Characterizing the functional role of testicular proteins in fertility and epididymal proteins in oncogenesis

Thesis submitted to the University of Hyderabad for the award of Doctor of Philosophy in the Department of Animal Biology

By

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This is to certify that this thesis entitled **"Characterizing the functional role of testicular proteins in fertility and epididymal proteins in oncogenesis"** is a record of bonafide work done by **Ms. Aisha Jamil,** a research scholar for Ph.D. programme in the Department of Animal Biology, School of Life Sciences, University of Hyderabad under my guidance and supervision. This thesis is free from plagiarism and has not been submitted in part or in full to this or any other University or institution for the award of any degree or diploma. Parts of the thesis have been:

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1. **Aisha, J.**, & Yenugu, S. (2022). Characterization of SPINK2, SPACA7 and PDCL2: Effect of immunization on fecundity, sperm function and testicular transcriptome. *Reproductive Biology*, *23*(1), 100711. doi:10.1016/j.repbio.2022.100711

B. Presented in the following conferences:

1. **Aisha Jamil** and Suresh Yenugu**,** Knockdown of Sperm associated antigen 11 A (*Spag11a*) enhances the susceptibility of epididymis and prostate to chemically induced carcinogenesis. Oral presentation at International Conference on Reproductive Biology, Comparative Endocrinology & Development, 14th-16th September, 2022, CSIR-CCMB, Hyderabad.

2. **Aisha Jamil** and Suresh Yenugu**,** Effect of immunization against SPINK2, SPACA7 and PDCL2 on fertility and testes transcriptome analysis in male rats. Presented poster at International Conference on

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Further, the student has passed the following courses towards the fulfilment of the coursework requirement for Ph.D.

Course Code	Name	Credits	Pass/Fail
AS 801	Analytical Techniques	4	Pass
AS 802	Research Ethics, Data Analysis and Biostatistics	3	Pass
AS 803	Lab Work and Seminar	5	Pass

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DECLARATION

I, **Aisha Jamil**, hereby declare that this thesis entitled **"Characterizing the functional role of testicular proteins in fertility and epididymal proteins in oncogenesis"** submitted by me under the guidance and supervision of **Prof. 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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Table of contents

Abbreviations

BM	: Basal membrane
BSA	: Bovine serum albumin
BTB	: Blood-testis barrier
cDNA	: Complementary DNA
CRISP	: Cysteine rich secretory protein
DAPI	: 4,6-diamidino-2-phenylindole
DEN	: Diethyl nitrosamine
DEPC	: Diethyl pyrocarbonate
DNA	: Deoxyribonucleic Acid
GAPDH	: Glyceraldehyde 3-phosphate dehydrogenase
HE2	: Human epididymal protein2
HRP	: Horseradish peroxide
Ig-G	: Immunoglobulin G
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
LB	: Luria broth
MRE	: Mean residual ellipticity
OPD	: O-Phenylene diamine
PBS	: Phosphate buffer saline
PBS-T	: Phosphate buffer saline with tween
PDCL2	: Phosducin like 2
ppm	: Parts per million
RNA	: Ribonucleic acid
rpm	: Revolutions per minute
RT-PCR	: Reverse Transcription-Polymerase chain reaction
SNP	: Single nucleotide polymorphism
SPACA7	: Sperm acrosome associated 7
SPAG11A	: Sperm associated antigen 11a
SPINK2	: Serine protease inhibitor kazal type 2

GENERAL INTRODUCTION

Male reproductive system

The male reproductive system consists of the gonads i.e. a pair of testes and other ductal structures, namely, epididymides, vasa deferentia, seminal vesicles, ejaculatory ducts and the penis (**Figure 1**). The testis is protected inside a sac like structure called scrotum. Accessory glands of the male reproductive tract include the prostate and the bulbourethral (Cowper) glands. The function of the testis is the production of haploid gametes i.e. spermatozoa and the male steroid hormone, testosterone for the development of secondary sexual characters. Glands of this system release major part of seminal fluid, which provides various nutrients required for the gametes during their movement in the female reproductive tract to enable fertilization.

