Identification and Functional Characterization of Prostate and Testis Expressed (*Pate*) Genes in the Rat

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By

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DECLARATION

I, Rajesh Angireddy, hereby declare that this thesis entitled "Identification and functional characterization of prostate and testis expressed (*Pate*) genes in the rat" submitted by me under the guidance and supervision of Dr. Suresh Yenugu is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "Identification and functional characterization of prostate and testis expressed (*Pate*) genes in the rat" is a record of bonafide work done by Mr. Rajesh Angireddy, a research scholar for Ph.D. programme in the Department of Animal Biology, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Dr. Suresh Yenugu
Supervisor

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CONTENTS

ABBREVIATIONS

AR	: Acrosome reacted
BSA	: Bovine serum albumin
CD	: Circular Dichroism
cDNA	: complementary DNA
CFU	: Colony forming unit
CRISP	: Cysteine rich secretory protein
CTC	: 5-Chlorotetra cyclin
CTC	: 5-cyano-2, 3-ditolyl tetrazolium chloride
DAPI	: 4,6-diamidino-2-phenylindole
DHT	: Dihydrotestosterone
$DioC_2(3)$	: 3,3′-Diethyloxacarbocyanine, iodide
DNA	: Deoxyribonucleic Acid
FITC	: Fluorescein isothiocyanate
Fluo-3, AM	: 1-[Amino-5- (2,7-dichloro-6-acetomethoxy-3-oxo-3*H*-xanthen-9-yl) phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-N,N,N',N'-tetra acetic acid
GAPDH	: Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	: Hydrogen peroxide
HE2	: Human epididymal protein2
HRP	: Horseradish peroxide
Ig-G	: Immunoglobulin G
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
KCl	: Potassium chloride
LB	: Luria broth
LPS	: Lipopolysaccharide
MP	: Membrane potential
Na_2HPO_4	: Disodium hydrogen phosphate
NaCl	: Sodium chloride
$NaHCO_3$	: Sodium bicarbonate
PATE	: Prostate and testis expressed
PBS	: Phosphate buffer saline
PBS-T	: Phosphate buffer saline with tween
PNA-FITC	: FITC conjugated Lectin from *Arachis hypogaea*
RNA	: Ribonucleic acid
rpm	: Revolutions per minute
RT-PCR	: Reverse transcription-Polymerase chain reaction
TO-PRO-3	: 3-methyl-2- (3-{1-[3-(trimethylammonio) propyl] quinolin-4 (1*H*)-ylidene}prop-1-en-1-yl)-1,3-benzothiazol-3-ium di iodide
TRITC	: Tetramethylrhodamine

ABSTRACT

Sperm maturation is a complex process that occurs in the epididymis and thought to be facilitated by a wide variety of proteins. Among them, the cysteine rich proteins that belong to different families, such as defensins, cystatins, cysteine rich secretory proteins, whey acidic proteins, etc. Recently a new class of cysteine rich proteins that belong to prostate and testis expressed (PATE) family were identified. The PATE proteins identified till date are thought to resemble the three fingered protein/urokinase-type plasminogen activator receptor proteins and are not well characterized in many species including rats. In this study, we report the identification and characterization of *Pate* genes in the rat. The rat *Pate* genes (*Pate-P, Pate-Q*, *Pate-F*, *Pate-A, Pate-C, Pate-E, Pate-N, Pate, Pate-Dj*, *Pate-B*) are clustered on chromosome 8 and their corresponding predicted proteins retained the ten cysteine signature characteristic to TFP/Ly-6 protein family. Though *Pate* gene expression is thought to be prostate and testis specific, we observed that the rat *Pate* genes are also expressed in seminal vesicle and epididymis and in tissues beyond the male reproductive tract. In the developing rats (20 – 60 day old), expression of *Pate* genes seem to be androgen dependent in the epididymis and testis. In the adult rats, androgen ablation resulted in down regulation of the *Pate* genes in the epididymides. PATE and PATE-F proteins were found to be expressed abundantly in the epididymis and on the sperm.

In this study, for the first time, we report that rearrangement of PATE and PATE-F proteins occur on the rat sperm head during capacitation. *In vitro* neutralization of PATE and PATE-F on the rat sperm using specific antiserum resulted in significant reduction of capacitation and acrosome reaction. To further understand the role of PATE and PATE-F proteins in sperm function, rats were immunized with these proteins to achieve neutralization of these proteins endogenously. In the immunized rats, a very high antibody titer was observed in the serum, epididymal and testicular fluids. Spermatozoa obtained from the PATE-F immunized rats failed to undergo capacitation and acrosome reaction, where as their counterparts obtained from PATE immunized animals displayed these properties. Concomitant with the loss of sperm function, PATE-F animals when mated with females produced reduced litter size. Basing on the results obtained using immunization strategies, the role of these proteins was further examined by knocking down the mRNA expression of *Pate* in the cauda. Spermatozoa obtained from these cauda failed to undergo capacitation and acrosome reaction. Further, infertility was observed in rats when *Pate* was knock down in cauda. These results suggest that PATE proteins may have role in sperm function and thus fertility. Besides role in sperm function, their contribution to immune function was analysed. PATE exhibited potent antibacterial activity and was inducible during LPS challenge both *in vitro* and *in vivo*. PATE-F on the other hand was not antibacterial. In conclusion, we report that PATE and PATE-F proteins in the rat contribute to sperm function innate immunity as well.

Technological advances have increased knowledge of gene expression profiles in reproductive system. However, mere gene expression profile is rarely enough to reveal the *in vivo* significance of a gene. Therefore, experiments to study individual genes and proteins in a living organism are needed. *Pate* genes are identified in humans and mouse and they have 10 conserved cysteines like TFP proteins. Though these genes were identified in the humans and mouse, their function in general and in the male reproductive tract is partially understood. The aim of the present study is to characterize rat *Pate* genes using *in silico*, *in vitro* and *in vivo* approaches. To accomplish this, present study was carried out with following objectives:

1. **Identification and *in vivo* characterization of rat *Pate* genes.**
2. **Functional importance of PATE proteins in the male reproductive system.**

INTRODUCTION

Male reproductive system

Male reproductive system is composed of testis, epididymis and accessory glands like seminal vesicles, prostate and cowpers gland. The main function of male reproductive system is to produce mature spermatozoa. Testis is located outside the body in a scrotal sac and produce male sex hormone testosterone, which is responsible for secondary sexual characters and also stimulates spermatogenesis. Spermatogenesis takes place in the seminiferous tubules of the testis and this process converts spermatogonial stem cells (diploid) to spermatozoa (haploid) by a number of meiotic divisions. Spermatozoa produced in the testis transported to epididymis where sperm maturation takes place. Male accessory glands secrete their fluids into the epididymis, which activates and nourishes sperm. Semen is composed sperm (1%), seminal fluid (70%), prostatic fluid (25%), and bulbourethral fluid (4%) (Mann *et al.,* 1957).

The epididymis

The mammalian spermatozoa are produced by extensive cellular differentiations, which takes place in the testis by a process called spermiogenesis. However, testicular spermatids are non-motile and infertile and require post gonadal maturation to be able to fertilize eggs (Dacheux *et al.,* 2003). Epididymis is an accessory organ of the male reproductive system and is responsible for post-testicular sperm maturation in mammals. It is located between the efferent ducts and the vas deferens, and is characterized by a single coiled tubule duct (Robaire *et al.,* 2001). Epididymis is divided into initial segment, caput, corpus and the cauda (Figure 1). Each region consists of a lumen and a polarized epithelium composed mostly of principal and basal cells (Dacheux *et al.,* 2005). The four regions of the epididymis are clearly differentiated in most mammals but their histology and ultra structure varies among different mammals (Smithwick *et al.,* 2001).

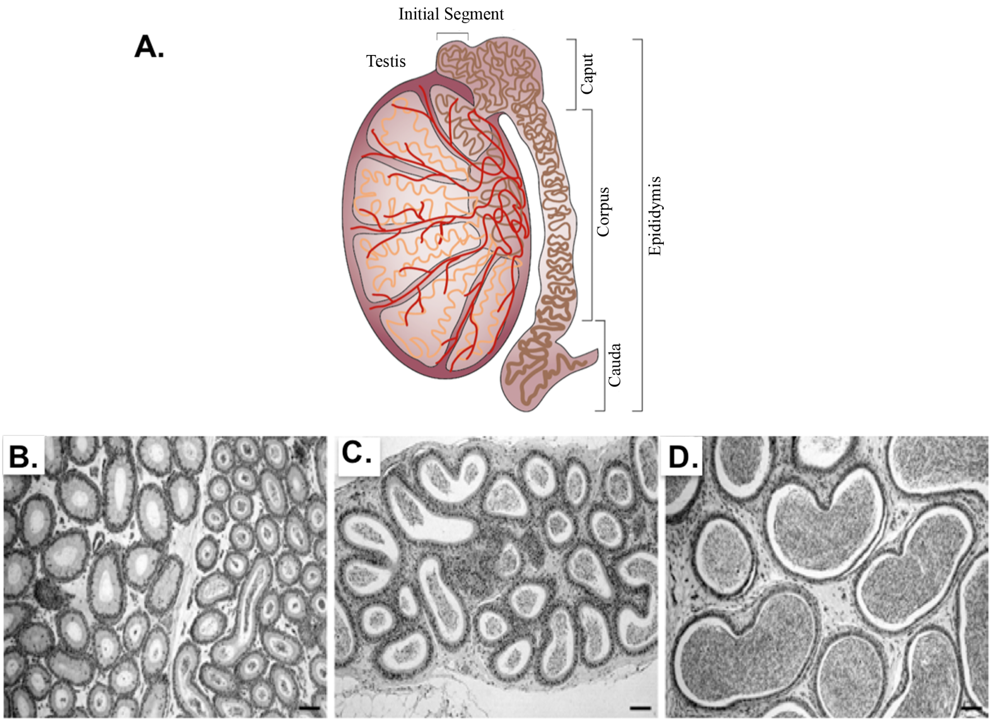

Figure 1. *Anatomy of the epididymis.* ***A.*** *Different regions of the epididymis* ***B.*** *Cross sections of the initial segment and the caput* ***C.*** *corpus* ***D.*** *cauda. Scale bar: 50µm. (Modified from Tollner et al., 2012; Robiare and Hinton, 2001(eBook)).*

Epididymal epithelium forms tight junctions with adjacent cells to store spermatozoa and create blood epididymal barrier. This barrier restricts the passage of ions, solutes and macromolecules to make luminal environment amiable for sperm maturation. Further, tight junctions protects spermatozoa from being exposed to outer environment because spermatozoa are immunogenic and contain proteins on their surfaces that would be recognized as non-self if they leave the epididymis (Robaire *et al.,* 1988, Dacheux *et al.,* 2005). The epididymis is composed of two types of epithelial cells namely the principal cells and the basal cells. Other cell types such as apical, narrow, clear and halo cells are also present in a segment specific manner (Figure 2). Principal cells are the most abundant and extensively studied cell type, which constitute approximately 80% of the total epithelial cell population in the initial segment and their percentage decreases in other parts of the epididymis (Robaire *et al.,* 1988). Principal cells secrete most of the proteins in the epididymal lumen and controls luminal protein concentration. These cells form tight junctions with adjacent principal cells and from blood-epididymis barrier (Robaire *et al.,* 2006, Cornwall, 2009). Basal cells constitute 15-20% of the total epididymal epithelium and are second largest population of epithelial cells (Robaire *et al.,* 2000, Cornwall, 2009). Although basal cells are extra tubular in origin, they play role in the processes of the epithelial immune system and in the regulation of electrolytes by principal cells (Robaire *et al.,* 2006). Apical cells produce enzymes of the carbonic anhydrase family and regulate pH of the lumen and sperm (Hermo *et al.,* 2005). Narrow cells protects spermatozoa from changing environment of harmful electrophiles by degrading specific proteins and carbohydrates in their lysosomes and also in the process of intracellular transport between lumen and epithelial cells (Adamali *et al.,* 1996, Robaire *et al.,* 2006). Clear cells are endocytic cells and they take up the contents of the cytoplasmic droplets released by the spermatozoa as they transit through the duct (Hermo *et al.,* 1994 and 2005).

All the different cell types of epididymis are involved in different functions such as protein secretion and absorption (principal cells), endocytosis (clear and apical cells), acidification of the luminal fluid (clear cells and narrow cells), immune defense and phagocytosis (halo cells) and the production of antioxidants (basal cells) (Robaire *et al.,* 2000, França *et al.,* 2005). These different cells perform different functions based on their location to modify epididymal secretions (Robaire *et al.,* 2000 and 2006). This epididymal regionalization in turn leads to diverse patterns of gene expression, which is critical to the formation and maintenance of the functions of the epididymis (Suzuki *et al.,* 2004).

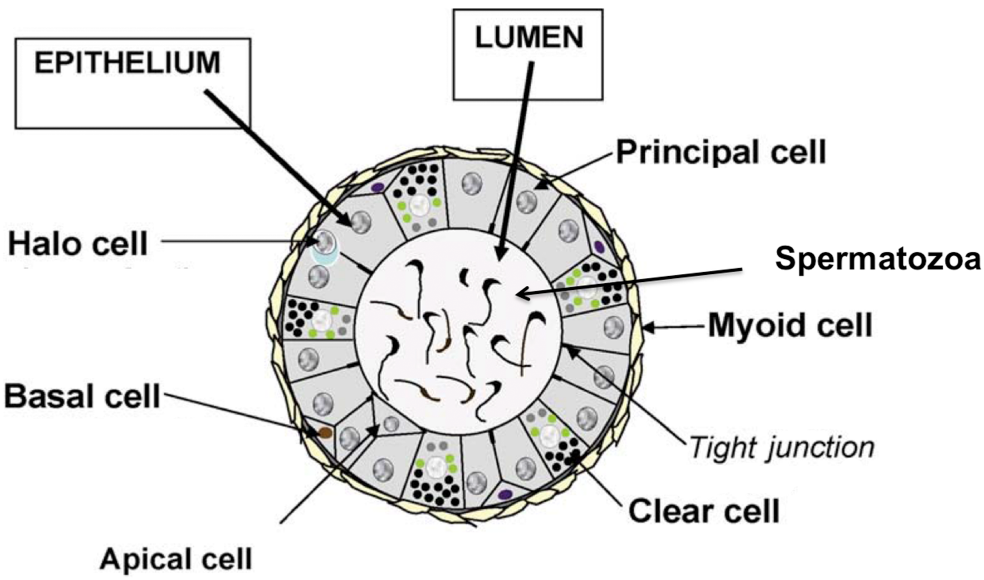

Figure 2. *Schematic representation of different cell types in epididymis as observed in light microscope. The different epididymal compartments as well as distribution of different cell types are illustrated. (Modified from Cornwall, 2009).*

Spermatids from the testis pass through the proximal caput epididymidis, where they interact with secreted protein to modify their membrane protein and lipid composition. Epididymal duct also protects the developing sperm from pathogens by secreting antimicrobial proteins (Hall *et al.,* 2002, Li *et al.,* 2001), and acts as storage for the mature sperm until ejaculation (Jones, 1999, Hinton *et al.,* 1996).

The Blood–Epididymis Barrier

Spermatozoa are immunogenic and they contain proteins on their surfaces that would be recognized as foreign if they leave the epididymis. Hence, a blood-epididymis is formed to protect sperm form leaving epididymis and seen as extension to blood-testis barrier (Cyr *et al.,* 2002, Robaire *et al.,* 2006). The junctional complex between adjacent epithelial cells composed of gap, adherens, and tight junctions. Tight junctions are formed between adjacent principal epithelial cells at their luminal surface to form the blood–epididymis barrier, whereas gap junctions allow communication between adjacent principal cells (Cyr *et al.,* 1995). Cadherins are involved in the formation and maintenance of tight junctions and their cytoplasmic domain forms tight complexes with several other proteins, which are involved in signal transduction or links cadherins to cytoskeleton (Gumbiner, 1996, Takeichi, 1990, Cyr *et al.,* 1992). Gap junctions mainly composed of connexins, and these proteins couple adjacent cells by allowing small molecules to pass from cytoplasm to cytoplasm (Soranzo, 1982). The blood–epididymis barrier maintains a specialized luminal microenvironment for the maturing spermatozoa by restricting the passage of a number of ions, solutes, and macromolecules across the epididymal epithelium (Hinton, 1985, Hinton, 1995). It also carefully controls the microenvironment so that the spermatozoa are bathed in an appropriate fluid milieu at each stage of maturation as they travel through each segment of the epididymis (Turner, 1991).

Epididymal lumen

The epididymal lumen is composed of complex mixture of water, inorganic ions, small organic molecules and proteins, which create hyperosmotic relative to serum (Turner, 2002), which is required for spermatozoa to acquire the capacity to regulate cell volume and protect sperm from osmotic shock caused by iso-osmotic secretions of the female tract (Cooper *et al.,* 2003, Cooper, 2007). Epididymal luminal fluid is result of secretory and absorptive functions of the epithelial cells, which line the lumen, and is characteristic to different epididymal regions. So luminal fluid composition is a balance between secretion and absorption, and reflects the function of different regions pertaining to maturation or storage of the spermatozoa.

The epididymis is a highly secretory organ and the epididymal lumen contains most complex fluid found in any exocrine gland due to continuous changes in composition as well as the presence of components in high concentrations (Dacheux and Dacheux, 2002). Approximately 99 % of testicular fluid that accompanies the spermatozoa is reabsorbed in the proximal caput epididymis (Mann *et al.,* 1981). Water absorption and secretion are secondary effects resulting from ion transportation mediated by various Na^+/H^+ exchangers on the apical surface of epithelial cells, which leads to exceptionally dehydrated luminal conditions (Wong *et al.,* 1978, Wong, 1988, Bagnis *et al.,* 2001). Several epididymal proteins have a highly conserved distribution of cysteine-residues, between which the structure stabilizing disulfide bridges are formed. Structural stability is needed in the dehydrated epididymal luminal fluid, which possibly explains the abundance of secreted cysteine-rich epididymal proteins (Cornwall *et al.,* 2007).

In addition to fluid transportation, ions, specifically bicarbonate ($HCO3^-$) and Na^+, affect the pH of the luminal fluid. Spermatozoa that are synthesized in seminiferous tubule with pH of 7.3 and has higher potassium concentration and lower sodium concentration then blood plasma (Hinton *et al.,* 1993), move to an environment in the initial segment of the caput which has pH 6.5 and sodium, potassium concentration similar to blood plasma. Spermatozoa then move into a progressively lower ionic environment from the caput to the cauda but a progressively higher organic solute and protein environment (Hinton *et al.,* 1995, Rodriguez *et al.,* 2003, Turner, 1991). The physiological importance of the ionic microenvironment is unclear, but this marginally acidic environment is necessary to render the spermatozoa in an immotile state during maturation and storage in the epididymis (Turner, 2002). The epididymal lumen has high osmolality compared to blood plasma, reaching about over 250 mOsm/kg water in cauda due to the presence of organic solutes (Levine *et al.,* 1971). Organic solutes in epididymal lumen include glutamate, taurine, L-carnitine, myoinositol, glycerylphosphorylcholine, phosphorylcholine, sialic acids, and many other amino acids present in different concentrations through the epididymal duct (Hinton *et al.,* 1995, Rodriguez *et al.,* 2003, Turner, 2002). Although role of each individual solutes is not known, evidences suggest that these solutes play critical role in protection of spermatozoa and epididymal epithelium from osmotic stress similar to seen in kidney (Yeung, C. H *et al.,* 2004).

It is widely accepted that epididymis is an androgen regulated tissue, and steroid hormones of the testis are the most abundant group found in epididymal lumen. Although androgens diffuse through vasculature, it was reported that concentrations of testosterone and dihydrotestosterone (DHT) are 100 fold higher in epididymal lumen compared to serum (Turner, 2002). Although androgens are present in higher concentrations, the physiological significance of luminal androgens are not well understood. Spermatozoa do not express androgen receptor, which implies that they may have role in tubular epithelium. Androgens in epididymal epithelium are complexed with luminal androgen binding protein (ABP) and bind to receptor on epithelial cell membrane to induce signaling cascade of events that are required for epithelial cell function (Gerard *et al.,* 1988). Estradiol is synthesized by the germ cells and also converted from androgens by cytochrome P450 aromatase in the epididymis. Estrogens are present in much lesser concentration than androgens (Ganjam *et al.,* 1976). Estrogen enhances sperm maturation in the epididymis (Meistrich *et al.,* 1975), and enhances the secretion of selected proteins in cauda epididymidis (Tony *et al.,* 1999). Additionally, the estrogen-receptor knock-out mouse shows mal development of the efferent ducts and epididymis (Hess *et al.,* 1997).

Glucose and other reducing sugars are found only in traces (White, 1973) and epididymal spermatozoa utilize glycerol to produce CO_2 (Cooper *et al.,* 1981). Different amino acid concentrations vary between amino acids, within the same region of the epididymis, and within amino acids, between different regions (Hinton, 1990). Many amino acids exist in luminal fluid in concentrations higher than in blood plasma. Glutamate and taurine are involved in the protection of sperm from oxidative stress (Fellman *et al.,* 1987). Other important components of the epididymal lumen include phospholipids and their substrates. Among lipids glycerylphosphorylcholine (GPC) concentration is 5-6 times higher in lumen compared to blood plasma, and speculated that it play important role in maturational developments in the sperm membrane (Turner, 2002).

A variety of proteins have been identified in the epididymal lumen (Hall *et al.,* 2002) and most of the epididymal proteins are expressed in a region specific fashion, which adds to the complexity of the epididymal proteome. Proteins that are secreted into the lumen are mostly derived from epididymal epithelial cells and those proteins originated from the testis are mostly reabsorbed by the epithelial cells in the proximal epididymis (Dacheux *et al.,* 2009). Epididymal proteins can be bound on spermatozoa, encased in small vesicles secreted by the epithelial cells known as epididymosomes or unassociated in the luminal fluid (Sullivan *et al.,* 2007). Major classes of secreted proteins include glycosidases, antioxidants, proteases, protease inhibitors, clusterin, cysteine-rich secretory proteins, and proteins that bind small hydrophobic molecules, lipids and iron (Dacheux *et al.,* 2002).

Epididymal lumen proteome and secretome

Spermatozoa take several days to weeks to travel through epididymal duct, depending on the species, and stored in the cauda and vas deferens till ejaculation. During their transit through epididymal duct the proportion of mature spermatozoa increases, but complete maturation takes place only when they reach the cauda epididymidis, where they acquire their fertilizing ability, which involves progressive motility, the ability to undergo capacitation and hyper activation, and the capacity to recognize and to bind to the zona pellucida of the oocyte (Turner, 2002). The milieu surrounding the spermatozoa in epididymal duct is a complex fluid that resulted from continuous and progressive changes in its composition throughout the duct and the presence of different components in unusually high concentrations, some of them are not found in blood plasma. This specificity is maintained not only by secretion and reabsorption of different cell of epididymal duct but also presence of blood-epididymis and blood-testis barrier. Recent advances in genomic, proteomic, and transcriptomic studies have reveled new information on functions and regionalization of the epididymis and shed some insights into the complexity of epididymal fluid. The different genes and proteins expressed by the epididymis have roles in sperm maturation and sperm protection (Guyonnet, 2011).

Sperm maturation in the epididymal duct is a result of sequential interactions of sperm with continuous change in fluid milieu along the duct, especially with the proteins secreted by epididymal epithelium (Dacheux *et al,* 2003, Robaire *et al,* 2006; Turner, 2008; Cornwall, 2009). The change in protein composition is a result of two opposite activities of the epithelium, namely protein reabsorption and degradation. Reabsorbitive activity is mostly seen in proximal caput, where fluid is mainly composed of testicular fluid and few epididymal proteins. For example, proteins from testis such as albumin, transferrin, and testicular clusterin, are rapidly reabsorbed in the efferent ducts, and almost all testicular proteins are removed from the fluid before sperm reaches to posterior part of the epididymis and this activity is variable among different species (Clulow *et al.,* 1994; Syntin *et al.,* 1996; Dacheux *et al.,* 2003, 2006 and 2009).

Analysis of protein secretory activity of epididymis by ^{35}S methionine labeling revealed that caput has more secretory activity compared to corpus and cauda (Brooks, 1987, Holland *et al.,* 1988). Although hundred of ^{35}S labeled proteins are visualized in two-dimensional gel electrophoresis only 10-20 major secretory proteins constitute 70% to 80% of this secretome (Dacheux *et al.,* 2009). Lactoferrin (LTF), Niemann-Pick disease type C2 (NPC2), cathepsin D, beta-N -acetyl-hexosaminidase (HEXB), preproprotein (CTSD), mannosidase, galactosidase, prostaglandin D2 synthase (PTGDS), glutathione peroxidase (GPX5), clusterin (CLU), epididymal retinoic acid–binding protein and cysteine-rich secretory proteins (such as CRISP1) are most abundant in epididymal secretome but their concentration and regionalization vary among different specie. After secretion, these proteins adsorbed on the membrane during maturation and play different roles, such as antioxidant, antibacterial, and antiviral activities (LTF) (Syntin *et al.,* 1996, Jenssen *et al.,* 2009).

Among different proteins secreted by epididymal epithelium, soluble proteins are the most extensively studied, because they constitute the bulk and readily available for proteomic and secretomic studies. Till date, proteins that are described in all mammalian epididymal fluid are considered as secretory proteins. Identification and characterization has been done for most easily detectable proteins and their number varies to 15 different species (Turner, 2002). Of all the proteins, ERABP (androgen-dependent protein B/C) and PGDS (Prostaglandin-D synthase) are proteins binding to small hydrophobic molecules specifically secreted in proximal caput, exists in different isoforms and binds to small hydrophobic molecules like retinoic acid and PGH2 (Newcomer *et al.,* 1990; Tanaka *et al.,* 1997). PEP, (phosphatidylethanolamine-binding protein) is a lipid binding protein, found in variety of species and has high binding affinity for phosphatidylethanolamine, which acts to remodel the spermatozoa membrane during maturation (Bernier *et al.,* 1986). Another lipid binding protein HE1, acts as cholesterol-binding protein and regulates the lipid composition of sperm membrane during maturation (Larsen *et al.,* 1997). Lactoferrin is a major iron binding protein found in corpus and cauda epididymis and it is one of the major sperm coating antigens of different species (Wichmann *et al.,* 1989). Mannosidase, p hexosaminidase, p galactosidase, a-glucosidase, p glucuronidase are glycosidases secreted by epididymal epithelium, are involved in the degradation of luminal and sperm surface glycoproteins (Turner, 2002). Glutathione peroxidase (GPX) is an anti oxidant enzyme secreted in proximal epididymis and GPX5 is an epididymal specific isoform (Hall *et al.,* 1998) bound to sperm acrosome and prevent sperm to undergo premature acrosome reaction in epididymis (Okamura *et al.,* 1997). Other antioxidant enzymes gamma glutamyl transpeptidase (GGT), Superoxide dismutase (E-SOD) and catalase are secreted in different regions of epididymis and protect sperm from oxidative damage (Turner, 2002).

Clusterin is a major protein secreted from epididymal epithelium and found in most species. It represents 25-30% of the total secretions and is the most intensively secreted protein in different mammals (Cheng *et al.,* 1988). Most of the secreted clusterins reabsorbed by the epithelium, except for one isoform secreted in the distal part of the epididymis that stays in the fluid and its function is unknown but overexpressed in numerous degenerative diseases, may represent a stress protein. Clusterins may play a role in cholesterol homeostasis and facilitate sperm maturation by plasma membrane remodeling (Turner, 2002). Other important soluble proteins like glycosylphosphatidylinositol-anchored protein CD52 (Kirchhoff, 1996), the prion protein (Gatti *et al,* 2002), the serine protease PRSS21 (Netzel- Arnett *et al.,* 2009) are secreted from epithelium and transported to spermatozoa. Recent studies indicated the presence of epididymosomes (for ‘‘epididymal exosomes’’) and micelle-like structures in the luminal fluid (Frenette *et al.,* 2002, Saez *et al.,* 2003). Epididymosomes transfer proteins from cell-to-spermatozoa (Frenette *et al.,* 2002, Oh *et al.,* 2009). On the other hand, micelle-like structures transfer of hydrophobic proteins from the fluid to the spermatozoa (Kirchhoff, 1998). Apart from soluble proteins, a large number of functionally important proteins are synthesized and secreted in a regionalized manner, which interact with spermatozoa, and facilitate sperm maturation. These include the CRISP family proteins, defensins and sperm associated antigens (Spag11), protease inhibitors (such as EPPIN, HongrES1, etc.), and number of unknown proteins (Cornwall, 2009, Caballero *et al.,* 2011).

Functions of the Epididymis

Important functions of epididymis are transport of spermatozoa through its ductal system, creation of specialized luminal environment to the spermatozoa to acquire motility and fertilizing ability (Robaire, 2006). Once spermatids are released into the lumen of seminiferous tubule, transport begins through efferent ducts to epididymis and this process was estimated by incorporating labeled isotopes into DNA of germ cells at spermatogonia stage and fallow the progression of labeled spermatozoa till cauda epididymis. These studies reveled that epididymal transit of spermatozoa takes approximately 10 days irrespective of the size of the animal, including humans (Orgebin-Crist, 1962). In efferent ducts movement of spermatozoa is possibly by the beat of ciliated cells in the duct (Crabo, 1965), where as in epididymis it is primarily due to rhythmic muscular contractions of the smooth muscle lining the epididymal tubule (Markkula *et al.,* 1979, Jaakkola *et al.,* 1982 and 1983). Movement of spermatozoa in epididymal duct is influenced by factors like testosterone, estrogen, prostaglandins, oxytocin or vasopressin and temperature (Meistrich *et al.,* 1975, Hib *et al.,* 1978, Hib, 1974, Jaakkola *et al.,* 1980).

Studies with rabbit, rat and rams indicated that spermatozoa first develop the ability to fertilize eggs and then further transit through the epididymis acquire the ability to produce complete litter of viable off springs (Nishikawa *et al.,* 1952, Fournier *et al.,* 1979). These observations further confirmed in mice that only 8% of oocytes fertilized by caput epididymal spermatozoa were capable of developing into blastocysts *in vitro*, compared with 48% of oocytes fertilized by cauda spermatozoa (Wazzan *et al.,* 1990). In a larger series of experiments with intra cytoplasmic sperm injection (ICSI) using testicular, epididymal, and ejaculated spermatozoa, testicular spermatozoa showed statistically significant lower fertilization rates (53.4%) than epididymal (58.5%) or ejaculated spermatozoa (64.8%), but even statistically lower yields of good-quality embryos (47%, 40%, and 59%, respectively) (Steirteghem *et al.,* 1998). All these clinical studies indicate that, mammalian sperm acquire optimal fertility *in vivo* during epididymal transit, and by passing any of the critical steps leads to fertilization with immature spermatozoa.

Along with fertilizing ability, other important features of epididymal maturation include acquiring for motility, and this involves quantitative increase in the percentage of motile spermatozoa and a qualitative difference in motility pattern (Bernard Robaire, 2006). Testicular spermatozoa are either immotile or display only a faint twitch of the flagellum, and caput sperm swim in a circular pattern, whereas spermatozoa released from the cauda move progressively and vigorously forward. Apart form capacity for progressive motility, epididymal spermatozoa develop the capacity to undergo the acrosome reaction, recognize and bind to the zona pellucida, and fuse with the vitelline membrane of egg (Lakoski *et al.,* 1988, Yeung *et al.,* 1997). Along with functional changes, epididymal sperm undergo structural changes as well, which include migration of the cytoplasmic droplet along the sperm flagellum, changes in the sperm plasma membrane, acrosomal reshaping and changes in the sperm nuclear chromatin (Bedford 1973, 2004).

Another important function of epididymis is storage of spermatozoa, which takes place in cauda epididymis. Although epididymal transit of spermatozoa is approximately 10 days, spermatozoa can be stored in cauda beyond 30 days (Orgebin-Crist *et al.,* 1975) and this extended storage leads to loss in fertilizing ability followed by loss in sperm motility (Depeiges *et al.,* 1985). Number of studies on different mammalian species reveled that that 50% to 80% of spermatozoa present in the excurrent ducts were found in the cauda epididymidis and that approximately 50% of these spermatozoa were available for ejaculation (Amann, 1981). Apart from live spermatozoa, abnormal-appearing or dead spermatozoa often appear in cauda (Weissenberg *et al.,* 1995), and the ability to recognize such spermatozoa and then develop a mechanism to neutralize or destroy them has been proposed recently. In this regard different mechanisms has been proposed. Epididymal principal cells identify and phgocytose defective cells by sperm cell-surface ubiquitination and provide sperm quality control (Sutovsky *et al.,* 2001). Fibrinogen-like protein (fgl2) specifically produced in cauda and coats nonviable luminal sperm and activates specific mechanism, which envelops defective spermatozoa with protein complex containing fgl2 (Olson *et al.,* 2004). Both of these novel mechanisms require epithelial cells to recognize and tag defective spermatozoa need to be further validated to show that this is normal function of cauda epididymis. Jones hypothesized that androgen withdrawal, either by orchidectomy or as a result of seasonal variation in seasonal breeders, activates a death pathway that leads to dissolution of spermatozoa so that cauda epididymis is cleansed of defective and dead spermatozoa in preparation for the next breeding period. But how this androgen withdrawal activates death pathway remains to be validated (Ezer *et al.,* 2003).

