intact sperm to the ZP (Bleil and Wassarman, 1983; Florman et al., 1998). ZP3 triggers a transduction pathway resulting in the fenestration and fusion of the sperm plasma membrane and the outer acrosome membrane at multiple sites of the anterior region of the sperm head, sequentially releasing the acrosomal contents at the site of sperm–zona binding and the exposure of the inner acrosomal membrane. It should be noted that the ability of mouse ZP3 (mZP3) to serve as an acrosome reaction inducer depends on the glycan moiety(ies) as well as on the polypeptide backbone of the molecule. Leyton and Saling (1989) did an ingenious experiment to show that ZP3 induced the acrosome reaction by aggregating the sperm membrane receptors for it. They capacitated mouse sperm and incubated them with fragments of ZP3 that could bind to the sperm. Although these fragments of ZP3 were bound, the acrosome reaction did not take place. However, when these fragments were crosslinked with antibodies against ZP3, the acrosome exocytosis occurred. Therefore, it appears that ZP3 induces the acrosome reaction by crosslinking its receptors. Binding of sperm to ZP3 activates sperm G proteins and voltage-sensitive Ca^{2+} channels and results in a depolarization of the sperm membrane and a significant increase in intracellular Ca^{2+} levels.

H. Carbohydrates

Carbohydrates are involved in fertilization through their addition to the protein core to form a glycoprotein which performs the specific functions. It is generally accepted that interaction of the opposite gametes is a carbohydrate-mediated receptor–ligand event. Several sperm surface antigens from various species have been proposed to function as receptor molecules on acrosome intact spermatozoa. There are two types of glycosylations: N-linked and O-linked. For N-linked glycans, carbohydrates are linked to proteins through the nitrogen of asparagines. N-linked oilgosaccharides are thought to play role in protein secretion and dimerization of ZP protein (Sareneva, et al., 1994). In O-linked glycans, carbohydrates are linked to proteins through beta-hydroxyl groups of serine and threonine, and to less extent, of tyrosine, 5-hydroxylysine and 4-hydroxyproline, through a glycosyl-phosphotidylinositol anchor or through a carbohydrate-peptide chain (Benoff, 1997). There is overwhelming evidence that species-specific sperm-ZP interaction is a carbohydrate-mediated receptor-ligand binding event, which initiates a signal transduction pathway resulting in the exocytosis of acrosome content, ZP3 acts as the primary receptor for sperm, and the O-linked glycans but not the protein backbone, are responsible for the primary binding. It appears that the protein backbone does not play a direct role in sperm receptor function (Florman et al., 1984; Florman and Wassarman, 1985; Miller et al., 1992). Certain synthesized O-linked related oligosaccharides inhibit binding of sperm to eggs in vitro (Litscher, et al., 1995; Johnston et al., 1998). ZP2, on the other hand, acts as the secondary sperm receptor, binds to a trypsin-

like proteinase associated with the sperm inner acrosomal membrane of the acrosome, and so makes the sperm bind more tightly to the zona pellucida (Bleil et al., 1988).

Extensive studies in the mouse have resulted in the identification of multiple receptors on spermatozoa and their complementary glycan units (ligands) on the homologous ZP (Tulsiani et al., 1997). The terminal sugar residues suggested to be recognized by the spermatozoa include mannosyl (Cornwall, et al., 1991), sialyl (lambert, 1984), glucosaminyl (Shur, 1993), a-galactosyl (Bleil et al., 1988), and b-galactosyl (Abdullah et al., 1989). Although a terminal fucosyl residue has not been implicated in mouse sperm–zona binding, its presence appears to be obligatory for an oligosaccharide to bind spermatozoa with high affinity (Johnston et al., 1998). Recent evidence from several laboratories (Johnston et al., 1998; Thaler et al., 1996) strongly suggests that sperm–egg binding leading to the acrosomal exocytosis is a complex event that likely reflects interaction between multiple sperm surface receptors and multivalent ZP3.

In the human system, in collaboration with other investigators, Dr. Oehninger has published numerous reports related to the involvement of carbohydrates (simple and complex) on human gamete interaction (Oehninger et al., 1990, 1991, 1992; 1995; Patankar et al., 1993). Initial studies were performed using hapten inhibition tests demonstrating the involvement of fucosylated and sialylated complex-type glycans (including probably biantennary-bisecting structures) (Clark et al., 1995, 1996; Dell et al., 1995; Morris et al., 1996;

Oehninger et al, 1998). More recently, we performed ZP lectin-binding, chemical treatment and enzymatic studies in order to assess the presence of carbohydrates on the human ZP. For the first time, we were able to provide direct evidence for the involvement of specific carbohydrate sequences (terminal sialic acid and other fucosylated and sialylated structures) in human gamete interaction (Patankar et al., 1997; Ozgur et al., 1998).

I. Putative zona binding proteins on sperm

Although several components have been suggested as putative sperm binding proteins on the surface of the male gamete, the identity and unambiguous function of these molecules remains to be elucidated. A number of ZP3-binding molecule candidates have been proposed including potential carbohydrate-binding proteins such as sp56, p95, β-1,4 galactosyltransferase and α-D-mannosidase (Bleil and Wassarman, 1990; Leyton and Saling,1989; Miller et al., 1992; Cornwall et al., 1991). However, these dada are from murine system, no persuasive data from human is available now.

Sp56 is one critical zona-binding protein of the murine sperm appears to be a protein that specifically binds to the galactose residues of ZP3. Bleil and Wassarman (1980) have shown that one of the critical carbohydrates of the ZP3 glycoprotein is a terminal galactose group. If this terminal galactose is removed or chemically modified, sperm-binding activity is lost. These researchers later isolated this protein by binding ZP3 covalently to beads and passing the proteins isolated from mouse sperm membranes over them in a column (Bleil and

Wassarman, 1990). Most of the proteins passed through the column, but one, a 56-kDa peptide, bound to the ZP3-coated beads. It did not bind to ZP2-coated beads in a similar experiment. This protein was found to be exposed in the sperm membrane, and it bound to galactose residues, strongly suggesting that it is a sperm receptor binding to terminal galactose moiety on the ZP3 glycoprotein. Purified sp56 binds to the zona pellucida of unfertilized (but not fertilized) eggs and blocks sperm-egg binding (Bookbinder et al., 1995).

Galactosyltransferase is another sperm protein that appears to be important for sperm-zona binding is a sperm cell membrane glycosyltransferase enzyme. Shur's laboratory has shown that this receptor for the zona is an enzyme that recognizes the sugar N-acetylglucosamine on ZP3 (Shur and Hall, 1982; Lopez et al., 1985; Miller et al., 1992). This enzyme, N-acetylglucosamine:galactosyltransferase, is embedded in the sperm plasma membrane, directly above the acrosome, with its active site pointed outward. The enzymatic function of this 60-kDa enzyme would be to catalyze the addition of a galactose sugar (from UDP-galactose) onto a carbohydrate chain terminating with an N-acetylglucosamine sugar. The sperm surface galactosyltransferase appears to recognize a carbohydrate group on the ZP3 protein of the mouse zona pellucida. The aggregation of these galactosyltransferases causes the activation of a G protein that may be important in initiating the acrosome reaction (Gong et al., 1995). Sperm from mice that have this enzyme knocked out have impaired fertility. They are unable to undergo the acrosome reaction in response to ZP3 and they do not penetrate the zona well (Lu and Shur, 1997).

Alpha-D-mannosidase is another sperm surface molecule that has been suggested as the potential sperm receptor. This enzyme has been identified in the plasma membrane of rat, mouse, hamster and human sperm (Tulsiani et al., 1989; Chen et al., 1995). This enzyme differs from other mannosidase present in other tissues, including the mannosidase found in the acrosome (Tulsiani et al., 1989)

The 95 kDa tyrosine kinase receptor is still a questionable candidate for ZP3 among different species. The 95 kDa tyrosine kinase receptor was first characterized in mice and has been shown to be phosphorylated in response to zona protein and to bind ZP3 directly (Leyton and Saling, 1989). In humans, a monoclonal antibody (mAb 97.25) has been shown to inhibit sperm–zona binding and immunoprecipitation experiments revealed that this antibody recognized a 95 kDa phosphotyrosine-containing protein (Moore et al., 1987; Burks et al., 1995). A dual cloning strategy using the mAb 97.25 and anti-phosphotyrosine monoclonal antibodies was used subsequently to identify the cDNA (hu9 clone) encoding a novel human 95 kDa sperm tyrosine kinase receptor, named the zona receptor kinase (ZRK), and is presumed to be similar to the mouse protein (Burks et al., 1995). However, there has been much controversy regarding the 95 kDa tyrosine kinase receptor/ZRK. It was reported to be a tyrosine-phosphorylated hexokinase in mice, but this has been disproved (Saling et al., 1995). There is also much uncertainty about the cDNA of human ZRK, as the sequence exhibits close similarity to the proto-oncogene c-*mer* even though it is more closely related to mouse than to human c-*mer* (Bork et al., 1996). The fact

that recombinant human ZP3 has also been shown to cause an increase in the tyrosine phosphorylation of a human 95 kDa protein is further evidence that such a moiety is somehow involved in initial gamete recognition (Burks et al., 1995; Brewis et al., 1998). Such zona-induced changes in tyrosine phosphorylation of a 95 kDa have also been demonstrated unequivocally in cats (Pukazhenthi et al., 1996). Therefore, it appears that this effect is conserved in mammalian spermatozoa and the 95 kDa protein remains the only human candidate for which there is evidence for interactions with ZP3.

J. Recombinant ZP3 production

Data from the mouse and pig demonstrate that the first stage of sperm-zona interaction is the binding of spermatozoa to ZP3 (Wassarman, 1992). Recombinant DNA technologies has revolutionized our understanding of many biological systems, consequently, the production of bioactive recombinant ZP3 has raised great interest for several groups around the world. Cloning cDNAs encoding ZP3 has made the expression of recombinant ZP3 in tissue culture cell lines possible and represents a potential option to obtain large amounts of ZP3. The expression of biologically active recombinant ZP3 has been reported, at least, in the mouse (Kinloch et al., 1991; Beebe et al., 1992) and human (van Duin et al., 1994; Barratt et al., 1994; Burks et al., 1995). In the mouse, some of these proteins have demonstrated partial or full biological activity in ligand-receptor or acrosome reaction assays. Expression of recombinant ZPs is not restricted to those of the mouse and human species. Prasad et al., (1996)

demonstrated that recombinant rabbit 55 kD protein (which is thought to be the rabbit homologue of mouse ZP1) purified from a baculovirus expression system could be used to generate a polyclonal antiserum which was then employed to study the localization of the native 55 kD protein in rabbit zonae. On the other hand, recombinant human ZP3 has been expressed using several approaches, i.e., Escherichia coli (Chapman and Barratt, 1996), in vitro transcription and translation (Whitmarsh et al., 1996), Chinese hamster ovary (CHO) cells (van Duin et al, 1994; Barratt and Hornby, 1995; Brewis et al., 1996) and in green monkey kidney (COS) cells (Burks et al., 1995). In the human, however, full biological activity has not been demonstrated, which is possibly due to inadequate or incomplete glycosylation of the recombinant protein (Chapman and Barratt, 1997).

With regard to the human system, production of a recombinant purified glycosylated ZP3 in a biologically active form is fraught with technical difficulties. In vitro transcription and translation systems and expression in Escherichia coli have led to the production of recombinant forms showing variable acrosome reaction-inducing activity. However, no direct or specific sperm-binding ability using homologous sperm-ZP bioassays have been reported for such non-glycosylated products. In addition, protein solubility has been a major difficulty encountered (Chapman and Barratt, 1997). The CHO cell product has been shown to possess acrosome reaction-inducing activity. However, no data are available related to sperm binding in validated assays (van Duin et al., 1994;

Whitmarsh et al., 1996). Again, this points to inadequate glycosylation of the protein core by the host cells.

In our previous studies, and considering that protein expression is tissue and species specific, human ovarian teratocarcinoma PA-1 cells were chosen as host cells. We have expressed and purified recombinant ZP3 and characterized its bioactivities. The rhZP3 from PA-1 cell has been demonstrated to possess both sperm binding activity and acrosome reaction inducing activity in human sperm (Dong et al., 2001). The binding activity was characterized by an indirect way by using the Hemizona Assay (HZA), and no direct binding of rhZP3 to human sperm has been demonstrated. This was the first report that showed rhZP3 had full spectrum of bioactivity as compared to the native zona pellucida.

SPECIFIC AIMS

A. Significance of the proposed study

Male reproductive dysfunction accounts for about 30-50% of infertility cases and may represent the most common single defined cause of infertility (Irvine, 1999). Despite of the fact that contemporary therapies have enhanced the opportunities for infertile couple to have offspring, unfortunately, male infertility still remains a mystery, and it is considered "idiopathic" in a great proportion of cases. In order to understand and eventually treat male infertility, there arises a pressing need to uncover the "secrets" behind male infertility. An abnormal sperm-zona pellucida interaction is often observed in males with unknown infertility etiology. In the human, ZP3 is important as both a potential target for contraceptive vaccine development and as a possible tool in the diagnosis and treatment of male infertility. In order to explore this clinical potential of ZP3 and to study the cellular mechanism by which human spermatozoa becomes activated on making contact with ZP3, generating bioactive recombinant ZP3 is necessary. Defects of sperm-zona pellucida binding can be diagnosed in a high proportion of infertile men in the presence or absence of abnormalities of the basic sperm parameters (Oehninger et al., 1991, 1997). The Hemizona Assay (HZA) developed at the Jones Institute (Burkman et al., 1988) is currently widely used in the clinical arena to assess the direct and tight binding of sperm with the oocyte's ZP. It is one of the most important functional tests for the assessment of sperm fertilizing capacity (Oehninger et

al., 2000). The HZA evaluates the tight binding of sperm to the zona pellucida, a crucial step that triggers the physiological acrosome reaction leading to fertilization and early embryo development. The specificity of the interaction between human spermatozoa and the human zona pellucida in the HZA are being documented by the fact that the sperm tightly bound are acrosome reacted (Oehninger et al., 1991, 1993, 1997 and 2000). The HZA can be used as a test of choice for human gamete interaction of both sperm-zona binding and zona-induced acrosome reaction (Oehninger et al., 1992, 1993, 1995). However, the HZA is practically limited, because it requires a constant supply of human oocytes. Although sperm-zona pellucida binding and acrosome reaction bioassays have been validated in the clinical arena, the development of simpler, more standardized, and universally applicable diagnostic methods is warranted. Consequently, the development of bioassays based on the use of a recombinant, biologically active human ZP3 would have a tremendous impact in the diagnosis of male infertility. In this proposal, a fusion protein (GFP/ZP3) will be generated by molecular biology techniques, and subsequently applied in a sperm-zona pellucida binding test using the fluorescent protein as the marker. The results from this project will not only provide useful data for studying the feasibility of diagnosing male infertility by using recombinant human ZP3, but will benefit the study of gamete interaction mechanisms by using the recombinant protein.

B. Rationale of the proposed research project

In mammals, fertilization takes place in the ampulla of the oviduct where individual gametes fuse to form a one-cell zygote. Mammalian gametes recognition and binding of spermatozoa to the egg is mediated by complementary molecules associated with the exterior of the egg and the sperm plasma membrane (Yanagimachi, 1994; Aitken, 1995). During mammalian fertilization, capacitated spermatozoa bind to the extracellular matrix of the egg (zona pellucida) and then undergo the acrosome reaction (AR). The AR is an essential exocytotic event which exposes the acrosomal contents and inner acrosomal membrane of the spermatozoon, allowing secondary binding, zona penetration and fusion with the oolemma. The predicted molecular masses of the protein cores of ZP proteins are about half of the apparent molecular mass of each ZP protein indicating significant post-translational modification. Much of this appears to be glycosylation that would permit the matrix to maintain a high state of hydration and may be important for sperm penetration through the zona pellucida. In-vitro biochemical studies have suggested functions for individual zona pellucida proteins in mice. ZP3 is considered the primary sperm receptor and induces sperm acrosome reaction after sperm bind to the zona pellucida. ZP2 is the secondary sperm receptor involved in the post-acrosomal induction binding. Due to the paucity of eggs for research, little is known about initial events of human sperm-egg recognition and induction of the AR. To obviate the problems of obtaining native human zonae, recombinant DNA technology has been used to produce zona proteins.

To date, the expression of recombinant zona pellucida proteins (rZPs) from a range of mammals has facilitated the study of gamete interaction. The production of recombinant zona proteins has raised great interest among reproductive sciences investigators. Kinloch et al., (1991) first demonstrated that it was possible to express biologically active recombinant mouse ZP3 which can induce the sperm acrosome reaction. In the human system, cloning the human ZP3 gene (Dean, 1990) has facilitated the study of this glycoprotein in human gamete interaction. Recombinant human ZP3 has been successfully expressed through different approaches, such as in Escherichia coli (Chapman and Barratt, 1996, Harris, 2000), in-vitro transcription and translation (Whitmarsh et al., 1996), in mammalian cell lines (van Duin et al., 1994; Barratt and Hornby, 1995; Dong et al., 2001).