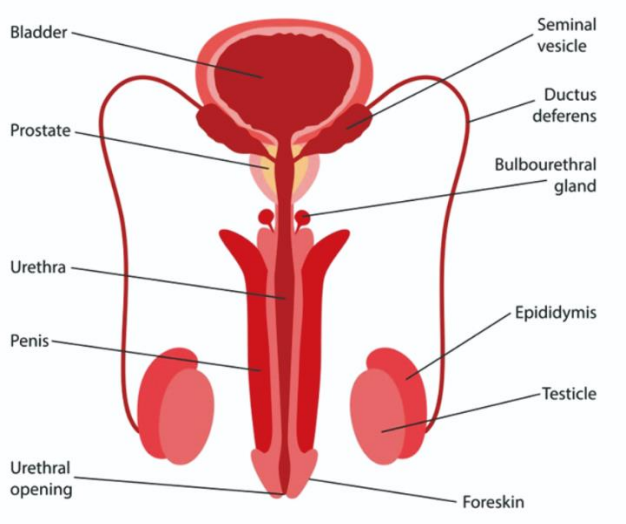

***Figure 1**. Schematic diagram of the male reproductive system (Adapted from: EMBIBE).*

Testis

The testes produce the male gametes and the male hormones and thus are considered as both endocrine and exocrine. The testis hosts the machinery for all the enzymatic events necessary for the synthesis of male steroid hormones and the generation of gametes (Swerdloff, Wang et al. 1992). The testes are extremely effective "sperm factories," producing a huge quantity of these complex cells. The scrotum is a shared musculo-cutaneous sac in which the testes, which are bilateral gonads, are located. The parenchyma (seminiferous tubules) is incompletely divided into about 250 lobules by septa that extend internally from the capsule. The interstitial (Leydig) cells, which produce hormones, are situated within the connective tissue stroma (tunica propria), which divides into 1-4 seminiferous tubules in each lobule (**Figure 2**). The seminiferous epithelium, a complexly stratified epithelium resting upon a basal lamina, contains two cell populations, namely, germ (spermatogenic) cells and sertoli cells (**Figure 2**). Seminiferous ducts are the sites of spermatogenesis, while Leydig cells, which are interspersed between

the seminiferous ducts, are the sites of androgen synthesis. Cells of the seminiferous ducts (spermatogonial stem cells), undergo meiosis during spermatogenesis. This process includes a variety of distinct processes that includes reduction in the number of chromosomes, nuclear condensation and elimination of extra cytoplasm. The spermatogonia at the base of the tubules are the starting point for the germ cells that are present in the germinal epithelium. During meiosis, these spermatocytes differentiate into haploid round spermatids before differentiating into elongated spermatids that are ready to be released into the lumen. The differentiating spermatogonia receive structural and functional support from the Sertoli cells. To acquire sperm motility and fertilisation ability, the sperm cells develop distinct physical traits and structures that includes the formation of flagellum and acrosome. These intricate processes that dictate spermatogenesis and steroidogenesis are reflected by the tightly controlled gene expression process in the testis (Dufau, Tsai-Morris et al. 2001, Walker 2009). Some genes that are specific to the testis have been identified. Examples include FAM46D (Bettoni, Filho et al. 2009), Adam31 (Liu and Smith 2000), PDHA2 (Pinheiro, Faustino et al. 2010), TEX101 and SPATA19 (Ghafouri-Fard, Abbasi et al. 2010), which have been shown to be important in the spermatogenesis and steroidogenesis. One of the most intricately specialised cell types ever identified is the spermatozoan. The goal of this specialised cell is fertilization of the female gamete.

1. Sertoli cells

Also referred to as sustenticular cells, these are the columnar, non-replicating (post-mitotic), and supporting cells (sustenacular cells) that stretch from the basal lamina to the lumen. They contain large apical and lateral projections that encircle the surrounding germ cells and make contact with surrounding Sertoli cells. Highly modified zonula occludens form the basal and lumenal compartments of the epithelium contribute to these interactions. The spermatagonial stem cells and early primary spermatocytes are located in the basal compartment that is located beneath the occluding junctions. The spermatocytes and spermatids in the later stages are housed in the luminal compartment. The blood-testis barrier is also established by the occluding junctions of the Sertoli cells. This barrier serves for both physiological and immunological purposes and thereby causing the luminal fluid to differ from the interstitial fluid. Further, the barrier provides the gametes with a more nutritive environment and prevents an immune reaction to the antigenic haploid spermatids. The Sertoli cells perform the role of nursing the maturing spermatozoa by exchanging metabolic and waste products with them. Additionally, they phagocytoze the leftover bodies created at the final stage of spermatogenesis. In order to increase the concentration of testosterone, they finally produce androgen-binding protein (ABP) into the luminal compartment (Mescher 2018).

2. Germ cells

The four to eight layers of germ cells in the testis are primarily involved in producing spermatozoa. The milieu of germ cells consists of spermatozoa, spermatocytes and spermatids. Primary spermatocytes are produced when the spermatogonial stem cells undergo mitotic division, and these cells further

differentiate as they proceed apically through the seminiferous epithelium to produce secondary spermatocytes, spermatids, and eventually spermatozoa. Spermatogenesis, also known as the generation of spermatozoa, is the process of cell division through mitosis and meiosis as well as the final differentiation of spermatozoa, also known as spermiogenesis.