Blood-epididymis barrier creates specialized luminal fluid microenvironment that is important for sperm maturation, and this barrier also protects maturing spermatozoa. Different mechanisms are developed in the epididymis to protect sperm from immune system, harmful xenobiotics and reactive oxygen species. These defense mechanisms include restricting the of entry of different types of compounds into lumen, synthesis and secretion of specific proteins like defensins and defensin like molecules, elimination of potential harmful components by synthesis and secretion of antioxidant and conjugating enzymes and synthesis and secretion of antioxidant compounds such as taurine and glutathione (Bernard Robaire, 2006). The epididymal components, which protect sperm, have been considered for the development of male contraception (Vernet *et al.,* 2004).

Sperm capacitation

Sperm capacitation generally refers to acquisition of fertilizing potential (Jaiswal *et al.,* 2002), and it also confers the ability of sperm to undergo acrosome reaction, which is further linked to sperm chemo taxis and hyperactive motility. In an ejaculate, at any given moment, only a portion of sperm will be capacitated and later become decapacitated (Cohen-Dayag *et al.,* 1994). The ratio between capacitated and decapacitated states are controlled by different capacitating and decapacitating factors because premature or over extended state of capacitation will deplete the energy reserves of the spermatozoon before fertilization and effect sperm fertilizing ability. Recent studies indicated few decapacitation factors like phosphatidylethanolamine binding protein 1 [PBP; also known as Raf kinase inhibitor protein-1 (RKIP-1) (Nixon *et al.,* 2006, Moffit *et al.,* 2007), HongrES1 (Ni *et al.,* 2009) and SERPINE2 (Lu *et al.,* 2011).

The physiological and functional changes upon capacitation in spermatozoa are well characterized (Figure 3). The requirements for capacitation vary between species but commonly include ionic concentration, pH, source of energy, and removal of de-capacitation factors (Jaiswal *et al.,* 2002). Bicarbonate and calcium are considered as the most important ions in capacitation, of which bicarbonate is key regulator of capacitation. Female reproductive tract fluids contain abundant bicarbonate, which is further linked to changes in sperm membrane, protein phosphorylation, and activation of cMAP signaling pathways (Bailey, 2010). During capacitation, spermatozoa undergo a major reorganization of their cell membrane's lipid and protein constitution. As the capacitation progress, sperm membrane cholesterol decreases which resulted in increased fluidity of the membrane, which enables further movements of membrane phospholipids and proteins (Cross, 1998). Subsequently, this grants the spermatozoa the ability to interact with the egg and enables the fusion of the plasma and outer acrosomal membranes during acrosome reaction (Cross, 1998, Jaiswal *et al.,* 2002). Other intracellular changes associated with capacitation includes, changes in ionic concentrations, extensive protein phosphorylation. However, several phosphorylated proteins have been identified and only a few have been elucidated for their roles in fertility (Urner *et al.,* 2003).

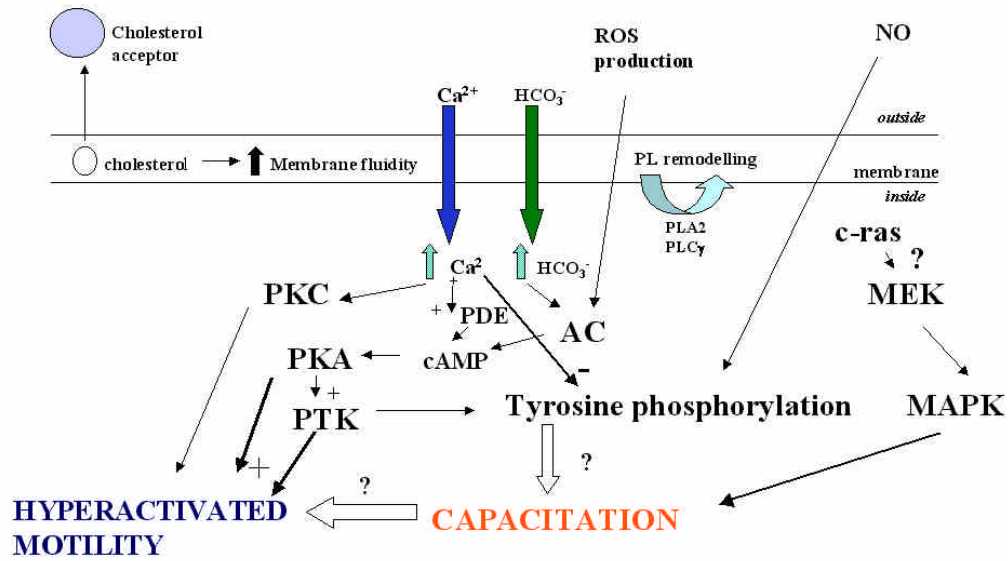

Figure 3. *Schematic representation of the main capacitation events, that leads to sperm hyperactive motility. (Baldi et al., 2000).*

The Acrosome Reaction (AR)

Acrosome reaction is an exocytotic process induced by the ligand, ZP3-receptor. Fusion between outer acrosomal membrane and the overlaying plasmamembrane of the egg, leads to the release of acrosomal enzymes and exposure of the molecules present on the inner acrosomal membrane surface, which facilitates fusion with oolemma. The sperm acrosomal membrane is derived from Golgi complex, consisits of an anterior cap and a posterior equatorial segment (Yanagimachi, 1985). Capacitation and acrosome reaction are sequentially and functionally linked such that only capacitated sperm are able to undergo AR. For instance, increase in intracellular calcium levels and tyrosine phosphorylations are essential for subsequent exocytosis in response to progesterone (Baldi *et al.,* 1991, Aitken *et al.,* 1998). Self aggregation of the sperm receptors for ZP may account for this spontaneous acrosome reaction (Saling, 1989) and wide variety of molecules present on sperm surface have been proposed as putative candidate for ZP3 receptor (Wassarman, 1999).

Many molecular mechanisms have been demonstrated to be active during AR, of which calcium plays a central role in receptor-mediated response and membrane fusion processes in spermatozoa (Yanagimachi, 1985, Wassarman, 1999). Both ZP3 and progesterone induced AR induces to increase intracellular Ca^{2+}, which is then associated with efflux of H^+ and a rise in intracellular pH (Florman *et al.,* 1989). This increase in intracellular calcium activates phospholipase C and phospholipase A2 (Roldan, 1998). Activation of these lipases leads to generation of lipid metabolites, such as arachidonic acid and lysophospholipids, which play important role in membrane fusion process that occurs during AR (Figure 4) (Baldi *et al.,* 1991).

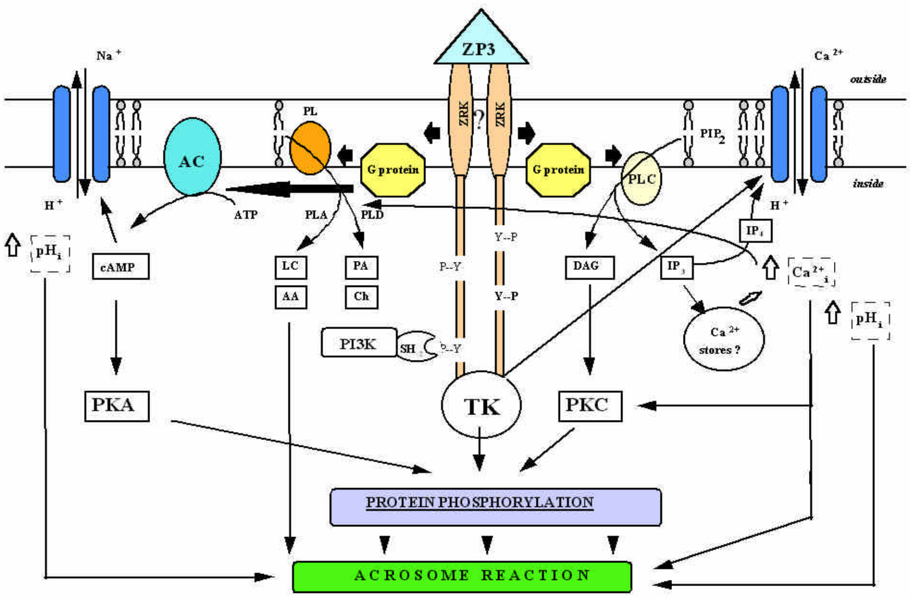

Figure 4. *Diagram illustrating the signal transduction pathways activated during the process of acrosome reaction induced by zona protein 3 (ZP3). (Baldi et al., 2000).*

Cysteine rich secretory proteins of the epididymis

Epididymis is highly secretory organ and secretes number of proteins but identification and characterization is done on the most easily detectable components. So total number of proteins secreted by the epididymis vary according to author and species (Dacheux *et al.,* 1998). All the species studied so far, the main characteristic of epididymal secretion is that some proteins are actively secreted and represent high proportion of the total secretion of the organ. The most abundant proteins in the epididymal luminal fluid are albumin, lactoferrin, PGDS, GPX, HE1/CTP, hexosaminidase and the secreted proteins. For example in boar and ram, 70% of the total epididymal secretion is linked to clusterin, Lf, GPX, and PGDS. Where as in rat cysteine-rich secretory protein (CRISP), Clusterin and retinoic acid- binding protein (E-RAPB) are the most abundantly synthesized and secreted (Turner *et al.,* 2000). Clusterin appears to be one of the most actively secreted proteins of the epididymis in different species, whereas the proportions of the other proteins seem to be species-specific.

Another important feature of epididymis is its region specific function owing to its region-specific gene expression (Daucheux *et al.,* 2003, Jalkanen *et al.,* 2006). Segment specific expression is observed for proteases, protease inhibitors, proteins with a disintegrin and metallopeptidase domain (ADAMs), defensins and cysteine-rich secretory proteins (CRISPs) (Cornwall *et al.,* 1997, Jalkanen *et al.,* 2005). Region specific expression of these proteins is responsible for creating region-specific luminal environment essential for epididymal sperm maturation. Epididymal cysteine-rich secretory proteins (CRISPs) have also been identified in venoms of various reptiles (Gibbs *et al.,* 2007). These proteins are characterized by presence of highly conserved cysteins with their specific disulphide-bonding pattern and are responsible for maintaining the structure (Bastolla *et al.,* 2007, Kaplan *et al.,* 2007). Based on their predicted structural properties these CRISPs may play a role in protecting epididymal lumen from micro-organisms by non-specific defense mechanism or maturation of spermatozoa by regulating certain ion channels, but their exact mechanism of actions are poorly understand. Recently, two research groups have independently identified cysteine rich secretory proteins with TFP/Ly- 6/uPAR domain, and named them Anlp (a neurotoxin-like protein) and *Pate* (prostrate and testis expressed) (Kaplan *et al.,* 2007, Levitin *et al.,* 2008). The TFP/Ly-6/uPAR domain of PATE proteins has 8-10 highly conserved cysteines, but their distribution is different from that of cysteine-rich domain of CRISPs (Ploug *et al.,* 1994).

Prostrate and testis expressed proteins (PATE)

To identify tissue specific target antigens for prostate cancer therapy, computer based screening was performed in human EST database and genes that are expressed specifically in prostate cancer, normal prostate, and testis were identified and hence named as *prostate and testis expressed* (*Pate*) (Bera *et al.,* 2002). Later RT-PCR analysis demonstrated that *Pate* genes are also expressed in epididymis and seminal vesicles (Soler-Garcıa *et al.,* 2005). Pattern search techniques revealed presence of human *Pate* gene locus within a genomic segment on chromosome 11q24 between *Acrv1* and *Ddx25* genes (Figure 6.A). Similar locus was also located in mice on chromosome 9qA4 with 0.8 Mbp of genomic DNA situated between the mouse *Acrv1* and *Ddx25* genes (Figure 6.B). The mouse genome had additional seven transcriptionally active *Pate* -like genes, designated *Pate-E, Pate-H, Pate-N, Pate-F, Pate-P, Pate-Q, and Pate-G*. All human and mouse *Pate* genes code for putative PATE-like proteins comprising a hydrophobic N-terminal signal peptide. Comparison of genomic and cDNA sequences revealed that in all PATE proteins, the N-terminal signal peptide is encoded by the first exon (exon 1), whereas protein domains containing cysteines 1–5 and cysteines 6–10 are encoded by two separate exons (exons 2 and 3, respectively) (Figure 6.C) (Levitin *et al.,* 2008).

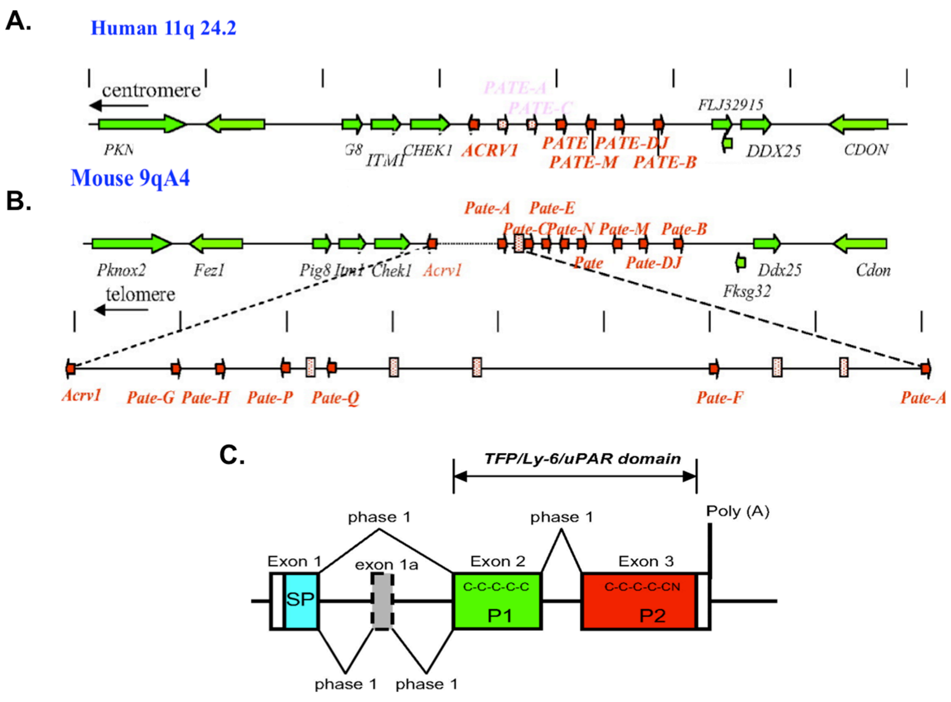

Figure 6. *Chromosomal location and exon structure of human and mouse Pate genes.* ***A.*** *Human Pate*

genes located within a genomic segment on chromosome 11q24 initiating at the centromeric side between Acrv1 and Ddx25. Arrows indicate direction of transcription. ***B.*** *Syntenic mouse Pate gene locus is on chromosome 9qA4 with 0.8-Mbp insertion between Acrv1 and Pate-A.* ***C.*** *The canonical exon structure of the PATE (Pate)-like genes in human and mouse. Optional extra exons are represented as dotted boxes. (Livitin et al., 2008).*

PATE proteins contains highly conserved 10 cysteine residues, with the C-terminal cysteine followed by asparagine (CN dipeptide) and forms two motifs namely C [XX]C[X7-8]C[X6]C[X7-8]C and C[X3]C[X15-16]CC[X4-5] CN, where X stands for any amino acid. The distribution of cysteine residues in PATE is also found in large family of three fingered proteins (TFPs), characterized by presence of specific disulphide bonding between 8 and 10 cysteine residues (Fry *et al.,* 2003, Ploug M 1994). This TFP architecture is also seen in uPAR and murine Ly-6 GPI-anchored proteins, so collectively called this domain as TFP/Ly-6/uPAR domain (Ploug, 1994). Similarly to CRISPs, the TFP/Ly-6/uPAR domain is also identified in different snake toxins (Figure 7) (Low *et al.,* 1976), but these toxins had no known enzymatic activity but binds to a wide range of cell surface receptors, ion channels and has antibacterial functions (Menez, 1998, Galat *et al.,* 2008). Interestingly, this TFP architecture is seen in transforming growth factor receptor family of proteins like BMP2, actvin receptors and extensive group of GPI-anchored, transmembrane, and secreted proteins also contains this domain (Figure 7) (Ploug *et al.,* 2003, Tsetlin *et al.,* 2004, Tremeav *et al.,* 1995, Kolbe *et al.,* 1993).

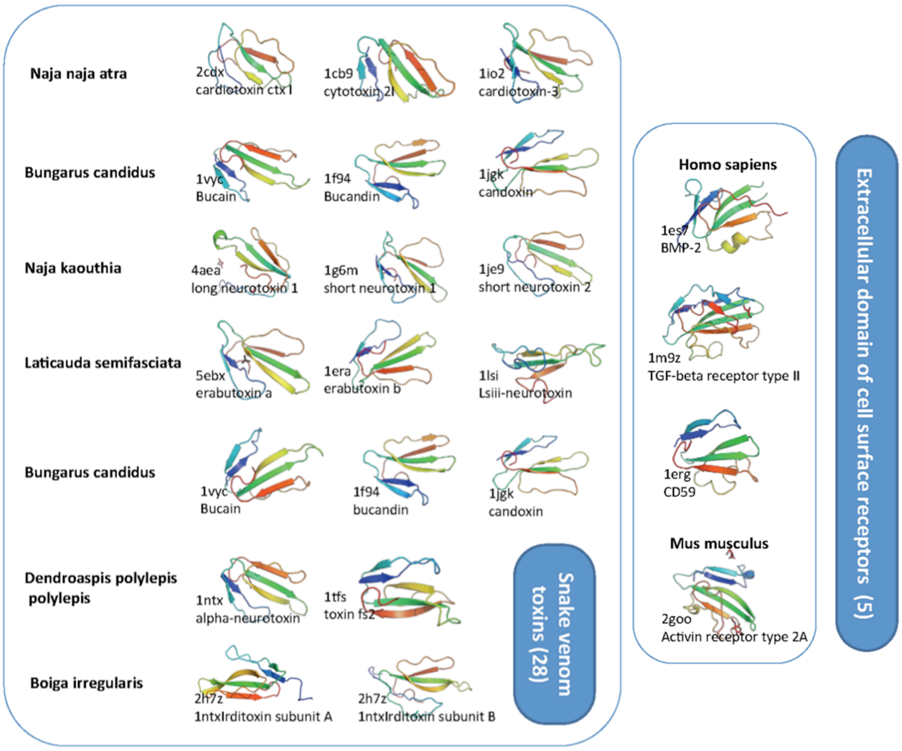

Figure 7. *Three fingered protein structure in different snake venom toxins and extracellular domain of cell surface receptors. Examples are shown with Protein Data Bank (PDB) ID. (Taken and modified from Tirosh et al., 2013).*

The only identified mammalian secretory TFP/Ly-6/uPAR proteins are SLURP-1 (secreted mammalian Ly-6/uPAR-related protein) (Adermann *et al.,* 1999) and SLURP-2 (Tsuji *et al.,* 2003), which are located on human chromosome 8q24.3 along with human Ly-6 like genes that code for GPI-anchored proteins. SLURP-1 regulates the calcium homeostasis by modulating α-7 nicotinic receptor (α-7 nAChR) (Chimienti *et al.,* 2003). Members of the secreted TFP/Ly-6/uPAR protein family, to which the PATE protein belongs, interact with membrane receptors. The human PATE gene is telomerically juxtaposed to the gene encoding acrosomal vesicle protein 1 (ACRV1), also known as the SP10 gene, also contains 10 cysteine residues like PATE, play a role in sperm binding and penetration to the oolemma (Freemerman *et al.,* 1995, Hamatani *et al.,* 2000).

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INTRODUCTION

Genome sequences as such do not provide straight away answers about the coding regions of proteins, regulating elements and the structural domains. With the advent of technology in the last two decades high-throughput sequencing data facilitated mapping of diverse genomes. The rat genomic sequence was first published in 2004 (Gibbs *et al.*, 2004). The strategy was a combination of whole genome shotgun sequencing and the bacterial artificial chromosome hierarchical approach. The rat strain used for the sequencing was the BN/ SsNHsd/Mcw (Brown Norway) and sequencing covered 90% of the genome. Once the genome is sequenced and the parts listed, next goal is to understand the function of each gene and the corresponding encoded protein (Gibbs *et al.,* 2004). Gene structures are predicted using homology search with the combination of *ab initio* modeling. Further, a broad spectrum of genetic, biochemical and computational approaches are employed for annotating the physiological function of hypothetical proteins encoded by predicted genes (Lubec *et al.,* 2005). It is very important to characterize hypothetical proteins whose information is available only in the form of primary sequence (Bhattacharjee *et al*. 2008, Berman *et al.,* 2000).

Developments in genomics and proteomics led to the identification of novel proteins in the male reproductive tract, thus allowing to understand the complex biological process involved in reproduction (Guyonnet *et al.,* 2011). Further, using various methods ranging from conventional protein separation and purification to monoclonal antibody generation and cDNA cloning, a considerable amount of information has been obtained on mammalian epididymal proteins. In general the predicted proteins are localized in the epididymal epithelium, epididymal lumen, vas deferens and on the surface of ejaculated spermatozoa (Kirchhoff, 1998).

In an attempt to identify tissue specific target antigens for prostate cancer therapy, expression analysis was used to identify genes that are expressed specifically in normal prostate and prostate cancer (Bera *et al.,* 2002). A human gene that transcribes into a 1.5 kb transcript, which in turn codes for a 14 kDa protein was named as *Pate* (expressed in prostate and testis). Similarly, in the mouse, 14 active *Pate*-like genes were identified and found to be expressed in epididymis and testis (Levitin *et al*. 2008).

However, the homologues of *Pate* genes in the rat are not identified. Though these genes were identified in the humans and mouse, their function in general and in the male reproductive tract is partially understood. The aim of the present study is to characterize rat *Pate* genes using *in silico*, *in vitro* and *in vivo* approaches.

METHODS

Sequence analysis

Mouse *Pate* genes containing the conserved 11 cysteine sequence [CX (2)CX (5,10)CX (3,8)CX (4,9)C and CX (2,4)CX (11,20)CCX (2,7)C, where X represents any amino acid] (Levitin *et al.,* 2008) were extracted from NCBI mouse EST database and blasted against rat genome (RGSC v3.4) and the blast parameters were assigned in such a way that least homologue DNA sequence in the rat was highlighted. The sequences were carefully observed for different exons and aligned using GU-AG rule between intron/exon boundaries. Once all the introns were aligned properly, initiation and termination codons were identified to deduce complete PATE protein sequence with the 11 cysteine signature. Primers were designed to amplify complete *Pate* coding sequence. The rat *Pate* genes were amplified using cDNA obtained from different tissues. All rat PATE cDNAs were directly sequenced from gel-purified RT-PCR DNAs. Corresponding exon/intron boundaries for different *Pate* mRNA were determined by aligning the cDNA with the genomic DNA sequence. The sequenced sequences were deposited in GenBank.

The sequenced sequences were translated *in silico* and the predicted physical features of the deduced amino acid sequences were analyzed using tools available at ExPASy proteomics server (http://ca.expasy.org/). All the PATE proteins were aligned using multiple sequence alignment tool to identify 11 conserved cysteines. Percent homology between different PATE proteins was calculated using CLASTALW (https://www.ebi.ac.uk/Tools/msa/clustalw2). Signal peptides were identified using SignalP (signal peptide prediction) algorithm (http://www.cbs.dtu.dk/services/SignalP). Phylogenetic analysis was done using Expasy phylogenetic tools (http://www.expasy.org/phylogeny_evolution).

Homology modeling

To generate the possible three-dimensional structures of PATE proteins, the protein sequences were submitted to BLAST against PDB database to search for homologous proteins. In the absence of any homologs to PATE proteins in the existing database even with the identity of TWILIGHT region, the sequences were submitted to FUGUE threading server (http://tardis.nibio.go.jp/fugue/) (Shi *et al.,* 2001). The parameters are set to generate the five best models and the best model was selected based on best Z score value obtained. This model was taken as the template to generate the final structure of PATE proteins using modeler 9.10. Among the 100 models with different energy constituents generated, the models with lowest energy constituents were selected. ModLoop database was used to address the amino acid residues that fall in the disallowed regions based on the Ramachandran plot values (https://modbase.compbio.ucsf.edu/modloop/) (Fiser *et al.,* 2000). The obtained structure was energy minimized in the Gromos Force field by Gromacs software (http://www.gromacs.org/) (Chris *et al.,* 2004).

Gene expression analysis

All procedures involving animals were approved by the institutional animal care and use committee of University of Hyderabad. All the animals used in the study were obtained from National Institute of Nutrition, Hyderabad, India. After asphyxiation using CO_2, tissues were immediately removed from adult male rat, and frozen in liquid nitrogen. Total RNA was isolated from caput, corpus, cauda, testis, prostate and seminal vesicles, non-reproductive tissues (brain, liver, lung, kidney, heart and spleen) and female reproductive tract tissues (cervix, ovary and uterus) using Trizol reagent. cDNA was synthesized using 2 μg of total RNA in the presence of MuMlv reverse transcriptase, and oligo dT primers, in a final reaction volume of 20 μl, at 42°C, for 30 min. One microliter of cDNA generated was used for PCR amplification of *Pate* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) with gene specific primers using Taq DNA polymerase (Table 1.1). Amplification was performed with a 2 min start at 94ºC, denaturation at 94°C for 30 sec, annealing at 56 to 60°C for 30 sec and extension at 72°C for 30 sec for 30 cycles, followed by a final extension at 72°C for 5 min. PCR amplicons were then analyzed on 2% agarose gels.

Gene expression in the male reproductive tract is tightly regulated by hormones, especially testosterone, whose levels are known to vary during development. To determine expression profile of *Pate* genes during development, epididymis and testes were collected from 20 to 60 day old male wistar rats. RNA was isolated and *Pate* genes expression assayed by RT-PCR. To study the direct effect of androgens in adult rats, epididymides were obtained from sham operated, castrated and castrated and dihydro testosterone supplemented rats. Total RNA was isolated and *Pate* genes expression analysed by RT-PCR.

Table 1.1. *Gene specific primers used to amplify different Pate genes.*

Gene Name	Forward primer (5' to 3')	Reverse Primer (5' to 3')
Pate-P	ATGGGAAAGGACATCTTGCTGCTC	TCACGGATATGGTTTATTGCAG
Pate-Q	ATGGAAAAGCACATCTTGCTGCTCC	TCAGGTATGTGCTTTATTGCAG
Pate-F	AGACTGAGATGGGCAAGCT	ACCTCTTGCATTGAAAAGATAG
Pate-A	GAGTCTGCAACCTGCTCTCATCTA	ATGTTGGGGTACAGCCAGA
Pate-C	ATGGAAAACCTACTGAAGCTGTGCC	CTAAGTCATTACGATTGGAAAACCG
Pate-E	ACCTGCTGCAAGAATTAGAAGATCC	AAGATGAGGAAGACTGGAGG
Pate-N	ATGGACTGGCTCCTGTTTCTTTTG	TTAGACAAAATTATTTTCTTTGAAGTG
Pate	ATGTTCAAGCCCCACTTACTGAG	CTAGAAAAAGAATGCTTTCCCTGTGAC
Pate-2	ATGCTGGTGATAGTCTGTTTGTTCTGCC	CTAGAGTCCCATTGGGAGGTT
Pate-Dj	ATGAACAGGCACTTCTTGCTGCTC	TTAAAGTCTGAAGTTACAAAAATC
Pate-B	CAAGCCACCTTCTAACATCCA	GCTTAAAACAACACGCAT

Cloning and recombinant protein production

Open reading frame of *Pate* genes without the signal sequence was amplified and cloned into pQE80 vector using standard restriction sites. Details of the *Pate* genes cloned and the restriction sites used are given in table 1.2. Constructed plasmids were transformed into BL21 competent cells and grown at 37˚C overnight on LB ampicillin plate. Colonies were collected and inoculated into 50 ml liquid LB and grown at 37˚C overnight in a shaking incubator. From the overnight culture, 10 ml was transferred to 500 ml LB medium and the bacteria grown to mid-log phase (OD A_{600}=0.5) and added IPTG (1 mM final concentration) to induce protein expression. After 3 h incubation at 37˚C, cells were harvested and protein isolated by Ni-NTA affinity column chromatography as per the manufacture instructions.

Table 1.2. *Details of cloning.*

Gene name	Region of ORF cloned	Restriction sites attached	Insert size
Pate	CDS with out signal peptide	Sal1 (GTCGAC), HindIII (AAGCTT)	302
Pate-F	CDS with out signal peptide	BamH1(GGATCC), HindIII(AAGCTT)	274

Generation of polyclonal antibodies

Recombinant proteins were further purified by electroelution to obtain maximum purity. For immunizations, 500µg of protein mixed with equal volume of Freund's complete adjuvant was injected intraperitonially to the rabbit. Booster doses were given at 20 day intervals over a period of three months with 200 µg of the same antigen using Freund's incomplete adjuvant. Blood was collected one week after each injection to check for antibody titer. Antiserum generated was used to detect recombinant protein by Western blotting to confirm the specificity of the antibody. Antiserum obtained at the third booster dose was found to have high titer and the same was used for further studies.

Immunolocalization

For immunofluorescent staining, epididymides and testes were fixed in 4% paraformaldehyde and Bouin's fluid respectively and embedded in paraffin. Five micron thick sections were taken and treated with xylene and graded alcohol (70–100%). The sections were subjected to antigen retrieval in citrate buffer (pH 6.0). PATE and PATE-F were detected by incubating the sections using polyclonal antibodies (1:250 dilution) raised in rabbit followed by TRIC (for PATE) or FITC (for PATE-F) conjugated secondary antibody (1:500 dilution) against rabbit IgG raised in goat. Sections were counter-stained with DAPI.

For immunostaining of the sperm, adult rat epididymides were dissected out and the spermatozoa were air dried and fixed on glass sides using methanol. Immunofluorescence on the sperm was detected by using PATE or PATE-F antiserum and anti-rabbit secondary antibodies tagged with FITC. Counter staining was done using DAPI. Photographs were taken using a color digital imaging system attached to a Leica Photomicroscope.

Circular dichroism

To understand the secondary structure of PATE proteins, recombinant proteins were purified and CD spectra recorded on a AVIV Circular Dichroism Spectrometer. The results were expressed as molar ellipticity ([Θ]; $deg \cdot cm^{-2}.dmol^{-1}$), with the mean amino acid residue mass (MRW) of 117.5 for rPATE and 114.95 for rPATE-F. The molar ellipticity was determined as $[\Theta] = (100\Theta \times MRW) / (C \times L)$, where C is the protein concentration in mg/ml, L is the light path length in cm and Θ is the measured ellipticity in degrees at a wavelength λ. The concentration of protein used was 0.6

mg/ml. Spectral data were submitted to DicroWeb for the analysis of percentage of different secondary structures present in the protein (Whitmore *et al.,* 2004, Lobley *et al.,* 2002).

RESULTS

In silico analyses

Using mouse *Pate* gene sequences, ten rat *Pate* mRNA transcripts were identified, amplified, sequenced and submitted to GenBank (Table 1.3). They were found to be localized on chromosome 8q21 and all genes were localized between the *Acrv1* and *Ddx25* gene, a characteristic feature observed in the humans and mice. Genomic localization of rat *Pate* genes were identified and depicted in Figure 1.1. All the *Pate* mRNAs were aligned with the corresponding predicted rat DNA sequences (Figure 1.2). Most of the genes have three exons except for *Pate* and *Pate-2*, which have four exons (Table 1.3). The first exon codes for signal peptide in of all *Pate* genes. Interestingly, we found alternate transcripts for *Pate* and *Pate-2* but they seem to have premature termination codon in the second exon (Figure 1.2).