The development of homologous functional bio-assay for sperm quality assessment has been a focal point of reproductive biologists. Burkman et al (1988) reported the hemizona assay (HZA), which used bisected human oocytes to assess the ability of sperm to bind to zona pellucida. The specificity of the human sperm-ZP interaction has been well documented (Oehninger et al., 1991, 1993, 1995, 1997 and 2000). Up to now, the HZA still remains a reliable functional test of choice for human gamete interaction. Another sperm-zona test is called competitive intact zona pellucida binding test using oocytes that failed in IVF to determine a sperm-zona pellucida binding ratio between control and teat spermatozoa (Liu et al., 1988). The test is based on competitive binding of two sperm populations (test patient and fertile control sperm donor) to several

oocytes. Test and control sperm are labeled with different fluorescent dye, which are FITC (green) and tetramethyl rhodamine isothiocyanate (red). Following labeling, sperm populations i.e. control and tests are simultaneously co-incubated with several oocytes, after which zona binding is assessed by counting zona bound sperm under fluorescent microscopy. The fact that sperm-zona binding follows a dose-response curve can be employed in a clinical setting as a diagnostic tool to establish the sperm concentration, which will ensure optimal zona binding under IVF conditions. However, the current available sperm-zona binding test requires a constant supply of human oocyte. This is very time-consuming, and, therefore, the introduction of a valid sperm binding assay using recombinant ZP3 would have significant impact on male infertility diagnosis.

Upon the availability of full-length cDNA of human ZP3 (Chamberlin et al., 1990), hZP3 expression has been conducted by different research groups. Despite several reports indicating the successful production of recombinant ZP3, no report has shown the rhZP3 to have direct binding activity with human sperm. Since glycosylation is crucial to the function of ZP3, the glycosylation level of in vitro expressed rhZP3 is an important aspect that has to be considered. Since gene expression is tissue- and cell specific, the glycosylation level also depend on cell type and species. In our previous study, after obtaining complementary DNA from mRNA isolated from human ovary, a biologically active recombinant zona pellucida protein 3 was produced by a human ovarian teratocarcinoma cell line (PA-1). The rhZP3 yield by our system showed similar biochemical characteristics to the native ZP3, including molecular size estimated by SDS-

PAGE and recognition by a polyclonal anti-human ZP3 antibody. This rhZP3 has been determined to possess both zona binding activity and acrosome reaction inducing activity (Dong et al., 2001). Again, the direct binding of rhZP3 with human sperm has not been documented.

In the present study, we intended to generate a fusion protein between GFP and ZP3 and investigate its biological activities. GFP is a monomeric protein of 238 amino acids with molecular weight of 27kD. A hexapeptide segment functions as a fluorescent chromophore that is formed upon crystallization of the residues Ser-dehydro-Tyr-Gly within the hexapeptide by post-translational modification. Since the chromophore forms automatically upon expression of the protein, and its fluorescence is highly stable and compatible with standard optics used to image fluorescein, GFP has rapidly gained popularity as a protein tag and cytoplasmic tracer dye (Steams, 1995). The project will focus on the characterization of direct binding activity of the fusion protein with human sperm. The fusion protein will combine the advantage of fluorescence characteristics of GFP and bioactivity of ZP3, thus providing an option to detect sperm binding. A six-histidine tag fused to the target protein has been widely applied in recombinant technology to facilitate protein purification for subsequent application. (Sisk et al., 1994; Petty et al., 1996). The expression and purification technique is based on the remarkable selectivity of Ni-NTA (nickel-nitrilotriacetic acid) for proteins with an affinity tag of six consecutive histidine residues — the 6xHis tag. The 6xHis tag is much smaller than most other affinity tags and is uncharged at physiological pH. It rarely alters or contributes to protein

immunogenicity, rarely interferes with protein structure or function, does not interfere with secretion, rarely requires removal by protease cleavage, and is compatible with denaturing buffer systems. This 6×his tag has a strong affinity to a Ni ion on Ni-NTA resin under protein binding conditions, thus when it is linked with target recombinant protein at either the C- or N-terminal, the exposed 6×His on the protein backbone is able to bind with Ni ion on Ni-NTA resin. The target protein can be captured and enriched on the resin, thereafter, the protein molecules bound on the Ni-NTA resin can be eluted from the resin under stringent conditions, using high concentrations of imidazole to compete with the six-his tag for binding to the resin. In our study, we put the 6xHis tag at the N-terminal of human ZP3 sequence for purification.

C. Specific aims:

Studies in animal models have well established that the zona pellucida protein 3 (ZP3) not only serves as the putative primary ligand for receptor on sperm, but also acts as a physiological inducer of the sperm acrosome reaction. It is generally accepted that carbohydrate epitopes displayed by ZP3 play a pivotal role in spermatozoa-ZP3 interaction. Human ZP3 cDNA sequence has been elucidated and cloned, and a biologically active recombinant ZP3 (rhZP3) with partial activity has been successfully expressed by several groups. In previous studies, we expressed, cloned and purified rhZP3 using PA-1 cells as hosts, then characterized the sperm binding activities through indirect way by inhibiting zona binding with rhZP3 under hemizona assay (HZA) conditions;

acrosome reaction agonistic activity of our rhZP3 was also identified (Dong et al., 2001). Till present, only our group in vitro expressed a bioactive rhZP3 with complete activities. However, the direct binding of rhZP3 with human sperm has not been shown. Therefore we have faced two problems: (1) to prove direct binding activity, (2) to establish a bioassays predicting of sperm zona-pellucida binding. In this current proposal, a GFP tag will be fused with ZP3 at the N-terminus, the hypothesis is that the fusion protein GFP/ZP3: a) allow direct identification of the green fluorescence protein on the sperm surface; b) will possess bio-activity to interact with human spermatozoa. The overall goal of the project is to produce fusion protein GFP/ZP3, which can be used in testing the human sperm-oocyte binding. Therefore the following specific aims are proposed:

Specific aim 1: To clone and express the fusion protein GFP/ZP3 in vitro

1a: To subclone the human ZP3 gene into a pEGFP-C1 vector.

1b:To express the fusion protein GFP/ZP3 in human ovarian teratocarcinoma cells (PA-1).

Specific aim 2: To identify, isolate and purify the recombinant fusion protein GFP/ZP3 from the culture medium of transfected PA-1 cells

2a: To isolate and purify GFP/ZP3 using affinity chromatography.

2b: To identify the purified GFP/ZP3 by immunobloting.

Specific aim 3: To characterize the direct binding activity of the fusion protein GFP/ZP3 to capacitated human spermatozoa, as well as its acrosome reaction inducing activity.

3a: To conduct flow cytometry to evaluate the binding of GFP/ZP3 to human spermatozoa.

3b: To apply fluorescence microscopy to visualize the binding of the fusion protein to human spermatozoa.

3c: To evaluate GFP/ZP3 as sperm acrosome reaction inducer.

MATERIALS AND METHODS

A. Design and construction of oligonucleotide primers

The following oligonucleotide primers were designed, synthesized and used in the project. These oligonucleotides were synthesized by GIBCO Life Technologies INC. (Grand Island, NY). All the cloning sites and some special features of the primers were indicated in the sequence as shown below.

B1 and B2, which contain signal peptide sequence of ZP3 and six histidine were designed for annealing to form an oligo DNA sequence (B1B2), the latter was used for insertion right upstream to GFP sequence in the pEGFP-C1 vector. The primers used were as follows:

A antisense: 5'-TAGGATCCTTATTCGGAAGCAGACACAGG-3'

C sense: 5'-

TACTCGAGACGACGACGACAAAAAATGGCAACCCCTCTGGCTCTTGCAG-3'

B1 sense: 5'-

CTAGCATGGAGCTGAGCTATAGGCTCTTCATCTGCCTCCTGCTCTGGGGTA

GTACTGAGCTGTGCTACCCCCAACATCATCATCATCATCATAAA-3'

B2 antisense: 5'-

CCGGTTTATGATGATGATGATGTTGGGGGTAGCACAGCTCAGTACTAC

CCCAGAGCAGGAGGCAGATGAAGAGCCTATAGCTCAGCTCCATG-3'

CH1 sense: 5'-ACCATGGAGCTGAGCTATAGG-3'

CH2 antisense: 5'-TTATTCGGAAGCAGACACAGG-3'

CH1 and CH2 were the primers used in our previous ZP3 subcloing (Chen, 1995).

B. Cell culture and medium collection

A human ovarian teratocarcinoma cell line, PA-1 was purchased from American Type Culture Collection (ATCC, Rockville, MD). PA-1 cells and stable transfected PA-1 cells were cultured in minimal essential medium (MEM, Sigma St. Louis, MO) supplemented with 5% Fetal Bovine Serum (FBS, Hyclone, Logan, Utah) and 100μg/ml geneticin (G418, Invitrogen, San Diego, CA) at 37 °C in 5% CO_2 in humidified air; medium was changed every 24 hr. After three weeks, cells were amplified and transferred from a 100mm culture dish to 150mm dishes. MEM containing 50% FBS and 5% DMSO (Sigma, St. Louis, MO) was used as a cytogenic medium for freezing the transfected PA-1 cells for the storage of cells (at –196 °C in liquid nitrogen). Since human ZP3 is a secreted protein, culture media from the stable transfected PA-1 cells were collected for further purification. Twenty-five 150 mm dishes were cultured and 20 ml of medium were collected from each plate every 24 hr. The collected medium was then centrifuged at 1000×g for 10 minutes to remove cellular debris, and stored at 4°C with the addition of protease inhibitors including 100μg/ml PMSF (Sigma), 2μg/ml Leupeptin (Sigma), 1μg/ml Pepstatin (Sigma) and 2mM EDTA (Sigma). The medium should be applied for purification within one week.

C. Subcloning of pEGFP-ZP3 plasmid construct

The subcloning was accomplished in two steps, In the first step, two (i.e., B1 and B2) oligonucleotides which includes the restriction site, ZP3 signal sequence and 6xHis were synthesized, the oligos were annealed to form double strand DNA as follows: Dispense 100µl aliquots of the mixed oligos into PCR tubes. The tubes were placed in a thermal cycler and set up a program to perform the following profile: (i) heat to 95°C and remain at 95°C for 2 minutes, (ii) ramp cool to 25°C over a period of 45 minutes, (iii) proceed to a storage temperature of 4°C. Briefly spin the tubes in a microfuge to draw all moisture from the lid. The samples were pooled into a larger tube and stored on ice or at 4°C until ready to use. After annealing the two oligonucleotides, the product B1B2 were digested with NheI and AgeI (New England Biolabs, Beverly, NA), then the digested B1B2 was ligated with pre-digested pEGFP-C1 vector (Clontech, Palo Alto, CA) with the same two enzymes. The ligation product was used for transformation; colony hybridization using radioactive probe and restriction enzyme digestion were used for the selection of possible positive clones. In the first-step subcloning, clone hybridization and restriction digest were applied to screen for E. coli clones with possible insertion of B1B2. Plasmid DNA (pEGFP-B1B2) isolated from the positive E. coli clone was utilized in the second step subcloning.

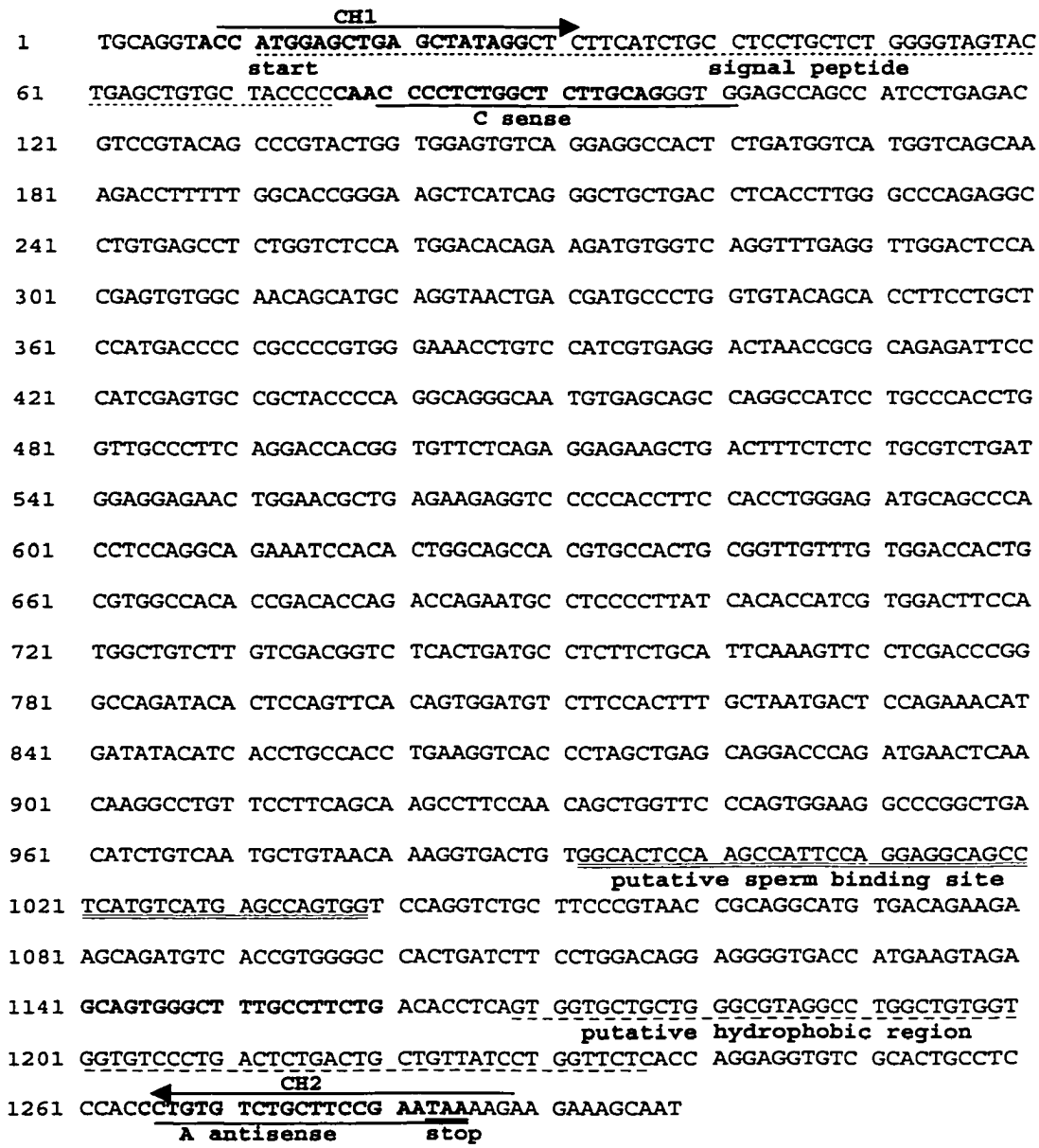
In the second step subcloning, a pentapeptide sequence (Asp-Asp-Asp-Asp-Lys) which can be recognized and cleaved by Enterokinase Max (Invitrogen, Carlsbad, CA) was inserted between GFP and ZP3, this procedure has several

advantages: 1) to ensure the free rotation of GFP and ZP3; 2) to prevent the possible blocking effect of GFP on ZP3 folding; 3) Enterokinase Max can be used to cleave off GFP in the final fusion protein product when GFP interfere rhZP3 binding with human spermatozoa. ZP3 sequence with Enterokinase Max sequence as well as cloning site were generated by PCR using A, C primer, ZP3 cDNA generated from human ovary tissue (Chen, 1995) was used as the template to produce our target sequence in this experiment. The PCR product digested with XhoI and BamHI (New England Biolabs) was then ligated with predigested pEGFP-B1B2, as above described, the possible positive transformants were selected by clone hybridization, PCR and restriction digestion. Detailed human ZP3 DNA sequence and primer information is shown in the following figure (Fig. 1).

D. Ligation and transformation

DNA ligations were performed by incubating DNA fragments (B1B2 or ZP3 DNA fragment from PCR) with appropriately liberalized cloning vector (pEGFP-C1), which has been digested with NheI and AgeI in the presence of buffer, rATP, and T4 DNA ligase (New England Biolabs). Ligations were done in a 10 ul volume as follows: X vol of insert (approx. 25 ng), Y vol of digested vector (approx 6 ng), 1 ul of ligation buffer, dH_2O to equal 9 ul, 1 ul of T4 ligase, overnight at 14°C. Meanwhile, the following ligation controls were set up: parallel ligations in the absence of insert DNA to determine the background clones arising from self-ligation. After ligation, transformation was performed: 1/2 of the

Human ZP3 mRNA total cDNA 1-1299



1 TGCAGGTACC ATGGAGCTGA GCTATAGGCT CTTCATCTGC CTCCTGCTCT GGGGTAGTAC

61 TGAGCTGTGC TACCCCCAAC CCCTCTGGCT CTTGCAGGGT GGAGCCAGCC ATCCTGAGAC

121 GTCCGTACAG CCCGTACTGG TGGAGTGTCA GGAGGCCACT CTGATGGTCA TGGTCAGCAA

181 AGACCTTTTT GGCACCGGGA AGCTCATCAG GGCTGCTGAC CTCACCTTGG GCCCAGAGGC

241 CTGTGAGCCT CTGGTCTCCA TGGACACAGA AGATGTGGTC AGGTTTGAGG TTGGACTCCA

301 CGAGTGTGGC AACAGCATGC AGGTAACTGA CGATGCCCTG GTGTACAGCA CCTTCCTGCT

361 CCATGACCCC CGCCCCGTGG GAAACCTGTC CATCGTGAGG ACTAACCGCG CAGAGATTCC

421 CATCGAGTGC CGCTACCCCA GGCAGGGCAA TGTGAGCAGC CAGGCCATCC TGCCCACCTG

481 GTTGCCCTTC AGGACCACGG TGTTCTCAGA GGAGAAGCTG ACTTTCTCTC TGCGTCTGAT

541 GGAGGAGAAC TGGAACGCTG AGAAGAGGTC CCCCACCTTC CACCTGGGAG ATGCAGCCCA

601 CCTCCAGGCA GAAATCCACA CTGGCAGCCA CGTGCCACTG CGGTTGTTTG TGGACCACTG

661 CGTGGCCACA CCGACACCAG ACCAGAATGC CTCCCCTTAT CACACCATCG TGGACTTCCA

721 TGGCTGTCTT GTCGACGGTC TCACTGATGC CTCTTCTGCA TTCAAAGTTC CTCGACCCGG

781 GCCAGATACA CTCCAGTTCA CAGTGGATGT CTTCCACTTT GCTAATGACT CCAGAAACAT

841 GATATACATC ACCTGCCACC TGAAGGTCAC CCTAGCTGAG CAGGACCCAG ATGAACTCAA

901 CAAGGCCTGT TCCTTCAGCA AGCCTTCCAA CAGCTGGTTC CCAGTGGAAG GCCCGGCTGA

961 CATCTGTCAA TGCTGTAACA AAGGTGACTG TGGCACTCCA AGCCATTCCA GGAGGCAGCC

1021 TCATGTCATG AGCCAGTGGT CCAGGTCTGC TTCCCGTAAC CGCAGGCATG TGACAGAAGA

1081 AGCAGATGTC ACCGTGGGGC CACTGATCTT CCTGGACAGG AGGGGTGACC ATGAAGTAGA

1141 GCAGTGGGCT TTGCCTTCTG ACACCTCAGT GGTGCTGCTG GGCGTAGGCC TGGCTGTGGT

1201 GGTGTCCCTG ACTCTGACTG CTGTTATCCT GGTTCTCACC AGGAGGTGTC GCACTGCCTC

1261 CCACCCTGTG TCTGCTTCCG AATAAAAGAA GAAAGCAAT

Fig. 1. Human ZP3 sequence (Chamberlin and Dean, 1990) and the position of the primers used in the study.

prepared ligation mixture and two controls were pipeted into eppendorf tubes. Competent cells (DH5α E. Coli cells) were plated alone on a selection plate as a negative control. The remainder of the ligation mix was stored at -20 °C and used as a back-up if necessary. The competent cells were placed directly on ice after removing from the -80 °C freezer. As the cells thawed, added 100 ul to each eppendorf tube containing the ligation products. The tubes were flicked to mix and placed immediately on ice for 30 minutes. Remove tubes from ice and incubate for two minutes at 37 °C in a water bath for a heat shock. Add 900 ul of sterile 1X LBM to each tube and continue incubating at 37 °C for 30 minutes. Plate 100-200 ul of each on LBM+ antibiotic using a glass spreader. Flame the spreader between plates and cool before each use. After inoculum has been absorbed (5 minutes) invert plates and incubate at 37 °C for 16 hours or until colonies were of the desired size. The remaining transformation culture was stored at 4 °C.