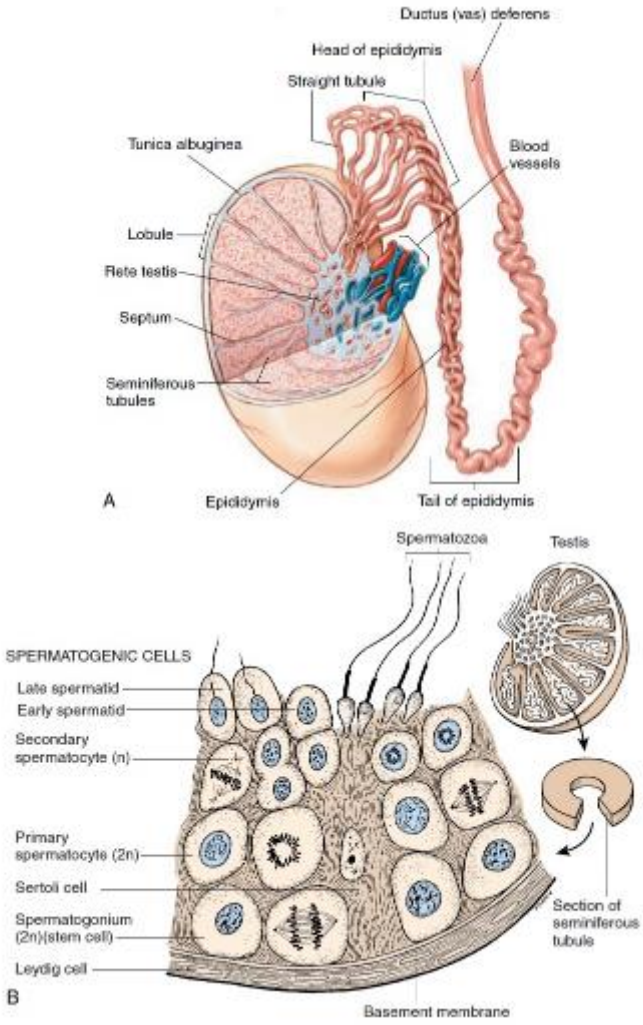

***Figure 2**. Schematic diagram of the testis. **A**. Sagittal and **B**. transverse section through a portion of seminiferous tubules (Adapted from The Massage connection ANATOMY AND PHYSIOLOGY).*

SPERMATOGENESIS

The process through which spermatozoa are created is known as spermatogenesis. The process starts with the spermatogonium, a primitive germ cell with a diameter of just 12 μm and a location close to the basal lamina of the epithelium. Spermatogonia start mitotic division when they reach sexual maturity, creating successive generations of cells (**Figure 3**). Three steps are identified in spermatogenesis (Belousov 2011).

1. **Spermatogonial phase:**

   Primary spermatocytes from stem cells are produced by mitosis in seminiferous tubules. The type Ap spermatogonia (daughter cells of type Ad) undergo recurrent mitotic divisions during the spermatogonial phase to produce many clones. Cytokinesis is incomplete during these divisions, and the daughter cells are all connected by a cytoplasmic bridge. The linked "cells" develop synchronously because of this cytoplasmic continuity. The associated type Ap spermatogonia develop into type B spermatogonia at the end of this phase.

2. **Spermatocyte phase:**

   To form haploid spermatids, primary spermatocytes divide twice throughout the meiotic process. Each type B spermatatagonia undergoes mitotic division during the spermatocyte phase, resulting in two primary spermatocytes that move into the luminal compartment of the seminiferous tubule. These cells copy their DNA so that the chromatids are doubled (4N) and the amount of DNA is increased before going through meiosis I. (2d). Crossover can happen when meiosis I progresses, and the maternal and paternal chromosomes are randomly separated during metaphase. Two secondary spermatocytes (2N, 1d) are produced at the end of meiosis I. The quick entry of the secondary spermatocytes into meiosis II (S phase) without DNA synthesis results in the production of spermatids (1N, 0.5d).

3. **Spermatid phase:**

   Spermatids develop into fully developed sperm cells (spermatozoa). Spermatids (immature gametes) transform into spermatozoa (mature gametes) while still being physically affixed to the Sertoli cells during the spermatid phase (spermiogenesis).