Table 1.3. *Accession numbers and number of exons for different Pate genes*

Gene Name	Accession Number	No of Exons
Pate-P	JQ031758	3
Pate-Q	JF412807	3
Pate-F	JF412806	3
Pate-A	JF412804	3
Pate-C	HQ687475	3
Pate-E	JF412805	3
Pate-N	HQ687476	3
Pate	JF412809	4
Pate-2	HQ687477	4
Pate-Dj	HQ916281	3

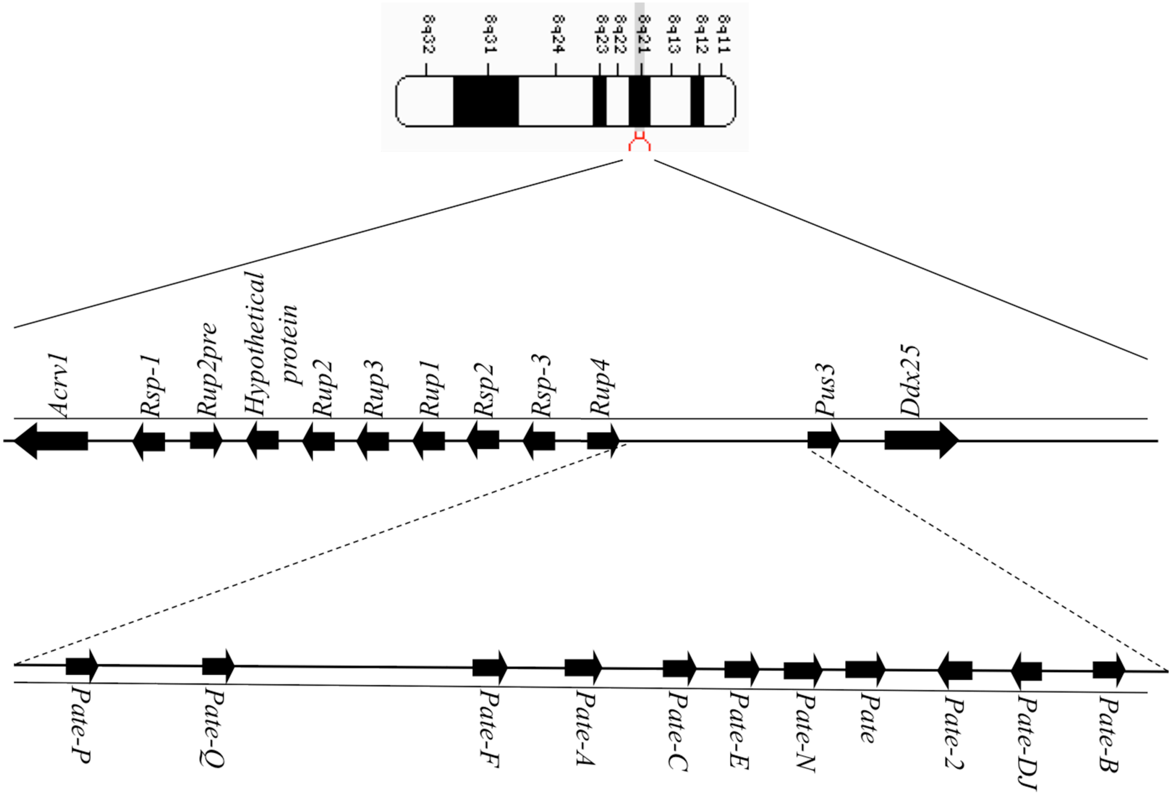

Figure 1.1. Orientation of Pate mRNAs between Acrv1 and Ddx25. *Arrows indicate direction of transcription. Positions were taken from the Mapview (RGSC v3.4) at the National Center for Biotechnology Information (NCBI) website. Distance between genes is not to scale.*

Pate-P

```
      1  gatttcactg caggatccgg aaaagacctg gaagcaccaa gggcgagatt aactctgact
     61  catttttcct tgaatttaag ttttgcctaa atggaagttt tacagatgat cattttagca
    121  ccattaagtt agtcaaaagt ctgtgctccc ctctggctat atgctcacct catttcctgg
    181  tatcccactt gccactcttc ctatcctagg ctacaatata tacctgcctt cctgatatga
    241  gtcctgagca ctctgtcttc tcctctgctg atttctggtc tgaagatcaa ttgcactgca

   (20)  M  G  K  D   I  L  L   L  L  L   G  L  S  L   L  V  S   S  M  Q
    301  ATGGGAAAGG ACATCTTGCT GCTCCTGCTG GGCCTGTCTT TGCTAGTGAG CTCCATGCAa
    361  ggtaagatcc tggcagacag cagtggttgg cactggtggg aaaacacact gatactacag
// 1081  tacaaacaac tcttttcctg gatattgctt cttcttttac cagagcaaca agttcagctc
   1141  ccgttggtgc ttgattttgg atcctgaatg agacccttat actggctttt gtcttttcAG
   (20)   A  L  T   C  I  K  C   E  R  F   N  S  Q   G  I  C  E   R  G  E
   1201  CTTTGACATG TATCAAGTGT GAAAGGTTCA ATTCTCAGGG GATTTGTGAG AGAGGAGAAG
   (18)   G  C  C   E  A  Q  P   G  E  K   C  A  S   L  I  T  Y   K
   1261  GTTGCTGTGA GGCTCAACCT GGTGAGAAAT GTGCCTCACT TATAACCTAT AAAGgtatgt
   1321  aagttcttag agctctgaaa gtcatctcgc ttgccctaca gtgatgaacc catctccagg
// 1681  tgcttatatt tcagatgtgc tttccatctg attctttact gactccccaa ctctttctac
    (7)                                              D  G  K   I  Q  F  G
   1741  ggatatttac atttgcctc  ttgactttct tcttctacag ATGGCAAAAT TCAGTTTGGA
   (20)   S  Q  R   C  A  D   L  C  Y  R   G  T  V   E  N  G   G  L  T  I
   1801  AGCCAGAGAT GTGCTGATCT TTGCTACAGA GGGACTGTTG AGAATGGAGG TCTGACAATA
   (16)   K  M  N   C  C  S   H  R  S  F   C  N  K   P  Y  P  *
   1861  AAAATGAACT GTTGCTCACA CAGGTCTTTC TGCAATAAAC CATATCCGTG Atacttaatt
   1921  gtactgttgg attttaggtg agttcatacg ctttccctgt gctcctgcaa tctcataact
   1981  ctgggcagtc agttgccagc actacacaaa ctaacaactg tattcactta gcattatgtg
   2041  ctgaagcatc aacagttcaa caggctagag agctctttca tgcttcccac aaaccctggt
   2101  gctccctcat tttcacatca tggtttcctt cccttttccc ctatatccct caaacacaat
   2161  agatatatgg cataagtttc ttcttctctt ccatctacac tgattcaaca aatacattct
   2221  gactgtcaaa cacagcactg
```

Pate-Q

```
      1  ttgaatttaa gttttcccta atgggaagct ttacagatga tcatttttag catgattaag
     61  ttagtgaaaa tctgtgctcc cctctggcta cttgctcagt ccatttcctg gtatcccaca
    121  tgctgttcat gctatcctag gctacaatat aaacctacct tcctgaatgg gtcctgagca
    (3)                                                          M  E  K  H
    181  tgctgtcctc tcccctgctg atttctcttc tgaagatcaa tctcactgca ATGGAAAAGC
   (17)   I  L  L   L  L  L   G  L  S  L   L  V  S   S  L  Q
    241  ACATCTTGCT GCTCCTGCTG GGCCTGTCAT TGCTAGTGAG CTCCCTACAA Ggtaagatcc
    301  tagcagatgg tagtgcttca cacaggtggg cagacacact gatactgcag attaggggac
// 1021  gctcttttac tggctattgt ttatttattt accagagcca caagctcagc acccattggt
    (3)                                                           A  L  T
   1081  gcttgagttt ggatcctgaa taagaccctt acactggatt tgtcttttca gCTTTGACAT
   (20)  C  I  T  C   E  R  F   N  S  R   G  I  C  E   R  G  E   G  C  C
   1141  GTATCACGTG TGAAAGGTTC AATTCTCGGG GGATTTGTGA GAGAGGAGAA GGTTGCTGTC
   (15)  Q  A  Q  P   G  E  K   C  A  S   F  I  T  Y   K
   1201  AGGCTCAACC TGGTGAGAAG TGTGCCTCAT TTATAACCTA TAAaggtatg taaggtctgg
// 1621  ttctggttta ctttccatct gactctttac taactcacca actcttttca cagacattta
   (10)                                  D  G  K  V   Q  Y  G   S  Q  K
   1681  cattttgcct cttgactttc ttcttctacA GATGGAAAAG TTCAGTATGG AAGCCAGAAA
   (20)  C  A  D  V   C  F  S   G  T  V   E  N  G  G   L  T  V   R  M  N
   1741  TGTGCTGATG TTTGCTTCAG TGGGACTGTT GAGAATGGAG GCCTGACAGT AAGAATGAAC
   (13)  C  C  S  H   R  S  F   C  N  K   A  H  T  *
   1801  TGTTGCTCAC ACAGGTCTTT CTGCAATAAA GCACATACCT GAtacttaat tgtactgttg
   1861  gatttttagg tgagtaccta ctccttccct gtgttcttgt aatctcataa ccctggacag
   1921  tcagttgcca gcacgtgtat ccacttagca tgagctgctg atgttttaac agttcaacag
   1981  gctagagaga tgttccctgc ttcccactag ccctggcact ccctcattgt tacatcatgg
   2041  tttccttcat ttttcccta  tgtccctcca acacaatagc tatatggcac aagttgcttc
```

Pate-F

```
   1 gatattggca aaacacccat gaacaggtgt gtcaggaagc tcctgcccac atcccaggag
  61 cagctcagag aagagtctac agtctgctct catctaccct gacccattct aggtacttgc
 (7)                                              M  G  K  L   L  L  L
 121 tgccagtgct ctgctgtgtc ttctgcaaac tcagactgag ATGGGCAAGC TCCTGCTCCT
(13)  L  L  L   L  G  A  F   V  L  L   L  I  Q
 181 GCTCCTGCTG CTAGGAGCTT TTGTTCTTCT GTTGATCCAA ggtgagaatg agtcttcaga
 241 ggctgaaaca ggggtgaagt caggagctcc acatagctga cagtaaactt aagatggtgg
// 8701 tcaggagtgc ccacttgatc aggcggtgag gtctctctcc cacggggttt gggagcagag
8761 agctgctgcc atgccttatt tcttaactgt tcttaccctt gttggtccta gctcaatcaa
 (9)                                         A  Q  E   R  I  C   M  S  C
8821 ccctggaatc gctcatttta tctctttttt ctaGCCCAAG AAAGAATATG CATGTCCTGC
(20) H  M  F  V   N  G  K   C  V  E   S  E  G  K   C  T  M   E  D  G
8881 CACATGTTTG TGAATGGCAA GTGTGTAGAA AGTGAAGGTA AATGCACCAT GGAGGACGGC
(15) G  A  C  R   T  R  D   I  Y  L   F  N  A  R   G
8941 GGTGCATGCA GAACCAGGGA CATCTATCTT TTCAATGCAA GAGGTaatgc taatatggaa
9001 tccacaaggg agtctttcaa ggaagtggtg tttcctgtgg aagagcaggc ttgtgtggat
9061 accaaagact ctcatacact gaatttcaaa tattattttc ttacattaaa tatcaatgtt
(14)           G   G  F  L   Y  N  H  T   M  L  E   C  S  K   S  C  K  A
9121 cttttccaGG TGGGTTTCTC TACAACCACA CTATGCTGGA GTGTTCTAAA TCTTGTAAGG
(20)  S  E  E   S  Y  F   H  L  K  I   S  T  F   C  C  K   S  Q  D  F
9181 CTTCAGAAGA GAGTTATTTT CATCTAAAAA TTTCAACTTT TTGTTGCAAA AGTCAAGACT
 (8)  C  N  K   Y  K  G   K  *
9241 TCTGCAATAA ATATAAAGGA AAGTGAacac atgtgctaat tgatgaccag ctccttcact
9301 tgcttcattt tcccttgtgt gcctgctgga tccccctcca tacacaatct gctgaagatg
```

Pate-A

```
   1 agagtctgca acctgctctc atctaccctg acccctcctg agtacttgct accagctgtt
(10)                                  M  G  K  L   L  F  L   L  L  L
  61 ctcctgtgtc ttctggtagc ccagactgag ATGGGCAAGC TCCTGTTCCT TCTCCTGCTC
(11) L  G  S  F   A  L  V   F  I  Q   V
 121 CTGGGGTCAT TTGCTCTTGT GTTCATCCAA Ggtaatagtg agtccttcga ggcaaaaaca
 181 ggggaaagga cagggactag agatggctga gagtaacggc agatgacagc ctgaatagct
// 4741 tttaatactc tagcattctt agtcactgca atcattttca cgccctttac atgtattttt
(19)       Q   A  T  V   C  M  V  C   N  S  F   K  S  G   H  C  L  A
4801 cccagTCCAA GCTACAGTGT GCATGGTTTG CAACTCTTTT AAAAGTGGAC ATTGTTTGGC
(20)  G  K  S   N  C  T  T   R  Y  K   P  G  C   R  T  R  N   F  F  L
4861 TGGCAAGAGC AACTGCACTA CAAGATACAA GCCTGGATGC AGAACCAGGA ATTTCTTCCT
 (5)  F  S  N   T  G
4921 ATTCTCCAAT ACAGGtaagg tgtttgaact agtctaaagg cataattgct gagaagtaga
4981 tgtactcctt ctttcaaaaa catgtttggt cctggggaga aagtcccctc acagtgaata
 (5)                                                   K  W   V  H  N
5041 ccagtatatt ttggacacta aatataaatg cttctatgtt ctaggCAAGT GGGTCCACAA
(20)  H  T  E   L  D  C  H   K  A  C   L  A  E   N  M  Y  L   G  A  L
5101 TCATACCGAA TTGGACTGCC ATAAGGCATG TCTGGCTGAA AATATGTATT TGGGAGCATT
(20)  K  I  S   T  F  C  C   K  G  E   D  F  C   N  K  Y  H   G  Q  V
5161 GAAGATATCT ACCTTTTGCT GCAAAGGTGA AGATTTCTGT AATAAATATC ATGGCCAAGT
 (6)  V  K  K   K  I  Y  *
5221 AGTGAAGAAG AAAATTTACT AActactgac tccttcatca ttgtcactgt gtctcagttt
5281 cccatttcct cttcctagaa tttctcaaca attccctctg tacatttaac ttgaagtaaa
```

Pate-C

```
        1  agcttacttt gagatttgct tctgagaaat ctaatacaca ctcttctttg tacatgcctg
       61  catggattat aaattcacta gagactggtc ttttctctga gtcattgctt ctcatcagct
      (7)                                               M  E  N  L   L  K  L
      121  gactggtact aacacagctg gaagaatctg cttaggagaa ATGGAAAACC TACTGAAGCT
     (10)   C  L  F   L  L  C  F   E  T  G
      181  GTGCCTCTTT CTTCTCTGCT TTGAAACAGG tattgcctgc aggttgctcc cttatctgct
      241  gtacgaagaa aagtaaatta gctcttattc cccactcctc tgtctgcggt gttgggacat
      301  gttgactgga gcatatctgt aagccaaaat attggtgcag ccaagctatc cagaggtcag
      361  atgatctatg ttgtctttga gaaagaacag tgacagtgat gataccctcc tcctctcatc
     (14)                        F   P  V  Q   C  V  K  C   N  S  Y   K  N  G
      421  agtctttaca ctttaggCTT CCCTGTACAG TGTGTGAAAT GTAATTCTTA CAAGAACGGA
     (20)  K  C  A  G   S  Q  Q   T  C  T   T  R  A  G   E  M  C   M  I  R
      481  AAATGTGCTG GAAGTCAGCA GACGTGCACT ACAAGAGCTG GTGAAATGTG TATGATACGC
      (7)  R  T  W  Y   A  S  E   I
      541  AGAACCTGGT ATGCATCTGA AAgtaagtat gtgttctctg ctctggagaa tatctatttc
      601  tccttcagga ttctaagtac cctatctaag attctggact tgaagaaaaa gaatcaactg
//   1681  attctggctc ttccaaactc atatgagtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt
      (2)                                                                I
     1741  gtgtgtgtat acatacatat atatgtgtgt gtatattttt tttcttcttc cacagTTAAT
     (20)  K  L  L  H   A  E  T   T  C  M   G  S  C  K   I  E  E   K  T  S
     1801  AAACTGCTAC ATGCTGAGAC TACGTGCATG GGATCCTGTA AAATAGAAG AAAAACGTCT
     (20)   G  Y  L   T  I  H  T   Y  C  C   D  F  T   D  F  C  N   D  I  G
     1861  GGATATCTAA CAATACATAC CTACTGCTGT GATTTTACAG ATTTCTGCAA TGACATCGGT
      (6)  F  P  I  V   M  T  *
     1921  TTTCCAATCG TAATGACTTA Gcataagcta gtttcagtcg ttctcatcag tcctttcctt
     1981  ttcatttggt ctaaattttc ttacattgac ctctattttt actctggatt atagattttt
     2041  ctggtaatca gtattgtata agtcacagaa cccaggacа ttctttgctc ttctgaatgt
     2101  atatttgatc atatagtcta attcttatgc ttggcatccc tagccttatt accacgagta
```

Pate-E

```
     (11)                                 M   G  N  E   P  K  L  G   I  I  L
        1  acctgctgca agaattagaa gatcctcaAT GGGGAATGAA CCAAAGCTGG GCATCATTCT
      (6)   L  L  C   M  Q  T  A
       61  GTTACTCTGT ATGCAAACTG gtaaatattg ggaaaccggt ttcctagcag ctaaggaatc
      121  agtttaactt gaactctgac atatttttag taaatctttt gttttggggc tctatgggct
//    601  tgctacctga aatatatctc tgtaaggtaa aaccttaatg aggtcaagca gcctgggagt
      (5)                                                          L   A  L  L
      661  ttgagtactt ctcttttctt tcaagtcttt ctctttattt cttgcagCTT TAGCGCTTCT
     (20)   C  R  E   C  T  S  Y   L  H  Q   K  C  L   H  E  M  K   T  C  T
      721  GTGTAGAGAA TGCACATCCT ATTTACATCA GAAATGTCTA CATGAAATGA AAACATGCAC
     (18)   A  K  D   G  E  S  C   L  T  V   R  V  W   N  I  P  Y   S  E
      781  AGCCAAAGAT GGTGAATCCT GTCTAACTGT TAGAGTGTGG AATATACCTT ACAGTGgtaa
      841  gtagtgtgtg aagttaatgt gggagggacg gtgcctcttt ctcttcaagg cctcaaattt
//   1081  gtttgtcatt taatatgatc ttggtttact cttacttgga tttgtggcct gcttcattca
      (9)                                            Q   V  P  N  E   A  Y  S
     1141  aaacaatata ttaattctat ttttgtctcc cacagAACAG GTACCAAATG AAGCATACTC
     (20)   R  C  Q   K  N  C  T   T  D  E   Y  Y  Y   G  D  Y  T   V  M  I
     1201  TAGATGCCAG AAAAACTGTA CAACAGATGA ATATTACTAT GGTGATTATA CAGTAATGAT
     (20)   K  C  C   E  A  Y  D   F  C  N   D  L  L   V  P  I  S   E  W  S
     1261  AAAATGTTGT GAGGCATATG ACTTTTGTAA TGATCTTCTT GTGCCAATTA GTGAATGGTC
            *
     1321  CTGAagaacc ttcagattat cttatggctt cctccagtct tcctcatctt
```

Pate-N

```
      1 cctcattgtt cagagtctag gacattactc agagagtggg aggccagggg aaagtctcag
     61 taacagctcc atctcacatc ctgtccagag aacagttcct gagcacttgc tggagactga
    (7)                                             M  D  W  L   L  F  L
    121 ctgtttatct catccctctt ttattgtttt cctctaacaa ATGGACTGGC TCCTGTTTCT
   (15)  L  F  P   G  L  L  I   L  Y  K   S  H  T   M  G
    181 TTTGTTTCCA GGACTACTGA TTTTGTACAA GTCTCATACA ATGGGtgagt gttatggcaa
    241 gaaaacaaag aaggaagaga tctagggtat gggattgata taccaagcat catgatcaga
//  541 gtgaaatgag atctcctttt tcttgtcccc actgcatccc aggtcttttt tctcaacctt
    (1)                                                              E
    601 ctctttagca tcaatgccta ccccattgtt atgatgtact atctatgccc tataggGGAA
   (20) P  S  F  C   T  S  C   D  E  Y   V  D  D  T   C  R  R   N  L  G
    661 CCCAGTTTTT GCACTTCCTG TGATGAATAT GTTGATGATA CCTGCAGAAG AAACTTGGGA
   (20) V  C  H  P   R  Y  P   D  F  A   C  Q  T  K   E  V  Y   I  Q  L
    721 GTCTGCCATC CCAGATATCC TGACTTTGCC TGCCAAACCA AAGAAGTATA TATTCAACTT
    (3) N  T  G
    781 AACACTGGAg gtgaggtgaa ggccaaggga ggggagagag actattttat gacttggttt
// 1141 tcaagctgga agggaaagaa tggaaaattc atctacatct cttctcattg gtaaagactg
   (10)                                E  Y  L  Y   K  Y  S   I  L  G
   1201 tatatttatg tcactgccct actttcttta GAATATCTGT ACAAATATTC TATATTGGGC
   (20) C  P  R  R   C  V  E   Y  V  R   F  I  K  F   E  K  N   I  F  S
   1261 TGCCCAAGAA GATGTGTGGA ATACGTGCGC TTCATCAAGT TTGAGAAAAA CATCTTCTCC

   (20) C  C  N  E   S  Y  C   N  S  F   Q  A  K  H   T  H  F   K  E  N
   1321 TGCTGCAATG AAAGCTACTG CAACAGTTTT CAAGCAAAAC ATACTCACTT CAAAGAAAAT
    (3) N  F  V  *
   1381 AATTTGTCT AAgctcttcc tgttaggcac atcctgccca tttcttcctg aacaagcttc
   1441 aatgtttgga agtttcttca ctcccatcct acacccacaa tctgctgaga gccaaagggc
```

Pate

```
      1 actcatcact gtataaatag tggggcttcc tgtcctaacc tgaacctttc cacctccaaa
   (17)M  F  K  P   H  L  L   R  C  L   T  L  L  C   Y  L  R   V
     61 ATGTTCAAGC CCCACTTACT GAGATGCCTG ACCTTACTCT GCTATTTGAG GGgtgagtca
    121 tgaagagtca ccagttggtg agctatgtcc atgatcaaag aatggtgaga actcccatga
//  301 agcagatggt ttgccaacag actgagcgat tcctagaagc tgtgaggtgg gggctggaaa
    (4)                                                         F  F  G
    361 attatattct gcagatagta tgaattttac cttttggttt tttcaacagT TTTCTTTGGG
    (8) S  L  P  G   D  A  N   K  P
    421 TCGCTTCCAG GTGATGCTAA CAAACgtgag tagaaatggt tgtgattaat ggctacaaag
    481 atgggggagt tgagtggagc ttggtgggca ctggcagcta aaagcaaacc acagctaggt
    961 caaagggagg gtaaacctca gacccaactg cacgattgtt ctctttctct ctcacctgaa
   (11)     D  K   V  L  I   H  E  N  N   N
   1021 cagCTGATAA AGTACTCATT CATGAAAATA ACAATggtaa gtacaaaaag ccctagcctg
// 1201 tgctttgatg ggtgtggggg ttcaggacac tcccagctgt aagcaggatt cacacttgaa
    (2)                                                            V  V
   1261 gtagaggata ggctctttgc aggtagtaac ttctcctctc ccacctctct tcaaGTTGTG
   (20) E  I  V  Q   C  R  M   C  H  L   Q  F  P  G   E  K  C   S  R  G
   1321 GAAATTGTGC AGTGCAGGAT GTGCCACCTC CAGTTCCCAG GAGAAAAGTG CAGCAGAGGT
   (19) R  G  I  C   T  A  T   V  E  E   A  C  M  A   G  K  I   F  K  K
   1381 AGAGGAATAT GTACTGCAAC AGTAGAAGAG GCCTGCATGG CTGGAAAAAT CTTCAAAAgt
   1441 gagtggtggg atcaagaagc aaaaacagga aagagaaagt gggatgtgtg ggccttgagg
// 2041 tctgaatact ggagtatgaa ccacatgtgg tgatttttag ataccagtgt gcgttgggga
    (1)
   2101 gaagtagatt tacagaatcc tcttccaagg ttgctgatct gttccctttc ttctgcagAG
   (20)  D G  T  M   W  L  K   F  M  G   C  L  K  N   C  A  N   V  K  K
   2161 GATGGTACCA TGTGGTTAAA ATTCATGGGC TGCTTAAAGA ACTGTGCTAA TGTGAAAAAA
   (20) I  K  W  G   S  Y  L   V  D  F   R  C  C  R   G  H  D   M  C  N
   2221 ATAAAATGGG GCAGCTATCT GGTGGACTTC AGATGCTGCC GGGGCCACGA CATGTGCAAT
    (3)  E  R  F  *
   2281 GAAAGGTTTT AGacacttct gtctttctgg gctcccacca tgtgtgtgat gggatggcct
   2341 cttacaaaga cctccggagt ttaactctgc agaaaaggac tgcaaacaca tggctctgcc
```

Pate-alternate transcript

```
         1 gatctattct tttttttttt ttaacggttc acagacttta ttggtttaac aatgaacagc
//     361 cctagactca tcactgtata aatagtgggg cttcctgtcc taacctgaac ctttccacct
      (17)      M  F   K  P  H   L  L  R  C   L  T  L   L  C  Y   L  R  V
       421 ccaaaATGTT CAAGCCCCAC TTACTGAGAT GCCTGACCTT ACTCTGCTAT TTGAGGGgtg
//     661 gaggaagcag atggtttgcc aacagactga gcgattccta gaagctgtga ggtgggggct
       (2)                                                              F  F
       721 ggaaaattat attctgcaga tagtatgaat tttacctttt ggtttttca acagTTTTCT
      (10)   G  S  L   P  G  D   A  N  K  P
       781 TTGGGTCGCT TCCAGGTGAT GCTAACAAAC gtgagtagaa atggttgtga ttaatggcta
//    1261 tggtaggaaa ggctcaaaaa gaatccaaga taaaaggcaa aatgagccct gagggaagag
      1321 aggtgcaaag ggagggtaaa cctcagaccc aactgcacga ttgttctctt tctctctcac
      (11)            D  K  V  L   I  H  E   N  N  N   V
      1381 ctgaacagCT GATAAAGTAC TCATTCATGA AAATAACAAT Ggtaagtaca aaaagcccta
      1621 ttgaagtaga ggataggctc tttgcaggta gtaacttctc ctctcccacc tctcttcaag
      (20)   V  E  I   V  Q  C   R  M  C  H   L  Q  F   P  G  E   K  C  S  R
      1681 TTGTGGAAAT TGTGCAGTGC AGGATGTGCC ACCTCCAGTT CCCAGGAGAA AAGTGCAGCA
      (20)   G  R  G   I  C  T   A  T  V  E   E  A  C   M  A  G   K  I  F  K
      1741 GAGGTAGAGG AATATGTACT GCAACAGTAG AAGAGGCCTG CATGGCTGGA AAAATCTTCA
       (1)   T
      1801 AAAgtgagtg gtgggatcaa gaagcaaaaa caggaaagag aaagtgggat gtgtgggcct
//    2281 ctctcacagt ggtaatccca gatgcatcta agcctctcat gatggccttt ctacctctag
       (6)                                                 S  S   F  Q  S  F
      2341 taaaatctta aaaccactca ccatttattt tcttttgggt agCCTCCAGC TTCCAATCCT
      (20)   T  S  E   Y  W  S   M  N  H  M   W  *
      2401 TCACATCTGA ATACTGGAGT ATGAACCACA TGTGGTGATT TTTAGATACC AGTGTGCGTT
      2461 GGGGAGAAGT AGATTTACAG AATCCTCTTC CAAGGTTGCT GATCTGTTCC CTTTCTTCTG
      2521 CAGAGGATGG TACCATGTGG TTAAAATTCA TGGGCTGCTT AAAGAACTGT GCTAATGTGA
      2581 AAAAAATAAA ATGGGGCAGC TATCTGGTGG ACTTCAGATG CTGCCGGGGC CACGACATGT
      2641 GCAATGAAAG GTTTTAGaca cttctgtctt tctgggctcc caccatgtgt gtgatgggat
```

Pate-2

```
         1 gatcctggga actggaagta ctgttgttct gaaaaattta tgatgtaatt tagatgaata
        61 gcccaaggac aggtcaccgt ttcctttcaa gtgagaaaag agccagtgcc ttcctcagga
       (7)                                             M  L  V  I   V  C  L
       121 agctgttgaa gacaaggctt gcactatgtt tgctctgttc ATGCTGGTGA TAGTCTGTTT
       (6)  F  C  Q   Y  W  G
       181 GTTCTGCCAG TATTGGGGTa agtcctggtg ataaaggtac tcaagagtgg cccaatgctg
       241 gggaaggagg ggtttccaaa ctggctgtct gagattgtaa caaaggaaac tgcttttcct
       (7)               V  L  N   E  P  E   K  D
       301 tctcttccca ggtGTCCTTA ATGAACCTGA AAAAGgtacg atcatggctg gcataaagtc
//     781 tattctggga atgttatgac ccacagatca atagtaactt acagagaaag ctctaattgc
       (3)                                                            L  G
       841 agacgttttc tccagactgc attcactaag tacttctttc tgtgtctagt agATCTTGGA
      (20) T  M  C  Y   K  C  K   K  Y  H   L  G  L  C   Y  G  L   M  R  S
       901 ACAATGTGTT ATAAATGTAA GAAATATCAT CTTGGGTTAT GCTACGGACT CATGAGATCC
      (20) C  T  L  K   H  R  Q   S  C  A   A  E  N  F   Y  I  L   T  N  R
       961 TGCACACTGA AGCATAGACA GTCCTGTGCT GCTGAGAACT TTTACATACT CACGAACAGA
       (1)  G
      1021 GGtaacgtgg ggaatgggat gatgggcagc taaccagacc ttcccttcta aacagcaggg
//    1201 atcagttatg gagtacagga aattatggaa ttttgaggat aggttgttac cagaaacatg
       (2)                                                         Q  S  M
      1261 agagtgagca ataaattctt caaagttctt tcctctttca ctttctcagg GCAGAGCATG
      (20) Y  H  Y  S   R  L  S   C  M  T   N  C  E  D   I  N  F   L  S  F
      1321 TATCATTATT CAAGACTGTC ATGTATGACC AACTGTGAGG ACATCAACTT CCTGAGTTTT
      (20) E  R  R  T   E  L  I   C  C  K   H  S  S  Y   C  N  L   P  M  G
      1381 GAAAGGAGGA CAGAGCTAAT TTGTTGCAAG CACAGTAGCT ATTGCAACCT CCCAATGGGA
       (1) L  *
      1441 CTCTAGttct gaatttatta tgggtatggt atcattcttc aacttactac caactccctt
      1501 ttccccaaag tttgtattta ctctccccac taactaacaa taaatgggaa aggcatttgt
      1561 ccatgaaaag agaatcagtc atatgagaaa ctgggctggg agtttgtcct
```

Pate-2 alternate transcript

```
       1 tggaaatagg aaccaatgtg atcaattagg atttaggaat atatcctgag ggagtcaaac
      61 acgatgtctt gatcctggga actggaagta ctgttgttct gaaaaattta tgatgtaatt
     121 tagatgaata gcccaaggac aggtcaccgt ttcctttcaa gtgagaaaag agccagtgcc
     (3)                                                        M  L  V  I
     181 ttcctcagga agctgttgaa gacaaggctt gcactatgtt tgctctgttc ATGCTGGTGA
    (18)   V  C  L   F  C  Q   Y  W  G  K   S  W  *
     241 TAGTCTGTTT GTTCTGCCAG TATTGGGGTA AGTCCTGGTG ATAAAGGTAC TCAAGAGTGG
     301 CCCAATGCTG GGGAAGGAGG GGTTTCCAAA CTGGCTGTCT GAGATTGTAA CAAAGGAAAC
     361 TGCTTTTCCT TCTCTTCCCA GGTGTCCTTA ATGAACCTGA aaaaggtacg atcatggctg
     421 gcataaagtc tttggttcat ttgtgacacc ttcaggagta aacctccctt ccatttactg
//   901 ctctaattgc agacgttttc tccagactgc attcactaag tacttctttc tgtgtctagt
     961 aGATCTTGGA ACAATGTGTT ATAAATGTAA GAAATATCAT CTTGGGTTAT GCTACGGACT
    1021 CATGAGATCC TGCACACTGA AGCATAGACA GTCCTGTGCT GCTGAGAACT TTTACATACT
    1081 CACGAACAGA GGtaacgtgg ggaatgggat gatgggcagc taaccagacc ttcccttcta
    1141 aacagcaggg cctttcactc ttaggatgag tgggagagtg gagttgatgc agatgagcag
    1201 tcttgcaaag actgcagaga tggatctttc cttaacatag cactgagtga tggaggggcg
    1261 ggggtctctc atcagttatg gagtacagga aattatggaa ttttgaggat aggttgttac
    1321 cagaaacatg agagtgagca ataaattctt caaagttctt tcctctttca ctttctcagg
    1381 GCAGAGCATG TATCATTATT CAAGACTGTC ATGTATGACC AACTGTGAGG ACATCAACTT
    1441 CCTGAGTTTT GAAAGGAGGA CAGAGCTAAT TTGTTGCAAG CACAGTAGCT ATTGCAACCT
    1501 CCCAATGGGA CTCTAGttct gaatttatta tgggtatggt atcattcttc aacttactac
    1561 caactccctt ttccccaaag tttgtattta ctctccccac taactaacaa taaatgggaa
    1621 aggcatttgt ccatgaaaag agaatcagtc atatgagaaa ctgggctggg agtttgtcct
    1681 catttctaag caaatctttg ctaattcttt ttgtcatatc tagcagggca ttttgatctg
    1741 tggacagtcc tggtcatcat gagtaggtcc agaattgatc acctcgtgta caagcccacg
    1801 agaatttggc tcaaaatgtc atcccacctt tgtacaggga aatctgtaaa tatactctgt
    1861 gttggtctgc accaagtgtt ctgagttggg
```

Pate-Dj

```
       1 atgaataatc atcaactttt gctggaggag aaagcttgtc cctggagccc agcagactgg
      61 gtctttgtta gctttcctgt ggaaggatta gctgtcagac ttgtgcttgc agcccttcct
     (9)                                    M  N  R   H  F  L   L  L  V  S
     121 agctttgctc cacacaatac atctgggtcc aaATGAACAG GCACTTCTTG CTGCTCGTCT
     (7)   L  F  C   L  I  V   E
     181 CCCTCTTCTG CCTCATTGTG Ggtgagttcc agaggctcct ccctgaggag tgagcttcag
     241 tgtcaggaac aagagaactg gaggggaatc ttcacacgta gagccatgtc catgtggact
//  1381 agctctttat ctgcctaccc acccttggaa gatcccttca cccagctttg ctatctgtct
     (3)                                                           A  T  T
    1441 acaaattcca aatccatgtc ctcaagcgga gctgtgtgct ctctcataca gAAGCAACAA
    (20)   L  K  C   T  T  C   H  L  R  T   Q  S  D   H  C  R   R  G  F  G
    1501 CACTGAAGTG TACAACATGC CACCTCCGCA CACAGTCAGA CCATTGCAGA AGAGGCTTTG

    (19)   V  C  L   A  Q  K   H  E  T  C   M  S  L   R  I  Y   S  N
    1561 GTGTTTGCCT TGCTCAGAAG CACGAAACAT GCATGAGCTT GAGGATCTAC TCCAgtaagt
    1621 gtcagggttg ggtgcagtca tgtgaagcat gggcaacttc ttaaaaaggg ctatggcaga
//  1921 gggtgctctt gggaaggaga agctgggaag tcatccataa acctacctgg ttaaggcaat
    1981 gagtgttctg ccttggatac tgagatcact gccttaccac tgcttctttc ttattgtagA
    (20)  G  S  L   Q  T  S  Y   M  V  C   Q  R  F   C  K  N  L   A  Y  N
    2041 TGGCTCTCTC CAGACCTCAT ACATGGTGTG TCAGAGATTC TGCAAGAATT TGGCATACAA
    (20)  F  N  N   R  T  Y  I   H  K  C   C  N  Y   D  F  C  N   F  R  L
    2101 CTTCAACAAT CGAACTTATA TTCATAAATG TTGCAACTAT GATTTTTGTA ACTTCAGACT
          *
    2161 TTAAgatact ccccccaacc tcattctaga aggtcccaca tgtttttctt cttttacact
    2221 ctgtttatcc tgcctttcca ccctgtatac atcgaggatg ctcctgctct atccccatca
    2281 ttcatcctga ttcctttaat ttggatctca
```

***Figure 1.2**. Alignment of rat Pate mRNA (Pate-P, Pate-Q, Pate-F, Pate-A,Pate-C,Pate-E, Pate-N, Pate, Pate-2 and Pate-Dj) with genomic DNA. Upper case letters indicate exons and lower case letters indicate introns. Amino acids are indicated in single letters. Predicted signal peptide cleavage site is indicated in bold italics. The conserved ten cysteines are indicated in blue. Numbers in parentheses indicate amino acids of the protein.*

In silico protein translation analyses revealed that all the *Pate* mRNA transcripts, except for *Pate* alternate transcript and *Pate*-2 alternate transcript, encode for proteins that are cysteine rich and contain the characteristic TFP/Ly-6/uPAR domain with a highly conserved distribution of 10 cysteines in two motifs (Figure 1.3), which is in agreement with the predictions available in the rat genome. The rat PATE proteins are highly homologous to their mouse and human counterparts (table 1.4). All the *Pate* proteins identified in this study contain a signal peptide cleavage sequence and seem to be secretory in nature (Table 1.4). The predicted physical characteristics of the rat PATE proteins are given in table 1.4.