E. DNA isolation from E. Coli (mini prep)

After transformation of bacteria with the vector containing ZP3 sequence, bacteria were cultured for isolation of plasmid DNA. Inoculate 5 ml of LBM medium containing 50 μg /ml ampicillin (Sigma) with a single bacterial colony (transformed with vector containing ZP3 sequence) containing the desired plasmid. Bacteria were incubated at 37 °C overnight on a roller drum. Next day, cells were spun down at 2500 rpm in Beckman low speed centrifuge (e. g. the J-6) for 15 minutes. Each pellet was resuspended in 200 μl GTE solution and

transferred to labeled eppendorf tubes. Incubated at room temperature for 5 minutes. Add 400 µl of a freshly prepared solution of lysis solution. Mix gently. Place on ice for 5 minutes. Add 300 µl of an ice-cold solution of 3 M potassium acetate. Mix gently. Place on ice for 5 minutes. Centrifuge for 1 minute at 4 °C. Transfer the supernatant to a clean tube. Add 0.6 volume (540 µl) isopropanol to the supernatant mix and incubate at room temperature for 2 minutes. Pellet the nucleic acid (1 minute microcentrifuge spin top speed). Discard the supernatant. Wash the pellet twice with 1 ml 70% ethanol. Spin and dry the pellet for 10 minutes in the lyophilizer or allow to air dry. Resuspend in 50 µl TE pH 7.5. Add 1 µl of a 10mg/ml solution of RNAase and incubate for 30 minutes at room temperature. Assay the DNA on a mini gel with appropriate concentration standards before restricting digestion the DNA.

F. Preparation of labeled probe for colony hybridization

Thaw alpha-^{32}P labeled dCTP (~1hr at room temp.) and also put on the heating for the 100 °C water bath. Denature 200 ng-1 mg oligonucleotide DNA (B1 which contains ZP3 signal peptide sequence and 6xHis) dissolved in 5-20 µl of dilute buffer in a micro centrifuge tube by heating for 5 min in a boiling water bath; then immediately cool on ice. Perform the following additions on ice: 1 µl 10 mM dATP, 1 µl 10 mM dGTP,1 µl 10 mM dTTP, 20 µl 2.5X Random Primers Solution, 5 µl (approximately 50 µCi) [alpha-^{32}P] dCTP, 3000 Ci/mmol, 10 Ci/µl , add distilled water to a total volume of 49 µl. Mix briefly. Add 1 µl of Klenow Fragment. Mix gently but thoroughly. Centrifuge 15-30 sec. Incubate at 37 °C for

10 ~ 30 min. Add 5 µl Stop Buffer. Determine cpm using Scintillation counter. Use appropriate volume such that 5E6 cpm (2-4x10^5 cpm per membrane) is incorporated into probe.

G. Colony hybridization to identify the colony with B1B2 insert

Plate the transformation mixture onto selective medium plates and leave in hood with cover off until agar surface is dry. Incubate at 37 °C for 12-14 hours (smaller colonies give cleaner hybridization signals. First wet the autoclaved filters with dH_2O and place between layers of Whatman paper. Then wrap in aluminum foil and autoclave at 15 lbs/sq.in. When filter was completely wet stab agar with needle dipped in India ink in >3 places peripherally to make asymmetric markings.

Four pieces of Whatman 3 MM paper were pepared for colony lysis and DNA transfer; these papers were laid in a plastic tray then saturated each with one of the following solutions: a. 10% SDS, b. 0.5 N NaOH 1.5 M NaCl, c. 1.5 M NaCl 0.5 M Tris-HCl pH 7.4, d. 2X SSC. Pour off any excess liquid for this will cause colonies to swell and blur subsequent hybridization signals. Peel the filter from the agar plate with blunt-tipped forceps and place upon the 10% SDS soaked paper colony side up. After three minutes transfer this to paper soaked in denaturing solution (b). After five minutes transfer to neutralizing paper (c). After five minutes transfer to paper soaked in (d). After five minutes of treatment with solution (d) dry the filters on dry 3 MM paper. Place filters between layers of 3 MM paper and bake in a vacuum oven for 1-2 hours. Store dry or wash in 0.1X

SSC 0.5% SDS at 65 °C for 30 minutes and store wet in seal-a-meal bags. Cross link the DNA under a UV light (Stratalinker). Rinse the filters in 2X SSC + 0.1% SDS on a rotator. Perform hybridizations as per the standard protocol. Prehybridize filters in a sealable bag containing pre-hybridization solution (25.0 mM KH_2PO_4, 5% Denhard's solution, 50ug/ml salmon sperm DNA, and 50% formamide in 5x SSC buffer) at 42 °C for 3 hours by gentle shaking. Remove pre-hybridization solution from the bag and transfer the membrane to another bag containing hybridization solution (same as prehybridization solution, but omitting Denhart's solution), the solution should be enough such that membranes move freely. Denature probe in boiling water for 5 minutes, rapidly chill on ice. Add the probe to the buffer. Incubate at 65 °C with constant shaking for 16hrs. Remove probe mix by pouring into 50 ml conical tube. Keep in freezer or discard in radioactive liquid waste container. Wash membrane with WSI in following sequence, monitoring with Geiger counter after each wash: 200 ml for 10 min. (discard in radioactive liquid waste) 200 ml for 10 min. For increased stringency (to decrease background) wash with WSII: 100 ml for 10 min. 200 ml for 10 min. Check membrane with Geiger counter. If background radioactivity is acceptable, allow filter to air dry between two 3MM Whatman filters (10-30 min.). Wrap membrane in Saran Wrap; develop in cassette with intensifying screen (white) in -70 °C freezer from 4 hr. to overnight.

H. Screen positive colony by PCR

Colonies were picked and put in 50µl PCR reaction mix (use new toothpick for each colony), boil 5' and spin briefly, add 0.5µl Taq Polymerase (2.5U) and overlay reaction with oil, amplify 30 cycles: 94 °C - 1', 42 °C - 2', 65 °C - 4'. Do final extension at 65 °C for 5', check on an agarose gel.

I. DNA isolation from transformed E. Coli. (max prep)

In order to obtain a greater amount of plasmid DNA containing positive transformed clone for subsequent application, those positive clones were amplified. Grow 30 ml of culture overnight at 37 °C in the appropriate antibiotic media in a 250 ml conical flask (to allow sufficient aeration). Normal broth concentration needs 50µg/ml (i.e. 1/1000 dilution). Transfer to Oak Ridge tubes and harvest the cells by spinning down at 5000 rpm for 5 minutes (Beckman; JA-20 rotor, 4°C). The cells were resuspended in 2.5 ml GTE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). Add 100 µl of 40 mg/ml lysozyme (in GTE) and incubate for 10 minutes at ambient. Add 5 ml of 0.2 M NaOH/1 % SDS, mix by vortexing and incubate on ice for 10 min. Add 3.75 ml 3M NaAcetate (pH 5.2) and vortex until a white precipitate forms. Incubate on ice for 15 minutes. Centrifuge for 15 minutes at 12,000 rpm. Transfer supernatant to a clean tube and add an equal volume of isopropanol. Incubate at ambient for 10 minutes. Centrifuge at 12, 000 rpm for 15 minutes, pour off supernatant and invert the tubes over a clean tissue to remove the remaining isopropanol (pellet = DNA). Resuspend the pellet in 5 ml of 2 M NH_4 Acetate and centrifuge at 12 000

rpm for 10 minutes. Add an equal volume of isopropanol (5 ml) to supernatant and incubate at ambient for 10 minutes. Centrifuge at 12 000 rpm for 15 minutes. The pellet was resuspended in 0.4 ml SDW and transfer to a clean microfuge tube. Add 20 µl of 10 mg/ml RNAse (heat treated) and incubate for 15 minutes at 37°C. Phenol extract the contents of the tube. Add $1/10^{th}$ volume of 3 M Na Acetate (40 µl), 2 volumes of 100 % ethanol and store tube at -20°C for at least 1/2 hour. Pellet the DNA for 15 minutes in microfuge (14,000 rpm), remove the ethanol and wash two times with 70 % ethanol. Dry gently and check plasmid by agarose gel electrophoresis (load 1 µl of DNA on a 1 % agarose gel).

J. Stable transfection

The day before transfection, 2–8 x 10^5 cells were seeded in 5 ml appropriate growth medium in 60 mm dishes. The cells were incubated under 37°C and 5% CO_2. On the day of transfection, 5 µg DNA (pEGFP-ZP3) dissolved in TE buffer pH 7 to pH 8 (minimum DNA concentration: 0.1 µg/µl) was diluted with cell growth medium containing no serum, proteins or antibiotics to a total volume of 150 µl. Twenty µl of SuperFect Transfection Reagent (QIAGEN, Valencia, CA) was added to the DNA solution. The samples were mixed well and incubated for 5–10 min at room temperature (15–25°C) to allow transfection-complex formation. While complex formation takes place, the growth medium was gently aspirated from the dish, and cells were washed once with 4 ml PBS. One ml of cell growth medium (containing serum and antibiotics) was added to the reaction tube containing the transfection complexes. The total volume were

mixed well and immediately transferred to the cells in the 60 mm dishes. Incubated cells with the transfection complexes for 2–3 h under 37 C and 5% CO_2. Medium containing the remaining complexes was removed from the cells by gentle aspiration, and washed cells 3–4 times with 4 ml PBS. Fresh cell growth medium (containing serum and antibiotics) was added, and incubated for 24–48 h. Passage cells at 1:10 to 1:15 into the appropriate selective medium. Cells were maintained in selective medium under their normal growth conditions until colonies appear.

A positive clone was selected by culturing transfected cells in 800ug/ml of G418 (GIBCO, Grand Island, NY) in MEM medium supplemented with 5% FBS for three months, single clone was picked, cultured in 96-well plate until each single cell form a single colony, then the cells grown in each were transferred to 24 –well plate followed by transferring to 6-well plate, when the confluence in each well reached 70-80%, cells were trypsinized and harvested, some of which were used for isolation of cytoplasmic mRNA, and the rest were frozen for storage. The isolated mRNA from each colony was then subjected to RT-PCR test, flow cytometry analysis to verify fusion protein expression, furthermore, the culture medium was harvested and checked for the presence of GFP by Western blot using monoclonal anti-GFP antibody.

K. Isolation of mRNA

RNeasy mini kit (QIAGEN, Valencia, CA) was applied in mRNA purification from transfected PA-1 cells. Direct lysis transfected PA-1 cells in cell-

culture dishes, add 1.0 ml of cold (4°C) RLT buffer (50 mM Tris Cl, pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40 (1.06 g/ml), just before use, add 1000 U/ml RNase inhibitor, 1 mM DTT). Gently using a rubber policeman to detach cells from the plates, and transferred to a centrifuge tube. Centrifuged lysate at 4°C for 5 min at 300–500 x g. Supernatant were transferred to an RNase-free 10–15 ml or 50 ml centrifuge tube, the pellet was discarded. After centrifuging, the pellet was warmed to 20–25°C, and appropriate volume of Buffer RLT was added. Mixed thoroughly by shaking or vortexing. Appropriate volume of ethanol (96–100%) was added to the homogenized lysate. Mixed thoroughly by shaking vigorously. The samples were applied to an Rneasy midi column placed in a 15 ml centrifuge tube. Centrifuge for 5 min at 3000–5000 x g, the flow-through was discarded, and centrifuge for 5 min at 3000–5000 x g. Add 2.5 ml or 10 ml Buffer RPE to the RNeasy column. The column was washed twice by centrifugation. To elute, after transferring the RNeasy column to a new 15-ml or 50-ml collection tube, appropriate volume of RNase-free water was directly added onto the spin-column membrane, after about 1 min, centrifuge for 3 min at 3000–5000 x g. Repeat the elution step as described with a second volume of RNase-free water.

L. RT-PCR

First Strand cDNA Synthesis: The isolated mRNA was applied for reverse transcription to generate cDNA. The reverse transcription was performed as follows: A 20 μl reaction volume was set up for 1μg of total RNA. Add the

following components to a nuclease-free microcentrifuge tube: 1 μl 50-250 ng of random primers, 1ug Total RNA, Sterile, distilled water to 12 μl. Heat mixture to 70 °C for 10 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add: 4 μl 5X First Strand Buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μl 0.1 M DTT, 1 μl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH). Mix contents of the tube gently and incubate at 42 °C for 2 min. Add 1 μl (200 units) of MLV reverse transcriptase, mix by pipetting gently up and down. Incubate 50 min at 42°C, then inactivate the reaction by heating at 70°C for 15 min. To remove RNA complementary to the cDNA, 1 μl (2 units) of E. coli RNase H was added and incubated 37°C for 20 min.

PCR Reaction: Only 10% of the first strand reaction was used for PCR. The following was added to a PCR reaction tube for a final reaction volume of 100 μl: 10 μl 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 3 μl 50 mM $MgCl_2$, 2 μl 10 mM dNTP Mix, 2 μl Amplification Primer 1 (10 μM), 2 μl Amplification Primer 2 (10 μM), 1 μl Taq DNA polymerase (2-5 U/μl), 2 μl cDNA (from first strand reaction, RNase H-treated), 80 ul Autoclaved, distilled water. The reaction mixture was mixed gently and layered 2 drops (~100 ul) of mineral oil over it. Heated reaction to 94 °C for 3 min to denature. The PCR reaction was performed as follows: denature DNA template at 94 ° C for 1 minute, anneal at 55 °C for 2 min, extension at 72 °C 1 min. Repeated cycles 34 times. A final extension step at 72 °C for 5 min. was performed to fill in any uncompleted polymerization. Then cooled down to 4-25 °C.

M. SDS-PAGE and Western blot analysis

In order to confirm the identity of protein expressed in vitro, monoclonal antibodies against GFP (Clontech, Palo Alto, CA), 6×His (Amersham, Piscataway, NJ) as well as polyclonal antibodies against human specific ZP3 peptide were used in Western Blot. The ZP3 antibody was produced by Biosynthesis (Lewisville, TX), which was raised from rabbit against a human specific ZP3 peptide (C-G-T-P-S-H-S-R-R-Q-P-H-V-M, ZP3 amino acid numbers 327–340, Chamberlin and Dean, 1990). For Western blot analysis, firstly, SDS-PAGE was performed on 8% gels to separate protein samples. For each sample, total five micrograms of purified protein was combined with equal volume of 2× protein loading buffer (12.5 mM Tris, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and boiled for five minutes, electrophoresis was carried out at 200 constant voltage and is continued until the tracking dye run out of the gel. After gels resolved the samples, gels were then either stained with Coomassie brilliant blue G-250 (Sigma), or electrophoretically blotted onto Nitrocellulose membrane by using the Trans-Blot SD Semi-Dry Transfer Cell column (Bio-Rad, Mercules, CA). Membranes were blocked with blocking buffer (5% non-fat dry milk, 0.1% Tween-20 in PBS, pH7.4) overnight at 4 °C with shaking. Primary antibody were diluted in the blocking buffer and incubated with the membranes for 1 hour at room temperature. Membrane was then washed with 0.4% Tween-20 in PBS for three times, each time 15 minutes. The membranes were then incubated with secondary antibody (anti-mouse IgG or anti-rabbit IgG) conjugated with Horse-Radish Peroxidase diluted in blocking

buffer (1:3000) for 1 hour at room temperature. After three time 15 min washes with PBS/0.3% Tween-20 and three time 5 min washes with PBS/0.1% Tween-20, the blots was developed using enhanced chemiluminescence detection with ECL kit (Amersham, Piscataway, NJ), according to the manufacture's instruction.