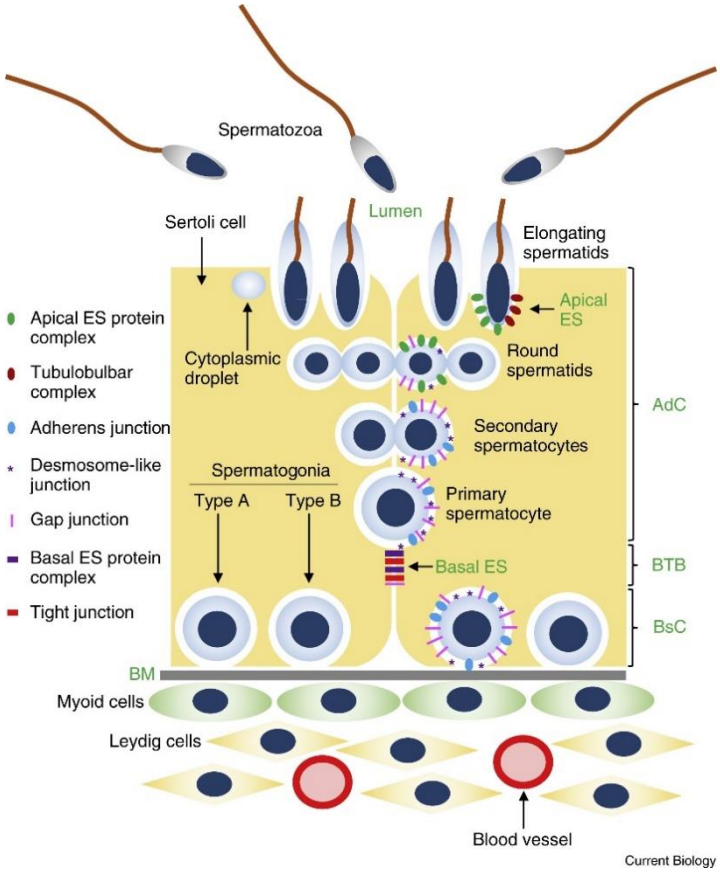

***Figure 3**. Spermatogenesis. Schematic representation of a cross-section of seminiferous tubule. AdC stands for adluminal compartment, ES for ectoplasmic specialisation, BsC for basal compartment, BM for basement membrane and BTB for blood-testis barrier (Nishimura and L'Hernault 2017).*

SPERMIOGENESIS

The process of producing spermatozoa culminates in spermiogenesis. The spermatids become spermatozoa, cells that are highly specialised to transport male DNA to the ovum, during spermiogenesis. During this step, no cell division takes place. The intricate process of spermiogenesis involves the creation of the acrosome, nucleus condensation and elongation, the production of the flagellum, and the loss of most of the cytoplasm. The mature spermatozoon is the final product, and it is subsequently ejected into the lumen of the seminiferous tubule. Formation of Golgi, cap, acrosome and maturation are the 4 stages that occur in this process (Toshimori 2009).

1. **Golgi phase**

   In this phase, the polarity of the spermatids is determined. A single, substantial acrosomal vacuole, which houses the acrosomal granules and is intricately coupled to the nuclear envelope at its core and to the proximal segment of the perinuclear theca at its periphery, is created from the trans-Golgi network's proacrosomal vesicles. The centrioles then move to the opposite end to create the posterior pole and start the construction of the flagellum.

2. **Cap phase**

In contrast to how the acrosomal vesicle (cap) progressively flattens and extends over the nucleus as spermatids mature, the Golgi apparatus travels distally toward the developing neck area. At this stage, round spermatids synthesize more than 200 different mRNA transcripts. The change from the cap phase to the elongation phase is the crucial step in acrosome biogenesis. Therefore, gene deletion (such as that of the GOPC and Herb genes) at this stage easily disrupts spermiogenesis. These mutants fail to fuse the Golgi-derived vesicles as the spermatids grow, and the acrosomal granules completely detach from the nucleus (Yao, Ito et al. 2002, Toshimori 2009, Yan 2009).

3. **Acrosomal phase**

   The spermatid shifts its orientation such that flagellum protrudes into the lumen and the acrosome points toward the basal layer. The cytoplasm travels posteriorly, the nucleus flattens and lengthens, and the mitochondria are concentrated around the flagellum. The connecting element is formed by the centrioles migrating back to the nucleus (neck). The cytoplasm gets thinner, the acrosome slowly turns to face the plasma membrane, and manchettes begin to form from the region around the nuclear ring as the spermatids elongate. The components of the acrosome eventually compact into an electron-dense matrix as the acrosomal cap lengthens. The next checkpoint is the change from the elongation (acrosome) phase to the maturation phase. The deletion of the IgSF protein RA175 gene leads to the disruption of the early elongating spermatid stage of spermiogenesis and oligoteratozoospermia (Fujita, Kouroku et al. 2006, Toshimori, Maekawa et al. 2006).

4. **Maturation phase**

   The acrosomal membrane is completely covered by the dense substance (acrosomal granule) in the acrosomal vesicles, which finally causes the acrosome to separate into the anterior and posterior acrosomes. During this period, basic molecules appear to flow and coalesce into the anterior and posterior acrosomes. Acrosomal matrix proteins show post-translational modifications in late spermatids, and they also show an unexpected rise in the synthesis of the acrosomal antigen MC41. Just prior to spermiation, the majority of the spermatids' cytoplasm and organelles are expelled in the cytoplasmic droplet, and the remaining spermatids are then released into the lumen as spermatozoa or sperm. The Sertoli cells eventually swallow the leftover bodies that spermatids discharge (Yoshinaga, Tanii et al. 2001).

SPERMATOZOA

In addition to delivering the paternal genome to the egg, mammalian spermatozoa have the capacity to stimulate the oocyte that has been halted at the metaphase of the second meiosis (MII). A mature spermatozoon is a highly differentiated haploid cell with a flagellum and a paddle-shaped head (tail). The sperm head is composed of an acrosome, a nucleus, and a little quantity of cytoplasm in the form of cytoplasmic layers. The paternal DNA is stored in the nucleus together with nuclear proteins called protamines. The mature sperm acrosome, a saccule that resembles a cap and includes hydrolyzing

enzymes and matrix proteins, covers the proximal portion of the sperm head. The cytoplasm thins out and forms confined gaps between the nucleus and the adjacent plasma membrane (Toshimori 2009).