Homologies among different PATE proteins were calculated using CLUSTAL-W. All PATE proteins have very low homology between them except for PATE-P, which has 83% similarity with PATE-Q (Table 1.5). Phylogenetic analysis of rat PATE proteins revealed that PATE-2 and PATE-N are branched out from all other members of the family (Figure 1.4).

```
PATE-P     ----MGKDILLLLLGLSLLVSSMQ-------------------ALTCIKCERFNSQGICE 37
PATE-Q     ----MEKHILLLLLGLSLLVSSLQ-------------------ALTCITCERFNSRGICE 37
PATE-F     --MGKLLLLLLLLGAFVLLLIQAQ-------------------ERICMSCHMFVN-GKCV 38
PATE-A     --MGKLLFLLLLLGSFALVFIQVQ-------------------ATVCMVCNSFKS-GHCL 38
PATE-C     ------MENLLKLCLFLLCFETGF-------------------PVQCVKCNSYKN-GKCA 34
PATE-E     -----MGNEPKLGIILLLCMQTAL-------------------ALLCRECTSYLH-QKCL 35
PATE-N     ----MDWLLFLLFPGLLILYKSHTMG----------------EPSFCTSCDEYVD-DTCR 39
PATE       MFKPHLLRCLTLLCYLRVFFGSLPGDANKPDKVLIHENNNVVEIVQCRMCHLQFPGEKCS 60
PATE-2     -----MLVIVCLFCQYWGVLNEPEKD----------------LGTMCYKCKKYHL-GLCY 38
PATE-DJ    -----MNRHFLLLVSLFCLIVEAT-------------------TLKCTTCHLRTQSDHCR 36
PATE-B     MNSMTKISILLIVALSFLCFTEAN------------------TNLICNTCNRSEN-SECK 41

PATE-P     RGEGCCEAQP-GEKCAS---LITYKDG---KIQFGSQRCADLCY-RGTVENGGLTIKMNC 89
PATE-Q     RGEGCCQAQP-GEKCAS---FITYKDG---KVQYGSQKCADVCF-SGTVENGGLTVRMNC 89
PATE-F     ESEGKCTMED-GGACRTRDIYLFNARGGGFLYNHTMLECSKSCK-ASEESYFHLKISTFC 96
PATE-A     AGKSNCTTRY-KPGCRTRNFFLFSNTG-KWVHNHTELDCHKACL-AENMYLGALKISTFC 95
PATE-C     GSQQTCTTRA-GEMCMIRRTWYASEIN---KLLHAETTCMGSCK-IEEKTSGYLTIHTYC 89
PATE-E     HEMKTCTAKD-GESCLTVRVWNIPYSE--EVPNEAYSRCQKNCT-TDEYYYGDYTVMIKC 91
PATE-N     RNLGVCHPRYPDFACQTKEVYIQLNTG-EYLYKYSILGCPRRCV-EYVRFIKFEKNIFSC 96
PATE       RGRGICTATV-EEACMAG--KIFKKDG---TMWLKFMGCLKNCANVKKIKWGSYLVDFRC 114
PATE-2     GLMRSCTLKH-RQSCAAENFYILTNRG-QSMYHYSRLSCMTNCE-DINFLSFERRTELIC 95
PATE-DJ    RGFGVCLAQK-HETCMS---LRIYSNG---SLQTSYMVCQRFCK-NLAYNFNNRTYIHKC 88
PATE-B     NGTGQCTAPE-GGSCST----ISIYHG--QRHVLSKQMCLGHCE-EKPHYNGDFMIYVMC 93

PATE-P     CSHRSFCNKPYP----------- 101
PATE-Q     CSHRSFCNKAHT----------- 101
PATE-F     CKSQDFCNKYKGK---------- 109
PATE-A     CKGEDFCNKYHGQVVKKKIY--- 115
PATE-C     CDFTDFCNDIGFPIVMT------ 106
PATE-E     CEAYDFCNDLLVPISEWS----- 109
PATE-N     C-NESYCNSFQAKHTHFKENNFV 119
PATE       CRGHDMCNERF------------ 125
PATE-2     CKHSSYCNLPMGL---------- 108
PATE-DJ    CN-YDFCNFRL------------ 98
PATE-B     CS-KNLCNSF------------- 102
```

Figure 1.3. Multiple sequence alignment of PATE proteins*. The characteristic ten cysteines are indicated in bold and underlined. The conserved amino acid residues are shaded.*

Table 1.4. *General characteristic features of PATE proteins*

Name of the protein	Length (aa)	MW (kD)	pI	Signal peptide	Net charge	O-Gly sites	Myristyltion	Phosphorylation			Homology with mouse counterpart	Homology with human counterpart
							sites	Ser	Thr	Tyr		
												--
PATE-Q	101	10.99	8.32	1 to 21	3	Nil	-Nil-	66	84	57	71%	--
PATE-F	109	12.27	8.54	1 to 21	4	Nil	-Nil-	79,83,86	Nil	58,87,106		--
PATE-A	115	12.98	9.27	1 to 23	10	Nil	-Nil-	Nil	63	Nil	89%	--
PATE-C	106	11.9	6.68	1 to 19	0	Nil	-Nil-	27,72,80	68	28,82	73%	--
PATE-E	109	12.55	4.77	1 to 20	-6	Nil	-Nil-	Nil	27,51	29,68,80,82,95	28%	--
PATE-N	119	14.14	6.86	1 to 22	0	Nil	-Nil-	18,29,101	28,37,56	33,49,60,84,102	88%	--
PATE	125	14.35	9.07	1 to 21	9	Nil	-Nil-	Nil	69	Nil	84%	76%
PATE-V1	99	11.3	8.53	1 to 21	3	Nil	-Nil-	87,90	69	92	80%	62%
PATE-2	108	12.6	8.45	1 to 16	4	Nil	-Nil-	66,74,99	45	58,70,101	83%	70%
PATE-DJ	98	11.56	9.08	1 to 18	8	Nil	-Nil-	Nil	20,63	77	86%	63%
PATE-P	101	11.04	8.31	1 to 21	-3	Nil	-Nil-	66	84	57	72%	--
PATE-4	102	11.27	6.85	1 to 23	0	Nil	-Nil-	Nil	Nil	Nil	57%	38%

__Table 1.5.__ CLUSTAL-W scores of different PATE proteins

Column1	PATE-P	PATE-Q	PATE-F	PATE-A	PATE-C	PATE-E	PATE-N	PATE	PATE-2	PATE-Dj	PATE-B
PATE-P		82	28	26	25	24	16	21	17	27	22
PATE-Q			27	27	22	30	17	22	15	27	22
PATE-F				50	30	22	22	17	18	23	27
PATE-A					33	22	16	16	20	19	24
PATE-C						30	13	22	21	20	22
PATE-E							14	20	17	27	19
PATE-N								15	22	26	17
PATE									17	26	21
PATE-2										22	18
PATE-DJ											23
PATE-B											

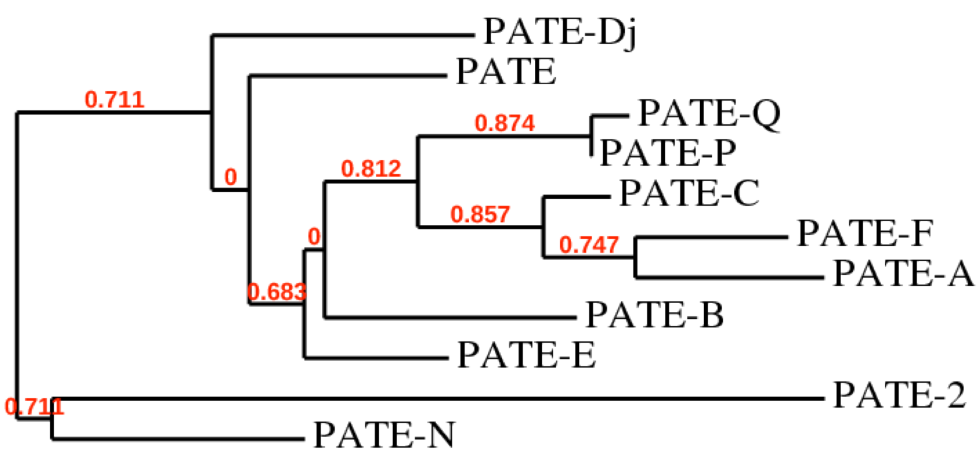

Figure 1.4. Phylogenetic analysis for different PATE proteins. *Numbers on the branch indicates the branch length, which is proportional to the number of substitutions per site.*

To understand the structure-function relationship, PATE and PATE-F models were generated. The PATE and PATE-F modeled proteins have structures similar to the three-fingered toxin, bucandin (Figure 1.5), a neurotoxin. Ramachandran plot analyses revealed that the percentage of amino acids in the allowed regions for PATE and PATE-F was 78.9 and 79.5 respectively. There were no amino acids (0 percent) in the disallowed regions. The G factor values for PATE and PATE-F were -0.57 and -0.29 respectively.

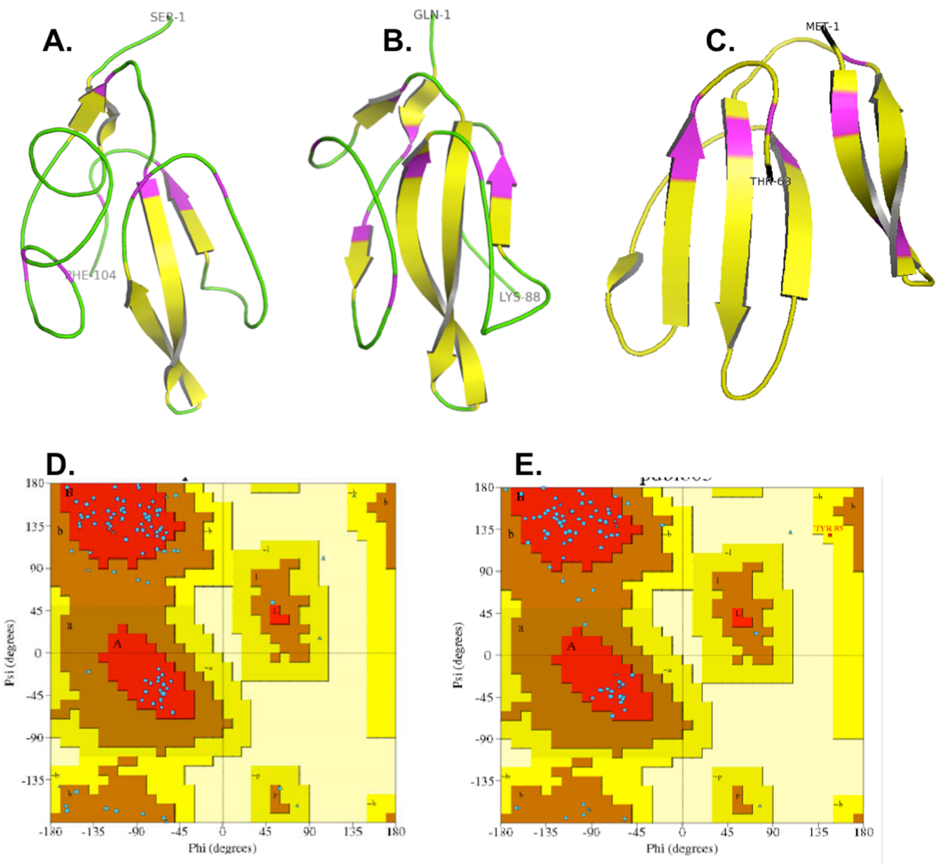

***Figure 1.5. Three dimensional protein structure prediction**. **A**) rat PATE, **B**) rat PATE-F and **C**) Bucandin carrtoon models. Ramachandran plot for the rat PATE (**D**) and PATE-F (**E**).*

The modeled proteins have structural similarity with the three fingered toxin bucandin, which has X-Ray crystallographic structures with PDB code 1F94. By CLUSTAL-W analysis, the similarity score between these proteins was found to be only 11 and 14 for PATE and PATE-F respectively (Figure 1.6). Though there is less of sequence similarity among these proteins, they seem to have conserved 10 cysteine residues, which may result in their structural similarity. To further understand their similarity with bucandin, PATE and PATE-F structures were superimposed. Both PATE and PATE-F exhibit extensive superimposition of their three dimensional structures with bucandin (Figure 1.7).

```
PATE-F      --------------QER-------ICMSCHMFVN-GKCVESEGKCTMED-GGACRTRDIY 37
PATE        SLPGDANKPDKVLIHENNNVVEIVQCRMCHLQFPGEKCSRGRGICTATV-EEACMAGKIF 59
BUCANDIN    -----------------------MECYRCGVS-G---CHLKI-TCSAEE--TFCY----- 23

PATE-F      LFNARGGGFLYNHTMLECSKSCKASEESYFHLKISTF-CCKSQDFCNKYKGK 88
PATE        ----KKDGTMW-LKFMGCLKNCANVKKIKWGSYLVDFRCCRGHDMCNERF-- 104
BUCANDIN    ----KWLNKISNERWLGCAKTCTEIDT--WN--VYNK-CCTTN-LCNT---- 63
```

Figure1.6. CLUSTAL-W alignment of PATE, PATE-F and Bucandin. *All the cysteines were shaded with different colors.*

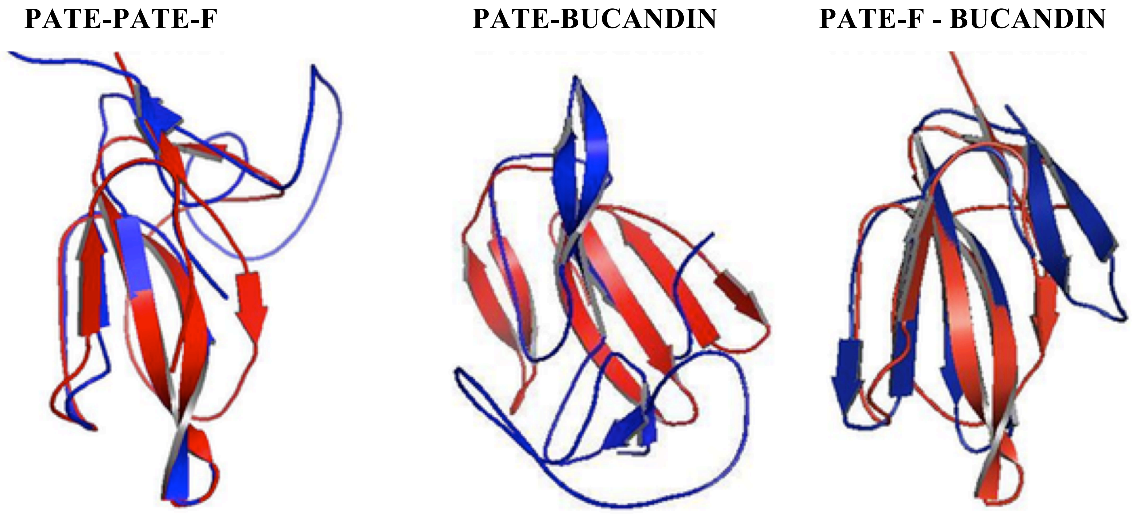

Figure 1.7. Super imposed models of PATE, PATE-F and Bucandin.

Pate gene expression in the rat

Using RT-PCR analyses, the expression pattern of rat *Pate* genes was determined in the male reproductive tract. Though *Pate* gene expression is thought to be prostate and testis specific, we found them to be expressed in the epididymis and seminal vesicle (Figure 1.8). *Pate-Q* was expressed in the caput, seminal vesicle and prostate. Epididymis specific expression was observed for *Pate-F*, *Pate-Dj* and *Pate-A*. *Pate-C*, *Pate-N* and *Pate*-2 were found to be expressed in all the tissues analyzed. *Pate* and *Pate-E* expression was found to be present in the epididymis and prostate. *Pate-P* expression was not found in any of the tissues analyzed (Figure 1.8). To determine if the expression pattern of *Pate* mRNA transcripts is male reproductive tract specific, RT-PCR was performed in a variety of tissues obtained from male and female rats (Figure 1.9). *Pate*, *Pate-F*, *Pate-A*, *Pate-E* and *Pate-N* were not expressed in any of the non-reproductive and female reproductive tract tissues. *Pate-Q*, *Pate-2* and *Pate-Dj* was expressed only in liver. We found tissue specific expression of *Pate-P* in the ovary. *Pate-C* expression is seen in most of the non-reproductive and female reproductive tract tissues analyzed.

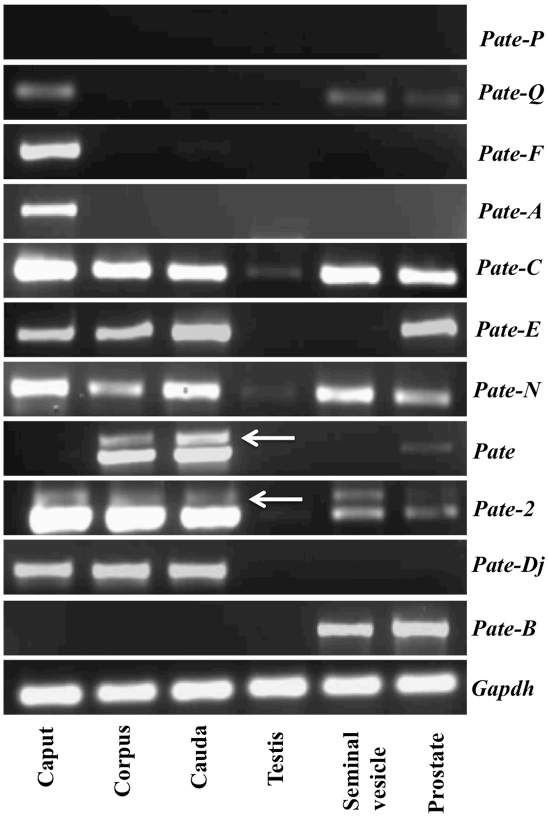

Figure 1.8. Expression of Pate genes in the male reproductive tract of rat. *Pate genes were amplified using gene specific primers using total RNA isolated from caput, corpus, cauda, testis, seminal vesicle and prostate.*

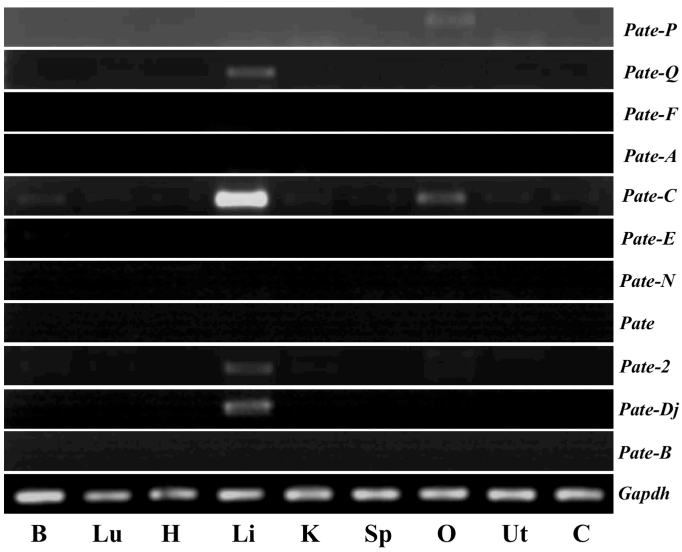

Figure 1.9. Tissue distribution of Pate genes in the rat. *RT-PCR analysis was performed using total RNA isolated from Brain, Heart, Lung, Liver, Kidney, Spleen, Ovary, Uterus and Cervix.*

Androgen dependent expression

Gene expression in the male reproductive tract is under the influence of androgens. To elucidate the effect of androgen variation, PCR analyses for *Pate* genes were carried out using total RNA isolated from the epididymides of 20-60 day old rats. *Pate-Q*, *Pate-C*, *Pate-2*, *Pate-E* and *Pate-Dj* were found to be expressed in the epididymides throughout the development, where as *Pate-N* and *Pate* were expressed starting from 30 days (Figure 1.10). *Pate-F* expression in the epididymis seems to appear from 40 days during development. The absence of certain *Pate* transcripts in the caput or cauda during development (Figure 1.10) seems to correlate with the expression profile observed in the male reproductive tract tissues obtained from the 90 day old rats (Figure 1.8). These results indicate that expression of some of the *Pate* genes in the epididymis seems to be suppressed during development. *Pate-P* and *Pate-B* were not found to be expressed in the epididymides obtained from all the age groups, which is in consistent with their absence in the epididymis of adult rat.

Though majority of the *Pate* genes were not expressed in the adult testis (Figure 1.8), it is possible that they may be expressed in younger rats during development. To determine, whether *Pate* genes have a role in testis development PCR analyses was carried out using mRNA isolated from testis of 20-60 day old rats. *Pate-C* and *Pate-N* were found to be expressed starting from day 30 in the developing rats, whereas the other *Pate* genes were not detected at all the ages analyzed (Figure 1.11).

To gain further knowledge into the role of androgens in regulating *Pate* gene expression in adults, RT-PCR analyses was performed using epididymides obtained from androgen ablated rats with or with testosterone supplementation. Androgen ablation resulted in complete loss of *Pate* gene expression (Figure 1.12). Dihydrotestosterone supplementation reverted the expression of majority of the *Pate* genes except *Pate* and *Pate-F*. These results suggest that *Pate* genes are under the control of androgens in the epididymis.

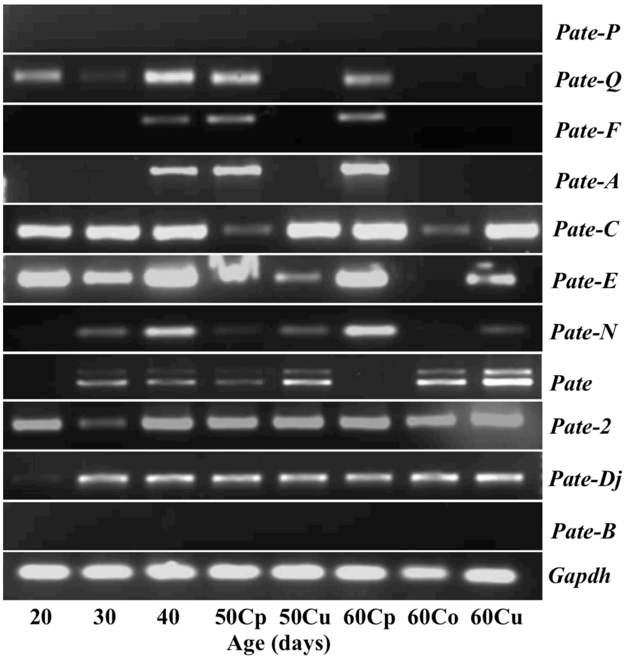

Figure 1.10.* *Pate gene expression in the epididymides of developing rats. *RNA was isolated from the epididymides of 20 - 60 day old rats, reverse transcribed and PCR for Pate genes performed. Caput, corpus and cauda obtained from 50 and 60 day old animals are indicated as Cp, Co and Cu respectively.*

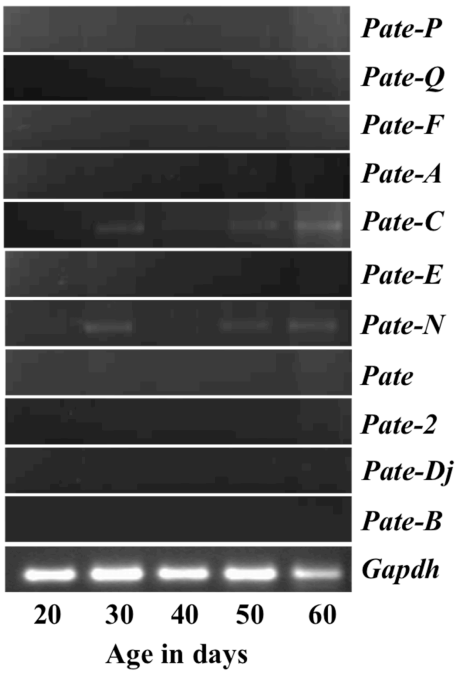

Figure 1.11.* *Pate gene expression in the testis of developing rats. *RNA was isolated from the epididymides of 20 - 60 day old rat testes, reverse transcribed and PCR for Pate genes performed.*

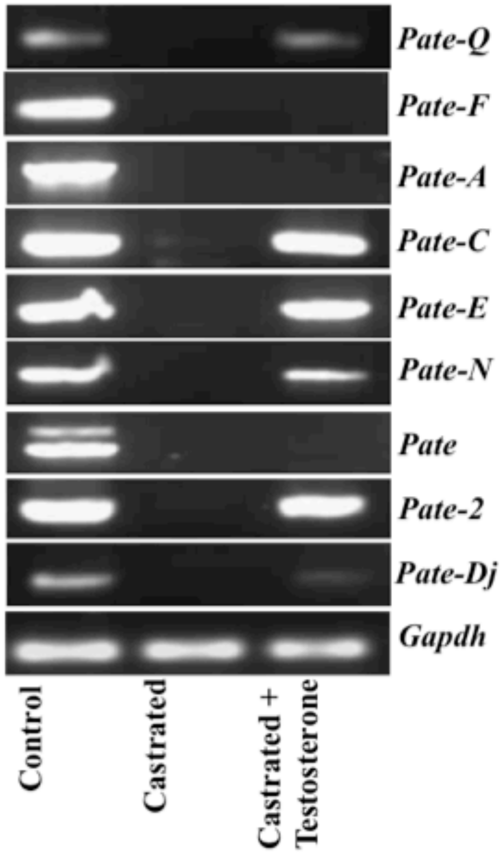

Figure 1.12. Androgen regulation of Pate gene expression in the adult rats. *Adult male Wistar rats were either sham operated or castrated or castrated and DHT supplemented. Two weeks after castration total RNA was isolated from the epididymides and PCR analyses carried out for the expression of Pate genes.*

Immunolocalization

To allow further insight into the functional role of PATE proteins in male reproductive tract function and to confirm whether their mRNA is translated into proteins, we cloned *Pate* and *Pate-F* genes into pQE80 vector (Figure 1.13). PATE and PATE-F proteins were over expressed and purified using Ni-NTA purification. Purified proteins were injected to rabbit and polyclonal antibodies were generated (Figure 1.14). To understand cross reactivity between PATE and PATE-F antisera, Western blotting was performed. We observed no cross reactivity between PATE and PATE-F antisera (Figure 1.14). To understand PATE and PATE-F protein localization, immunofluorescence was performed in rat testis and epididymis. PATE protein was found to be abundantly localized in the caput and cauda and appears to be present throughout the epithelium, suggesting that it may be secreted into the lumen (Figure 1.15). PATE-F protein was also found to be expressed abundantly in the caput (Figure 1.16). Similar to PATE, PATE-F is localized throughout the epithelium and on the spermatozoa. Results of this study indicate that PATE proteins may have roles in both spermatogenesis and sperm maturation.

Localization of PATE and PATE-F proteins on the sperm was analyzed using immunofluorescence. PATE was found to be present throughout the sperm surface (Figure 1.15), whereas, PATE-F localization was restricted to the head region (Figure 1.16). Presence of PATE proteins on the sperm surface indicates their possible role in spermatogenesis and sperm maturation.

CD spectral data of rPATE and rPATE-F were submitted to DichroWeb and analyzed using K2D2 method. For analysis of different secondary structural components for the proteins, data points from 190 to 260 nm was given. From the K2D2 analysis rPATE has 5% α-helix, 47% β-sheet and 48% random coil structures (Figure 1.17A), where as PATE-F has 29% α-helix, 18% β-sheet and 57% random coil structures (Figure 1.17B).