N. BCA assay:

When each batch protein was isolated and purified from culture medium, the protein concentration was determined by a BCA assay kit from PIERCE (Rockford, IL). A standard curve using BSA was generated each time when measuring the protein concentration. BSA at the concentration of 0, 100, 200, 300, 400, 500, 600, 700, 800, 1000, 1200μg/ml is prepared freshly. The BCA working solution was prepared by combining 5 ml of solution A and 100 μl of solution B, for both sample and standard protein, 12.5 μl was added to each well on a 96 well plate, duplicate samples were used, and 250 μl of BCA reagent was added to each well, the plate was thereafter incubated at 37 °C for 30 minutes; right after incubation, the microtiter plate was read at 570nm to get OD values of each standard and sample, finally the protein concentration was determined by the standard curve generated from the OD of BSA with serial dilutions versus BSA concentration.

O. Protein purification by affinity chromatography

The first step in isolation and purification is to ultra-filtrate cell culture medium to a small volume. An apparatus from Amicon (Bedford, MA) using a

filter membrane with 30-kD molecular weight cut-off was applied to concentrate five hundred miniliters of culture medium to about 16-20 ml. The concentrated medium was then dialyzed against 5 liter of Ni-NTA binding buffer (50 mM Na_2HPO_4, 300mM NaCl, pH 8.0) three times, with changing buffer every 12 hours, the medium was then ready for protein purification.

A 6×His sequence has been inserted right upstream to the fusion protein sequence to facilitate protein purification; therefore the affinity column Ni-NTA which specifically binds with 6×His was applied for protein purification. All the procedures for protein purification were performed in cold room (4 °C). The resin from Qiagen (Valencia, CA) was packed on a column, ten volumes of Ni-NTA buffer was allowed to pass through the resin to equilibrate the resin. The dialyzed concentrated medium was then applied to the Ni-NTA column to pass through and collect "pass" fraction; the resin is then washed with 10 column volume (CV) of 5-8 mM of imidazole in binding buffer. Finally, elution with Ni-NTA buffer containing imidazole was performed to obtain bound protein on the resin.

P. Preparation of motile sperm and spermatozoa capacitation:

Sperm samples were obtained from healthy donors according to World Health Organization (WHO) standards. Semen samples donated for research have been approved by the Institutional Review Board at Eastern Virginia Medical School. Sperm samples were collected in the morning around 8-9:00 am, and the quality of semen was assessed by the andrology lab. Freshly ejaculated spermatozoa from healthy donors were obtained by masturbation after

at least 3 days of abstinence. After liquefaction by putting in 37 °C for at least thirty minutes, Computer-Assisted Sperm Analysis (CASA) was performed to assess spermatozoa motility and concentration. The Hamilton-Thorn Research Sperm Analyzer was routinely used to evaluate sperm motion parameters. Samples having >40 million sperm/mL, >60% initial progressive motility and >14% normal morphology (strict criteria) were used in the experiments. Spermatozoa used in the experiment are motile and were selected by discontinuous centrifugation. Spermatozoa were isolated by discontinuous Percoll (Pharmacia, Peapack, NJ) gradient separation (90% and 40% layers) using human tubal fluid as diluent medium. Two milliliters of semen was carefully placed on Percoll layers, after centrifugation at 400×g for 20 minutes, purified population of highly motile (from 90% layer) is recovered, washed in HTF/0.5% HSA (Irvine Scientific, Santa Ana, CA) medium and centrifuged at 380×g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in HTF medium supplemented with 0.5% HSA. The final suspension was then readily subjected to motility analysis, generally, at least 90% motility should be obtained. Motion analysis was conducted after suspending sperm pellet in HTF medium, Then sperm samples was incubated at 37°C in 5% CO_2 for 3 more hours in the same medium, to allow capacitation.

Q. Acrosome Reaction:

Sperm treated with GFP/ZP3 followed by acrosome reaction evaluation was performed to test the second bioactivity of GFP/ZP3. The sperm

concentration for the immunofluorescence detection of acrosome reaction is set at 2 million motile sperm/ml. A series of 100 µl sperm aliquots (positive control: calcium ionophore A23187 at 5 µM; negative control: sperm culture medium; test: GFP/ZP3) will be prepared in Eppendorf vials and incubated in the 95% air, 5% CO_2 37 °C incubator for 30 minutes. Triple slides were made for each assay. Hoechst 33258, a DNA-specific stain, that will enter the nuclear membrane of dead spermatozoa giving a fluorescent counterstain, was used for determination of viability (live sperm). Fluorescence isothiocyannate conjugated Pisum Satvum Agglutinin (FITC/PSA, Sigma, St Louis, MI) staining techniques was employed to evaluate the acrosome status of spermatozoa in spot slides. The epifluorescent microscope will be used to read the spot slides at a power of 400 magnification, blind reading will be required for evaluation of immunofluorescence patterns and at least 100~200 cells from 10 random fields were evaluated per spot on the slide. In some experiments, solubilized human ZP was used as described in Cross *et al*, 1988, Mahony *et al*, 1991 and Franken *et al*, 1996. The results were expressed as percentage of live, acrosome-reacted spermatozoa in the total population counted.

R. Using flow cytometry to test the binding activity of GFP/ZP3:

Flow cytometry was employed to test the binding activity of GFP/ZP3 to acrosome-intact spermatozoa. The motile sperm were used in all experiments, in each test, sperm only and sperm incubated with recombinant GFP (5ug/ml; Clontech, Palo Alto, CA) were set as control. Motile sperm (2 million) were

incubated with GFP or purified recombinant fusion protein GFP/ZP3 at 37 °C in 5% CO_2, 95% air for a certain time as needed in the experiment. The spermatozoa were then be divided into two aliquots, in which one as non-washed sample, the other will then be washed with PBS for two times, the wash was done by suspending the sperm pellet in 1 ml of PBS and centrifugation at 600g for 8 minutes. Both unwashed and washed sperm sample were resuspended in 600ul of PBS for analysis by FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Measurement was performed at a sample flow rate of 250-300 events/second and 15,000 events were analyzed for each sample. A gate was set on dot plot distributions of forward versus 90 degree scatter to exclude debris and clumps. Sperm that gave fluorescent signals above the control EGFP were considered to be positive. Data were expressed as the percentage of fluorescing sperm.

S. Assessment of the binding of GFP/ZP3 to human spermatozoa in Indirect Immunofluorescence Study

More than ninety percent motile spermatozoa were collected from capacitated human spermatozoa by the gradient Percoll centrifugation method as described above. Motile spermatozoa were incubated with recombinant GFP/ZP3 (20 μg/ml) in HTF containing 0.5% (w:v) HSA for 3 h (5% CO_2 in air at 37°C). Sperm aliquots were centrifuged at 800 x *g* for 5 min; the pellets were washed twice with PBS, then the pellets were spotted onto 8-well multitest slides (Cel-Line Inc., CA), air dried, and fixed with 1% paraformadehyde for 30 min for

subsequent staining. The specimen was blocked with PBS containing 3% (w:v) BSA in a humidified box for 1 h at RT (all subsequent incubations were carried out at RT for 1 h). After washes with PBS, the specimen was then incubated with Alexa488 conjugated anti-GFP antibody (1: 200) for 1 hour. After three times of washes with PBS, the specimen was examined using a fluorescence microscope (Nikon, Garden City, NY) at x400 and x1000 magnification.

RESULTS

A. Subcloning of human ZP3 cDNA sequence into pEGFP-C1 vector

The binding activity of the putative ZP3 has been demonstrated to be attributed to the C-terminus of ZP3 backbone, and the sequence responsible for the binding activity has also been identified in mouse, which is from amino acids 329-334 (Chen et al., 1998). ZP3 is a secreted protein, and the signal peptide is vital for the protein secretion process as well as post-translational modification. Purification of recombinant ZP3 has been facilitated by inclusion of six-His tag at the C-terminal of ZP3, but still could not reach ZP3 with high purity and was not good enough for downstream study using rhZP3 (Dong et al., 2001). We want to in vitro express a fusion protein with ZP3, this fusion protein could provide a tag for detecting sperm binding, so green fluorescence protein (GFP) was selected for this study to fuse with human ZP3. Keeping the binding regions on ZP3 backbone exposed to sperm was another issue that has to be concerned in expressing the fusion protein. Due to the above reasons, we have to fuse the ZP3 downstream to the C-terminus in order to maintain the binding activity of ZP3; the signal peptide sequence of ZP3 was put upstream to the GFP gene to allow the secretion of fusion protein. Since we do not know whether the protein conformation of the fusion protein will interfere the binding activity of ZP3 to human sperm, a proteinase was inserted between ZP3 and GFP; this will allow us to cleave off GFP in case the structure of fusion protein would disturb ZP3-sperm interaction.

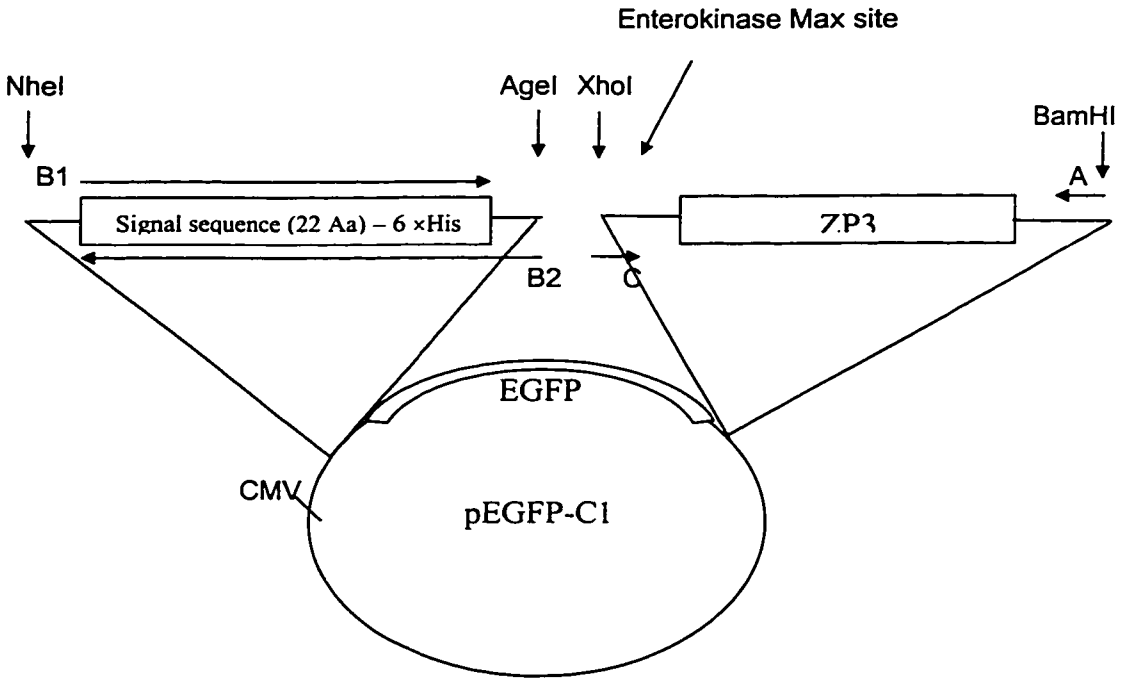


Fig. 2. The strategy of subcloning. The subcloning was accomplished in two steps. In the first step, two (i.e., B1 and B2) oligonucleotides which includes the restriction site, ZP3 signal sequence and 6xHis were synthesized, after annealing the two oligonucleotides, the product B1B2 were digested with NheI and AgeI, then the digested B1B2 was ligated with pre-digested pEGFP-C1 vector (Clontech, Palo Alto, CA) with the same two enzymes. The ligation product was used for transformation; clone hybridization using radioactive probe and restriction enzyme digestion were used for the selection of possible positive clones. Plasmid DNA (pEGFP-B1B2) isolated from the positive E. coli clone was utilized in the second step subcloning. In the second step subcloning, a pentapeptide sequence (Asp-Asp-Asp-Asp-Lys) which can be recognized and cleaved by Enterokinase Max (Invitrogen) was inserted between GFP and ZP3. ZP3 sequence with Enterokinase Max sequence as well as cloning site were generated by PCR using A, C primer, ZP3 cDNA generated from human ovary tissue (Chen, 1995) was used as the template to produce our target sequence in this experiment. The PCR product digested with XhoI and BamHI was then ligated with predigested pEGFP-B1B2.

As such, the ZP3 subclonig was accomplished in two steps. The strategy of subcloning of the ZP3 gene into the pEGFP (enhanced green fluorescent protein) vector is outlined in Fig. 2.

A1: Insertion of the oligo B1B2 into the pEGFP-C1 vector

The general idea of this experiment is shown in Fig. 1. In the first step, synthesized oligonucleotide B1B2 (95 bps long) which includes the restriction site, ZP3 signal sequence and 6×His in that order were inserted into the pEGFP-C1 vector. Here, the introduction of a six-histidine sequence was for the purpose to facilitate later protein purification. Oligonucleotides B1 and B2 introduced NheI and AgeI respectively at 5' and 3' end for the insertion of annealed oligo DNA sequence B1B2 into pEGFP vector. The pEGFP-C1 vector was digested with AgeI and Nhe!, then the oligo DNA sequence B1B2 was inserted to the digested vector by ligation. The resultant plasmid construct was transformed into competent cells. Colony hybridization and PCR were employed to screen for possible positive clones with insert. Fig. 3 shows the results from colony hybridization, by using the radiolabelled (^{32}P) B1 as probe; the dark dots represent possible clones with the insert of oligo sequence B1B2 into pEGFP-C1 vector.

In order to further verify and select positive clone with the B1B2 sequence, DNA restriction digestion was conducted. Because the size of B1B2 is small (95 bps), this will give some difficulty in visualizing the band on agarose gel, so instead of directly obtaining the 95 bps band, NheI and another restriction enzyme site downstream to GFP sequence was selected to digest the plasmid to

Fig. 3. Step I construct and screening positive colony by colony hybridization. The left and right panels were step I construct and colony hybridization respectively. Colony hybridization was applied to screen insertion of oligo DNA sequence B1B2 into pEGFP-C1 vector. A ^{32}P labeled oligonucleotide B1 was used as probe to detect the presence of the insert; the strong dark dots in the picture indicated the possible successful outcome.

obtain a DNA fragment which includes GFP and B1B2, therefore in this experiment, NheI and XhoI were used to digest the plasmid with the insertion of B1B2. The original size of DNA sequence between restriction site XhoI and NheI is 752 bps long, there are 9 bps between NheI and AgeI, after digestion the vector with NheI and AgeI, B1B2 (95 bps long) was inserted into digested vector, so when the positive plasmid with B1B2 insert was digested with NheI and XhoI, the size of DNA sequence between these two sites should be 838 bps long. Those clones showing positive signal in colony hybridization were amplified and subject to plasmid DNA isolation, the isolated DNA was then digested with two enzymes (NheI and XhoI), and an agarose gel was run to verify whether there was an insert of B1B2, vector only without insert was used as a control, plasmid isolated from a negative clone from colony hybridization was also used as another negative control. The picture below (Fig. 4) indicated two clones (shown on the left of the picture) had the insert of B1B2. The resultant positive clone was designated pEGFP-B1B2.