The acrosomal area and the postacrosomal region (PAR) are the two main portions of mammalian sperm head. The anterior acrosome and the posterior acrosome are the two subdomains that make up the acrosome area. The acrosomal reaction involves the anterior acrosome. Gamete membrane fusion appears to be facilitated by the posterior acrosome. The neck area is formed by the PAR, which extends from the posterior acrosome end and the head's distal end's posterior ring (connecting piece). The posterior ring reveals a belt-like restricted zone of plasma membrane. It joins the underlying nuclear envelope through fusion. It is also assumed that the proximal portion of the PAR participates in egg activation (Toshimori 2009).

Axoneme, mitochondria, and cytoskeletal elements like fibrous sheaths (FSs) and outer dense fibres (ODFs) are all found in the tail. The tail is organised structurally into four main parts: the neck, middle portion (midpiece), main piece, and end piece. The neck area has a complicated structural makeup. The basal plate, a redundant nuclear envelope, neck mitochondria, and a connecting portion are its main parts (including the segmented columns attached to the ODFs, the centrosome, and the capitulum). The proximal centriole and several pericentriolar matrix proteins are found in centrosomes. When the sperm reaches the egg, they play a role in microtubule production, and during fertilisation, they morphologically change into the sperm aster. The proximal portion of the axoneme develops from the distal centriole (Toshimori 2009).

The nucleus flattens and condenses during the last stage of spermiogenesis as protamines, a type of nonhistone basic protein, replace the normal histones associated with nuclear DNA, nucleosomal structure is lost and the transcriptional activity in the spermatid is silenced (Eddy, Sauterer et al. 1993). At the same time, the remaining cytoplasm is also condensed into a cytoplasmic droplet. The testicular spermatozoa that are expelled from Sertoli cells are morphologically and genomically developed. The spermatozoa that enter the caput epididymidis are developmentally young; they lack the abilities to go forward and recognise the zona pellucida.

The ribosomes and endoplasmic reticulum (ER) of the sperm are almost completely missing when they are discharged into the lumen of the tubule. All of the components that sperm will need for ascending the female reproductive system must be supplied from the outside, for example, by the cells of the cauda epididymis, as they lack the ability to create proteins. The alkaline nature of sperm prevents them from reaching their maximum motility (hypermotility) until they reach the vagina, when the acidic pH of the vaginal secretions neutralises the alkaline character of the sperm. It takes 20 to 30 minutes for this progressive process. During this period, a clot made of fibrinogen from the seminal vesicles secures and safeguards the sperm. Fibrinolysin from the prostate breaks the clot just as the sperm start to become

hypermotile, enabling optimal sperm movement (Loveland and Schlatt 1997). About 75 days are needed for a spematogonium to produce mature sperm (JM, DillFJ et al. 1992).

FACTORS AFFECTING SPERM MATURATION

The environment that spermatozoa experience as they travel through the human epididymis is diverse in terms of the proteins they come into touch with. Sperm are exposed to proteins involved in membrane remodelling and enzyme activity in the proximal epididymis. To enable the absorption of GPI-anchored zona binding proteins P34H and CD52, the middle region of the sperm membrane may be altered by a distinct group of dominant proteins and enzymes. As sperm move farther away, their survival before ejaculation is aided by the action of lytic enzymes, proteins associated in zona binding and oocyte fusion, the major maturation antigen CD52, antimicrobial activity, and decapitation factors (Cooper 2002).

Epididymosomes

There is evidence that sperm cells pick up proteins devoid of a signal peptide during their transit through the epididymis, suggesting an unique secretion mechanism. In actuality, the P34H protein family, which is unique to the human epididymis, has been described. This glycosyl phosphatidylinositol (GPI) anchor-attached protein is found on the surface of sperms and is necessary for the zona pellucida to be recognised. The "prostasome-like particles" or "epididymosomes," which are epididymal membranous particles found in the lumen, are what anchor the P34h to the sperm surface. The maturation of the sperm cells' epididymis is directly influenced by epididymosomes (Saez, Frenette et al. 2003).

Prostasomes and modifications to post-ejaculatory sperm: At the time of ejaculation, the prostate secretes the "prostasomes," which are combined with the semen. It has been demonstrated that prostasomes contain a wide variety of proteins, some of which have enzymatic characteristics. Prostasomes have an immunosuppressive effect, improve sperm motility, and have an impact on the sperm capacitation process on the maturation of spermatozoa after the testis (Saez, Frenette et al. 2003). The presence of many complement inhibitory molecules, including CD55, CD46, and CD59 (protectin), which prevent the formation of the membrane assault complex, is what gives prostasomes their immunosuppressive properties. These compounds may serve to prevent spermatozoal phagocytosis by white blood cells in the female genital canal. Sperm motility is also influenced by prostasomes. Due to changes in the sperm milieu, they improve spermatozoa's progressive motility (Saez, Frenette et al. 2003). These vesicles include a calcium-dependent ATPase.