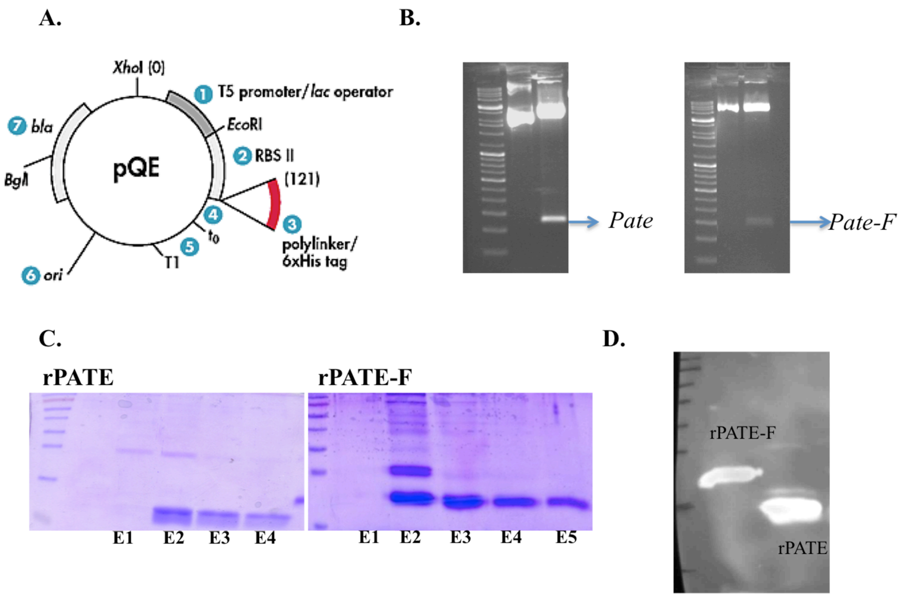

Figure 1.13. Cloning and recombinant protein purification. A. *Vector map of pQE80 plasmid.* ***B.*** *Clone confirmation by double digestion.* ***C.*** *SDS-PAGE showing the purity of elutions after Ni-NTA protein purification.* ***D.*** *Western blotting to confirm identity of the protein purified.*

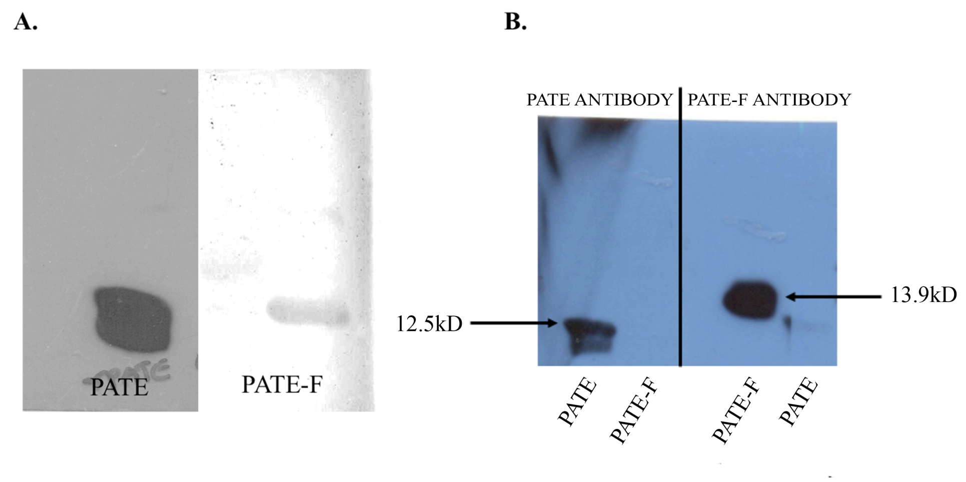

Figure 1.14. Generation of polyclonal antibodies against PATE and PATE-F proteins. A. *Detection of rPATE and rPATE-F using polyclonal antibodies.* ***B.*** *Cross reactivity between PATE and PATE-F polyclonal antibodies.*

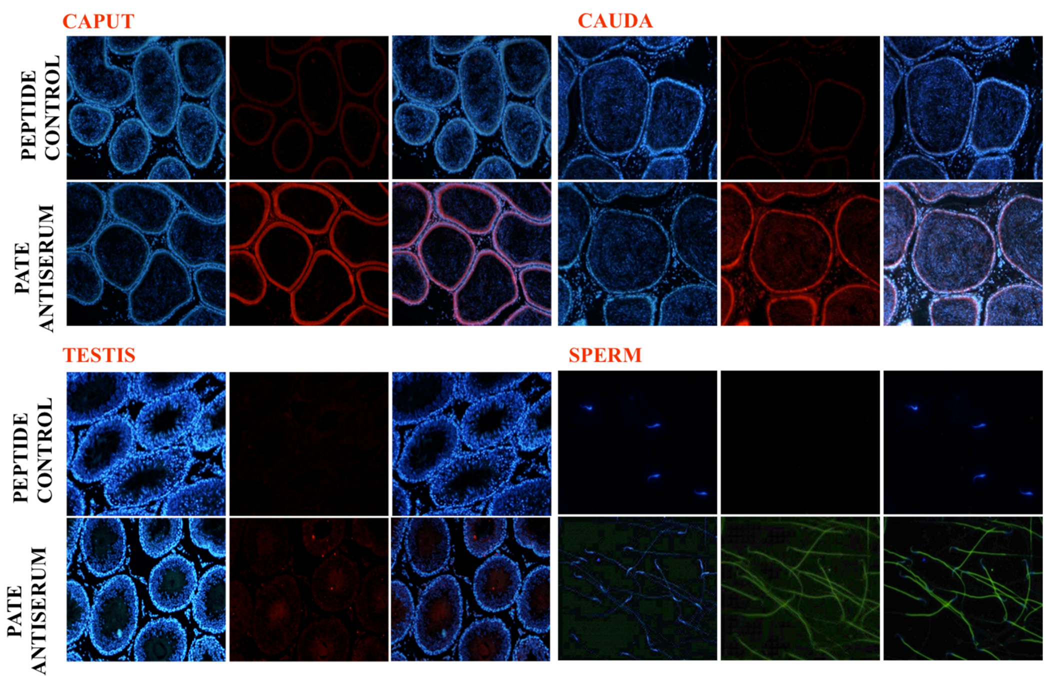

Figure 1.15. Immunolocalization of rat PATE. *Serial sections of the rat tissues or smears of spermatozoa were incubated with preimmune serum or immune serum against PATE. Localization was detected by incubating with secondary antibodies conjugated to TRITC and FITC.*

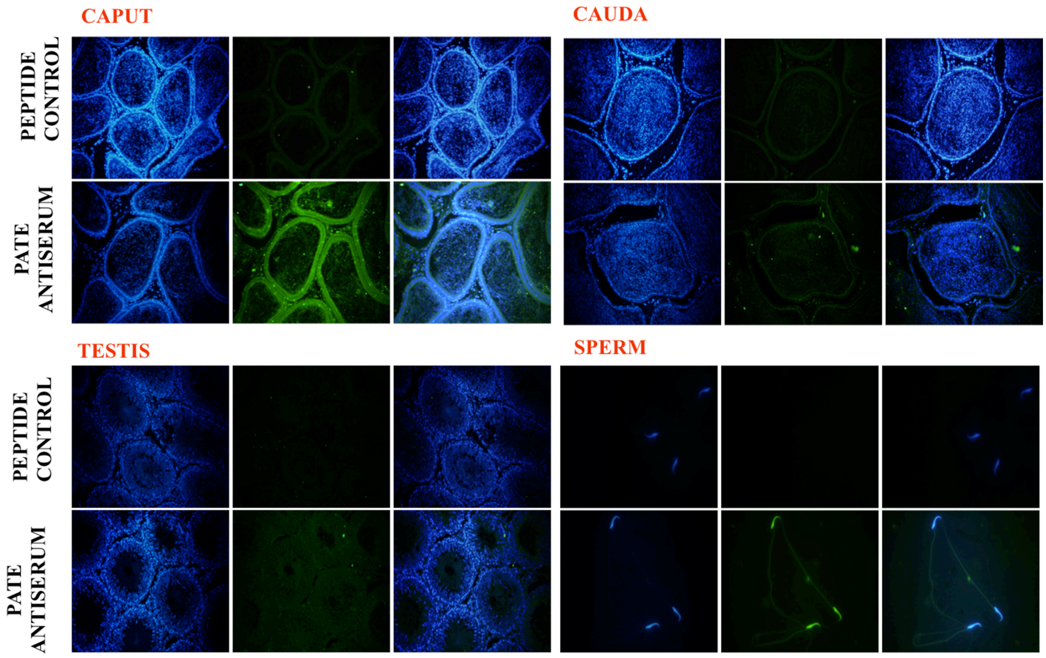

***Figure 1.16**. **Immunolocalization of rat PATE-F.** Serial sections of the rat tissues or smears of spermatozoa were incubated with preimmune serum or immune serum against PATE-F. Localization was detected by incubating with secondary antibodies conjugated to FITC.*

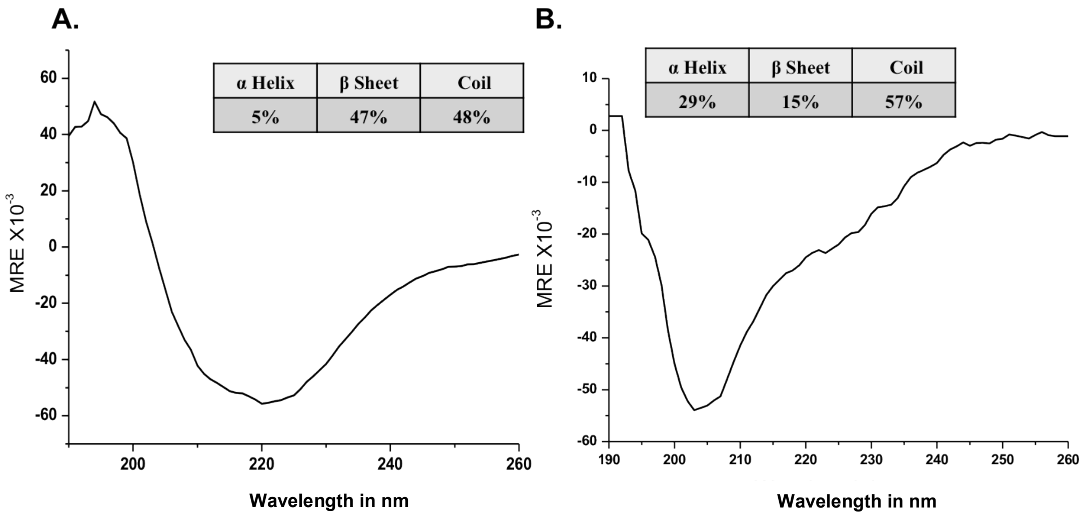

***Figure 1.17. Circular dichroism spectra of PATE and PATE-F.** rPATE and rPATE-F were dissolved in 10 mM phosphate buffer (pH 7.4) at room temperature and spectra was measured in AVIV Circular Dichroism Spectrometer. **A.** CD spectrum of rPATE. **B.** CD spectra of rPATE-F.*

DISCUSSION

Epididymal fluid is a complex mixture of proteins (Metayer *et al.,* 2002) that is thought to modify sperm surface during epididymal transit and maturation. PATE proteins are reported to be present in the male reproductive tract of humans and mouse. However, their role in this organ system still remains elusive. The expression pattern of *Pate* genes and proteins are not characterized in the rat. In this study, we analyzed their mRNA and protein expression to determine their possible roles in male reproductive tract function and immunity.

In silico analyses revealed that all the ten *Pate* genes identified in this study are clustered on chromosome 8 within a 2.5 kb segment present between the *Acrv1* and *Ddx25* genes. Similar organization of *Pate* genes is observed in the mouse and humans (Levitin *et al.,* 2008, Turunen *et al.,* 2011). Further, majority of the *Pate* mRNAs are encoded by three exons, whereas *Pate* and *Pate-2* are coded by five and four exons respectively. This is in agreement with the gene structure available at GenBank. Similar exon structure was reported in the human and mouse, wherein *Pate* and *Pate-2* were encoded by five and four exons respectively and the rest of the *Pate* genes are encoded by three exons, adding further evidence that these gene are highly conserved among the species. Sequence analyses of the alternate transcripts of rat *Pate* and *Pate-2* revealed that they encode for truncated proteins lacking the ten cysteine signature and. Could have functional roles that are different than their counterparts. Rat PATE proteins can be classified to the secreted Ly-6 family, because of their 10 cysteine signature and the presence of three fingered protein structure. The similarity of PATE and PATE-F three dimensional structures to the toxin bucandin a 63 amino acid neurotoxin isolated from the Malaysian krait (*Bungarus candidus*) (Torres *et al.,* 2001) gives a clue to understand the role of PATE and PATE-F in nerve function. The RMSD value (1.9) obtained when PATE and PATE-F 3D structures were superimposed is a clear indication that these proteins share structural similarity. PATE-Bucandin and PATE-F-Bucandin protein superimpositions resulted in RMSD values of 3.5 and 2.9 respectively; values that do not indicate a high degree of similarity between PATE proteins and Bucandin. In our analyses, we did not apply in depth the various factors (compactness, hydrogen bonding, percent secondary structure, principal component analyses, etc) that affect the RMSD and this could have resulted in high values.

To the best of our knowledge, we report for the first time the expression pattern of *Pate* mRNA transcripts in the rat. *Pate* gene expression is not restricted only to the prostate and testis, as indicated by their nomenclature. However, organ (epididymis) specific expression of rat *Pate* (*Pate-F and Pate-A*) was observed in our study similar to the mouse, wherein *Pate-F* and *Pate-Dj* were testis specific and *Pate-Q* and *Pate-P* were restricted to the placenta (Levitin *et al.,* 2008). On the same lines, the human *Pate-Dj* mRNA was detected only in the testis. Reproductive tract specific expression was also demonstrated for other genes such as *Spag11e* (Zhou *et al.,* 2004), DEFB118 (Liu *et al.,* 2001) and members of the HE2 family (Hamil *et al.,* 2002). The expression of rat *Pate* genes in non-reproductive and female reproductive tissues suggests that they may have functions beyond male reproductive tract physiology. Such observations were reported for the mouse and human *Pate* genes (Levitin *et al.,* 2008, Turunen *et al.,* 2011).

Hormonal regulation of a wide variety of genes due to fluctuations of androgens at various stages of development in the male reproductive system is well reported (Rodriguez *et al.,* 2001). Androgen levels in the epididymis of rat decline from birth until 20 days and a normal level of 10 ng/g tissue (35 nM) is maintained until approximately 40 days after which, the levels begin to increase to that of the adult, between 15–20 ng/g (Charest *et al.,* 1989). Serum testosterone levels in the young rat remain low and do not begin to increase to adult levels until 35–40 days of age (Nayfeh *et al.,* 1966). Absence of *Pate-A, Pate-F, Pate-N* and *Pate- Dj* in the epididymides during early development (20–30 days) correlates with reported low levels of androgens. *Pate-Q, Pate-F* and *Pate-A* in the adult rat (90 days old) was restricted to the caput. In the developing rat epididymis also, their expression was restricted to the caput in the 50 and 60 day old rats. The expression observed in the 30 and 40 day old animals could be due to the use of whole epididymides. It is possible that the expression of these genes could be restricted to the caput in the younger animals also. Presence of some of the *Pate* mRNA transcripts at all the stages of development indicated their androgen independent expression in the epididymis. Variation in testicular androgens during development in the rat is quite different from the epididymis. A steady increase in testosterone levels occurs in the rete testis of 30–130 day old rats (Harris 1974, Bartke 1981). Though majority of the *Pate* mRNA were not detected in the adult rat, we analyzed their expression in the testis to determine whether they are expressed in the developing rats and whether they have a possible role in testicular development. Among the *Pate* genes

(*Pate-N, Pate-C* and *Pate-2*), whose expression was barely detected in the adult rats, *Pate-C* and *Pate-N* were found to be expressed at very low levels in the testes of developing rats starting from day 30. The expression pattern of *Pate* transcripts in the testis is androgen dependent since it correlates with the minimal androgen levels from day 20 to day 40 and increased androgen in the adult (Charest *et al.,* 1989).

The role of androgens in governing *Pate* gene expression was evident since a down regulation was observed in the epididymidis of castrated rats and that DHT supplementation reverted the mRNA levels. *Pate* gene expression in relation to androgens was reported in the human and mouse. In the human dorsal prostate, PATE-B and PATE-E were found to be up regulated in castrated rats, whereas in the ventral prostate, no changes were observed for PATE-H under the same conditions (Levitin *et al.,* 2008). In the mouse, mixed responses in *Pate* gene expression was observed in the initial segment, caput and proximal epididymis of gonadectomized mice (Turunen *et al.,* 2011). Androgen regulation of *Pate* genes seem to vary among the species and in the organs within species, suggesting a more complex network of regulatory mechanisms that may include the testicular factors.

PATE and PATE-F proteins were found to be abundantly localized in the male reproductive tract and on the spermatozoa. Similar PATE protein expression in the reproductive tract and on the sperm is reported in the mice and humans (Bera *et al.,* 2002, Levitin *et al.,* 2008, Turunen *et al.,* 2011) implicating that they may have similar functions. Further, the exact role of PATE proteins in the male reproductive tract remains elusive, though their possible role in calcium transport to regulate acrosome reaction is reported (Coronel *et al.,* 1992). In this study, PATE was found to be predominantly localized on the sperm tail, whereas PATE-F was restricted to the sperm head. Human PATE and PATE-B were found to be localized only on the sperm head (Levitin *et al.,* 2008, Turunen *et al.,* 2011). The presence of PATE-F specifically on the sperm head suggests that it may be involved in fertilization, whereas PATE localization on the tail sperm surface may contributes to motility.

Based on the results of this study, we conclude that *Pate* genes are abundantly expressed in the male reproductive tract and are androgen dependent. Their presence on the sperm implicates a role in sperm function.

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INTRODUCTION

Technological advances have increased knowledge of gene expression profiles in reproductive system. EST databases and microarray studies have suggested reproductive tract specific expression of several genes, and these have subsequently been confirmed with *in vitro* and *in vivo* methods. However, mere gene expression profile is rarely enough to reveal the *in vivo* significance of a gene (Lawrence *et al.,* 2000). Therefore, experiments to study individual genes and proteins in a living organism are needed. Comparison of the electrophoretic profiles from epididymis and other body fluids suggest that many proteins present in epididymal fluid were not found in other secretions and might be tissue specific. Further comparative studies with epididymal fluid from different species confirmed that the epididymal proteins are both tissue and species specific. Such proteins are responsible for the array of changes undergone by spermatozoa during maturation (Holland *et al.,* 1998). Thus a complete characterization of all the proteins secreted by the epididymis will give important information for understanding the molecular events of sperm maturation.

Spermatozoa produced in the testis are immature and lack fertilizing ability and undergo maturation during their passage through the epididymis. This process is facilitated by a number of proteins secreted by the epididymal epithelial cells in to the luminal fluid, which then bind on to the sperm surface (Ducheux *et al.,* 2003). Further, a variety of factors secreted in the seminal vesicles and prostate also aid in the formation of functional spermatozoa (Peitz, 1988, Peitz and Olds-Clarke, 1986, Robert and Gagnon, 1996). Defensins (Yenugu *et al.,* 2004, Yenugu *et al.,* 2006, Travis *et al.,* 2000), members of the sperm associated antigen 11 family (Hamil *et al.,* 2000), lysozymes (Zhang *et al.,* 2005, Mandal *et al.,* 2003), lipocalins (Hamil *et al.,* 2003), cathelicidins, protease inhibitors (Hamil *et al.,* 2002, Blankenvoorde *et al.,* 1998, Hiemstra *et al.,* 1996), inhibitors of complement lysis (Collard and Griswold, 1987, Griswold *et al.,* 1986), and the cysteine rich proteins such as CRISPs (Jalkanen *et al.,* 2005, Luo *et al.,* 2012) and members of the prostate and testis expressed (PATE) family (Bera *et al.,* 2002, Soler-Garcia *et al.,* 2005, Levitin *et al.,* 2008, Turunen *et al.,* 2011) are some examples of a wide range of proteins that bind to the sperm surface (Hall *et al.,* 2002).

Though few studies have reported that the PATE proteins are localized in the male reproductive tissues and spermatozoa, their precise function is not yet known

(Soler-Garcia *et al.,* 2005, Turunen *et al.,* 2011, Margalit *et al.,* 2012, Levitin *et al.,* 2008). Our studies indicated that the rat PATE and PATE-F proteins are abundantly expressed in the male reproductive tract and on the sperm (Rajesh and Yenugu, 2012). PATE proteins are known to display neuromodulatory activity (Levitin *et al.,* 2008) and inhibition of calcium uptake in the spermatozoa (Coronel *et al.,* 1992).

Spermatozoa must undergo acrosome reaction and capacitation in order to fertilize the ovum. These complex processes involve changes in calcium dynamics and reorganization of proteins and lipids on the sperm membrane (Rahman *et al.,* 2014). Further, a variety of factors produced in the male and female reproductive tracts contribute to the acrosome reaction and capacitation. Epididymal and testicular proteins contribute to sperm maturation and function (Chan and Zhang, 2005). For example, BIN1b, a β-defensin like protein, contributes to the calcium mediated initiation of sperm motility (Zhou *et al.,* 2004). The bovine actin-binding protein is involved in intracellular signaling and membrane modeling during epididymal sperm maturation and acrosome reaction. (Howes *et al.,* 2001). Cystatin-related epididymal spermatogenic protein, p14, nerve growth factor and MUPP1 are some of the few examples of sperm bound proteins that are involved in capacitation and acrosome reaction of caprine spermatozoa (Nandi *et al.,* 2012, Chau and Cornwall, 2011, Jin *et al.,* 2010, Ackermann *et al.,* 2008). Though the human PATE proteins were found to be involved in sperm-oolemma fusion and penetration but not sperm-zona binding (Margalit *et al.,* 2012), their role in sperm maturation and capacitation are not yet reported. In this study, we analyzed the role of two rat PATE proteins, namely, PATE and PATE-F in sperm function.

METHODS

Antibacterial assay

Colony forming units (CFU) assay was performed to test the antibacterial activity (Yenugu *et al.,* 2003). *E.coli* grown to mid-log phase (A_{600}=0.4-0.5) diluted with 10 mM sodium phosphate buffer (pH 7.4) was used in the assay. Varying concentration of rPATE and rPATE-F proteins (10-100 µg/ml) was added approximately 2×10^6 CFU/ml of bacteria and incubated at 37˚C for 30-120 min. After incubation, the assay mixtures were serially diluted with 10 mM sodium phosphate buffer (pH 7.4) and 100 µl of each was spread on a Luria-Bertani agar plate and incubated at 37˚C overnight to allow full colony development. The resulting colonies were hand counted and plotted as log CFC/ml.

Triple staining for bacterial viability and membrane permeability

A triple-staining method was performed to visualize the total number and live *E. coli* cells with membrane permeabilization induced by recombinant protein on bacteria (Maria Luisa Manogni *et al.,* 2004). The three dyes DAPI (to stain all bacterial cells), CTC (to stain live-metabolically active bacteria) and FITC (to detect dead bacteria with membrane disruption) are used. Log phase bacteria were incubated with rPATE and rPATE-F in 10 mM phosphate buffer PH 7.4 for 2 h at 37˚C with agitation. After protein treatment, the bacterial cells were incubated with 900 µl of CTC (5 mM in PBS) for 90 min at 37^0C with agitation. The cell suspension was poured on to poly (L-lysine) coated glass slides and the plates were kept at 30˚C for 45 min to allow the adhesion of *E. coli* cells to the glass slides. The plates were then washed with sodium phosphate buffer, and DAPI solution (10 µg/ml in PBS) was added to cells. After 30 min at 30˚C, the DAPI solution was removed and the slides were washed with sodium phosphate buffer. FITC solution 6 µg/ml in sodium phosphate buffer was added and incubated for 30 min in 30˚C. The slides were then washed and Phase-contrast images as well as images under fluorescence light were recorded using a Zeiss Axioplan 2 optical microscope equipped with an oil-immersion objective (100X).

Measurement of bacterial membrane potential and permeability

Membrane potential (MP) of bacteria measured accurately by using two different dyes $DiOC_2(3)$ and TO-PRO-3. Assay uses the ratio of red to green fluorescence of

$DiOC_2(3)$ (David J. Novo *et al.,* 2000). The green fluorescence of $DiOC_2(3)$ due to emission from single dye molecules depends on bacterial cell size or clump but independent of MP, where as red fluorescence is due to emission from dye aggregates and depends on both size and MP. So the ratio provides a cell size-independent measure of MP. Far red (>695 nm) fluorescence of TO-PRO-3, which is permeable to dead bacteria, is an indicator of membrane permeability. Normalizing TO-PRO-3 fluorescence with the green $DiOC_2(3)$ fluorescence signal produces discrimination between cells with impermeable and permeable membranes. Bacteria were diluted in sodium phosphate buffer pH 7.4 to a final concentration of 10^6 to 10^7 per ml and then incubated with 100 μg of rPATE and rPATE-F or streptomycin (0.5 μg/ml) or CCCP (15 μM) for 2 h in shaking incubator at 37˚C. Cells were then washed in phosphate buffer and incubated with 30 μM DiOC2(3) and 100 nM TO-PRO-3 for 5 min at room temperature. Then bacterial cells were washed thrice with same buffer and samples were analyzed in a flowcytometer (BD LSR Fortessa).

LPS binding assay

LPS from *E. coli* was dissolved in sterile water to a final concentration of 40 μg/ml and transferred 50 μl to a 96-well microplate and air-dried at room temperature overnight. The plates were then incubated at 60°C for 30 min to fix the ligands, and the wells were blocked with 1 mg/ml BSA in PBS at 37°C for 2 h. After washing four times with PBS-T, added different concentrations (10, 25, 50, 100 μg/ml) of recombinant PATE or PATE-F or EP2E. After incubation at room temperature for 2 h, the wells were washed three times with PBS-T and blocked wells with 1% skimmed milk at 37°C for 2 h and added polyclonal antibody (1:250 dilutions) corresponding to each recombinant protein and incubated for 2 h at room temperature. Washed thrice with PBS-T and incubated with HRP conjugated anti-rabbit secondary antibody (1: 3000) for 2 h at room temperature, washed, and reacted with 75 μl of 0.4 mg/ml O-phenylenediamine in 51.4 mM Na_2HPO_4, 24.3 mM citric acid, and 0.045% H_2O_2 (pH 5.0) at 37°C for 20 min. Subsequently, 25 μl of 2 mM H_2SO_4 was added to each well to terminate the reactions, and absorbance at 492 nm was monitored by a micro plate reader. As a negative control, BSA at the same concentrations was treated similarly.

Scanning electron microscopy

E.coli cells were grown in LB broth to exponential phase, harvested by centrifugation, washed twice with 10 mM sodium phosphate buffer and resuspended in the same buffer. Approximately 10^8 CFU/ml cells were incubated at 37^0C for up to 2 h with 100 µg of rPATE or rPATE-F proteins. Cells were washed and resuspended in 10 mM sodium phosphate buffer (pH 7.4) and were fixed with an equal volume of 4% glutaraldehyde. Then the cells were dehydrated through a graded series of ethanol (30, 50, 75 and 100%) and finally resuspended in acetone. Cells were poured and dried on to glass slides of 1x1 cm length. The slides were mounted on aluminum specimen supports with carbon adhesive tabs, and coated with 15 nm thickness of gold-palladium metal (60:40 Alloys). Samples were examined on ESEM XL-30 scanning electron microscope using an accelerating voltage of 20 kV.

***In vitro* and *in vivo* endotoxin treatments**

The effect of endotoxin challenge on *Pate* expression was investigated *in vitro* and *in vivo*. For the *in vitro* experiments, caput, cauda and testis from adult rat were dissected and cut into two longitudinal halves. One half of the tissue was used as control, and the other was treated with LPS. Tissue were transferred to 2 ml nutritive media (136.89 mM NaCl, 5.63 mM KCl, 180 mM NaH_2PO_4, 14.88 mM $NaHCO_3$ and 5.55 mM glucose pH 7.6-7.8) and cultured at 30˚C with aeration. After 15 min of incubation, tissues were transferred to nutritive solution with or without LPS (1µg/ml) and incubated for 0-9 h. During these incubations nutritive solution with or without LPS were renewed every 30 min. The tissues were collected, rinsed with PBS, frozen in liquid nitrogen and stored in -80˚C until use.

For the *in vivo* LPS challenge, adult male Wistar rats (90-days old), maintained on a 12L:12D lighting schedule, at 22-25˚C, with food and water ad libitum, were injected intraperitoneally with LPS (1 mg/g body weight) or saline (control). LPS dose and site of injection was chosen based on previous reports (Biswas and Yenugu, 2011). Rats were sacrificed at 3, 6, 9, 15 and 24 h after LPS treatment. Testis, caput and cauda epididymis and seminal vesicle were identified, stripped of connective tissues, frozen in liquid nitrozen, and kept at -80˚C until use.

Assessing the role of PATE and PATE-F proteins on sperm functions

Numerous reports have demonstrated that a variety of proteins are expressed on the surface of mammalian spermatozoa are involved in the process of fertilization (Talbot *et al.,* 2003). Our previous studies indicated that both *Pate* and *Pate-F* proteins are produced from epididymal epithelium and attached to the sperm surface (Rajesh and Yenugu, 2012). Till date no studies were conducted to evaluate role of PATE proteins on sperm capacitation and acrosome reaction. We adopted three different approaches to determine role of PATE and PATE-F proteins in male reproduction.

1. Neutralization of PATE / PATE-F on sperm *in vitro* using antiserum raised in Rabbit.
2. Generating autoantibodies to block endogenous PATE / PATE-F *in vivo*.
3. Knock down *Pate / Pate-F* mRNA using shRNA.

Sperm cell preparation

Spermatozoa were collected from the cauda epididymides of male wistar rats and minced in 10 ml of M2 medium and incubated in 5% CO_2 at 37˚C for 10 min to allow sperm to swim out. After spermatozoa were released from the cauda epidldymides, they were capacitated in M2 medium; at 37°C in 5% CO_2 and 97% air for 5 h. For induction of acrosome reaction, capacitated sperm were induced with 10 μM calcium ionophore A23187 and incubated at 37°C, 5% CO_2 and 95% air for 30 min.

Surface PATE protein expression by indirect immuno fluorescence

To understand the distribution of PATE and PATE-F proteins on sperm surface during capacitation and acrosome reaction, immuno fluorescent staining was performed in fixed or live spermatozoa. Spermatozoa were capacitated and acrosome reacted *in vitro* and smeared on poly lysine coated slides (0.05 mg/ml) and fixed in 2% p-formaldehyde for 10 min at room temperature. Slides were extensively washed in PBS and spermatozoa were incubated with 5% goat serum in PBS for 30 min at room temperature. Then spermatozoa were incubated with rabbit anti PATE or PATE-F antiserum (1:100 in PBS) over night at 4°C. Rabbit pre-immune serum and PATE or PATE-F antiserum pre-absorbed with corresponding recombinant proteins were used as controls. After washing three times with PBS, PBST (0.1% tween in pBS) and PBS for

10 min, spermatozoa were incubated with FITC conjugated goat anti-rabbit IgG (1:200) for 40 min at room temperature. Then slides were washed with PBS, PBST and PBS, mounted with anti-fade containing DAPI solution (5 μg/ml) and sealed with nail polish.

For staining live spermatozoa, control, capacitated and acrosome reacted sperm were taken in 1.5 ml tubes and washed with PBS-BSA (4 mg/ml) in 500g at 37°C for 5 min and resuspended in 200 μl of 1:50 diluted anti PATE or PATE-F antiserum in PBS-BSA and incubated for 1 h at room temperature. Then spermatozoa were washed thrice with PBS-BSA and resuspended in 1:100 diluted (PBS-BSA) FITC conjugated goat anti-rabbit IgG for 30 min at room temperature. Then washed thrice with PBS-BSA and resuspended in PBS containing 5 μg/ml DAPI solution. 10 μl of the suspension was taken on slide and mounted with antifade and observed under fluorescent microscope.

1. Neutralization of PATE/PATE-F on sperm *in vitro* using antiserum raised in rabbit.

Cauda epididymides of adult male wistar rats were minced in 10 ml of M2 medium. To test the effect of PATE and PATE-F antibodies on capacitation and A23187-induced acrosome reaction, spermatozoa were resuspended in M2 medium containing PATE or PATE-F antiserum (1:100 dilution), or pre-immune sera (1:100 dilution) for 5 h. Spermatozoa were then treated with the ionophore A23187 (10 μM final concentration) at 37°C, 5% CO_2 and 95% air for 30 min to induce acrosome reaction. Solvent control containing equal amount of DMSO that was used to dissolve the ionophore were included in the assays. The effect of PATE or PATE-F antiserum on capacitation and acrosome reaction was measured by microscopy (CTC, flilipin and PNA-FITC staining), Western blotting (sperm protein tyrosine phosphorylation) and flow cytometry (intracellular calcium concentration by staining with Fluo-3, AM and the binding of PNA-FITC to sperm surface).

Evaluation of sperm capacitation and acrosome reaction by CTC staining assay

Following incubation with antibodies, an aliquot (50 μl) of the sperm suspension was treated with an equal volume of CTC solution for 30 sec, followed by the addition of 8 μl of 12.5% paraformaldehyde in 0.5 M Tris–HCl (pH 7.4). The CTC solution was prepared using 750 μM CTC, 5 mM DL-cysteine, 130 mM NaCl, and 20 mM tris (hydroxymethyl) aminomethane (pH 7.8). Ten microliter of the fixed sperm suspension was placed on a glass slide and mounted with one drop of antifade solution. 100 to 200 spermatozoa on each preparation were assessed under fluorescent microscope in DAPI region. Three kinds of CTC staining patterns were identified: an F pattern, with uniform fluorescence over the whole head (uncapacitated spermatozoa); a B pattern, with a fluorescence-diminished band in the postacrosomal region and a relatively bright fluorescence in the acrosomal region (capacitated spermatozoa); and an AR pattern, with almost no fluorescence over the whole head except for a thin band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa).

Evaluation of sperm capacitation by filipin staining

Filipin binds to cholesterol with high affinity and can be exploited to assess the cholesterol distribution in the sperm membrane. Spermatozoa were fixed in 4% p-formaldehyde washed twice in PBS followed by incubation with 50 µg/ml filipin for 30 min at room temperature to stain the membrane cholesterol. Spermatozoa stained with filipin were smeared on glass slides and observed under fluorescence microscope with excitation and emission wavelength of 380 nm and 510 nm respectively (Barboni *et al.,* 2011). Decreased fluorescence intensity indicates efflux of cholesterol from the sperm membrane. The spermatozoa are compared with uncapacitated spermatozoa.