A2: Subcloning of ZP3 sequence without the signal sequence into pEGFP-B1B2

Full length human ZP3 cDNA has been generated from total mRNA isolated from surgically removed human ovary using guanidinium thiocyanate extraction method in our previous study (Chen, 1995). Plasmid DNA isolated from the positive clones from step I was used in the step II subcloning. ZP3 sequence with Enterokinase site right upstream to it was generated by PCR using primer A and C, the digested PCR product by restriction enzyme was inserted into pEGFP-B1B2 by ligation reaction. In turn, the ligated product was

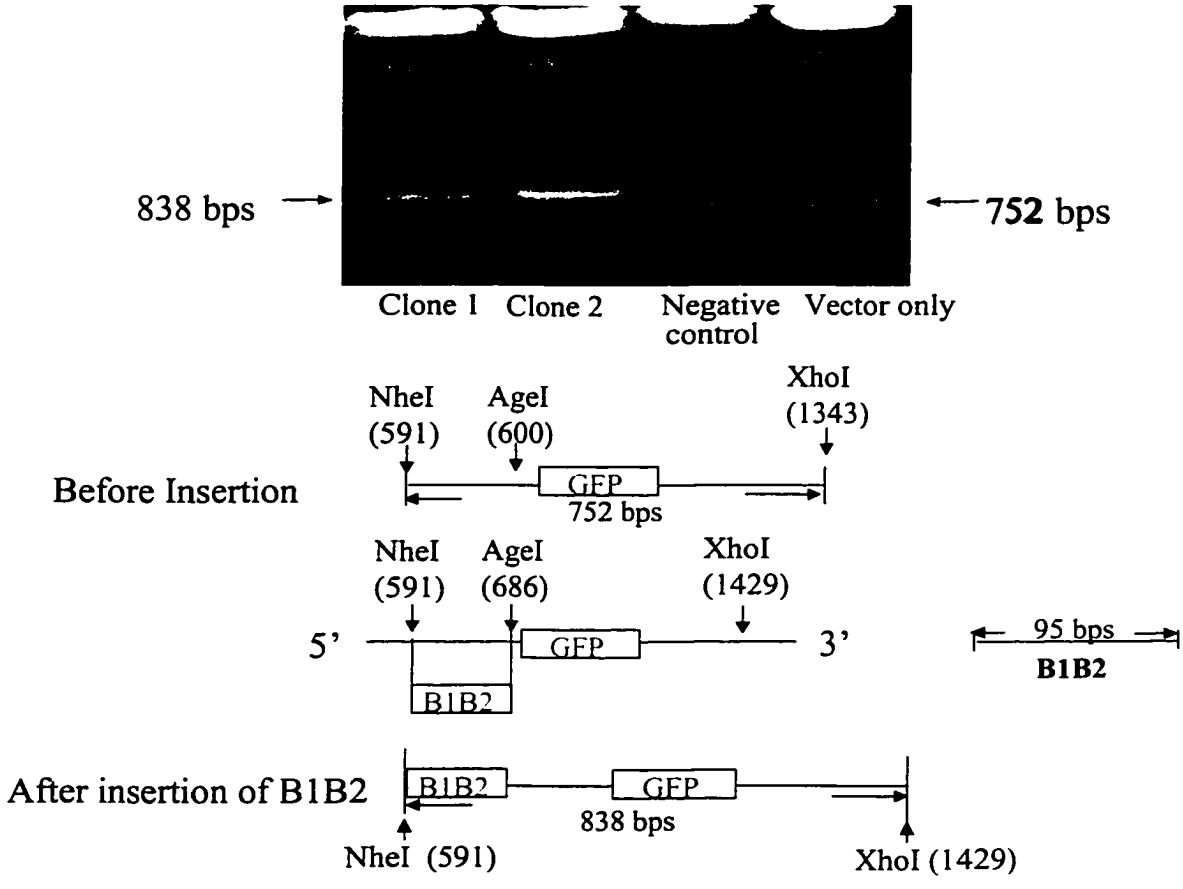


Fig. 4. Confirmation of the insertion of B1B2 by restriction digestion with NheI and XhoI. The left two lanes represent two positive clones, the right two lanes represent sample from controls. There were two negative controls in the experiment, Lane 3: plasmid isolated from a negative clone from colony hybridization; lane 4: Vector only without insert.

used in transformation to obtain positive E. coli clones with insert of ZP3 sequence. In order to verify the integration of the target cDNA (human ZP3) into the vector pEGFP-B1B2, PCR using three pairs of primers (B1 and CH2, CH1 and CH2, CH1 and A) was conducted. Fig. 5 showed the data from PCR of one clone with correct insert. Three pairs of primers were applied in the PCR to confirm the insertion of ZP3. Primers B1 and CH2, CH1 and A, CH1 and CH2 were chosen to perform PCR, plasmid DNA from vector only was used as a negative control. With all three pairs of primers, one clone displayed strong signal showing positive insert of ZP3, the size from PCR was about 2070 bps, which is the size of combined sequence from GFP and ZP3, so it was concluded that ZP3 has been inserted into the vector. By now, the subcloning of ZP3 into pEGFP-C1 vector was successful and can be used in DNA sequencing to check if there is any mutation happened during subcloning. So before using the positive plasmid construct in transfection, DNA sequencing was performed to check the authenticity of inserted sequence, by comparison the sequence hZP3 obtained from the positive clone with that of published, the result indicated the sequence was correct and identical to original published sequence (data not shown).

Therefore, based on the convincing results from PCR and DNA sequencing, it can be deduced that the human ZP3 cDNA has been correctly inserted into pEGFP-C1 vector and ready for transfection and expression in mammalian cells.

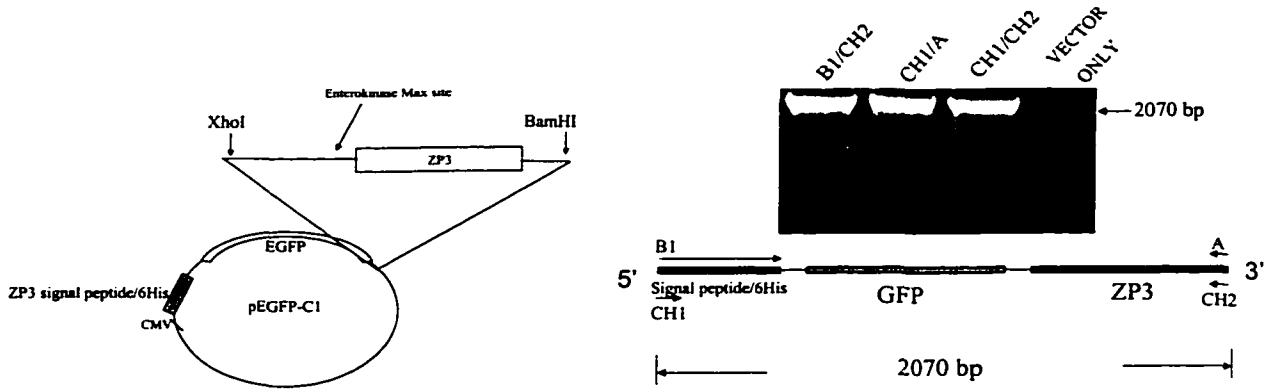


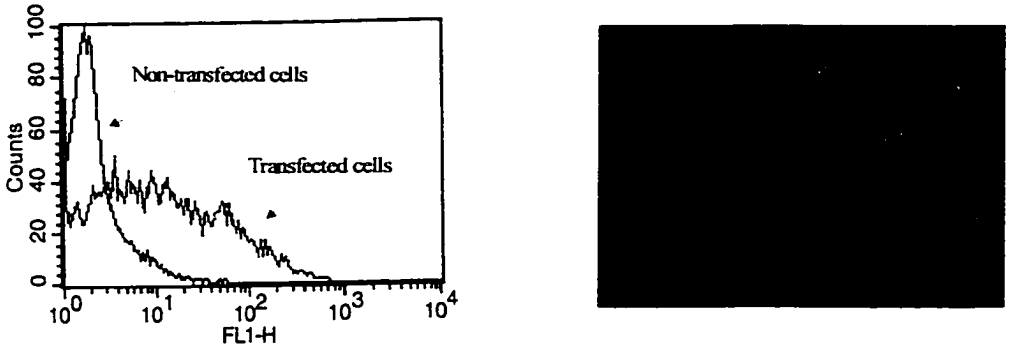
Fig. 5. Step II construct and the determination of the insertion of ZP3 cDNA by PCR. The insertion of ZP3 into vector pEGFP with B1B2 was verified by PCR using three pairs of primers (right). Vector only was used as the negative control. All three pairs of primers gave a bright band of approximately 2070 bps, which is the size of fusion protein GFP/ZP3.

B. Stable transfection and expression of fusion protein GFP/ZP3

When the subcloning was completed, the plasmid DNA isolated from the positive clones was used for transfection into PA-1 cells with the aim to express fusion protein GFP/ZP3. Positive clones were selected by culturing cells in 800μg/ml of G418 in MEM medium supplemented with 5% FBS, single clone was picked and amplified for RT-PCR test, flow cytometry analysis, furthermore, the culture medium was harvested and check for the presence of GFP by Western blot. A stable transfected human ovarian cell line was established by lipid-mediated transfection followed by antibiotic selection by growing the cells in medium containing G418. Total seven clones were later identified to be positive transfectants among those isolated single clones.

B1. Determination of the expression of GFP/ZP3 by flow cytometry and fluorescence microscopy

Firstly, in an easier and convenient way, flow cytometry was performed to identify the positive transfectants since a fluorescent marker (GFP) should be present inside the cells. In flow cytometry, measurements of cells or other biological particles are made as the cells or particles flow in single file in a fluid stream past optical and/or electronic sensors. Modern flow cytometers typically use lasers as light sources and measure light scattered by cells, which provides information about their size and internal structure, and fluorescence in several spectral regions emitted by dyes or labeled probes or reagents which bind specifically and stoichiometrically to cellular constituents such as antigens and nucleic acids. Flow sorting allows cells with pre-selected characteristics to be



A. Flow cytometry B. Fluorescence microscopy

Fig. 6. Determination of the expression of GFP/ZP3 by flow cytometry and fluorescence microscopy. Panel A: Flow cytometry of transfected PA-1 cells versus non-transfected cells. Total 15,000 cells were analyzed per sample. An obvious shift was observed in the transfected cell population. FL1 denotes fluorescence one, which in here is green fluorescence, the positive transfected cells shifts to the right of non-transfected cells due to the measurement of fluorescence from GFP/ZP3 inside the transfected cells. Panel B: Stable transfected PA-1 cells were grown on cover slips, and then the cells expressing GFP were visualized by fluorescence microscopy.

diverted from the stream and collected for further analysis. With the characteristics of flow cytometry, it could be used to detect the green fluorescence and thereby differentiating positive transfectants with GFP/ZP3 from non-transfected cells. If the GFP/ZP3 was expressed in PA-1 cells, besides those proteins secreted into culture medium, green fluorescence would appear inside cells because of some retaining fusion protein inside the cells. A typical result from flow cytometry telling the difference between positive transfectants and non-transfected cells is shown in Fig. 6. The picture clearly demonstrated the expression of GFP/ZP3 in PA-1 cells, in the histogram overlay, FL1 denotes fluorescence 1, which in here is green fluorescence, the histogram of positive transfected cells shifts to the right of non-transfected cells due to the measurement of fluorescence from transfected cells.

B2. RT-PCR to identify the expression of fusion protein GFP/ZP3

When the green fluorescence can be detected in the transfected PA-1 cells, RT-PCR was conducted to further identify the expression of fusion protein in PA-1 cells. Both cultured transfected and non-transfected cells were harvested for RNA isolation, the isolated RNA was then subjected to RT-PCR. As shown in Fig. 7, RT-PCR using two pairs of primers A, C and CH1, CH2 was performed, non transfected PA-1 cells showed no signal, seven clones were found to show distinct specifically amplified sequence. Fig. 7 just showed the results from two of the seven clones, which displayed strong bands corresponding to approximately 1245 bps (ZP3) and 2070bps (EGFP/ZP3) respectively. Non-transfected PA-1 cells did not show any RT-PCR result. So

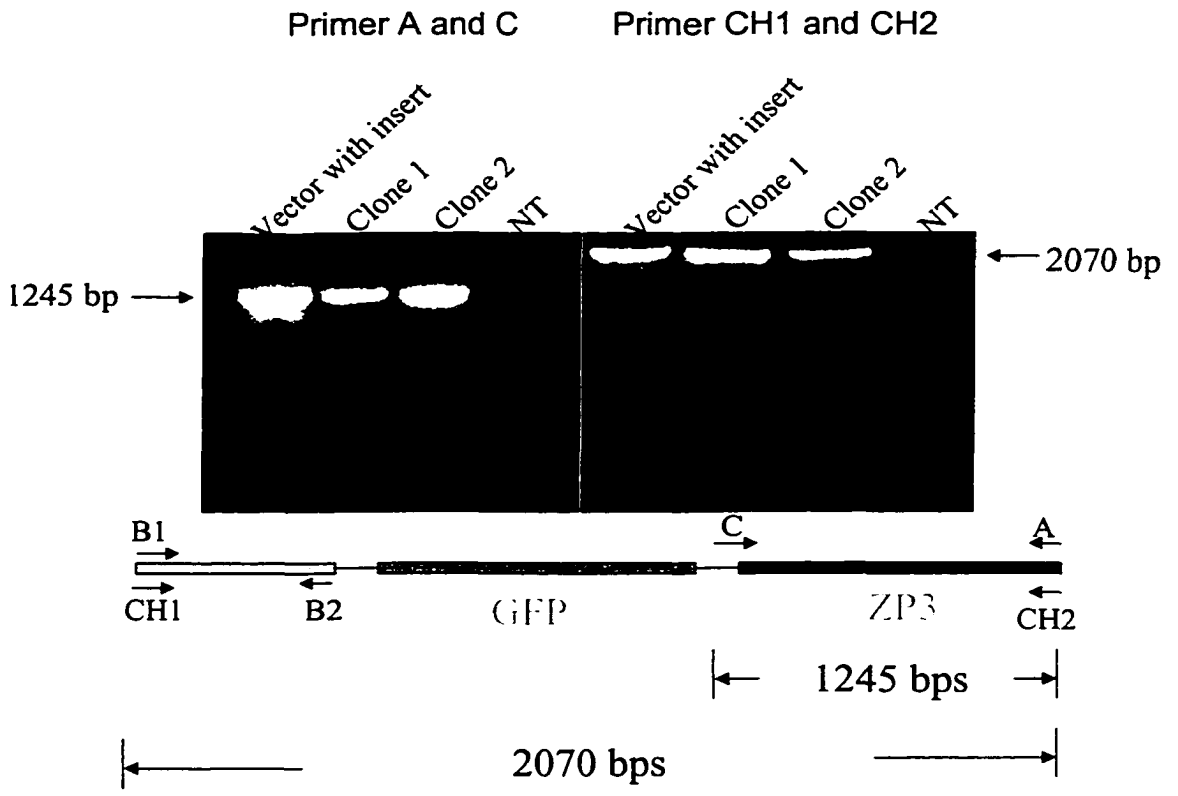


Fig. 7. Verification of the expression of fusion protein GFP/ZP3 in stable transfected PA-1 cells with human GFP/ZP3 cDNA by RT-PCR. Clone 1 and 2 displays a high level of expression of ZP3/GFP by RT-PCR assay. Left panel: using primer A, C; Right panel: using CH1, CH2 primer. The GFP/ZP3 cDNA was used as positive control and non-transfected PA-1 cells (NT) for the negative control. Primer A, C amplified the sequence of ZP3 without signal peptide (22 amino acids, 66 bps), but with the sequence of Enterokinase (15 bps) and sequence of restriction enzyme sites (12 bps), plus trp (3 bps) and lys (3 bps), so the resultant size is 1245 bps. Primer CH1 and CH2 amplified the sequence of ZP3 and GFP.

again, these RT-PCR results confirmed the expression of insert ZP3 as well as fusion protein EGFP/ZP3 in the mRNA level in the host PA-1 cells, and no hZP3 mRNA was detected in the non-transfected cells.

B3. Western blot using monoclonal anti-GFP antibody to demonstrate the expression of EFGP/ZP3 protein

After positive RT-PCR results showing expression of mRNA of EGFP/ZP3 was obtained, the expression of fusion protein at protein level also has to be confirmed. In order to demonstrate the production of recombinant fusion protein GFP/ZP3 in the transfected PA-1 cells and its secretion into the medium, western blot using monoclonal anti-GFP antibody was performed. Cultured cells were treated with lysis buffer to obtain protein from transfected cells; meanwhile, culture medium from transfected and non-transfected cells were harvested and the total protein from medium were precipitated down by 10% TCA method. The resultant protein from transfected cells lysis, transfected and non-transfected cell culture medium were applied to SDS-PAGE, after being transferred to nitrocellulose membrane, the protein samples on the membrane were probed with monoclonal anti-GFP antibody. The result is shown in figure 8, anti-GFP monoclonal antibody recognized a protein of about 90 kD in the cell lysis sample from transfected cells as well as sample from culture medium of transfected cells, no signal was identified in the non-transfected cells. The detection of GFP is very specific, since no background signal was picked up in the molecular weight ladder as well as in other sample. The 90kD protein detected in the sample from cultured transfected PA-1 cells is about the size of fusion protein (GFP/ZP3).

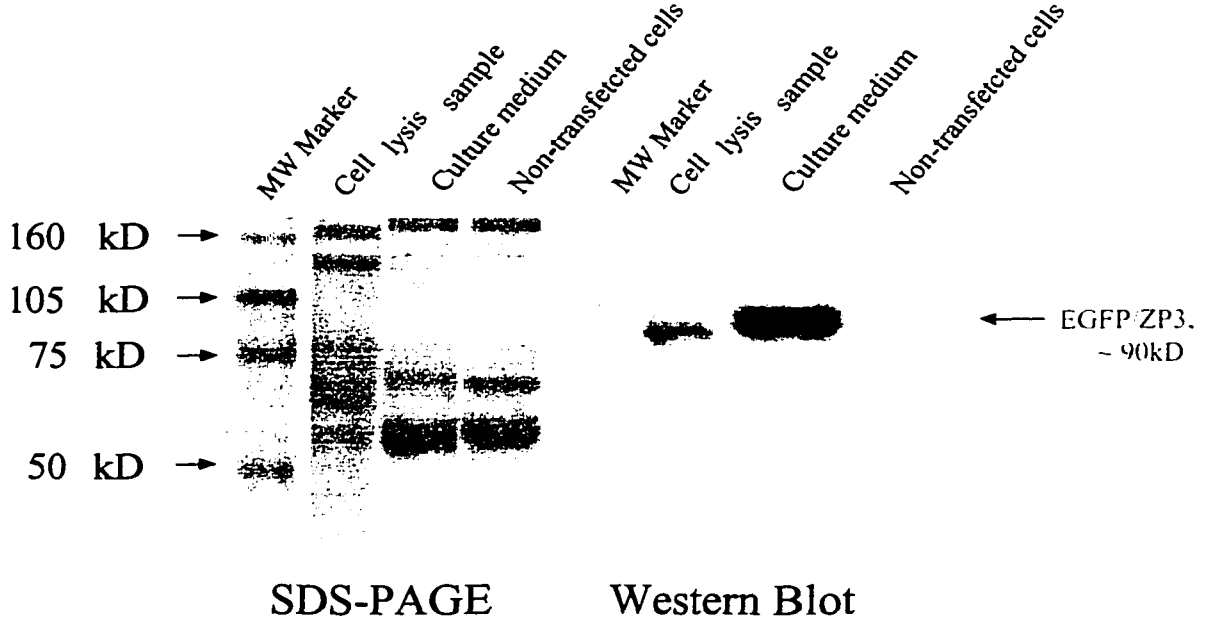


Fig. 8. SDS-PAGE and western blot of non-purified fusion protein sample. The left and right panel represents SDS-PAGE and western blot respectively. In each panel, from left to right, molecular weight marker, cell lysis from transfected cells, partially purified protein from culture medium, medium from non-transfected cells. Monoclonal anti-GFP antibody was used in the western blot.

The transfected cell lysis sample showed positive signal, which make sense and fit into our hypothesis, the production and secretion of GFP/ZP3 is actually a continuous process, so there should be some fusion protein present inside the cells. These results indicated that GFP/ZP3 was expressed in the transfected PA-1 cells.