Acrosomal reaction and capacitation

Before fertilisation, a number of hydrolytic enzymes, including hyaluronidase, neuraminidase, acid phosphatase, and a protease with trypsin-like activity, are released from the acrosome, a cap-like structure covering the anterior region of the sperm nucleus. These enzymes are known to separate

corona radiata cells and break down the zona pellucida. At the same time, significant alterations happen in each sperm compartment (head and flagellum, membrane, cytosol, and cytoskeleton). Complex signal transduction mechanisms are started, proteins and membrane lipids are rearranged, and factors from seminal plasma and epididymal fluids are lost or redistributed. Acrosomal enzymes are released into the extracellular space when spermatozoa come into contact with an oocyte because the acrosome's outer membrane frequently fuses with the egg's plasma membrane. The acrosomal reaction, a crucial stage in fertilisation, is this process (Marsico, Pizzo et al. 1994, Wassarman 1999).

In mammals, sperm-egg interaction and mutual activation are mediated by the zona pellucida, the egg's glycoprotein coat. The spermatozoon connects to the zona pellucida while the plasma membrane is still intact via specialized receptors distributed throughout the anterior acrosomal area. When the sperm connects to the zona pellucida, an acrosome reaction occurs, allowing the sperm to penetrate the egg and fertilize it. The attachment of the sperm to the egg and occurrence of the acrosome response would only take place if the sperm had previously undergone capacitation in the female reproductive tract (Breitbart and Spungin 1997, Breitbart 2002, Breitbart, Cohen et al. 2005).

Proteome of the testis

It is well known that several different proteins are produced into the male reproductive tract's lumen. These secretions contain enzymes, protease inhibitors, antimicrobial peptides, enzymes, and host defence effector molecules (Hall et al., 2007). (Rang et al). Additionally, they produce proteins that are hypothesised to play a part in sperm maturation, protection, and production (Dacheux et al. 2003). Transcriptome analysis shows that 77% of all human proteins are expressed in the testis and many genes show an elevated expression in the testis compared to other tissue types. The majority of testicular proteins are involved in general metabolism function. 19 % of them are related to structural components and only 2 % are involved in immunity/defence function. 6% of testicular proteins are categorized as unclassified (Djureinovic, Fagerberg et al. 2014) (**Figure 4**). In the testis, a large number of genes are uncharacterized and functionally unknown. The proteins that show enhanced expression in the testes may have a role in spermatogenesis and fertility. Studying the uncharacterized protein with highly predicted expression may help in understanding the crucial role in testicular function.

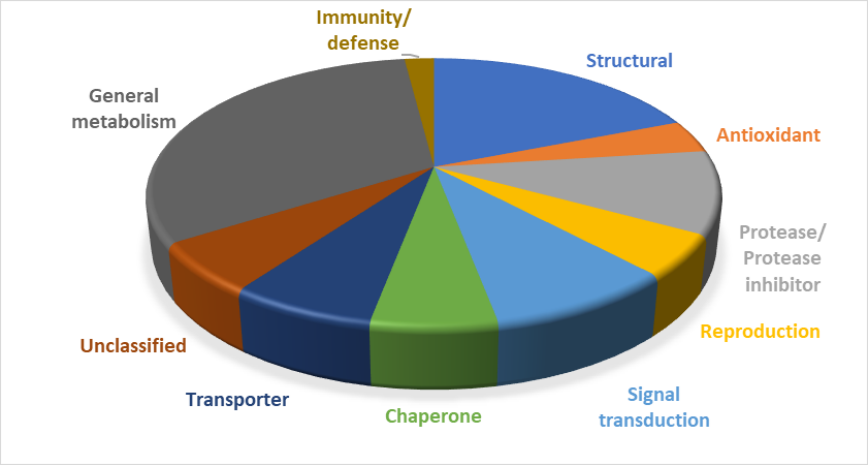

***Figure 4**. Functional classification of testicular proteome (Djureinovic, Fagerberg et al. 2014).*

Epididymis

One of the auxiliary organs, the epididymis is a lengthy, convoluted ductal structure physically split into the caput (head), corpus (body), and cauda (tail) parts (Belleannee, Thimon et al. 2012). (**Figure 5**). Testicular spermatozoa are immotile and incapable of fertilisation (Cosentino and Cockett 1986). They go through capacitation/acrosome response in the female reproductive tract and epididymal maturation in the epididymis to become fertile (Sullivan and Mieusset 2016). The 2 to 6 day epididymal transit, during which sperm mature in the epididymis, can take place. A variety of maturation factors that are produced into the lumen are known to express themselves differently in each epididymal area. Secretory proteins are added to the spermatozoa's surface during transit to speed up maturation, motility acquisition, and fertilisation.