Evaluation of sperm capacitation by detecting tyrosine phosphorylation

The sperm pellet was washed twice with 1 ml of PBS and resuspended in Laemmli sample buffer with β-mercaptoethanol (to a final concentration of 2.5%) and vortexed for 15 sec, centrifuged at 16,000 g for 10 min to remove insoluble material. Sperm proteins were separated by electrophoresis on 10% polyacrylamide-SDS gels, followed by transfer to a PVDF membrane. Non-specific binding to membranes was blocked in 5% BSA for 1 h at room temperature. Blots were incubated with the primary monoclonal anti-phosphotyrosine antibody, (1:1000, clone 4G10, Millipore, USA), followed by incubation with goat anti-mouse secondary antibody conjugated with horseradish peroxidase at 1:3000 dilution and the specific signals were detected by ECL (Western blotting detection reagents). To ensure that equal amounts of protein were loaded in each well of the gel, the same membrane was reprobed with a monoclonal anti-tubulin antibody using the same procedure.

Evaluation of sperm acrosome status by PNA-FITC staining

Peanut agglutinin (PNA) binds to the glycoproteins present on the sperm membrane and acrosomal matrix. During acrosome reaction there is loss of acrosomal membrane matrix, leading to reduced fluorescence, which can be used to assess acrosome reaction. To determine the role of PATE or PATE-F proteins on acrosome reaction, caudal spermatozoa were allowed to capacitate in M2 medium in the presence of PATE or PATE-F antiserum, after which acrosome reaction was induced by addition of 10 µM ionophore (A23187) for 30 min. Spermatozoa were then smeared on microscopic glass slides, air dried and incubated in absolute methanol for 15 min. Methanol-treated smears were incubated with FITC conjugated PNA (40 µg/ml) for 30 min at room temperature. The slides were then washed in PBS for 15 min to remove excess unbound probe. After drying, smears were examined immediately using a fluorescence microscope (DasGupta, Mills, and Fraser 1993; Mendoza *et al.,* 1992). Spermatozoa incubated with PBS were used as un-capacitated control.

Measurement of intracellular calcium concentration

The intracellular calcium ion concentration, $[Ca^{2+}]i$, of sperm was determined with fluo-3, AM by flow cytometry. Briefly, acrosome reacted spermatoza (treated with immune and pre-immune sera) were loaded with fluo-3, AM (10 mM) at 37°C for 20 min. After incubation, spermatozoa were washed twice with PBS at 500 g for 5 min to remove any free fluo-3, AM and $[Ca^{2+]}i$ content was analyzed by flow cytometry. The fluorescence of fluo-3 was excited at 488 nm and measured with a 515– 540 nm filter. The fluorescence intensity of spermatozoa was quantified for 10,000 individual sperm cells.

Evaluation of acrosomal membrane status by flow cytometry

This assay is based on the binding of FITC labeled PNA (PNA-FITC) to the glycosylated proteins of acrosomal region. As acrosome reaction proceeds, there is loss of acrosomal membrane proteins leading to reduced PNA binding. PNA conjugated to FITC was used to assess the extent of binding and the intensity of fluorescence is a direct estimate of the amount of PNA bound to sperm surface. The subpopulation of spermatozoa that undergo acrosome reaction will display decreased fluorescence intensity, which is quantified in terms of percentage in a flow cytometer. Spermatozoa ($5x10^6$ cells/ml) treated with or without PATE or PATE-F antiserum (1:100) or pre-immune serum was subjected to acrosome reaction with ionophore A23187 (10 μM final concentration). They were then washed with PBS and stained with PNA-FITC (50 μg/ml) to stain the acrosomal region. The decrease in the intensity of fluorescence over a period of time is a direct measure of the population of spermatozoa undergoing acrosome reaction. Fluorescence intensity was measured in a flow cytometer with excitation and emission at 495 and 515 nm respectively. The fluorescence intensity of sperm was quantified for 10,000 individual sperm cells.

2. Generating autoantibodies to block endogenous PATE / PATE-F *in vivo.*

Immunization schedule to generate autoantibodies

The immunization schedule is shown below (Figure 1). Male Wistar rats (n=4) aged 90 days were immunized with recombinant PATE or PATE-F protein. 100 μg of the recombinant protein in 50 μl was mixed with equal volume of Freund's complete adjuvant and administered intraperitoneally at multiple sites. Booster doses containing 100 μg of the recombinant protein mixed with Freund's incomplete adjuvant were given 3, 14, 28, 49 and 91 days after first immunization (Figure 1). Antibody titer was checked one week after the third booster dose. The last two immunizations were given one week prior to each mating. To determine the antibody titer, blood was collected from the tail vein prior to the antigen administration (day 0) and on day 35 (one week after last immunization). Spermatozoa were obtained from these animals after 2nd mating to assess acrosome reaction and for computer assisted sperm analyses.

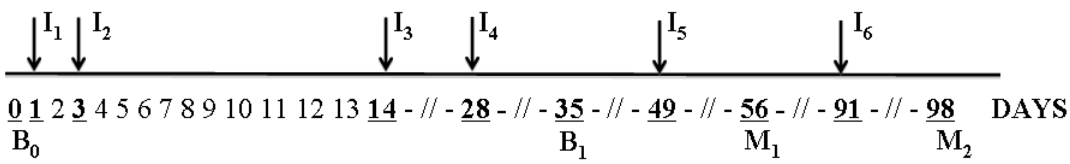

Figure 1. Immunization of rats with PATE proteins. *Male Wistar rats (n=4) were immunized with PATE or PATE-F protein mixed with Fruend's adjuvant. Bold and underlined numbers indicate the day on which a procedure was conducted. Arrows indicate the day on which the animals were immunized.* I_1, I_2, I_3, I_4, I_5 *and* I_6 *indicate immunizations.* B_0 *and* B_1 *– blood sample collection for pre-immune serum and antiserum respectively.* M_1 *and* M_2 *– matings after immunization.*

Collection of epididymal and testicular fluids

Tissue fluids were collected at the end of the second mating experiment. Control and PATE or PATE-F immunized rats were anesthetized and epididymis and testis removed by careful dissection. Caput, cauda and testis were separated and the tissue fluid was collected into a microtube by making incisions and squeezing. The fluids were centrifuged at 10,000 rpm for 10 min at 4˚C. The supernatant was quantified for protein concentration and equal quantity was used for measuring the antibody titer using ELISA.

Enzyme linked immunosorbant assay (ELISA)

The wells of microtitre plates were coated with 40 μg/ml of PATE or PATE-F recombinant protein and incubated at 37˚C and 60˚C overnight and 30 min respectively. The plates were blocked with 1 mg/ml BSA for 2 h and incubated with different dilutions of pre-immune or PATE or PATE-F antiserum or testicular fluid or epididymal fluid for 3 h followed by four washes with TBS-T (PBS with 0.1% Tween-20). They were then incubated with HRP conjugated secondary antibody (1:10000 dilution). After thorough washing, O-Phenylenediamine (OPD) was added and the intensity of the color developed was measured at 490 nm, which is a direct measure of the antibody titer.

Computer assisted sperm analysis (CASA)

The motility parameters of spermatozoa isolated from control and PATE or PATE-F immunized rats were assessed by CASA. Briefly, the spermatozoa diluted in capacitation medium were placed in a microscopic chamber slide maintained at 37°C and data (path velocity (VAP), progressive velocity (PV), track speed (TS), lateral amplitude (LA), beat frequency (BF), straightness (STR) and linear velocity (LV)) acquired with the following settings: frames acquired - 50; frame rate – 60 Hz; minimum contrast - 25; minimum cell size (pixels) - 3; low average path velocity cut off - 7.5 μm/sec; medium average path velocity cut off - 12.5 μm/sec; low straight line velocity cut off – 5 μm/sec; static head intensity limits - 0.2–1.47; static head-size limits - 0.12–7.37; static elongation limits - 1–98; magnification - 1.43 (4x); video frequency – 60 Hz; bright field - off. Spermatozoa with data point's ≥ 15, VCL >300 μm/sec, LIN <40%, ALH >12 μm were considered as hyper activated.

Evaluation of sperm capacitation and the acrosome reaction

We adopted protein tyrosine phosphorylation to measure sperm capacitation in control and immunized animals as already mentioned. For the induction of acrosome reaction, capacitated sperm were treated with 10 µm ionophore and incubated for 30 min. Sperm acrosome reaction was measured by staining sperm with Fluo-3, AM (binds to intracellular calcium) and PNA-FITC (binds to acrosomal membrane) using flow cytometry as described earlier.

Assessment of fertility

The fertility of the immunized animals was analyzed by natural mating. Rats that had displayed serum antibody titer were used for mating. One week prior to mating, a booster dose of the antigen was given (Figure 1). Each male rat was mated with two normal female rats. The resulting litter size was noted.

3. Knock down *Pate / Pate-F* mRNA using shRNA.

Cell culture and shRNA transfection

pGFP-V-RS vectors containing shRNA specific to rat *Pate* gene were commercially synthesized from OriGene technologies (Cat No: HC131280), USA. Two 29 nucleotide long shRNA sequences targeting the *Pate* are HT131280A: GAGGAATATGTACTGCAACAGTAGAAGAG (sh1 targeting 187-216 nucleotides), HT131280B: GCTATCTGGTGGACTTCAGATGCTGCCGG (sh2 targeting 319-348 nucleotides). One plasmid containing scramble negative control (Cat No: TR30013) was also synthesized. To confirm the knockdown efficiency, *Pate* sh1 and *Pate* sh2 plasmids were co- transfected with a pCDNA 3.0 vector expressing *Pate* into HEK 293 cells. The knock-down efficiency was determined by real-time PCR and Western blotting of extracts from cells 48 h after transfection.

DNA–lipid mixture and *in vivo* gene transfer

Rats were anesthetized with an intraperitoneal injection of Ketamine/Xylagine mixture (60 mg/kg body weight, 10 mg/kg body weight respectively) and the scrotal sac covering the testis and epididymis of male wistar rats (300, 350 g) cut open under anesthesia, the cauda epididymis with tunica was exposed out to inject shRNA and lipid mixture. The lipid FuGENE® 6 was employed as gene transfer medium. *Pate* sh1, sh2 (0.5 µg each) plasmids and FuGENE were mixed in 1:6 ratios and resuspended in 50 µl

Optimem medium. From the mixture, 2.5 µl (50 ng/cauda) solution was gently injected into the lumen of the cauda epididymis using 10 µl Hamilton syringe. The number of males injected with *Pate* shRNA was 9. Injected animals were kept for mating after 5 days and mated animals were then analyzed for sperm capacitation and acrosome reaction. Rats were injected with scrambled shRNA plasmid and untransfected animals were used as controls in this experiment.

Efficeiency of shRNA transfection as measured by TGFP mRNA and protein expression in cauda

Control and transfected animals were asphyxed with CO_2 and cauda tissues were immediately removed and frozen in liquid nitrogen. Total RNA was isolated using Trizol reagent. cDNA was synthesized from 2 µg total RNA in the presence of reverse transcriptase and oligo dT primers at 42°C for 30 min. One microliter of the cDNA was used for PCR amplification of *Tgfp* (Primer sequences were: *Tgfp* F.P 5'ATCCGGGGTCTTGAAGGCGTGCT3', *Tgfp* R.P 5'GGGCTACGGCTTCTACCAC TTCG3') and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) with gene specific primers using Taq DNA polymerase. Amplification was performed with a 2 min start at 94ºC, denaturation at 94°C for 30 sec, annealing at 60 to 60°C for 30 sec and extension at 72°C for 30 sec for 32 cycles, followed by a final extension at 72°C for 5 min. PCR amplicons were then analyzed in 2% agarose gels.

Control and transfected cauda epididymis were dissected, divided two pieces and treated as follows: (a) one piece was fixed in 4% paraformaldehyde overnight and washed and placed in 15, 30% sucrose solutions for 1 h each and washed with PBS and immersed in cryomedium. 5 µm cryosections were prepared by using Leica CM1860 cryostat and sections were mounted on to Lysine coated slides. These sections were stained with DAPI and mounted with Vectashield anti fade and observed under fluorescent microscope for TGFP expression. (b) Other piece, cauda tubules were mounted on slides and observed in low magnification objective lens for TGFP expression. Slides were observed and photographed in a fluorescence microscope at 10X magnification to capture tubule fluorescence.

Detection of PATE knock-down by RT-PCR and flow cytomerty

Polymerase chain reaction and flow cytometry were performed to determine the efficiency of *Pate* knockdown in transfected cauda and sperm samples collected after 7 days of transfection. Total RNA was isolated from control, scrambled and shRNA transfected animals using Trizol. cDNA was synthesized from 2 μg of total RNA using Invitrogen reverse transcription kit. PCR amplification for *Pate* was done using following primers F.P: 5'ATGTTCAAGCCCCACTTACTGAG3', R.P: 5'CTAGAAA AAGAATGCTTTCCCTGTGAC3'. Amplification was performed with a 2 min start at 94ºC, denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec for 32 cycles, followed by a final extension at 72°C for 5 min. PCR amplicons were then analyzed in 2% agarose gels.

Control and shRNA transfected rat spermatozoa were stained with PATE anti serum at a final concentration of 1:100 and as s negative control, PATE anti serum was replaced with pre-immune serum at same dilution. The samples were incubated for 1h at room temperature and washed thrice with PBS containing 4 mg/ml bovine serum albumin. Then spermatozoa sample were resespended in PBS containing FITC-conjugated goat anti-rabbit IgG at a dilution of 1:200 and incubated for 45 min at room temperature. After three washes with PBA-BSA, samples were analyzed on a flow cytometer (BD-FACS-FORTESSA). 10,000 individual spermatozoa were analyzed per sample for FITC fluorescence. Aliquots of the above samples were also analyzed in fluorescent microscope to check the staining pattern.

Evaluation of capacitation and the acrosome reaction

We adopted protein tyrosine phosphorylation to measure sperm capacitation in control and transfected animals as already mentioned. For the induction of acrosome reaction, capacitated spermatozoa from control and transfected animals were treated with 10 μm ionophore and incubated for 30 min. Acrosome reaction was measured by staining spermatozoa with Fluo-3, AM (binds to intracellular calcium) and PNA-FITC (binds to acrosomal membrane) using flow cytometry as described earlier.

Fertility

The mating experiments were performed 5 days after transfection of shRNA and each male rat was mated with 2 normal females. After 2 days, males were separated from cages and sampled for determining knockdown efficiency and measuring sperm functions. After birth, the number of viable off spring in a litter was recorded.

Statistical analyses

Statistical analyses were performed using Holm-Sidak test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are Mean ± S.D. * indicates $p<0.05$.

RESULTS

Antibacterial activity

Proteins with cationic nature or rich in cysteine content are known to exhibit antimicrobial activity. Because of their cationic nature (pI - 9.0 for PATE and pI - 8.54 for PATE-F) and high cysteine content they might be expected to exhibit antimicrobial activity. PATE at higher concentrations (50 and 100 μg/ml) exhibited bacterial killing activity, whereas PATE-F even at a concentration of 100 μg/ml did not display any antibacterial activity (Figure 2.1).

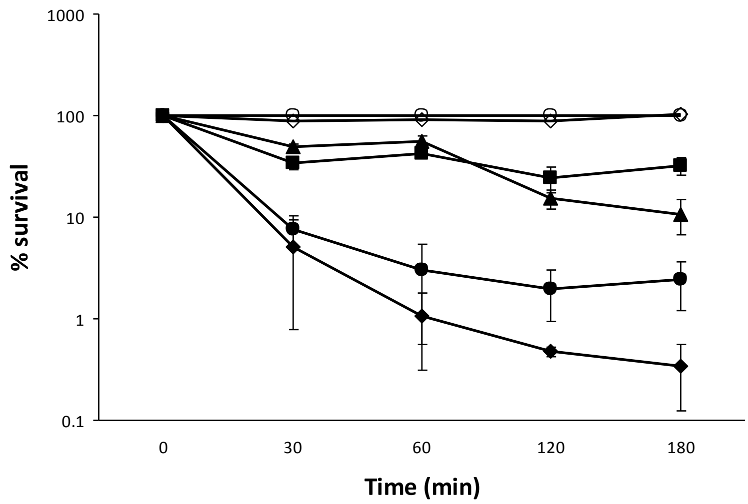

Figure 2.1. ***Antibacterial activities of recombinant PATE and PATE-F proteins and their response to endotoxin challenge.*** *Mid-log phase E. coli were incubated with 0 (○), 10 (■), 25 (▲), 50 (●) and 100 (♦) μg/ml rat recombinant PATE or PATE-F (◊) proteins for 0–180 min. Values shown are Mean ± S.D.*

Membrane permeability

Triple staining was performed using three different dyes (FITC, CTC and DAPI), to understand mechanism of action of rPATE or rPATE-F. CTC stains the live and metabolically active bacteria, FITC stains dead bacteria with permeable membrane, where as DAPI stains all bacteria. In *E.coli* treated with 100 μg of recombinant protein and incubated for two hours, FITC was accumulated in bacteria treated with rPATE, indicating that rPATE kills bacteria by increasing membrane permeablization. Where as bacteria treated PATE-F or bacteria without any protein treatment stained with CTC, indicating intact membrane. Phase contrast and DAPI staining visualized all bacterial cells irrespective of treatments (Figure 2.2).

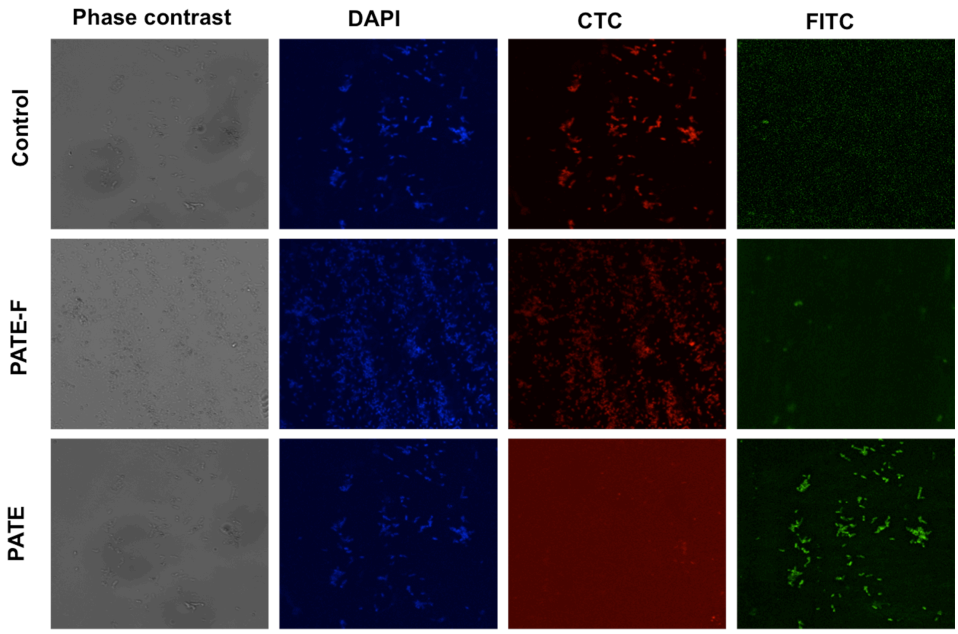

Figure 2.2. ***Bacterial membrane permeabilization.*** *E.coli cells were grown to mid log phase and treated with 100 µg/ml of rPATE or rPATE-F and stained with 5 mM CTC, 6 mM FITC and 10 µg/ml DAPI. Control - un treated bacteria; PATE-F - bacteria treated with recombinant PATE-F; PATE-bacteria treated with recombinant PATE.*

Bacterial membrane potential

Metabolically active bacteria with intact cytoplasmic membranes maintains difference of electrical potential across the membrane, with the interior negative, with respect to the exterior. A reduction in the magnitude of membrane potential (MP) is referred to as electrical depolarization and MP is reduced to zero if the membrane ruptures. The membrane potential was measured as the intensity ratio of the red fluorescence (a membrane potential-dependent signal) and the green fluorescence (a membrane potential-independent signal). We used CCCP (a proton ionophore that reduces membrane potential to zero with out altering membrane permeability), streptomycin (an antibiotic) as positive controls. Mean fluorescence intensities of FITC, Texas Red-A and perCP-Cy5-5A in bacteria treated with rPATE differed with untreated control (Figure 2.3.A). Red to green fluorescence ratio (indication of MP) of bacteria treated with rPATE was similar to CCCP and tetracycline, indicating a decrease of MP. However, rPATE-F treated bacteria had red to green fluorescence ratio similar to that of control (Figure 2.3.B).

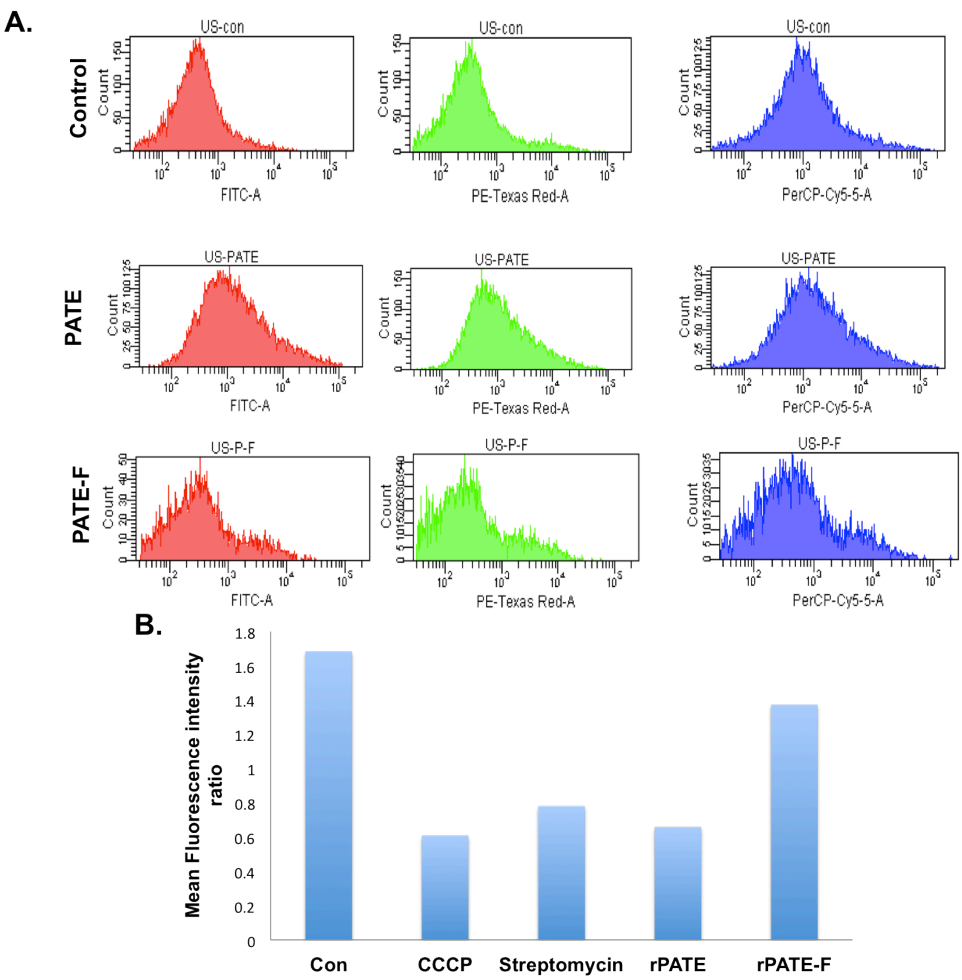

Figure 2.3. Effect of rPATE on membrane potential. *E.coli were treated with 100 µg/ml rPATE or CCCP (15µM) or streptomycin (0.5 µg/ml) for two hours and then stained with $DiOC_2(3)$ (30 µM) and TO-PRO-3 (100 nM).* ***A.*** *Flow cytograms of control, rPATE and rPATE-F treated bacteria.* ***B.*** *Red/green mean fluorescent ratios of different treatments. Con - un treated bacteria; CCCP - bacteria treated with CCCP; Streptomycin - bacteria treated with streptomycin; PATE - bacteria treated with rPATE; PATE-F - bacteria treated with rPATE-F.*

Scanning electron microscopy

From the previous experiments we understand that PATE protein kills bacteria by increasing membrane permeability. To observe the morphological changes, we performed scanning electron microscopy. Bacteria were treated with 100 µg/ml of recombinant protein and observed under scanning electron microscope. Untreated bacterial cells showed smooth surface morphology with out any evidence of cellular leakage. *E.coli* treated with rPATE-F showed intact bacterial membrane similar to that of control. When treated with rPATE, rapture of bacterial membrane and leakage of cytoplasmic contents was evident (Figure 2.4).

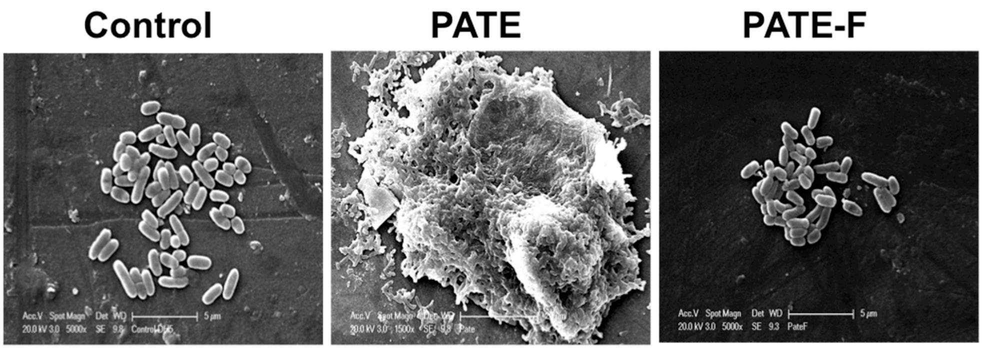

__Figure 2.4. SEM micrographs of untreated and recombinant protein treated bacteria.__ E.coli were treated with 100 µg/ml of rPATE or rPATE-F for two hours and observed under electron microscope. Control - un treated bacteria; PATE-F - bacteria treated with recombinant PATE-F; PATE-bacteria treated with recombinant PATE.

LPS binding assay

Our results indicate that rPATE kills bacteria by increasing membrane permeability. Since the first step in bacterial killing involves binding of protein with lipopolysaccharide (LPS) of bacterial cells, that ability of PATE proteins to bind LPS was assayed by the LPS binding assay. rPATE exhibited LPS binding activity similar to that of EP2C, a defensing like protein known to exhibit antibacterial activity by binding to LPS (Figure 2.5). PATE-F did not display any LPS binding activity. The negative control BSA also did not exhibit any LPS binding activity.

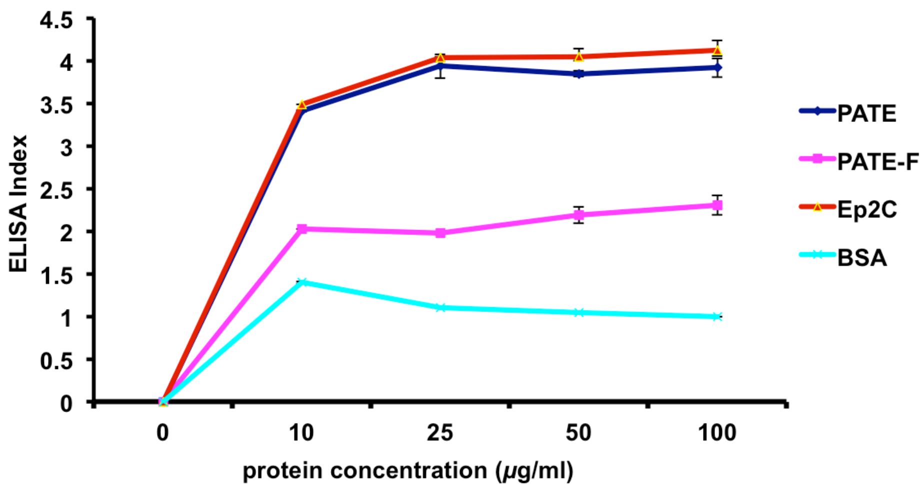

__Figure 2.5. LPS binding activity.__ LPS was coated to 96-well microplate and incubated with PATE or PATE-F or EP2C or BSA, followed by corresponding primary and secondary antibodies. Binding activity was measured at 490 nm by adding O-Phenylenediamine (OPD).

Endotoxin effect on *Pate* expression

Since the recombinant PATE protein showed potent antibacterial activity *in vitro*, its possible functional role during infection or under conditions that mimic an infection to counter pathogen attack in the male reproductive tract was investigated. To accomplish this, *Pate* expression was analyzed by semi-quantitative RT-PCR in the caput epididymides challenged with LPS *in vitro*. *Pate* expression was up regulated 3 hours after LPS challenge followed by a decline at the later time points (Figure 2.6 A). To determine whether, the effects observed *in vitro* are replicated *in vivo*, *Pate* expression was analyzed in the epididymides obtained from rats challenged with a single dose of LPS. In the epididymis, the levels of *Pate* mRNA were found to be increased 15 h after LPS injection (Figure 2.6 B). These results suggest that *Pate* may have a role in contributing to the innate immune responses of the male reproductive tract. Surprisingly *Pate-F* expression was down regulated after LPS treatment both *in vitro* and *in vivo*.

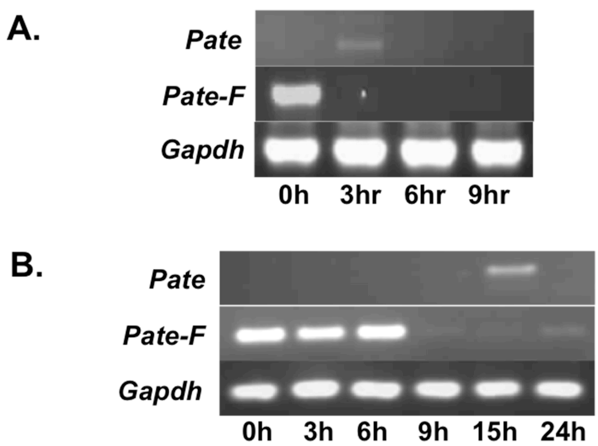

Figure 2.6. Endotoxin effect on *Pate* or *Pate-F* gene expression. ***A.*** *Rat epididymides were maintained in nutritive medium and challenged with LPS (1µg/ml). Expression of rat Pate in the caput epididymides was analysed by PCR.* ***B.*** *RNA was isolated from the caput epididymides of rats challenged with a single intraperitoneal dose (1 mg /kg body weight) of LPS. RT-PCR analysis was performed to determine Pate gene expression.*

Dynamics of PATE and PATE-F protein distribution on the spermatozoa during capacitation

Immunofluorescence was performed on caudal spermatozoa that were subjected to capacitation and acrosome reaction *in vitro*. In the uncapacitated sperm, PATE was distributed on the entire head region (including anterior acrosomal segment, equatorial segment and postacrosomal segment) and on tail with a bright fluorescance at anterior acrosomal segment. In capacitated sperm, PATE localization was seen only at anterior acrosomal segment, where as fluoresecence at equatorial segment and postacrosomal segment was completely lost, indicating capacitation induced rearrangement of PATE protein on rat sperm head. Immunofluorescence for PATE-F revealed that in uncapacitated sperm, fluorescence was seen at equatorial segment and on tail, where as in capacitated sperm, fluorescence from equatorial segment and tail was redistributed to dorsal acrosomal segment and post acrosomal segments. In the acrosome reacted sperm, PATE and PATE-F distribution was similar to that of capacitated sperm, indicating that acrosome reaction did not induce any further rearrangements. The capacitation induced rearrangement of PATE and PATE-F proteins on rat sperm head indicates that these proteins may play a role in sperm function.

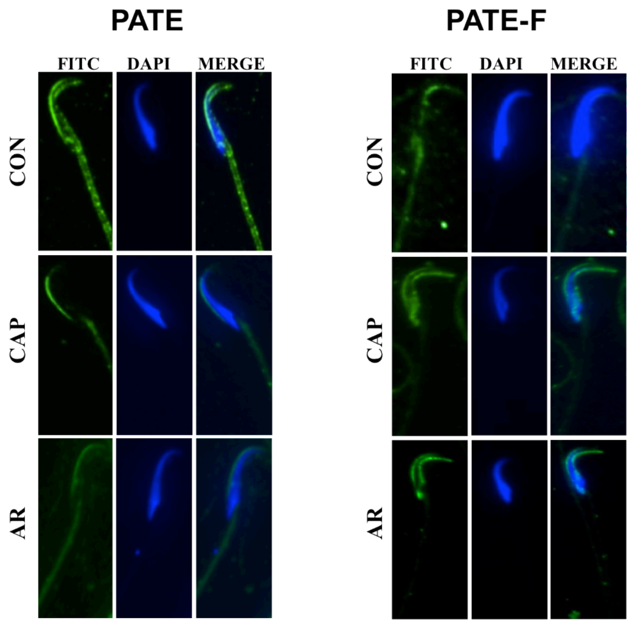

Figure 2.7. Immunofluorescence detection of PATE and PATE-F on rat spermatozoa during capacitation and acrosome reaction. *Cauda epididymal spermatozoa were capacitated, acrosome reacted and smeared on lysine coated slides and fixed in 2% PFA. They were labeled with PATE or PATE-F antiserum followed by FITC conjugated anti-rabbit IgG. CON – control uncapacitated spermatozoa; CAP – capacitated spermatozoa; AR – acrosome reacted spermatozoa.*

Microscopic examination of spermatozoa undergoing capacitation and acrosome reaction pre-incubated with PATE or PATE-F antiserum raised in rabbit

To test whether neutralization of PATE proteins may affect sperm function, rat spermatozoa were incubated with pre-immune serum or antiserum against PATE or PATE-F raised in rabbit. Capacitation was measured by staining spermatozoa with CTC (chlortetracycline fluorescent assay), filipin (an antibiotic that can aggregate unesterified sterols into complexes) and by detecting tyrosine phosphorylation of sperm proteins by Western blotting. Acrosome reaction was measured by staining spermatozoa with CTC and PNA-FITC (FITC conjugated peanut agglutinin binds to acrosome region).