Taken the convincing evidence from flow cytometry, RT-PCR and western blot together, it can be concluded that fusion protein GFP/ZP3 has been successfully expressed in the host PA-1 cells.

C. Purification of fusion protein from cell culture medium

As stated in the materials and methods section, a six-histidine was inserted upstream to the GFP to facilitate protein purification by Ni-NTA column. Protein with a six-His tag would bind strongly to the Nickle ion on the Ni-NTA resin, then the bound protein can be eluted from the resin by application of high concentration of imidazole to compete protein binding with nickel ion, and thus bound protein can be eluted from the column.

Before the purification of fusion protein GFP/ZP3, the culture medium from stable transfected PA-1 cells were subject to ultra-filtration to reduce the volume from 500ml to about 25 ml. Then the concentrated medium was extensively dialyzed against protein binding buffer so as to be ready for protein binding. The resultant medium was applied to equilibrated Ni-NTA resin. After protein bind with resin, the resin was washed with 4mM imidazole in protein binding buffer to remove non-specific bound protein from the resin. Firstly, a pilot experiment was

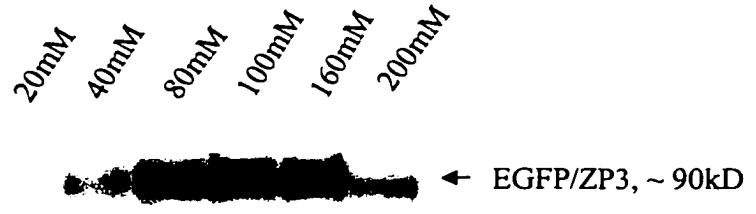


Fig. 9. The sequential protein elute with different concentration of imidazole from Ni-NTA column. Lane 1-6: Molecular weight marker, 20mM imidazole elute, 40mM elute, 80mM elute, 100mM elute, 160mM elute, 200mM elute. Monoclonal anti-GFP antibody was used in the western blot.

conducted to optimize the concentration of imidazole to be used for the elution of protein from column. Washed resin was eluted with a sequential elution buffer with different concentration of imidazole (20mM, 40mM, 80mM, 100mM, 160mM and 200mM). The eluted protein from each fraction was subjected to western blot to find out the optimal concentration of imidazole for elution through the identification of GFP in each fraction of protein. The result is shown in Fig. 9. This figure clearly indicated that fusion protein GFP/ZP3 started to come off from the resin under the condition with 40mM imidazole, and the protein seem to be elute from the resin in a step-wise way, with the increase of imidazole concentration, by using buffer containing imidazole from 40mM to 200mM, fusion protein can be eluted from the resin, but with the majority of protein eluted by 80 to 160 mM of imidazole, and until 200mM imidazole was used, less protein left on the resin was eluted, which meant most protein has been eluted from the column by lower concentrations of imidazole. From this data, protein-binding buffer with 200mM imidazole was applied for elution to completely remove protein from resin in subsequent experiment. And the eluted protein will be used in biological assays. By using the current protocol, the purity of our purified protein sample could reach 30-40%. (Fig. 10)

D. Identification of purified protein using western blot by three antibodies.

Upon the completion of partially purification, emphasis was placed to identify the purified protein by western blot to prove the presence of GFP, ZP3 and 6×His in the purified product. The anti-ZP3 antibody was generated by

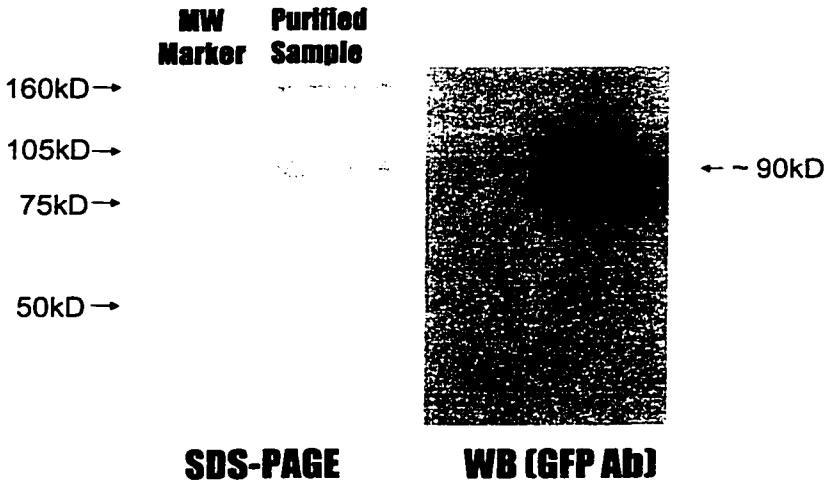


Fig. 10. Purification of GFP/ZP3 by Ni-NTA affinity column. The left and right panel were SDS-PAGE and corresponding Western Blot result. Lane 1: Molecular weight marker; Lane 2: Purified sample.

immunizing rabbit with a human ZP3 specific oligopeptide that spans 14 amino acids sequence at the C-terminus of ZP3 backbone. When using anti-ZP3 polyclonal antibody from rabbit, a pre-immune serum from rabbit was also applied in parallel to the western blot to see whether there were some endogenous antibody from rabbit could recognize ZP3, no similar signal to that identified by anti-ZP3 serum was observed by pre-immune serum in the purified fusion protein sample (data not shown). So the specificity of the polyclonal anti-hZP3 antibody was proved to be very good even use this antibody at 1:5000 dilution, the antibody can still recognize ZP3 and gave strong signal without any background signal. The fusion protein (GFP/ZP3) purified from the Ni-NTA column by affinity chromatography was then subject to western blot by three different antibodies (Fig. 11). In the blot using anti-6×His antibody, a His ladder was used as positive control for the immunoblot as well as molecular weight marker. As it can be seen from the three blots, apparently, by using three antibodies, a single band corresponding to about 90kD was recognized in the purified sample. Therefore, western blot analysis using three antibodies (anti-GFP, anti-6×His, anti-ZP3) strongly demonstrated the presence of GFP, ZP3 and 6×His in purified fusion protein. This gave solid evidence that fusion protein cDNA has been successfully integrated to the cell chromosome and produced correct protein that secured by our study. This is a further powerful support to the previous stable transfection and expression experiment.

An enterokinase site has been placed in between GFP and ZP3 sequence, interest was taken to see whether the GFP tag can be cleaved off and

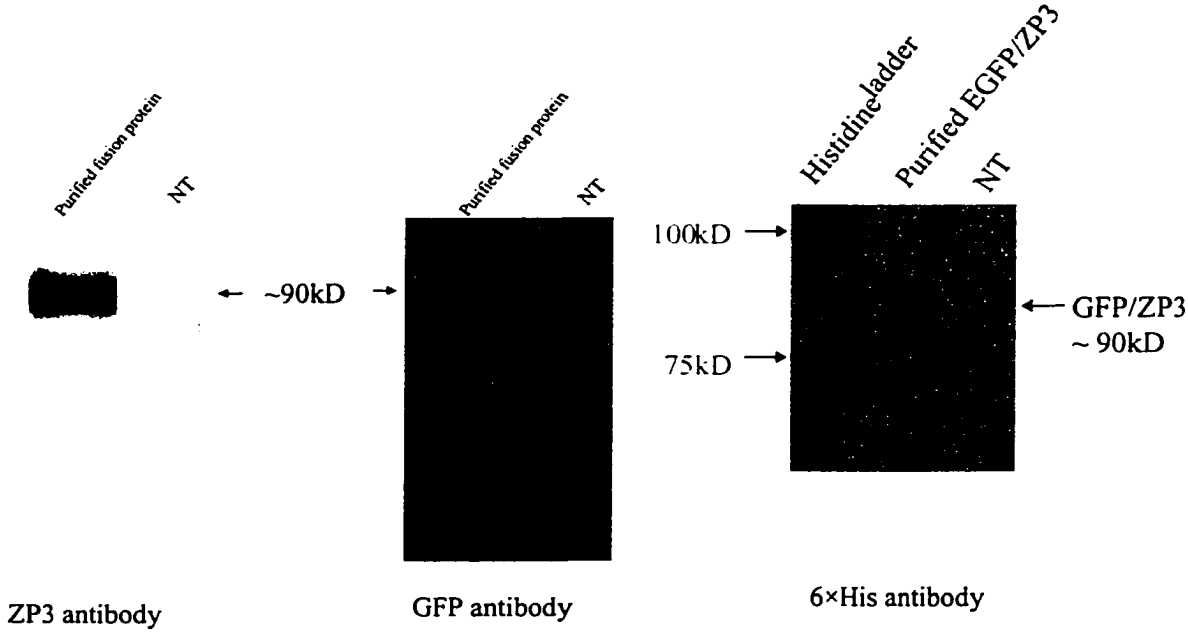


Fig. 11. Identification of GFP/ZP3 by three antibodies. Western blot of purified fusion protein by anti-human ZP3 polyclonal (left panel) and monoclonal anti-GFP antibody (center panel). In each panel, the left lane: purified fusion protein sample, the right lane: purified protein sample from non-transfected cell culture medium (NT). The right panel was the Western blot of fusion protein by monoclonal anti-6×His antibody, a histidine protein ladder from QIAGEN was used as a positive control and molecular marker, a band of about 90 kD was observed in the purified fusion protein sample.

result in separation of GFP and ZP3. So the enzyme digestion experiment was performed to address this issue. After enzyme digestion, western blot was conducted to check the digestion efficiency. The result is shown in Fig. 12. When using anti-ZP3 antibody, the uncut protein displayed a protein band at 90kD, whereas to the digested protein, the protein recognized by ZP3 antibody was about 60~65kD, which is the size of putative human ZP3 itself.

As a summary to the above results, the expression and identification of fusion is complete, and the data has been proved to be very convincing.

E. Characterization of the biological activities of the fusion protein (GFP/ZP3)

During fertilization in mammals, zona pellucida protein 3 (ZP3) is thought to be the primary zona protein that contributes to the initial sperm-egg recognition and induces sperm acrosome reaction. (Bleil and Wassarman, 1980; Wassarman, 1990; Wassarman, 1995). There is overwhelming evidence that species-specific sperm-ZP interaction is a carbohydrate-mediated receptor-ligand binding event, which initiates a signal transduction pathway resulting in the exocytosis of acrosome content, ZP3 acts as the primary receptor for sperm, and the O-linked glycans but not the protein backbone, are responsible for the primary binding. It appears that the protein backbone does not play a direct role in sperm receptor function (Florman et al., 1984; Florman and Wassarman, 1985; Miller et al., 1992).

So far, we have successfully expressed the fusion protein GFP/ZP3, so whether this protein possesses biological functions was vital to us. So bioassay

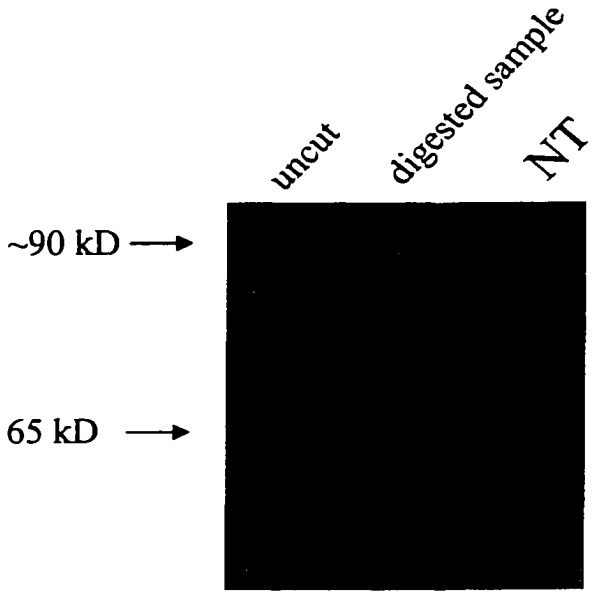


Fig. 12. Digestion of the purified fusion protein with enterokinse Max. In the picture, from left to right: un-digested purified GFP/ZP3, digested fusion protein sample and protein sample from non-transfected cells (NT), sample were probed with anti-ZP3 antibody.

should be performed to characterize the functions of GFP/ZP3. In this study, we applied flow cytometry and immunofluorescence technique to prove the concept that the purified fusion protein would be able to bind to human spermatozoa. In addition, although not a primary goal of this present project, we also tested the acrosome reaction inducing activity of GFP/ZP3 on human spermatozoa.

E1: Human sperm binding activity

It was well documented that ZP3 on mammalian oocytes is responsible for two important functions: as a sperm ligand and inducer of sperm acrosome reaction. First, we want to characterize the binding activity of the recombinant fusion protein GFP/ZP3. In the present study, flow cytometry and indirect immunofluorescence were applied to reach this specific aim. The semen samples used in the studies were from healthy donors unless otherwise indicated.

a. Flow cytometry

Taking advantage the GFP tag in the fusion protein, fluorescence activated cell sorting (FACS) can be used to identify if the fusion protein can bind to the human spermatozoa.

a1. Sperm binding time course study

Based on preliminary study using flow cytometry to identify the binding activity of GFP/ZP3 to human spermatozoa, the results turned out to be promising. To further characterize the binding activity of the purified fusion protein, more detained studies were conducted. Firstly, a time course test for sperm binding was performed. The motile sperm samples were incubated with

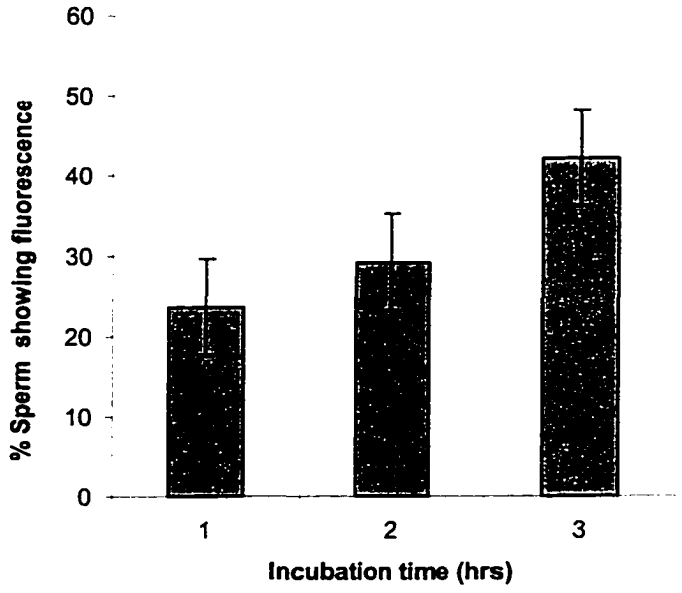


Fig. 13. Time course study of sperm binding with GFP/ZP3. Human spermatozoa were incubated with fusion protein in the presence of binding buffer for different period of time. There was an increase of sperm binding as identified by flow cytometry with the increase of incubation time. Each value is the mean ± SEM from three separate experiments.

GFP/ZP3 for different period of time, the binding of sperm and GFP/ZP3 was measured after one, two and three hours of incubation. It should be noted that due to the long time preparation before FACS and the availability of FACS facility, the longest time can be tested was 3hrs. After purification, the concentration of fusion protein was expressed as the amount of GFP in the protein sample ([GFP] per batch of fusion protein). In the experiment, GFP only was used as negative control, for GFP only and fusion protein treated sperm sample, GFP concentration was normalized to be the same (final 5μg/ml of GFP), GFP only should show no binding, on the other hand, GFP/ZP3 show binding with sperm. Fig. 13 illustrated the relationship between incubation period of sperm with protein and its corresponding percentage of sperm showing binding. Fusion protein showed sperm binding, whereas GFP only showed no binding (data not shown here, but will be addressed later). It is clear that with the period of incubation increases, the percentage of binding sperm increases, so there is a time dependency for human sperm binding with fusion protein.

a2. Dose dependency test

In order to determine how the concentration of fusion protein affect the binding of sperm with GFP/ZP3, a dose dependency assay was set up. Fig. 14 shows the sperm binding test result obtained from FACS performed for dose test: 1.0, 2.0, 3.0, 4.0, 5.0, 7.5, and 10.0, 12.5μg/ml of fusion protein. The result illustrates that there was a dose response for sperm binding with GFP/ZP3. The sperm binding increases with the increase of fusion protein concentration, and the binding curve starts to level off at around 5μg/ml of fusion protein GFP/ZP3.