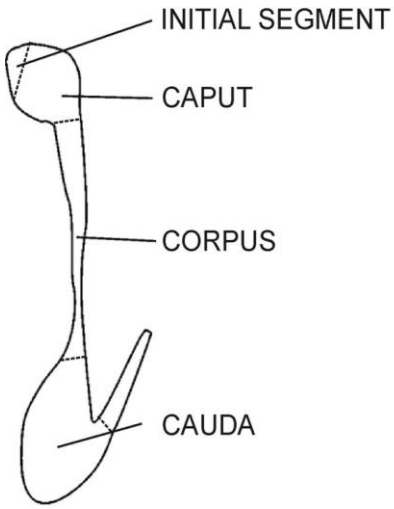

***Figure 5**. Schematic diagram of the rodent epididymis showing the different epididymal segments (Pinel, Mandon et al. 2019).*

The ability of spermatozoa to undergo capacitation in the female reproductive system is similarly influenced by epididymal maturation (Gervasi and Visconti 2017). The epididymis' secretory functions are tightly controlled in a region-specific way (Moser, Weiner et al. 2002). There are various androgen-regulated genes that are unique to the epididymis (Hu, Zou et al. 2014). Thus, androgen is required for sperm maturation in the epididymis (Pujianto, Loanda et al. 2013). Although the caput and corpus are where the majority of sperm maturation takes place, the functionally mature sperm are stored in the cauda area (Yeung and Cooper 2002).

Six separate cell types with specialised physiological roles make up the epididymis. While apical and narrow cells are only found in the proximal region, the primary cells, basal cells, halo cells and clear cells are prevalent throughout the epididymis (**Figure 6**) (Hermo and Robaire 2002). Principal cells, one of the six main types of cells in the epididymis, make up 80% of the total number of 4 cells and are crucial for the production and secretion of epididymal proteins (Abe, Takano et al. 1983). Epithelial cells create a luminal microenvironment by establishing a protective barrier and transporting ions, solutes, proteins, nutrients, and water. The reabsorption of numerous substances from the epididymal fluid is carried out by the main cells (Breton, Nair et al. 2019). The reabsorption of various components from the epididymal fluid is carried out by the main cells (Hermo, Dworkin et al. 1988). The second most prevalent cell type is clear cells, which are found in the caput and cauda. The transparent cells are crucial to endocytosis (Hermo, Dworkin et al. 1988). The beginning segment has narrow cells and apical cells, however it is unknown what they do (Sun and Flickinger 1980, Adamali and Hermo 1996). Basal cells provide a protective role in maintaining the structural integrity of the lumen by being joined to the major cells (Adamali and Hermo 1996).

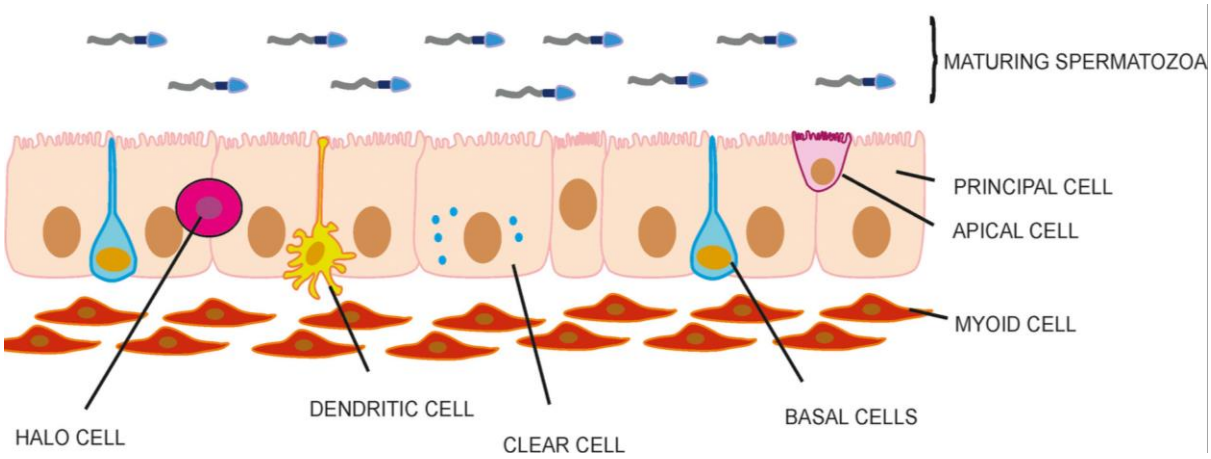

***Figure 6**. Schematic diagram of the epididymal epithelium showing the different cells types (Pinel, Mandon et al. 2019).*

Epididymal proteome

The epididymal luminal milieu is composed of a variety of proteins, the majority of which are secretory and are added to the surface of spermatozoa to help in sperm maturation and innate immunity (Tollner, Bevins et al. 2012). The distribution of epididymal protein expression varies across the regions (Dacheux, Belleannee et al. 2009, Belleannee, Belghazi et al. 2011). Most region-specific genes are found in the caput area of the epididymis (Zhang, Liu et al. 2006, Dube, Chan et al. 2007, Thimon, Koukoui et al. 2007). Many of them that belong to various families have been discovered and characterised (Cornwall 2009, Caballero, Frenette et al. 2010, Bjorkgren and Sipila 2019), including CRISPs, defensins, SPAG11 family, Cathelicidins, Serine proteases, and protease inhibitors. They support a number of processes, including sperm maturation, sperm stability in the female reproductive system, serving as markers for different malignancies, and innate immunity. For instance, the human epididymis protein 4 (HE4) is a recognised marker for numerous cancer forms (Anastasi, Marchei et al. 2010, James, Carrell et al. 2020). Furthermore, sperm survival and protection are aided by epididymal proteins. Additionally, antioxidant, antibacterial, and antiviral properties of lactotransferrin are known (Jenssen and Hancock 2009).