CTC staining

Spermatozoa in capacitation medium when stained with 5-chlorotetra cycline (CTC) will display three different staining patterns: an F pattern, with uniform fluorescence over the whole head (uncapacitated spermatozoa); a B pattern, with a fluorescence-diminished band in the postacrosomal region and a relatively bright fluorescence in the acrosomal region (capacitated spermatozoa); and an AR pattern, with almost no fluorescence over the whole head except for a thin band of fluorescence in the equatorial segment (acrosome reacted spermatozoa). Spermatozoa pre-treated with PATE antiserum also displayed similar staining pattern observed during capacitation and acrosome reaction in control spermatozoa (Figure 2.8). However, such staining pattern was not observed in spermatozoa pre incubated with PATE-F antiserum, indicating that blocking PATE-F may inhibit these processes (Figure 2.8).

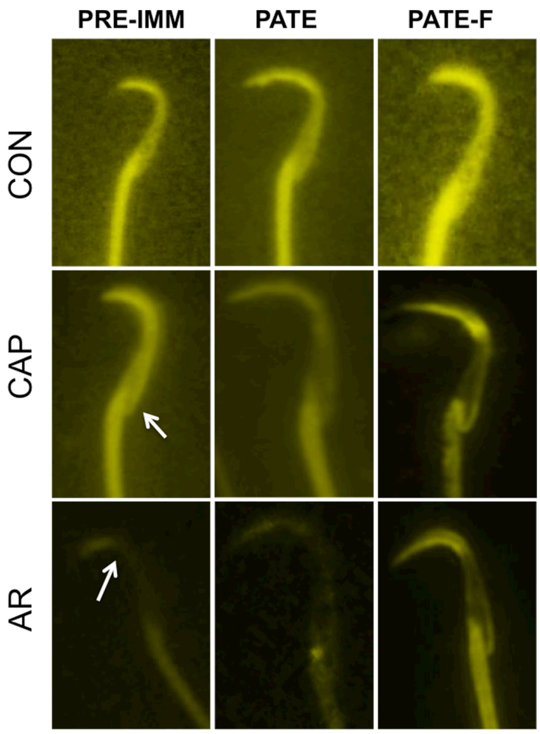

Figure 2.8. CTC staining pattern in rat spermatozoa pretreated with PATE or PATE-F antiserum. *Control rat spermatozoa were treated with pre-immune or PATE or PATE-F antiserum and allowed to undergo capacitation and acrosome reaction in vitro. Pre-Imm – spermatozoa incubated with pre-immune serum; PATE – spermatozoa incubated with PATE antiserum; PATE-F – spermatozoa incubated with PATE-F antiserum; CON – uncapacitated spermatozoa; CAP - capacitated spermatozoa; AR – acrosome reacted spermatozoa.*

Filipin staining

Capacitation is associated with loss of cholesterol from the sperm membrane and the same can be monitored by staining with the fluorescent dye filipin. As capacitation progresses, due to loss of cholesterol, filipin staining on sperm head will decrease. Such a staining pattern was observed in control capacitated spermatozoa (Figure 2.9). Similar staining pattern was also observed in spermatozoa pre-incubated with PATE antiserum (Figure 2.9). But, loss of fillipin staining was not observed in spermatozoa preincubated with PATE-F antiserum (Figure 2.9).

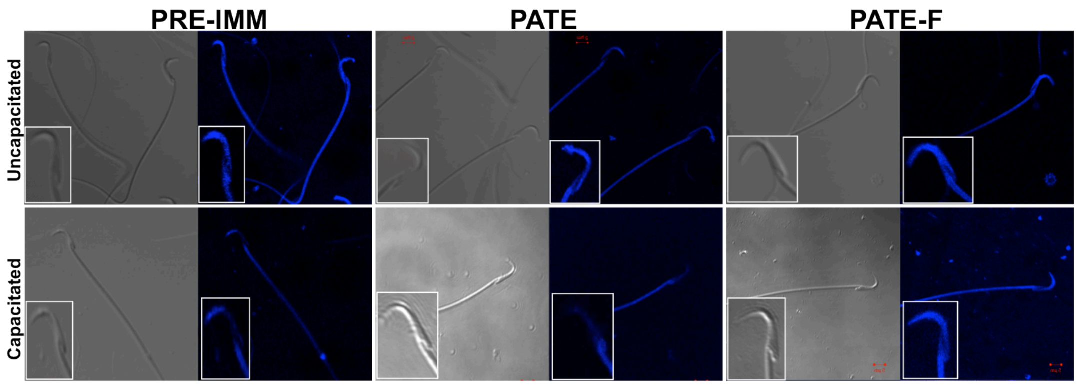

Figure 2.9. Filipin staining in rat spermatozoa pretreated with PATE or PATE-F antiserum. *Control rat spermatozoa were treated with Pre-immune or PATE or PATE-F antiserum and allowed to undergo capacitation in vitro. Spermatozoa were fixed in 4% PFA and stained with cholesterol binding dye Filipin to asses cholesterol efflux during capacitation. Insert shows higher magnification of the sperm head. Pre-Imm – spermatozoa incubated with preimmune serum; PATE – spermatozoa incubated with PATE antiserum; PATE-F – spermatozoa incubated with PATE-F antiserum.*

Tyrosine phosphorylation

Capacitation process can also be monitored by detecting the amount of tyrosine phosphorylation by Western blotting. As spermatozoa undergo capacitation, a number of sperm bound proteins are subjected to tyrosine phosphorylation. When spermatozoa were incubated with pre-immune serum or PATE anti serum, we observed increased phosphorylation similar to control capacitated sperm (Figure 2.10). Such an increase was not observed in case of spermatozoa pre-treated with PATE-F antiserum (Figure 2.10).

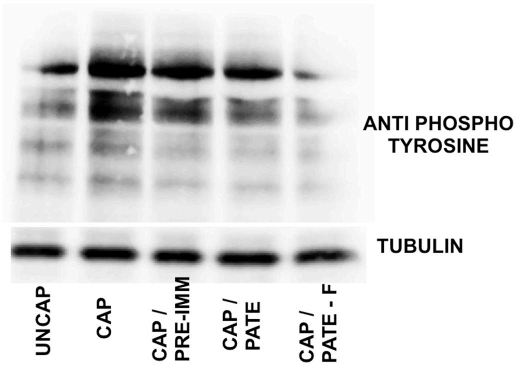

Figure 2.10. Tyrosine phosphorylation in PATE or PATE-F anti serum treated spermatozoa. *Protein tyrosine phosphorylation was measured by Western blotting after pre-incubation of spermatozoa with pre-immune or PATE or PATE-F antiserum. UNCAP- control uncapacitated; CAP – capacitated; CAP/PRE-IMM – incubated with preimmune serum and capacitated; CAP/PATE – incubated with PATE antiserum and capacitated; CAP/PATE-F – incubated with PATE-F antiserum and capacitated.*

PNA-FITC staining

Acrosome reaction can be assayed by staining with PNA-FITC, a fluorescent dye tagged protein, that stains glyocoproteins on the spermatozoa. Acrosome reaction is associated with loss of outer acrosomal membrane glycoproteins and thus results in loss of fluorescence exhibited by PNA-FITC. In spermatozoa pre-incubated with pre-immune or PATE antiserum, loss of fluorescence was observed indicating occurrence of acrosome reaction (Figure 2.11). Such a loss of fluorescence was not observed in spermatozoa pre-incubated with PATE-F antiserum, suggesting that PATE-F may be involved in acrosome reaction (Figure 2.11).

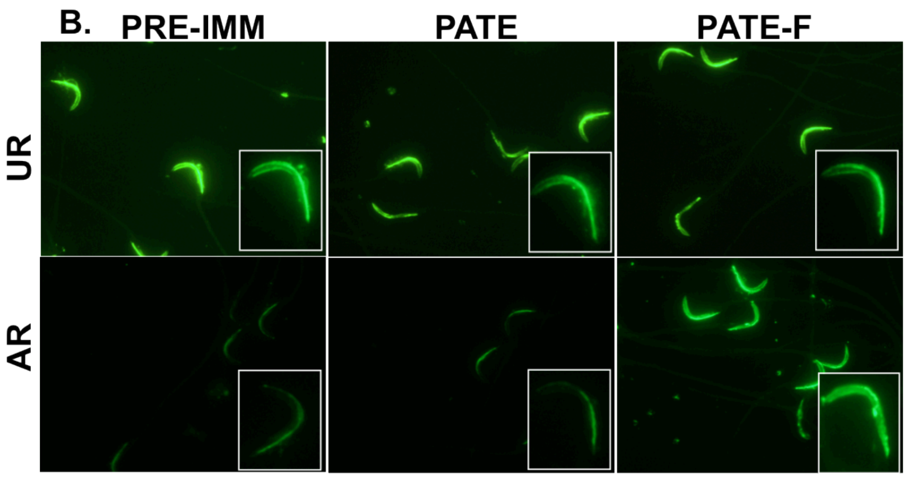

Figure 2.11. Effect of blocking PATE or PATE-F on acrosome reaction. *Spermatozoa were pre incubated with pre-immune or PATE or PATE-F antiserum and stained with 40 µg/ml PNA-FITC. Insert shows higher magnification of the sperm head. PRE-IMM – spermatozoa incubated with pre-immune serum; UR – unreacted spermatozoa; AR- Acrosome reacted spermatozoa; PATE – spermatozoa incubated with PATE antiserum; PATE-F – spermatozoa incubated with PATE-F antiserum.*

Assessment of acrosome reaction by flow cytometry in spermatozoa pre-incubated with PATE or PATE-F antiserum

Fluo-3, AM staining

The hall mark of acrosome reaction is the release of Ca^{2+} from intracellular stores and the extent of Ca^{2+} release can be measured by Ca^{2+} binding dye Fluo-3, AM. Increased fluorescence indicates the occurrence of acrosome reaction (Figure 2.12.A) shows a typical increase in fluorescence after acrosome reaction. We measured the changes in fluorescence intensity by flow cytometry. Capacitated spermatozoa after treating with ionophore showed increase in Fluo-3, AM fluorescence intensity. As indicated in the flow cytogram (Figure 2.12.B), fluorescence peak shifts towards the right in the acrosome reacted spermatozoa. In spermatozoa, pre-incubated with PATE or PATE-F antiserum and subjected to acrosome reaction, fluorescence intensity was not increased when compared to the unreacted spermatozoa (Figure 2.12.C), indicating that neutralizing of these proteins may inhibit Ca^{2+} release and thus acrosome reaction.

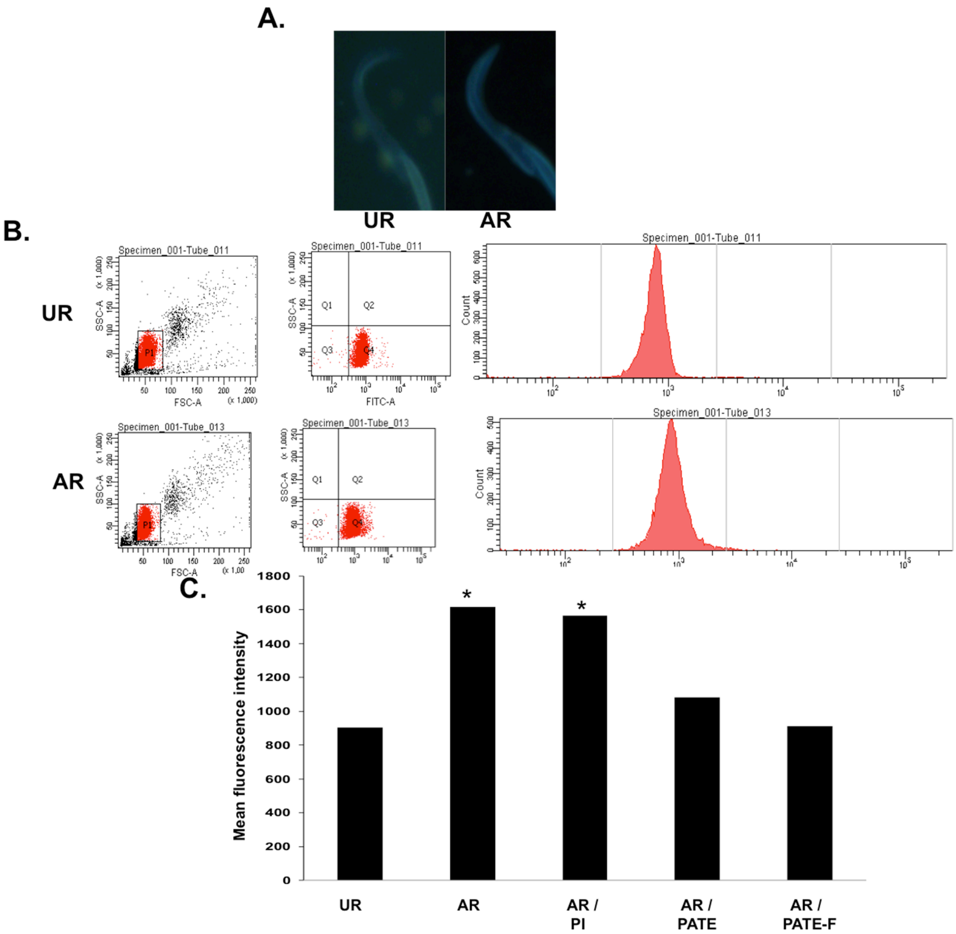

Figure 2.12. ***Assessment of acrosome reaction by flow cytometry.*** *Spermatozoa from control rats were treated with pre-immune or PATE or PATE-F antiserum raised in rabbit. Ionophore (A23187) induced acrosome reaction was assessed by measuring the fluorescence intensity of the Ca^{+2} binding dye, Fluo-3,*

*AM. **A**. Spermatozoa were stained with Fluo-3, AM and observed under fluorescent microscope. **B.** Flow cytograms of control and acrosome reacted spermatozoa. **C**. Mean fluorescence intensities of spermatozoa treated with different antibodies. UR- unreacted; AR – acrosome reacted; AR/PI – pre-incubated with pre-immune serum and acrosome reacted; AR/PATE – pre-incubated with PATE antiserum and acrosome reacted; AR/PATE-F – pre-incubated with PATE-F antiserum and acrosome reacted. Values shown are mean ± S.D. * indicates $p < 0.05$.*

PNA-FITC staining

Acrosome reaction was also monitored by measuring the fluorescence intensity of PNA-FITC bound to sperm surface. Acrosome intact spermatozoa exhibited higher fluorescence intensity whereas acrosome reacted spermatozoa exhibited lower fluorescence intensity due to loss of PNA-FITC binding and these two populations can be estimated by flow cytometry (Figure 2.13.A). To quantify the percentage of spermatozoa that have undergone acrosome reaction under different conditions, sperm were capacitated in M2 medium and incubated with ionophore to induce acrosome reaction. The percent of spermatozoa that underwent acrosome reaction was significantly increased after ionophore treatment when compared to the unreacted spermatozoa (Figure 2.13.B). Pre-incubation with PATE-F antiserum prevented increase in the percent of spermatozoa that underwent acrosome reaction (Figure 2.13.B). A similar effect was observed when pretreated with PATE antiserum. These results indicate that neutralizing PATE or PATE-F proteins on the sperm surface may affect acrosome reaction.

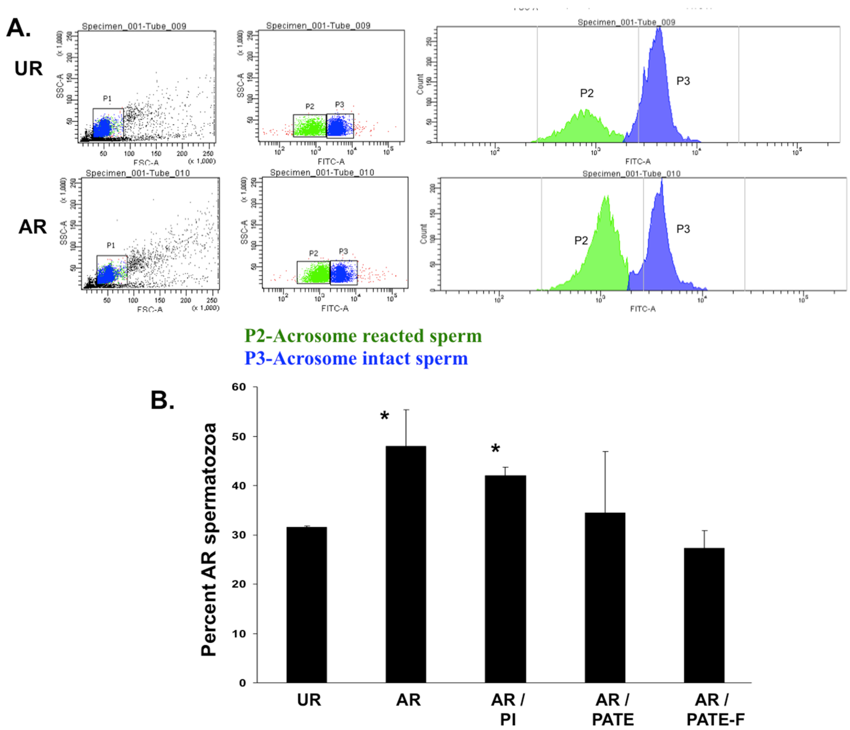

***Figure 2.13. Assessment of acrosome reaction by flow cytometry.** Spermatozoa from control rats were treated with pre-immune or PATE or PATE-F antiserum raised in rabbit. Ionophore (A23187) induced acrosome reaction was assessed by measuring the fluorescence intensity of the PNA-FITC binding. **A.** Gating of acrosome reacted (P2- with low fluorescence intensity) and acrosome intact (P3- with high*

fluorescence intensity) spermatozoa and the representative cytograms. ***B.*** *Percentage of acrosome reacted spermatozoa treated with different antibodies. UR- unreacted; AR – acrosome reacted; AR/PI – pre-incubated with pre-immune serum and acrosome reacted; AR/PATE – pre-incubated with PATE antiserum and acrosome reacted; AR/PATE-F – pre-incubated with PATE-F antiserum and acrosome reacted. Values shown are mean ± S.D. * indicates p < 0.05.*

Assessing the effect of PATE or PATE-F immunization on sperm function

The auto antigen model was generated by immunizing male Wistar rats with recombinant PATE or PATE-F proteins and the antibody titers were verified by ELISA. The possible function of PATE or PATE-F with regard to male reproduction was assessed.

Autoantigenicity

The role of PATE or PATE-F proteins on sperm function was assessed by generating autoantibodies in adult male rats. ELISA was performed using different dilutions (1:100, 1:500 and 1:1000) of serum or tissue (caput, cauda and testis) fluids to test the presence of antibodies. The antibody titers for both PATE and PATE-F were significantly higher in the serum and caput, cauda and testicular fluids obtained from immunized rats compared to those obtained from pre-immunized animals (Figure 2.14).

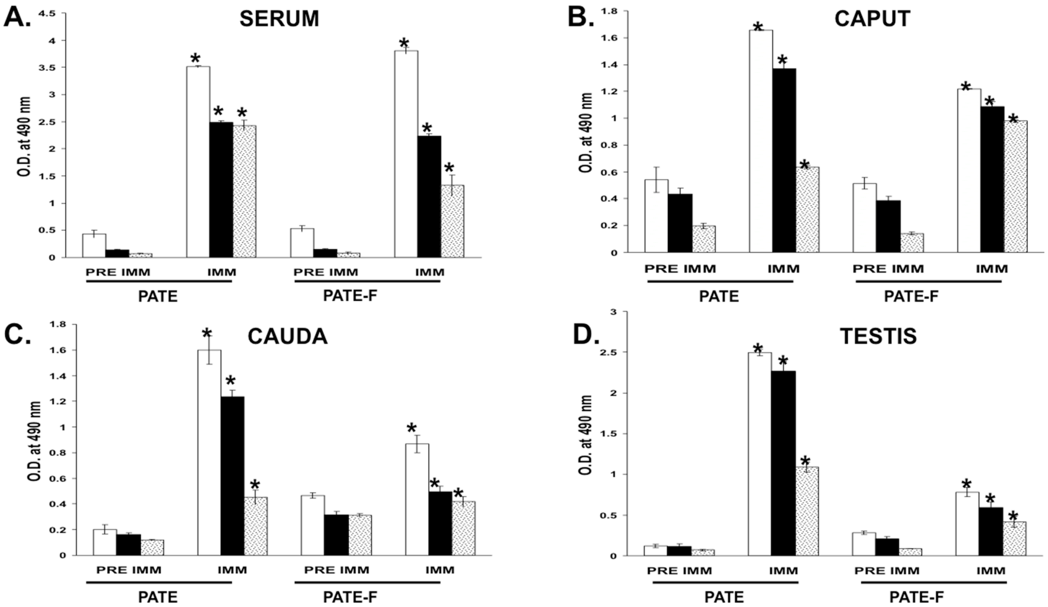

Figure 2.14. Antibody titer in the serum and tissue fluids. *Male Wistar rats (n=4) were immunized with PATE proteins and blood collected on 35th day after immunization. Tissue fluids were collected at the end of the second mating. The samples were diluted (□ - 1:100; ■ - 1:500; and ░ - 1:1000) and the antibody titer estimated by ELISA. PRE IMM - pre-immunized rats; IMM - immunized rats. Values shown are mean ± S.D. * indicates p < 0.05 compared to the respective pre-immune control.*

Histopathological evaluation

Cross sections of testes and epididymides obtained from control or PATE or PATE-F immunized rats were examined under microscope to determine the possible pathomorphological effects due to autoantibodies. The overall architecture of testis and epididymis were not affected and were similar to the unimmunized control (Figure 2.15).

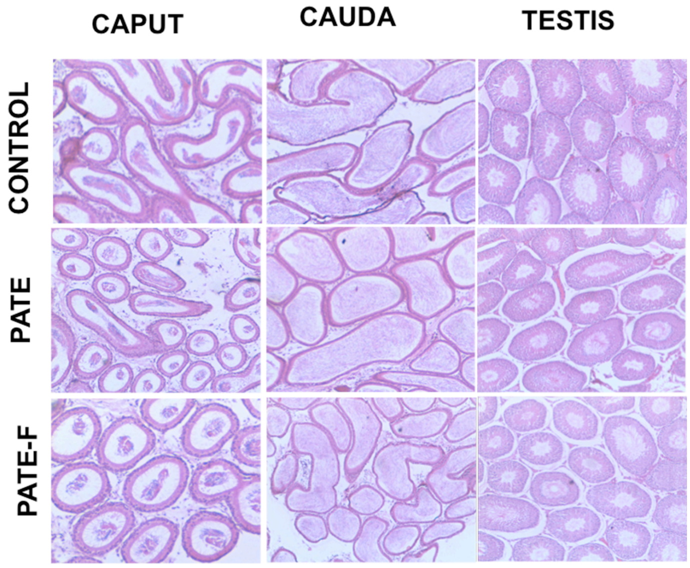

Figure 2.15. Morphological features of testis and epididymis in immunized rats. *Cross sections of testis and epididymis obtained from PATE or PATE-F immunized or control rats were stained with hematoxylin and eosin. Magnification – 10X.*

Immunolocalization of PATE and PATE-F proteins on rat sperm using serum from immunized rats

To determine weather the serum obtained from immunized rats is specific and can detect PATE or PATE-F proteins, immunofluorescence was performed. Pre immunized rat serum was unable to detect these proteins on rat spermatozoa. Serum obtained from PATE or PATE-F immunized rats was able to detect the respective proteins (Figure 2.16), thus confirming the authenticity of the antiserum generated.

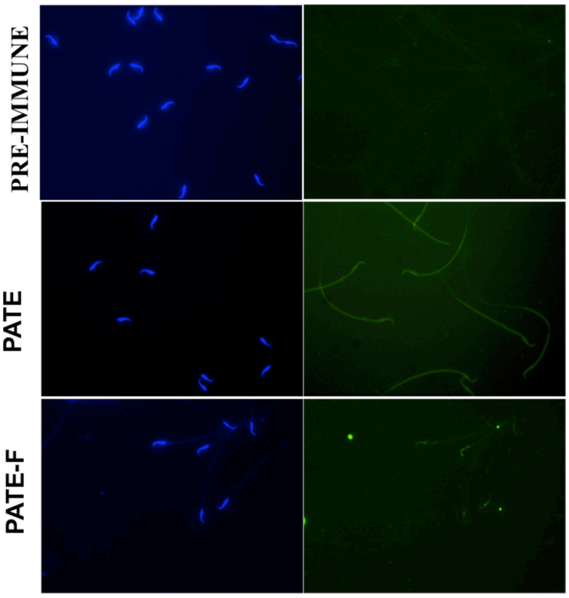

Figure 2.16. ***Immunofluorescence detection of PATE and PATE-F.*** *Spermatozoa from control rats were smeared on lysine coated slides, fixed and permeabalised with 0.1% triton-X100. They were treated with serum obtained from PATE or PATE-F immunized rats, followed by FITC conjugated anti rat IgG raised in rabbit. PRE-IMMUNE - spermatozoa incubated with pre-immune rat serum; PATE - spermatozoa incubated with serum obtained from PATE immunized rat; PATE-F - spermatozoa incubated with serum obtained from PATE-F immunized rat.*

Computer assisted sperm analyses

To determine the effect of PATE or PATE-F autoantibodies on sperm function, spermatozoa obtained from control and immunized rats were subjected to computer assisted sperm analyses. The path velocity (VAP), progressive velocity (PV), track speed (TS), lateral amplitude (LA), beat frequency (BF), straightness (STR) and linear velocity (LV) in the spermatozoa obtained from PATE immunized rats were similar to their counterparts obtained from unimmunized controls (Figure 2.17). However, in spermatozoa obtained from PATE-F immunized rats, the VAP, PV and TS were significantly reduced when compared to their unimmunized counterparts (Figure 2.17).

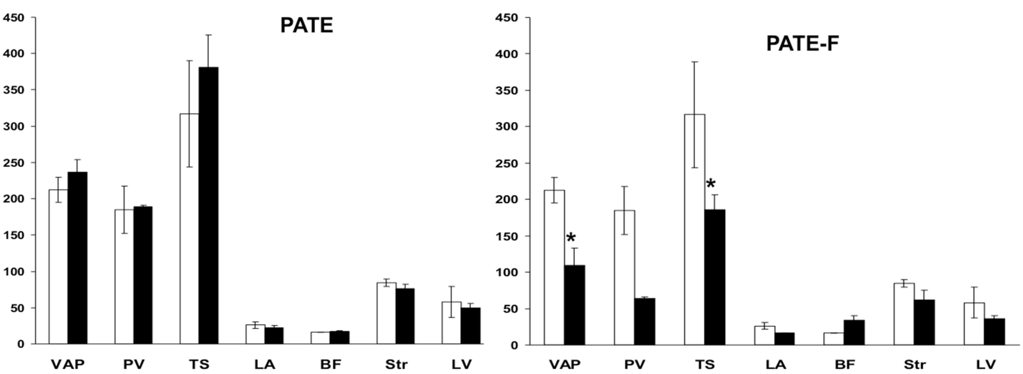

Figure 2.17. Computer assisted sperm analysis*. Spermatozoa obtained from control (n=3) and PATE or PATE-F immunized rats (n=4) were placed in a microscopic chamber slide of a computer assisted sperm*

*analyzer and the following parameters analysed: path velocity (VAP), progressive velocity (PV), track speed (TS), lateral amplitude (LA), beat frequency (BF), straightness (STR) and linear velocity (LV). Values shown are mean ± S.D. * indicates $p < 0.05$.*

Capacitation in spermatozoa of immunized rats

Tyrosine phosphorylation of the sperm proteins obtained from control or PATE or PATE-F immunized rats was analyzed. Increased tyrosine phosphorylation was observed in control spermatozoa that underwent capacitation (Figure 2.18). Similar phosphorylation pattern was also observed in spermatozoa obtained from PATE immunized rats (Figure 2.18.A). PATE-F immunization resulted in significant decrease in sperm protein tyrosine phosphorylation, indicating PATE-F may have role in capacitation (Figure 2.18.B).

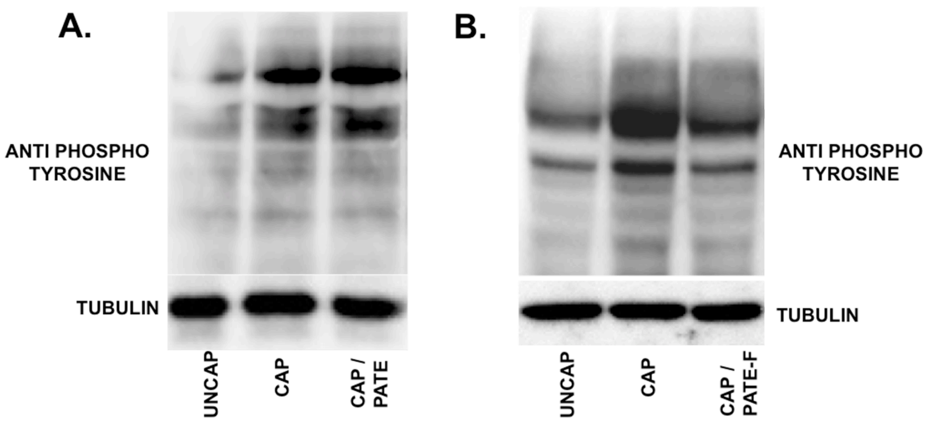

Figure 2.18. Capacitation in spermatozoa of immunized rats. *Adult male Wistar rats were immunized with PATE or PATE-F and the spermatozoa collected at the end of the experimental period. Capacitation was induced by incubating spermatozoa in M2 medium for 5 h and Western blotting was performed with anti-phosphotyrosine antibody.* ***(A)*** *Tyrosine phosphorylation of sperm proteins in PATE immunized rats.* ***(B)*** *Tyrosine phosphorylation of sperm proteins in PATE-F immunized rats. UNCAP – un capacitated spermatozoa; CAP – capacitated spermatozoa; CAP/PATE – spermatozoa obtained from rats immunized with PATE; CAP/PATE-F – spermatozoa obtained from rats immunized with PATE-F.*

Acrosome reaction in spermatozoa of immunized rats

We further investigated whether acrosome reaction will be compromised in the spermatozoa of PATE or PATE-F immunized rats. Increased fluorescence intensity due to binding of intracellular Ca^{2+} to Fluo-3, AM (Figure 2.19.A) and percentage of acrosome reacted spermatozoa (Figure 2.19.B) was observed in spermatozoa obtained from control as well as in PATE immunized rats. In spermatozoa obtained from PATE-F immunized rats, fluorescence intensity due to Ca^{2+} binding to Fluo-3, AM was similar to that of their counterparts obtained from control unreacted spermatozoa (Figure 2.19.C), indicating that acrosome reaction seems to be compromised in PATE-F immunized animals. Similarly the percentage of acrosome reacted spermatozoa in the PATE-F immunized rats was similar to that of that of control unreacted spermatozoa (Figure 1.19.D).