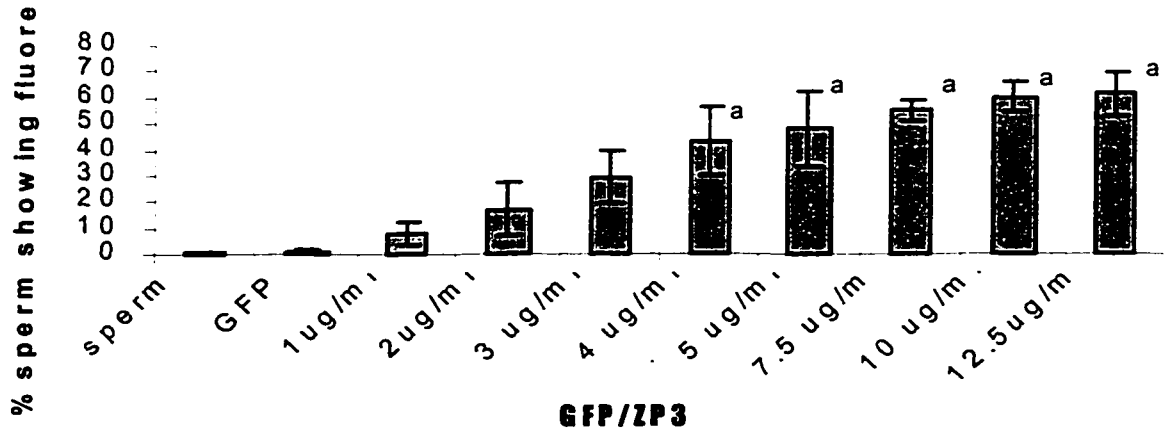


Fig. 14. Dose dependency test of sperm binding with GFP/ZP3. A series of doses were applied to human spermatozoa, the binding was analyzed by flow cytometry. There was a dose-response for sperm binding with GFP/ZP3. The binding curve starts to level off at around 5ug/ml of fusion protein GFP/ZP3. Each value is the mean ± SEM from four separate experiments. One-way ANOVA followed by LSD teat was used for statistics test. [a] indicated there was a significant difference compared with other samples ($p<0.05$).

a3. Effect of enterokinase digestion of fusion protein on sperm binding test

An important issue in the experiment is to prove the binding of recombinant ZP3 to human sperm to be true positive, in other words, the binding is a biological phenomena, it is due to bioactivity of ZP3 but not result from artificial causes like some other co-purified protein in the sample which may physically help recombinant GFP/ZP3 adhere to the human sperm surface. To answer this question, Enterokinase Max was applied to digest purified fusion protein GFP/ZP3. A pilot study was conducted first to find out optimal conditions for complete digestion of fusion protein, for a certain amount of protein used in digestion, a series of different units of enzyme were used in the digestion, the digestion was completed in a 37 °C water bath; meanwhile, another sample with same amount of protein but with no enzyme added was used as a control to see if some protease acts at 37 °C to cleave fusion protein. Western blot using ZP3 antibody was carried out to verify the efficiency of protein digestion. In Western Blot, besides all digestion protein samples, one sample with original undigested protein was used as a control to determine if fusion protein has been fully digested. In Fig. 15, the result from enterokinase digestion and the corresponding sperm binding test is shown. Two doses of enterokinse were used in digestion to find out if there was s dose effect on fusion protein digestion and then therefore sperm binding. From the western blot using ZP3 antibody, 1U of enterokinase completely cleave off GFP tag from fusion protein, and the sperm binding percentage decreased to 10%, while the undigested protein gave 53.6% binding sperm; for the 0.5U enterokinase digested protein sample, about 60%

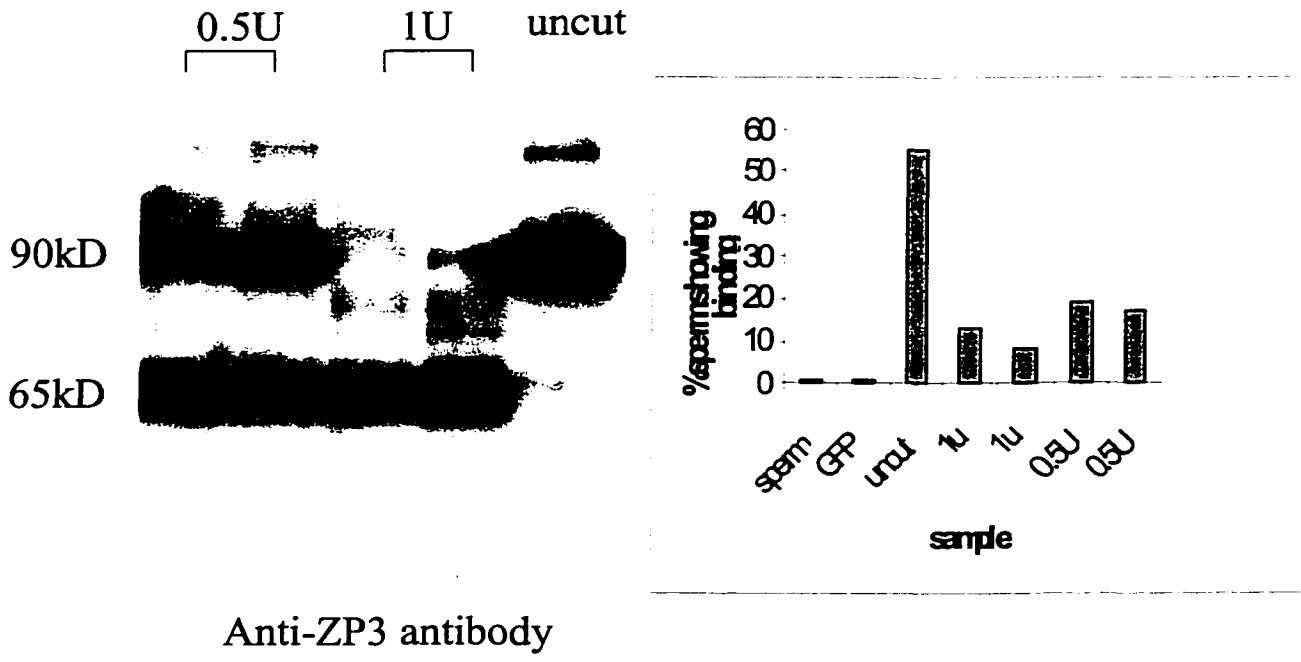


Fig. 15. Enterokinase digestion of fusion protein vs sperm binding test. Two doses of enterokinase were applied to the fusion protein; the digestion mixture was left at 37 °C for overnight. Then the undigested and digested proteins were used for the sperm binding test. The digested protein resulted in a decrease in sperm binding, complete digestion of fusion protein resulted in almost no sperm binding; furthermore, low dosage of enzyme resulted in incomplete digestion of fusion protein, and there were still about 20% sperm showing binding.

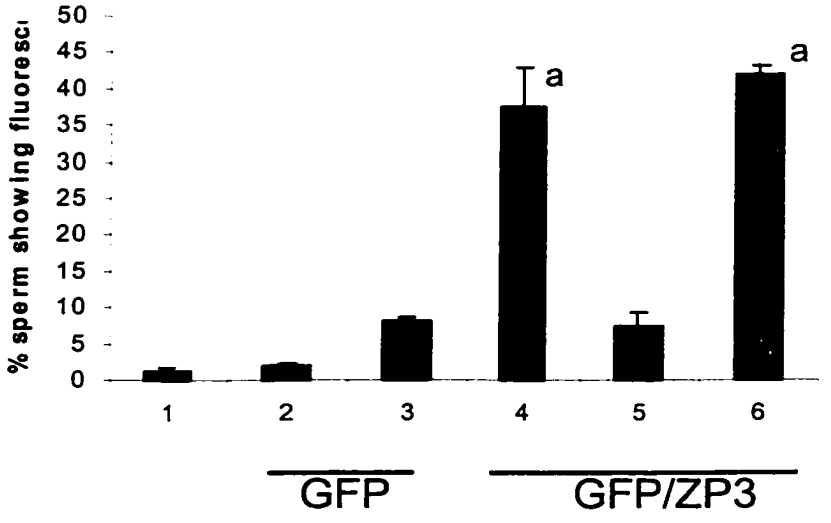


Fig. 16. Determination of the effect of enzyme digestion buffer on sperm binding. The samples are as follows: 1, sperm; 2, GFP only; 3, GFP with digestion buffer; 4, uncut protein; 5, protein cut with 1 unit of enterokinase; and 6, protein with digestion buffer only. When GFP protein was used, digestion buffer only resulted in some non-specific signal (about 10% of sperm showing fluorescence). Wilcoxon ranked sign test was used for comparison of means of samples with different treatment. [a] indicated there was a significant difference compared with other samples ($p<0.05$).

GFP was digested off from fusion protein, and the corresponding treated sperm sample showed 20% of sperm binding. These data indicated that the sperm binding analyzed by FACS is a ZP3 mediated phenomenon. There was a dose dependency relation between dose of enterokinase used and the sperm-binding outcome. One would ask why after complete digestion of fusion protein, the percentage of binding sperm did not return to basal line, which should be close to non-treated sperm only. It was postulated that the enzyme digestion buffer may some how create a certain level of background binding of sperm with GFP, to address this question, another experiment was performed. The result is illustrated in Fig. 16. As can be deduced from the data, GFP only with enzyme digestion buffer did give about 10% of background binding. However, there was no significant difference for sample treated with GFP and enzyme digestion buffer together and GFP only. A summary of the enzyme digestion vs sperm binding test is illustrated in Fig. 17, which is from four times repeat of same experiment. The result demonstrates that the sperm binding as assayed by flow cytometry using fusion protein GFP/ZP3 is mediated by ZP3.

A summary of the sperm binding activity of GFP/ZP3 test is outlined in Fig. 18. The same experiment was conducted for ten times to obtain the result as shown. The dose of GFP/ZP3 in this test was 5μg/ml, GFP (5μg/ml) only and NT + GFP were set as negative control. Sperm treated with GFP/ZP3 resulted in 50.02 ± 4.51% binding, whereas GFP only and NT + GFP did not show any binding.

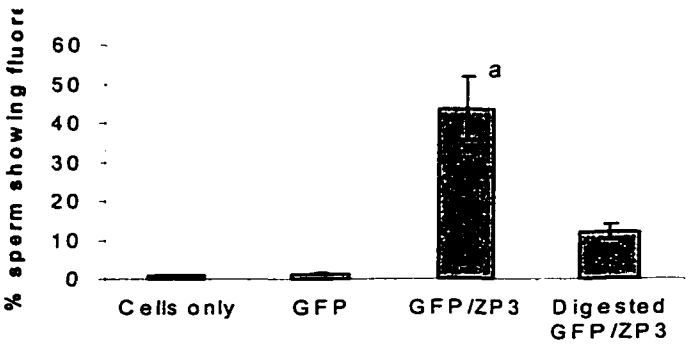


Fig. 17. Summary of enterokinase digestion vs sperm binding test. Four times of enzyme digestion were conducted and the sperm binding test by flow cytometry was performed. The digested fusion protein showed a dramatic decrease of sperm binding in the test. There was some non-specific signal detected in the digested fusion protein sample when analyzed by flow cytometry. Each value is the mean ± SEM from four separate experiments (n=4). Wilcoxon ranked sign test was used for comparison of means of samples with different treatment. [a] indicated there was a significant difference compared with other samples ($p<0.05$).

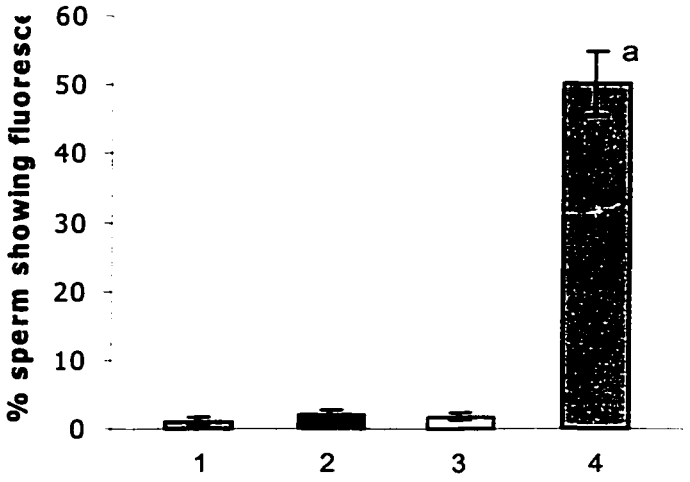


Fig. 18. Sperm binding test by flow cytometry. One million of sperm were used in each sample, samples were as follows: 1, sperm only; 2, GFP alone; 3, GFP + NT; 4, GFP/ZP3. Fusion protein dose used in the test was 5μg/ml, GFP only and NT + GFP were used as negative control. Each value is the mean ± SEM from ten separate experiments (n=10). Wilcoxon ranked sign test was used for comparison of means of samples with different treatment. [a] indicated there was a significant difference compared with other samples ($p<0.05$).

b. Immunofluorescence to identify sperm binding

As an alternative approach, immunofluorescence was performed to directly visualize sperm binding with GFP/ZP3. Since when fusion protein was incubated with sperm, this could not give us visible signal under fluorescence microscope, this may be due to the low amount of protein expressed and isolated, or the green fluorescence has been quenched during protein purification. Therefore, to further demonstrate that GFP/ZP3 could bind to human sperm head region, we carried out immunofluorescence by amplifying the signal through the application of anti-GFP antibody. A typical binding pattern of the GFP/ZP3 to human sperm is shown in Fig. 19. After incubation n of sperm with GFP/ZP3, anti-GFP-Alexa 488 was applied to stain the control and ZP3 treated human spermatozoa. With the treatment of GFP/ZP3, sperm showing binding to ZP3 was identified by observing green fluorescence over the sperm acrosome region, whereas sperm without ZP3 treatment showed no fluorescence. Again, this method ascertained the binding of GFP/ZP3 with human spermatozoa.

E2. Acrosome reaction induced by fusion protein GFP/ZP3

We also tested another activity of fusion protein GFP/ZP3 to act as sperm acrosome reaction inducer. A DNA dye Hoechst stain H33258 was applied to differentiate live/dead cells; only live cells were counted for computing sperm acrosome reaction percentage. Calcium ionophore A23187 was used a positive control; solubilized human zona pellucida (0.6ZP/μl) was also used as a positive control, due to the difficulty in obtaining human zona, solubilized human zona pellucida was only applied in acrosome reaction test once, so there was no error

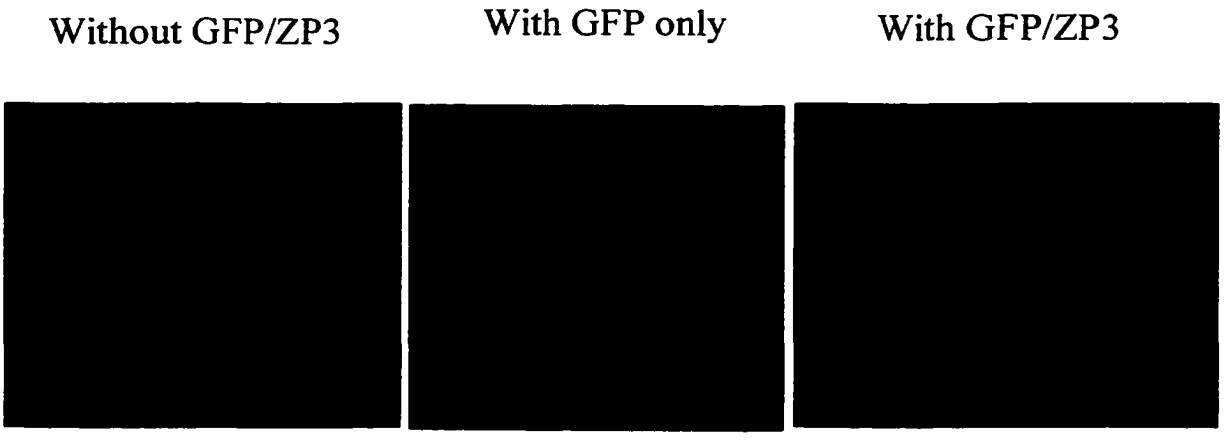


Fig. 19. Immunofluorescence microscopy to identify GFP/ZP3 binding with human sperm. After incubation of sperm with or without fusion protein, fixed sperm sample was probed with alexa 488 conjugated anti-GFP antibody, which gave green fluorescence under fluorescent microscope. With the treatment of GFP/ZP3, some sperm showed binding with ZP3, which can be identified by the green fluorescence over the sperm acrosome region; those without GFPZP3 treatment and those treated with GFP alone did not show any green fluorescence.

bar for HZP in the result (Fig. 20). Fusion protein gave a comparable acrosome reaction inducing activity to ZP; recombinant GFP/ZP3 could induce 19.16% (± 3.44%) sperm acrosome reaction, which is significantly higher than the spontaneous acrosome reaction (10.87 ± 2.89%).

In summary, both biological activities of GFP/ZP3 have been characterized. Fusion protein has been demonstrated to possess complete biological activities of native ZP3 in as a ligand for human sperm receptors and sperm acrosome reaction inducer.

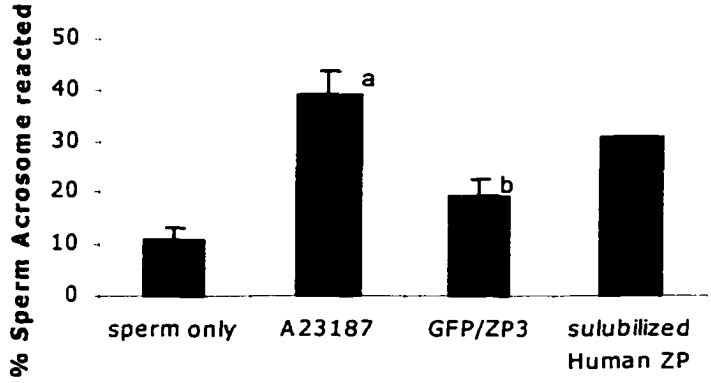


Fig. 20. Acrosome reaction induced by recombinant fusion protein GFP/ZP3, Calcium ionophore and solubilized human zona pellucida (HZP) were used as positive control (n=4). Each value is the mean ± SEM from four separate experiments. Fusion protein could induce capacitated human spermatozoa to undergo acrosome reaction at 19.16% ± 3.44%, which is significantly higher than that of spontaneous acrosome reaction. Wilcoxon ranked sign test was used for comparison of means of samples with different treatment. [a, b] indicated there was a significant difference compared with other samples ($p<0.05$).