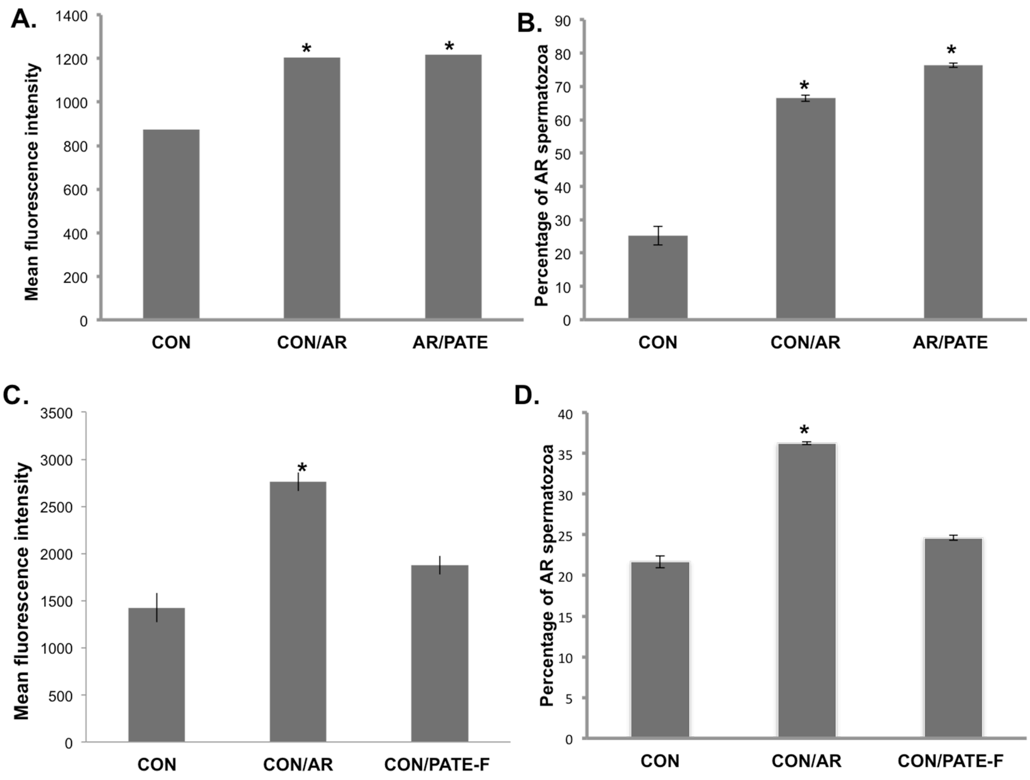

Figure 2.19. Acrosome reaction in spermatozoa of immunized rats. *Adult male Wistar rats were immunized with PATE or PATE-F and the spermatozoa collected at the end of the experimental period. Acrosome reaction was induced with A23187 and the fluorescence intensity of* ca^{2+} *bound Fluo-3, AM and acrosome bound PNA-FITC was measured in a flow cytometer.* ***A.*** *Mean fluorescence intensities of control and PATE immunized rats.* ***B.*** *Percentage of acrosome reacted spermatozoa in control and PATE immunized rats.* ***C.*** *Mean fluorescence intensities of control and PATE-F immunized rats.* ***D***. *Percentage of acrosome reacted spermatozoa in control and PATE-F immunized rats. CON –unreacted spermatozoa; AR – acrosome reacted spermatozoa; AR/PATE – spermatozoa of rats obtained from PATE immunized rats and subjected to acrosome reaction; AR/PATE-F – spermatozoa of rats obtained from PATE-F immunized rats and subjected to acrosome reaction. Values shown are mean ± S.D. * indicates* $p < 0.05$.

Assessment of fertility

Since the antiserum obtained from PATE or PATE-F immunized rats affected capacitation and acrosome reaction, we also analyzed fertility in these animals. Females mated with unimmunized rats had an average litter size of 9.6 and 9.5 in the first and second mattings respectively (Table 1). The litter size was 11.13 and 11.37 in rats immunized with PATE protein. A significant decrease in the litter size (6.75 and 5.75 respectively for 1^{st} and 2^{nd} mating) was observed in rats immunized with PATE-F.

Table 1. Litter size in PATE or PATE-F immunized rats

Antigen	No. of Males	No. of Females	No. of Pups in 1st mating	Average litter size 1st mating	No. of Pups in 2nd mating	Average litter size 2nd mating
Control	3	6	58	9.6	57	9.5
PATE	4	8	89	11.13	91	11.37
PATE-F	4	8	54	**6.75**	46	**5.75**

Effect of transient *Pate* gene knock down on rat sperm capacitation, acrosome reaction and fertilization

Gene targeting is a convenient strategy for functional studies, however, gene knock out is not yet practical in rats and so gene silencing techniques are useful choice. Epididymis is located outside the body in a scrotal sac, which makes it convenient for local injection procedures. Thus, we adopted shRNA approach to knock down *Pate* gene expression in rat cauda epididymis. Two shRNAs (*Pate* sh1 and *Pate* sh2) was designed. *Pate* sh1 is a 29 nucleotide sequence targeting the 3rd exon and *Pate* sh2 is a 29 nucleotide sequence targeting the 4th exon (Figure 2.20.A). These two shRNAs targeting the different sites of *Pate* gene and a non silencing scrambled control were synthesized and cloned into pGFP-VRS vector (OriGene technologies, USA).

Effect of shRNA transfection on *Pate* mRNA and protein expression *in vitro*

HEK 293 cells were transfected with plasmids containing scrambled or *Pate* sh1 or *Pate* sh2 sequences (Figure 2.20.A). The transfection efficiency was assessed over the delivery by observing the GFP protein expression in the cells. We observed similar GFP expression in cells transfected with plasmids containing scramble or sh1 or sh2 sequences (Figure 2.20.B). These results indicate that the plasmids are transfectable. The ability of shRNA to knock down *Pate* mRNA and protein expression was assayed by co-transfecting HEK cells with plasmids that encode *Pate* (*Pate* - pCDNA3.1) and plasmids that encode the two shRNAs. *Pate* mRNA and protein levels were reduced in HEK cells transfected with plasmids encoding *Pate* sh1 and sh2, where as these levels were unaltered with plasmids containing scrambled sequence (Figure 2.20.C and D).

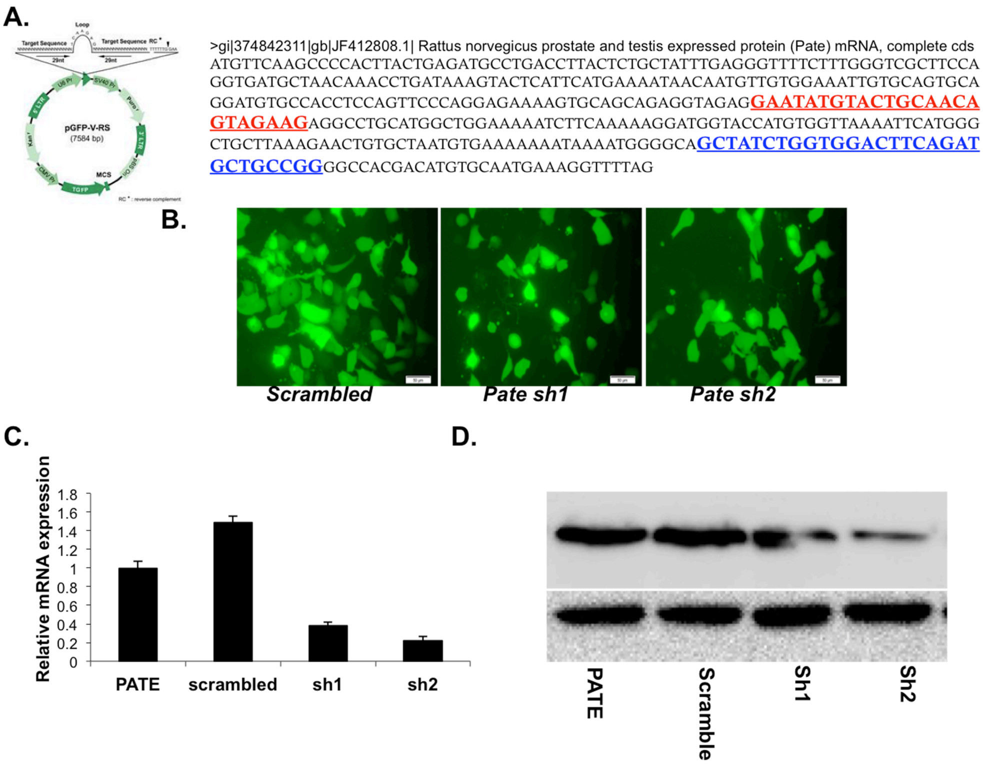

Figure 2.20. Design of shRNA against Pate and their ability to knock down expression. A. *Vector map of pGFP-VRS plasmid and potential shRNA sequences targeting two regions of Pate mRNA are underlined.* ***B.*** *HEK cells were transfected with pGFP-VRS plasmid containing scrambled or sh1 or sh2 sequences.* ***C.*** *mRNA expression in co-transfected cells.* ***D.*** *Protein expression in co-transfected cells. PATE - cells transfected with pCDNA3.1 encoding Pate; Scramble - cells co-transfected with pCDNA3.1 encoding Pate and pGFP-VRS encoding scramble; sh1 - cells co-transfected with pCDNA3.1 encoding Pate and pGFP-VRS encoding Pate sh1; sh2- cells co-transfected with pCDNA3.1 encoding Pate and pGFP-VRS encoding Pate sh2.*

Effect of shRNA transfections on *pate* expression *in vivo*

Plasmids that encode scrambled or shRNA targeting *Pate* mRNA were mixed with FuGENE (Promega, USA) and was locally injected into the rat cauda epididymis. Transfection was confirmed in the cauda tubules by the presence of green fluorescence of the TGFP protein encoded by the pGFP-VRS plasmid (Figure 2.21.A). We further confirmed transfection by amplifying *Tgfp* gene expression in the cauda (Figure 2.21.B). Compared with the scrambled or sham operated control, *Pate* mRNA levels were significantly suppressed in the cauda transfected with plasmids encoding *Pate* shRNA (both sh1 and sh2) (Figure 2.21.C). A considerable reduction in PATE binding to sperm head was observed, as indicated by immunofluorescence staining (Figure 2.21.C) and flow cytometry (Figure 2.21.C).

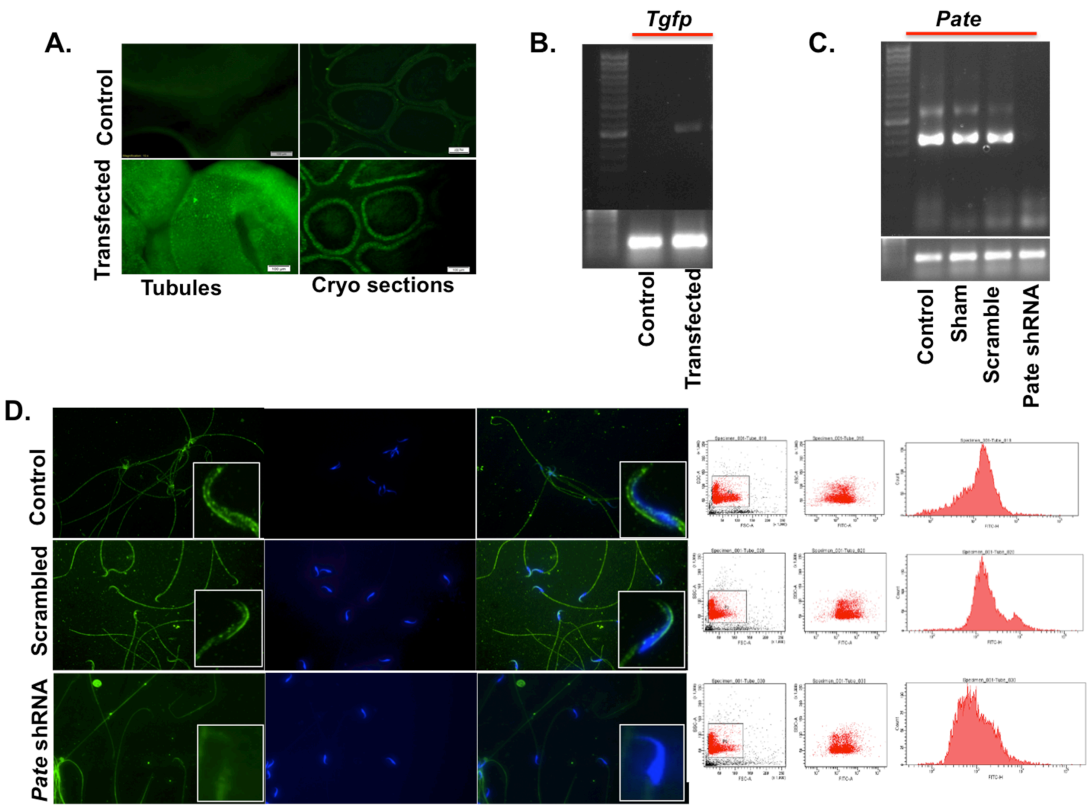

Figure 2.21. In vivo transfections. *Rat cauda epididymis was injected with 50 ng scramble or Pate shRNA (both sh1 and sh2) containing plasmid and FuGene mixture and analyzed for Tgfp or PATE mRNA and protein expression.* ***A.*** *TGFP expression in cauda epididymal tubules and in epithelial cells was detected by green fluorescence.* ***B.*** *Tgfp mRNA expression in transfected cauda epididymis.* ***C.*** *Pate mRNA expression in control or sham operated or scrambled or Pate shRNA transfected cauda.* ***D.*** *Immunofluorescence staining of spermatozoa obtained from control or transfected with scrambled or Pate shRNA caudae. The corresponding binding fluorescence intensities was measured by flow cytometry. Insert shows higher magnification of the sperm head. Control - untransfected cauda; Scrambled - cauda transfected with pGFP-VRS containing scramble; Pate shRNA - cauda transfected with pGFP-VRS containing Pate sh1 and Pate sh2 sequences.*

Effect of *Pate* gene knockdown on sperm capacitation, acrosome reaction and fertilization

The effect of *in vivo* knock down of PATE in the cauda on sperm function was analysed at multiple levels. Elevated tyrosine phosphorylation of sperm proteins is a hallmark of capacitation. Western blot analysis showed that in spermatozoa obtained from cauda transfected with *Pate* shRNA, phosphorylation levels were similar to uncapacitated sperm (Figure 2.22.A), indicating the down regulation *Pate* mRNA and protein affected sperm capacitation. Similarly, acrosome reaction was measured in spermatozoa obtained from transfected cauda. Increased fluorescence intensity due to binding of intracellular calcium to Fluo-3, AM and increased percentage of spermatozoa that display loss of fluorescence due to PNA-FITC binding is a key feature during acrosome reaction. Both mean fluorescence intensity and percentage of acrosome reacted spermatozoa were significantly reduced due to *Pate* knock down when compared to scramble or untransfectd controls (Figure 2.22 B&C).

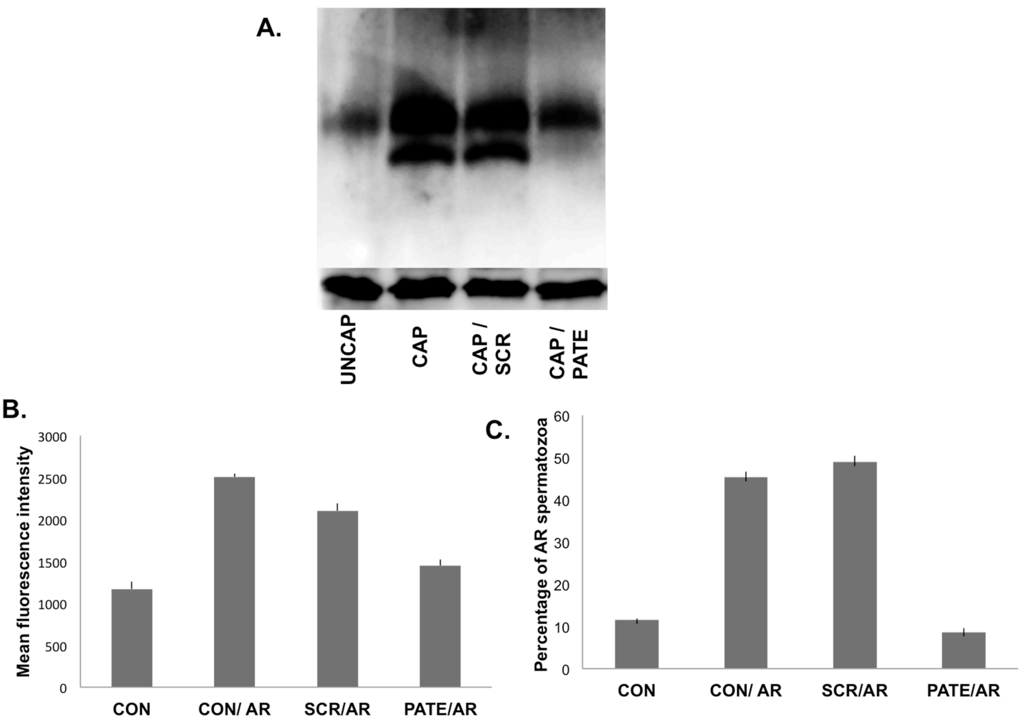

Figure 2.21. Capacitation and acrosome reaction in spermatozoa obtained from cauda transfected with Pate shRNA. *The cauda of Wistar rats were transfected with scramble or PATE shRNA (both sh1 and sh2) and the spermatozoa were collected at the end of the experimental period. Spermatozoa were capacitated and acrosome reaction was induced with A23187 ionophore.* ***A.*** *Capacitation was measured by detecting tyrosine phosphorylation levels.* ***B.*** *Mean fluorescence intensities in the spermatozoa obtained from control and rats where cauda were transfected with scramble or PATE shRNA (both sh1 and sh2).* ***C.*** *Percentage of acrosome reacted spermatozoa in control and rats where cauda were transfected with scramble or PATE shRNA (both sh1 and sh2). UNCAP – uncapacitated spermatozoa; CAP – capacitated spermatozoa; CAP/SCR - spermatozoa of rats obtained from pGFP-VRS scramble plasmid transfected rats and subjected to capacitation; CAP/PATE - spermatozoa of rats obtained from pGFP-VRS plasmid with Pate shRNA (both sh1 and sh2) transfected rats and subjected to capacitation; CON – control unreacted spermatozoa; CON/AR – acrosome reacted spermatozoa; SCR/AR – spermatozoa of rats obtained from pGFP-VRS scramble plasmid transfected cauda and subjected to acrosome reaction; PATE / AR – spermatozoa of rats obtained from pGFP-VRS plasmid with Pate shRNA (both sh1 and sh2) transfected cauda and subjected to acrosome reaction.*

Finally, we performed mating experiments to evaluate the fertility when *Pate* was down regulated in the cauda. As shown in table 2, out of 5 animals where cauda were transfected with *pate* shRNA, three were completely sterile and two had reduced litter size compared to scramble or untransfected controls. This difference in fertility among the *Pate* shRNA transfected animals may be due to differential levels of plasmid transfections or knock down efficiency.

Table 2. Fertility analysis

Male	Female	No of pups
Control	Rat-1	10
	Rat-2	10
Scr shRNA	Rat-1	10
	Rat-2	10
Pate shRNA (Rat-1)	Rat-1	6
	Rat-2	--
Pate shRNA (Rat-2)	Rat-1	--
	Rat-2	--
Pate shRNA (Rat-3)	Rat-1	--
	Rat-2	--
Pate shRNA (Rat-4)	Rat-1	--
	Rat-2	--
Pate shRNA (Rat-5)	Rat-1	7
	Rat-2	--

DISCUSSION

Cysteine rich proteins belonging to the WFDC family, eppin and defensins are known to exhibit potent antimicrobial activity and the mechanisms involve permeabilization of bacterial membranes and inhibition of macromolecular synthesis (Yenugu *et al.,* 2004, Rajesh *et al.,* 2011). Further, snake toxins that contain the Ly-6 domain are shown to be involved in defense against microbes (Kaplan *et al.,* 2007). In this study, we report that rat PATE exhibited potent antimicrobial activity, a property that is highly conserved in LY-6 family of proteins; and PATE may function to confer antimicrobial defense mechanisms in the male reproductive tract. PATE-F, on the other hand, did not exhibit any antimicrobial activity, suggesting a varied functional nature among the PATE proteins. PATE binds LPS of gram-negative bacteria and increases membrane permeability, alters membrane potential and causes leakage of cytoplasmic contents, which leads to bacterial aggregation, as evidenced by bacterial triple staining, LPS binding assay and SEM. Since PATE protein displayed potent antimicrobial activity, its epididymal expression was analyzed in response to LPS *in vitro* and *in vivo*. We observed an induction of *Pate* gene expression during LPS challenge, suggesting that the innate immune responses in the male reproductive tract under these conditions may involve alterations in *Pate* mRNA expression. Ours is the first study that provides evidence on the possible involvement of *Pate* genes in the male reproductive tract immunity. However, the expression profile of PATE protein in response to LPS challenge or bacterial infection and its ability to clear the invading pathogens needs further investigation.

Proteins bound to sperm surface are implicated to have role in acquisition of motility, capacitation, acrosome reaction and fertilization (Naz, 2011). Identification of these proteins contributed to the understanding of the mechanism of sperm function and also to develop potential male contraceptives. In the last decade, studies that employed active immunization of animals with sperm surface antigens to determine the contraceptive potential are emerging (Naz, 2011). This strategy is based on induction of antibodies against epididymal or testicular proteins so that they do not associate with sperm surface or mask them to prevent interaction with other proteins for maturation. Examples include: antisperm antibodies (Vazquez-Levin *et al.,* 2014), CRISP1(Luo *et al.,* 2012), eppin (O'Rand *et al.,* 2006), Bin1b (Li *et al.,* 2001), FA-1 (Naz and Zhu, 1998), LDH-C4 (O'Hern *et al.,* 1997), P10G (O'Rand *et al.,* 1993), A9D (Lea *et al.,* 1998), SP56 (Hardy and Mobbs, 1999), 80 kDa HSA (Khobarekar *et al.,* 2008), YLP12 (Naz and Chauhan, 2002), zona pellucida proteins (Eade *et al.,* 2009) and Izumo (Naz, 2008, Wang *et al.,* 2009). We previously demonstrated that the rat PATE proteins are localized on the sperm surface and predicted to have role in sperm function (Rajesh and Yenugu, 2012). As an initial step, sperm were capacitated and acrosome reacted *in vitro* and localization of PATE or PATE-F proteins on uncapacitated and capacitated sperm were studied. Because capacitation induces rearrangement of sperm proteins on sperm head (Rochwerger *et al.,* 1992, Larissa *et al.,* 2010). Under normal conditions, sperm capacitation take place in the female reproductive tract and this process can induced *in vitro* by incubating cauda epididymal sperm in a defined medium (Toyoda *et al.,* 1974, Davis, 1981, Davis *et al.,* 1980, Dow *et al.,* 1989), containing high concentrations of bovine serum albumin (BSA) which mimics oviduct fluid, which is essential for sperm capacitation (Yanagimachi, 1994). BSA in the medium helps to remove cholesterol from sperm plasma membrane and increases the sperm membrane fluidity (Cross, 1998). This increases membrane permeability towards Ca^{2+} and $HCO3^-$, results in increased levels of Ca^{2+} and $HCO3^-$ and activates cAMP signaling, which leads to increase in protein tyrosine phosphorylation. This increase in protein tyrosine phosphorylation is an indicator of sperm capacitation (Robert *et al.,* 2003, Visconti *et al.,* 1999). Then acrosome reacted sperm were treated with progesterone or ionophore to induce acrosome reaction. We observed capacitation induced rearrangement of PATE or PATE-F protein on sperm surface, which gives us clue that these proteins might play role in sperm capacitation.

Using antiserum raised in rabbit against rat PATE or PATE-F proteins, the effect of neutralization of PATE or PATE-F on spermatozoa and its relation to sperm function was analyzed. We observed that the spermatozoa had failed to undergo capacitation and ionophone induced acrosome reaction in case of PATE-F neutralization. PATE neutralization did not alter capacitation but inhibited ionophore induced calcium release. This observation provided the first clue that PATE and PATE-F proteins play a crucial role in sperm function in the rat. A clear relationship between reduction in fertility and sperm antigen immunization has been demonstrated. For example, immunization with the epididymal protein DE significantly affects fertility in both male and female rats (Ellerman *et al.,* 2008). Similarly, cysteine-rich secretory protein 1 (CRISP1) immunization affected fertility in male and female mice (Munoz *et al.,* 2012). To implicate the role of PATE and PATE-F proteins in sperm function and fertility, immunization studies were performed. The high titer in the serum, testicular and epididymal fluid suggest that the antibodies generated due to immunization indeed crossed the blood testis and epididymal barrier; and thus may neutralize the PATE and PATE-F proteins. In this study, a significant decrease in litter size was observed in rats immunized with PATE-F and not with PATE. Similar results were obtained in rats subjected to short term immunization. We earlier demonstrated that PATE protein exhibits potent antibacterial activity and is inducible during endotoxin challenge (Rajesh and Yenugu, 2012). In contrast, PATE-F did not display antibacterial activity. These results indicate that though these two proteins belong to the same family, their functional roles seem to be different, with PATE playing an important role in innate immunity whereas PATE-F is involved in sperm function. Such a differential role for proteins belonging to the same family was also reported for CRISP1 and CRISP2, wherein only CRISP1 had the potential to reduce fertility (Munoz *et al.,* 2012).

CASA analyses indicated loss of motility parameters in spermatozoa obtained from PATE-F immunized rats and not in PATE immunized rats. Loss of these vital features could have contributed to the reduced fertility in PATE-F immunized animals. In spermatozoa treated with mouse Izumo antibody, sperm-egg fusion was inhibited *in vitro*, but immunization of mice with Izumo had no effect on fertility *in vivo* (Wang *et al.,* 2008). Such controversial results were observed in our study. Ionophore induced acrosome reaction *in vitro* was inhibited in rat spermatozoa incubated with antiserum against PATE or PATE-F raised in rabbit. In contrast, spermatozoa obtained from PATE immunized rats were able to undergo acrosome reaction *in vitro* and the fertility was not affected in these rats. It is possible that in the immunized rats, though PATE was neutralized, its effect could not have been significant because its role could have been taken over by other testicular or epididymal proteins. In conclusion, we report that immunization of rats with PATE-F protein affects fertility. No significant loss of fertility was observed with PATE protein immunization. The loss of fertility could be due to impaired sperm motility and inability to undergo capacitation and acrosome reaction.

RNAi mediated gene silencing is a powerful tool to study function of different genes in reproductive system. Few studies have reported the gene function in tissues with blood barriers such as testis or brain (Shoji *et al.,* 2005, Kumar *et al.,* 2007). *In vivo* transfection methods have shown varying periods of transgene expression and it is depends on type of tissue and DNA stability (Leibiger *et al.,* 1991, Sullivan *et al.,* 1997, Valenzuela *et al.,* 2002). Reports indicated that epididymal epithelium has low mitotic activity and retains trans gene expression for extended period of time (Sun and Flickinger, 1982). Recently electroporation or liposome mediated gene transfer methods were used to transfer DNA into different organ systems especially testis and epididymis (Kirby *et al.,* 2004; Yang *et al.,* 2006). We adopted shRNA mediated gene silencing in cauda epididymis and pGFP-VRS plasmid containing shRNA were transfected to cauda epididymis using liposome mediated gene transfer method. pGFP-VRS with scramble or *Pate* shRNA were mixed with FuGENE and delivered to interstitial space of cauda epididymis. FuGENE6 mediated gene transfer retained p-GeneGRIP plasmid green fluorescence for 7 days and started diminishing from 19 days in mouse cauda epididymis (Esponda and Carballada, 2009). Similarly, we observed *Tgfp* gene and protein expression (associated with pGFP-VRS) in transfected rat cauda. Furthermore, RT-PCR, immunofluorescence and flow cytometry experiments showed decreased *Pate* mRNA in cauda and protein on spermatozoa obtained from *Pate* shRNA transfected cauda. These results are in agreement with the previous groups and confirm that the transfection protocol we adopted is indeed authentic.

Pate gene-knock down in male wistar rats resulted in decrease capacitation and acrosome reaction *in vitro*. We observed that knock-down of *Pate* in the cauda resulted in the animals being either infertile or have reduced litter size and this correlates with decreased sperm functions *in vitro*. Similar results were observed when knockdown of HongrES1 *in vivo* results in reduced fertility, along with deformed appearance of fetuses and pups in rats (Zhou *et al.,* 2008). *Bin1b* knock down by anti-sense oligonucleotides resulted in considerable attenuation of sperm motility and progressive motility in rats (Chen *et al.,* 2004). Further, knock down of *Defb15* led to a reduction in fertility and embryonic development failure in rats (Yue *et al.,* 2004). Although immunization of rats with PATE protein did not alter sperm capacitation, acrosome reaction and fertilization, *Pate* knock down can inhibit sperm functions and fertilization. It is possible that in the immunized rats, though PATE was neutralized, its effect could not have been insignificant because its role may have been compensated by other epididymal proteins. Where as in knock down rats, targeted *Pate* mRNA suppression and further loss of protein binding on sperm head is sufficient to effect sperm functions and fertilization.

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SUMMARY

Spermatozoa produced in the testis are immature and lack fertilizing ability and the maturation takes place in the epididymis. Epididymal maturation of spermatozoa is facilitated by a number of proteins secreted by the epididymal epithelium in to the luminal fluid, which then bind on to the sperm surface. *Pate* genes were first identified as tissue specific target antigens in prostate cancer and further studies indicated that these genes are present between *Acrv1* and *Ddx25* in human and mouse, and abundantly expressed in male reproductive tract. However, the homologues of *Pate* genes in the rat are not identified. Though these genes were identified in the humans and mouse, their function in general and in the male reproductive tract is partially understood. In the present study we try to identify and characterize rat *Pate* genes using *in silico*, *in vitro* and *in vivo* approaches.

Chapter 1. Identification and *invivo* characterization of rat *Pate* genes

We identified ten rat *Pate* mRNA transcripts, amplified, sequenced and submitted to GenBank. Similar to human and mouse, rat *Pate* genes are clustered between the *Acrv1* and *Ddx25* genes and have three exons except for *Pate* and *Pate-2*. Interestingly we found alternate transcripts for *Pate* and *Pate-2* but they have premature termination codons in the fourth exon. All the *Pate* mRNA transcripts, encode for characteristic TFP/Ly-6/uPAR domain with a highly conserved distribution of 10 cysteines in two motifs. All the *Pate* proteins identified in this study contain a signal peptide cleavage sequence and seem to be secretory in nature. Homologies among different PATE proteins were very low. The PATE and PATE-F modeled proteins have structure similar to the three-fingered toxin, bucandin.

Although named as prostate and testis specific, RT-PCR analyses revealed that the rat *Pate* genes are expressed in epididymis and seminal vesicle except for *Pate-P,* which is expressed in ovary. Reproductive tract specific expression of *Pate* genes indicates that they might play role in male reproduction. Further we found the expression of *Pate-Q*, *Pate-2, Pate-C* and *Pate-Dj* in the liver suggesting an ubiquitous role. In developing rats, *Pate-A, Pate-F* and *Pate-N* expression was observed starting from 30 days of age implicating that the *Pate* gene expression may be determined by the androgen levels. Androgen ablation in adult rats resulted in complete loss of *Pate* gene expression indicating that the *Pate* genes are under the

control of androgens in the epididymis. Immunofluorescence studies revealed that PATE and PATE-F proteins were found to be abundantly localized in the epididymis and on the spermatozoa. Presence of PATE proteins on the sperm surface implicates their possible role in sperm function.

Chapter 2. Functional importance of PATE proteins in male reproductive system

Proteins with cationic nature or rich in cysteine content are known to exhibit antimicrobial activity. Recombinant PATE and PATE-F were tested for antimicrobial activity using CFU assay. PATE exhibited bacterial killing, whereas PATE-F did not. PATE binds to LPS of *E. coli* and its mechanism of action involves alteration of membrane permeability, membrane potential and rupture of bacterial membrane and leakage of cytoplasmic contents. We also observed *Pate* mRNA up regulation in the caput, when rats were challenged with LPS both *in vitro* and *in vivo*.

Numerous reports have demonstrated that a variety of proteins expressed on the surface of mammalian spermatozoa are involved in the process of maturation and fertilization. We observed rearrangement of PATE and PATE-F proteins on sperm head, when spermatozoa are capacitated *in vitro*, indicating that these proteins may play a role in sperm function. The role of PATE proteins in sperm function was studied by three different approaches namely, neutralizing PATE proteins on sperm surface using anti serum *in vitro*, neutralizing endogenous levels by immunizing rats with PATE proteins and *Pate* mRNA knock down by shRNA. *In vitro* neutralization of PATE and PATE-F resulted in inhibition of sperm capacitation and acrosome reaction. Immunizing rats with PATE or PATE-F resulted in production of autoantibodies and these antibodies were found in high titers both in the serum and tissue fluids, indicating that these antibodies crossed blood testis barrier. Immunization did not induce any inflammation in the reproductive tissues as evidenced by H&E staining. Spermatozoa obtained from PATE-F immunized rats showed significant reduction in sperm motility, capacitation and acrosome reaction. Further PATE-F immunized rats showed decreased litter size indicating that the potential role played by this protein in male reproduction. On the other hand, immunizing rats with PATE did not alter sperm function and litter size.

Gene targeting is a convenient strategy for functional studies and we adopted shRNA approach to knock down *Pate* mRNA levels in rat cauda epididymis. Co-transfecting HEK cells with *Pate* pCDNA3.1 and *Pate* shRNA expressing plasmids resulted in decreased mRNA and protein levels, indicating that *Pate* shRNA is able to down regulate its expression *in vitro*. Expression of *Tgfp* mRNA and protein in transfected cauda indicates successful transfection of shRNA plasmids *in vivo*. Further *Pate* shRNA transfection inhibited *Pate* mRNA and protein levels *in vivo*. In spermatozoa obtained from *Pate* mRNA knock down rats, decreased capacitation and acrosome reaction was observed. Further, *Pate* mRNA knock down in cauda resulted in the animals being either infertile or have reduced litter size. These results suggests that PATE and PATE-F may have important role in sperm function.