DISCUSSION

Apart from a number of important steps in the fertilization process, the sperm-zona pellucida binding capacity of a given sperm population has been shown to be a crucial event during mammalian fertilization. The results of sperm-zona binding assays provide important evidence on the recognition event leading to fertilization (Franken et al., 1989, 1993; Gamzu, et al., 1994; Oehninger, 2000). Systematic examination of the interaction between sperm and zona pellucida has been the most promising of the new approaches. The development of homogenous functional bio-assay for sperm quality assessment has been a focal point of reproductive biologists. Originally, human sperm-oocyte interaction was defined in an assay developed to evaluate zona penetration; the methodology of this assay formed the cornerstone of the future sperm-oocyte interaction test (Overstreet et al., 1976). There are two well-established bio-assays that have the advantage of providing a functional homologous test for sperm binding to the zona pellucida, these two assays thus can compare populations of fertile and infertile spermatozoa in the same assay. These two methods are: Hemizona Assay (HZA) (Burkman et al., 1988) and a competitive intact zona pellucida-binding test (Liu et al., 1990).

The zona binding test is not widely used because of the difficulty in obtaining sufficient numbers of human zona. In theory the paucity of materials can be overcome by the production of recombinant proteins. Recombinant DNA technology made the production of larger quantities of protein in vitro feasible.

There are strong evidence from mouse model which concluded that ZP3 was responsible for the primary binding, and ZP2 attributes to secondary binding (Wassarman, 1988, 1992), what's more, recombinant DNA technology also demonstrate that in vitro expressed ZP3 and ZP2 possess the biological activities to bind to human spermatozoa (Whitmarsh et al., 1996; Tsubamoto et al., 1999). However no data are available related to sperm binding in validated assays. (Chapman, et al., 1996, van Duin, et al., 1994). There is a genuine need for the production of a recombinant human ZP3 that expresses the full spectrum of sperm binding and acrosome reaction induction of native ZP3. The production of a purified glycosylated recombinant human ZP3 in a biologically active form is fraught with technical difficulties.

The study carried out by our group has moved the rhZP3 production forward in that the rhZP3 has been characterized to possess binding activity and induce acrosome reaction (Dong et al., 2001). The successful production of biologically active rhZP3 by PA-1 cell line apparently closely resembles the native protein. In the present study, major objective was to characterize the sperm receptor activity of rhZP3, fluorescent dye has been widely applied in identification of molecule for different purpose in science; however, although through directly linking the fluorescent dye to ZP3 may allow the direct detection of binding of ZP3 with sperm, based on our previous experience work on ZP3, high background due to the difficulty and efficiency in removing free dye when preparing the fluorescent dye-tagged ZP3, and the results were not reproducible, these limit the application of ZP3 in the clinical diagnosis.

The cloning of the wild-type GFP gene (wtGFP; Prasher et al., 1992; Inouye and Tsuji, 1994) and its subsequent expression in heterologous systems (Chalfie et al., 1994; Inouye and Tsuji, 1994; Wang and Hazelrigg, 1994) established GFP as a novel genetic reporter system. When expressed in either eukaryotic or prokaryotic cells and illuminated by blue or UV light, GFP yields a bright green fluorescence. The detection of GFP and its variants can be performed in living cells and tissues as well as fixed samples. GFP has been expressed as fusions to many proteins. In many cases, chimeric genes encoding either N- or C-terminal fusions to GFP retain the normal biological activity of the heterologous partner, as well as maintaining fluorescent properties similar to native GFP (Flach, et al., 1994; Wang and Hazelrigg, 1994; Marshall et al., 1995; Stearns, 1995). The use of GFP and its variants provide a "fluorescent tag" on the protein, which allows for in vivo localization of the fusion protein. GFP can provide enhanced sensitivity and resolution in comparison to standard antibody staining techniques (Wang and Hazelrigg, 1994).

The first specific aim of this present study was to express fusion protein GFP/ZP3 in PA-1 cells. As mentioned above, ZP3 has been demonstrated to be responsible for the primary binding of sperm with egg. The properties of GFP may provide an alternative way to give a fluorescent tag to ZP3, that is the fundamental idea in this study to in vitro express fusion protein GFP/ZP3, and consequently use it in the clinical diagnosis of male infertility. Thus by taking the advantage of the availability of green fluorescent protein, we expressed fusion protein GFP/ZP3 and then use this novel fusion protein to characterize ZP3's

sperm binding activity and eventually will try to develop a kit for diagnosing male sperm quality in clinical arena. The test may be used to predict fertilization failure and influence the decision to perform intracytoplasmic sperm injection as opposed to in vitro fertilization.

For fusion protein GFP/ZP3 expression, several points have been considered, first the fusion protein should be secreted; second, protein can be easier isolated and purified; third, the protein should present biological activity. Protein expressed inside the cell are sorted to different destination, ZP3 is secreted protein, and its secretion requires the signal peptide, which is a 22 amino acids peptide. In the in vitro system, there are reports showing that using the signal sequence from another secreted protein, normally non-secreted heterologous protein was able to secret into the medium (Braspenning et al, 1998). So in the experiment design, the original ZP3 secretion signal peptide sequence followed by a 6×His sequence is inserted before the 5' end of GFP sequence, the 6×His sequence is used for protein purification by Ni-NTA affinity column.

The vector used in the expression of fusion protein includes the cytomegalovirus (CMV) intermediate early enhancer and the simian virus 40 (SV40) promoter to generate high levels of expression on mammalian cells. According to the murine model, the binding site of ZP3 to sperm is located at the C terminal; therefore, to maintain the binding site exposed on the fusion protein, the 5' end of ZP3 was fused with the 3' end of GFP, as shown in Fig. 2; the signal peptide of human ZP3, with a six-histidine residue at its 3' end, was

inserted between the CMV promoter and the 5' end of the GFP; the final construct was transfected into PA-1 cells, and treated with geneticin (G418) to obtain stable transfected cells.

Here, again, GFP shows its advantage in the selection of positive clones and monitoring the expression of fusion protein. As a live marker, GFP operates independently of cofactors and can be detected rapidly and easily. GFP fusion protein provides a versatile tool for tracking, identifying and purification of rhZP3. In cell culture, phenol-red free medium was used, the expression of fusion protein can be identified and tracked by reading the culture medium by a fluorometer, when the reading is higher, the protein yield is correspondently higher. Since the protein secretion is a continuous process, so we could actually still visualize the green fluorescing cell under fluorescence microscope. If the cells do not grow well, and the UV reading from cell culture remains very low, the medium will not be applied to protein purification. So GFP tag also provide a quality control of our culture system to monitor and ensure proper cell growth. However, we still keep in mind that the impact of 27kD protein on ZP3 structure and function has not yet been addressed, this will have to wait until purity of GFP/ZP3 increases to more than 80%, and protein structural study can be performed.

DNA sequencing of the subcloning product gave confident data showing that there was no mutation during subcloning of ZP3 cDNA into pEGFP vector, and the signal peptide of ZP3 was correctly put right upstream to the GFP sequence. The expression of GFP/ZP3 has been successful, as derived from

the strong evidence from fluorescence miceoscopy photograph of cells expressing fusion protein GFP/ZP3, flow cytometry, RT-PCR and western blot using three antibodies, anti-GFP, anti-His, anti-ZP3. RT-PCR using two pairs of primers unequivocally demonstrates the mRNA expression of fusion protein, and the cDNA size inferred was correct, the size of cDNA for both ZP3 only and GFP/ZP3 were confirmed by this approach. Western blot using three antibodies gave a specific and strong band corresponding to molecular size of about 90kD, which is the size that adds those of ZP3 and GFP together. So the origin of translated protein was confirmed by the convincing western blot data. There is no doubt that we have obtained recombinant ZP3 with a GFP tag. This is fundamental and important for us because all the following experiments are based on this; in other words, an authentic in vitro recombinant ZP3 product is available for study in biological assay. We still face the problem to increase protein yield as well as purity. Since there is 5% of serum present in the culture medium, a serum free medium may be applicable in cell culture to decrease the co-purified protein. Meanwhile, cell growing in a large-scale apparatus such as bioreactor will eventually be set up to upgrade protein production. Cells will be grown in a suspension instead of monolayer on culture plate. The CMV promoter for increasing mammalian protein expression may be silenced or decreasing in efficiency with longer time of cell culture, this may be circumvented by replacing the CMV promoter by some other promoters that are more stable in promoter activity, the use of EF-2 promoter will be an option to fulfill this mission.

Furthermore, GFP appears to increase protein solubility and stability (Rucker, et al., 2001; Waldo et al., 1999). Solubility has been a common problem in rhZP3 expression and purification for several groups (Chapman and Barratt, 1997). So through the production of fusion protein GFP/ZP3, the solubility problem may be circumvented. For long-term preservation of purified GFP/ZP3, more work need to be done to address this issue. When the yield reaches a certain high level, the protein can be lyophilized; or if the protein has to be stored in solution, the addition of some oligosaccharide can be the alternative option.

The data from protein expression provides us firm basis for the further development of a sperm function test using rhZP3. Next, Sperm receptor activity and acrosome reaction inducing ability of GFP/ZP3 were examined.

The study on human zona-sperm binding are restricted to the application of solubilized zona or oocyte (Morales et al., 1994; Henkel et al., 1995; Bastiaan et al., 1999; Henkel et al., 2001), this is due to the unavailability of recombinant ZP proteins with closest characteristics to the native zona proteins. As a result, little is known about the ZP-mediated signal transduction in the human.

Flow cytometry is a method for quantitating components or structural features of cells primarily by optical means. Although it makes measurements on one cell at a time, it can process thousands of cells in a fluid stream passing through the path of laser beams in a few seconds. Since different cell types can be distinguished by quantitating structural features, flow cytometry can be used to count a large population of single cells of different types in a mixture. In our

study, we aimed to distinguish ZP3-bound and unbound sperm through the fluorescent tag linked to ZP3. So through flow cytometry, the basic two different populations of spermatozoa in the reaction system can be differentiated and the percentage of fluorescing sperm corresponding ZP3-binding sperm can be calculated. Under our experimental conditions, fusion protein GFP/ZP3 showed the activity to bind to human spermatozoa, the binding was demonstrated to be a time-dependent and protein dose dependent event, which fit in the biological phenomena. Furthermore, to prove whether the binding as detected by flow cytometry was the effect of ZP3, removal of GFP tag from fusion protein was conducted and the resultant sample was applied in the binding test, as it was expected, uncut fusion protein still showed about 50% sperm binding, but the GFP removed protein sample resulted in no sperm binding as analyzed by flow cytometry. Furthermore, different units of enterokinase were applied to digest fusion protein and the digestion efficiency was different, western blot was performed to check the digestion for reference in sperm binding test. When higher unit of enzyme was used, the enzyme completely cleaved off GFP from fusion protein, and the binding assay using this product gave negative result (no binding was observed); when less enzyme unit was used, only a portion of the fusion protein was digested, and accordingly, this digested fusion protein sample still resulted in human sperm binding but to a less level as compared with that of uncut fusion protein. Importantly, this experiment was repeatable. The consistent and reproducible binding test from protein enzyme digestion and its corresponding binding test clearly indicated that the binding of fusion protein with

human spermatozoa identified by flow cytometry was a true result reflecting the activity of ZP3, but not from the artificial effect from GFP. So it can be inferred from these data that ZP3 can detect the putative sperm receptor on sperm plasma membrane.

Flow cytometry can characterize the binding activity of ZP3 with human sperm, but in the practical point of view, this approach may not be very useful, but at least it can be used to prove our hypothesis that the recombinant GFP/ZP3 possess the biological binding activity to bind to human sperm. In order to give more direct evidence that identifying the binding between GFP/ZP3 and human sperm, immunofluorescence was conducted. Attempts to detect direct binding between sperm and ZP3 by fluorescence microscopy failed, this may be due to the fluorescence quenching during purification and the sensitivity of fluorescence microscopy was not good enough to capture the fluorescent signal on the sperm plasma membrane despite of the fact that fusion protein bind to the sperm; because we are still working very hard on protein purification, the purity and protein yield remains low, then another reason for undetectable fluorescence for binding can be the amount of the purified fusion protein was not high enough to give visible signal. By the time when protein purity and yield reaches a certain level, these problems will be automatically solved. Therefore, numerous trials were done to evaluate sperm binding by the amplification of fluorescence signal through fluorescent dye conjugated anti-GFP antibody, or by indirect immunofluorescence. Since GFP antibody is more versatile than ZP3, the GFP tag, again, gave us the privilege to use it as an epitope for sperm binding

detection. The data from immunofluorescense gave us exciting result that with the treatment of fusion protein, human sperm displayed fluorescence over the sperm acrosome region, but not tail or post-acrosomal region; on the contrary, sperm without ZP3 treatment showed no fluorescence over acrosomal region. In practically all cases, motile sperm bound to the head region as revealed by immunofluoscence microscopy. Spermatozoa bind to the zona pellucida via the head region where complementary receptors for ZP3 are specifically located (Moore, 1995; Burks et al., 1995). The similarity in binding between zona and recombinant ZP3 strongly support that rhZP3 from our lab is biologically active and resemble that of the native zona. The result from immunofluorescence is in concert with flow cytometry to prove the binding activity of GFP/ZP3. Sperm binding assay using immnobead is also underway, form the preliminary data (not included in this dissertation), human sperm were found to be able to bind to the immunobead crosslinked with fusion protein, more work need to be done to find out best binding condition and result interpretation. Whitmarsh et al. (1996) evaluated their rhZP3 from in vitro transcription and translation by coating bead with rhZP3, the biological activity was expressed by using the percentage beads bound by capacitated sperm, however, and no further report is available. Although the data from our lab is still very premature, this approach is very promising; it can be very applicable when the method is set up.

A series of studies over the years have demonstrated that in murine ZP3 a small number of unique polypeptide sequences which are heterogeneously O-glycosylated are responsible for sperm-ZP3 binding. It has been postulated that

recombinant proteins, which are glycosylated, may serve as better ligands for sperm receptor than non-glycosylated proteins. This may be due to the ability of glycosylated proteins to attain a more biologically appropriate conformation to complementary receptors on sperm plasma membrane. With the ability of recombinant proteins which lack carbohydrates to undergo successful binding to zona components (Zhu et al., 1997; Chapman et al., 1998) in vitro, as well as the ability of relevant transgenic/gene-ablated mice to undergo fertilization in vivo (Thall et al., 1995; Asano, et al., 1997), it is becoming apparent that, while carbohydrates may be involved in sperm-egg interactions, their absolute and essential role in primary sperm-egg binding still remains in question. Maybe the protein backbone of ZP3 has a more significant role to play in sperm binding and subsequent acrosome reaction in the human than the mouse. Despite the argument on the role of carbohydrates and protein backbone in ZP3 bioactivity, the fusion protein GFP/ZP3 has been proved to successfully expressed and contains the ZP3 polypeptide portion; and furthermore, the rhZP3 produced in our lab should be glycosylated, as the protein was expressed in mammalian cells, this can also be inferred from the molecular size of fusion protein (about 90kD), if the protein is not glycosylated, the size would be only 72kD, which indirectly indicate the fusion protein is glycosylated, however it is certain that further evidence from glycosylation study need to be obtained in future experiments, this will have to wait until when recombinant protein of high purity is ready. With both carbohydrates and protein backbone of ZP3 present in the

fusion protein product, the bioactivities characterized for the fusion protein in this study fit in well-documented bio-function of native ZP3.

The binding activity of recombinant zona proteins has also been demonstrated by some other groups, the N-terminal polypeptide portion of human ZP2 has been shown to contain a binding site for acrosome-reacted spermatozoa and to play an important role in secondary sperm binding and penetration into ZP (Tsubamoto et al., 1999); in bonnet monkey, purified recombinant ZPB from E coli. has been demonstrated to be able to bind to capacitated as well as acrosome-reacted spermatozoa (Covind et al., 2001). Interestingly, the proteins from these reports were from bacteria and thus non-glycosylated, whilst they still possess the activity to bind to sperm.

Recombinant fusion protein GFP/ZP3 can induce capacitated human sperm to undergo acrosome reaction. Induction of acrosome reaction was not observed when the rhZP3 was incubated with human spermatozoa for a short period of time (30 min). However, significant induction of acrosome reaction was observed with longer incubations (1 hr, up to overnight). At present, we can only speculate about these difference. The rhZP3 product, although able to initiate binding and an acrosome reaction, may not have been sufficiently close to the native molecule to induce a rapid acrosome reaction. Solubilized human zona pellucida could initiate high percentage of acrosome reaction (1ZP/ul) within 30 min incubation (Franken, 1996, 2000). Experience use rhZP3 from other laboratories produced by CHO cells to initiate acrosome reaction was different. High levels of acrosome reaction (50%) could occur with short incubation times

(30 min) (Barratt and Hornby, 1995; Brewis et al., 1996), while van Duin et al (1994) reported that overnight capacitation/or longer incubation periods with rhZP3 are necessary to achieve a significant response. Non-glycosylated rhZP3 from in-vitro coupled cell-free transcription-translation system lacking pancreatic microsomes (Whitmarsh et al., 1996) or in Escherichia coli (Champan et al., 1998) also induced the human sperm acrosome reaction, albeit at a considerably slower rate than its glycosylated homologue. To the fusion protein GFP/ZP3 presented here, study on the comparison of non-glycosylated form from Escherichia coli and glycosylated form from PA-1 cells would be interesting. Further experiments examining induction of sperm acrosome reaction with GFP/ZP3 are necessary, such as dose response and varying the capacitation condition.

CONCLUSIONS

1. Recombinant fusion protein GFP/ZP3 was successfully expressed in PA-1 cells as demonstrated by flow cytometry, fluorescence microscopy, RT-PCR and immunoblotting.
2. The molecular weight of the expressed fusion protein was 90~92 kD, digestion of this protein by enterokinase further confirmed the successful protein expression as ZP3 of 65kD was identified in immunoblotting.
3. GFP/ZP3 showed binding activity to human spermatozoa as analyzed by flow cytometry and fluorescence microscopy.
4. The binding of GFP/ZP3 to human spermatozoa was time- and dose-dependent.
5. When GFP was removed from fusion protein, the resultant product displayed no detectable binding in flow cytometry assay.
6. The protein was also able to induce human sperm acrosome reaction.

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