Defensins

Antimicrobial peptides such as defensins are crucial components of innate immunity (Lehrer 2004). According to their secondary structure and disulphide bond pairing, the three defensin subtypes in humans are known: alpha-defensins (human neutrophil peptides), beta-defensins, and theta-defensins (Ganz 2003, Lehrer 2004, Selsted and Ouellette 2005). Small antimicrobial peptides known as -defensins are mostly expressed in the epididymis and testis and are conserved across species (Patil, Cai et al. 2005, Oh, Lee et al. 2006). 52 mouse, 43 rat, and 39 human beta-defensin genes have been found (Patil, Cai et al. 2005). Defensins have antibacterial, antifungal, and antiviral properties that they use to combat several pathogens. Permeabilization of cell membranes and interference with fundamental metabolic processes are part of their mode of action (Selsted and Ouellette 2005). Both in vivo and in vitro evidence of their antibacterial action points to this protective effect (Garcia, Krause et al. 2001, Salzman, Ghosh et al. 2003, Yenugu, Hamil et al. 2003, Yenugu, Hamil et al. 2004, Yenugu, Hamil et al. 2004). Defensins play a role in motility, capacitation, sperm maturation, and male fertility in addition to their antibacterial and host defence functions (Zhou, Zhang et al. 2004, Yudin, Generao et al. 2005, Hall, Yenugu et al. 2007, Tollner, Venners et al. 2011). Male reproductive tracts express defensins (Yamaguchi, Nagase et al. 2002, Rodriguez-Jimenez, Krause et al. 2003, Semple, Rolfe et al. 2003, Patil, Cai et al. 2005), which aid in sperm maturation and capacitation (Yudin, Tollner et al. 2003, Zanich, Pascall et al. 2003, Zhou, Zhang et al. 2004). Seven different β-defensin (Defb) gene location and expression are investigated (Jelinsky, Turner et al. 2007, Guyonnet, Marot et al. 2009). On

chromosomes 8 and 20, the human Defb genes are grouped together (Yenugu, Hamil et al. 2006). Rodents have Defb genes, which are similar to human Defb genes (Froy, Hananel et al. 2005).

Sperm associated antigen 11 family members

The humans chromosome 8p23 harbours the *SPAG11* gene, often referred to as human epididymal protein 2 (*HE2*) and epididymal protein 2 (*EP2*) (Osterhoff, Kirchhoff et al. 1994). The *SPAG11A* gene, which has two promoters, was created as the result of the fusion of two primordial *Defb* genes. Two promoters enable alternative splicing, which results in the translation of more than 20 different protein isoforms (Frohlich, Po et al. 2000). Three distinct *Spag11* genes, *Spag11a* (also known as *Spag11e* or *Bin1b*), *Spag11c*, and *Spag11t*, are found on chromosome 16 in the rat and have been functionally described (Yenugu, Hamil et al. 2006). Rat SPAG11A protein is crucial for sperm maturation and innate defence against pathogens (Li, Chan et al. 2001, Zhou, Zhang et al. 2004). The six-cysteine motif signature found in -defensins is present in the proteins produced by the *Spag11c* and *Spag11a* genes (Yenugu, Hamil et al. 2006) and these proteins have strong antibacterial activity. Their method of action, which is similar to that of -defensins, involves membrane permeation and the suppression of macromolecular formation (Yenugu, Hamil et al. 2004).

Epididymis and cancer

The leading cause of death worldwide is cancer. This disease still poses a major hazard despite improvements in detection, diagnosis, and treatment. Men may have a higher chance of developing cancer because of abnormalities in the Y chromosome, which determines sex, which cause numerous male-specific genes to lose function (Cannon-Albright, Farnham et al. 2014). Male malignancies of the prostate, lung, colorectal, melanoma, and testicles are the most prevalent (Siegel, Miller et al. 2020).

Although most of a man's organs are susceptible to cancer, epididymal carcinoma is extremely uncommon (Yeung, Wang et al. 2012). Young men are more likely than older men to have testicular cancer (Manecksha and Fitzpatrick 2009), and older men are more likely to develop prostate cancer (Jones, Young et al. 1997, Ganem, Jhaveri et al. 1998, Odrzywolski and Mukhopadhyay 2010, Munoz, Wheler et al. 2015). Epididymal cancer is rare, accounting for up to 0.03% of all male cancer cases, however there have never been any reported cases of the epididymal malignancies (Yeung, Wang et al. 2012). Despite sharing a common embryonic beginning, the kidney is 100 times more likely than the epididymis to acquire cancer (Yeung, Wang et al. 2012). This increases the appeal of the physiology of the epididymis significantly.

Epididymal tumours are divided into two groups based on their pathological characteristics: adenomatoid and cystamatoid types (Jones, Young et al. 1997, Pan, Song et al. 1998, Almohaya, Almansori et al. 2021). Adenomatoids are uncommon benign mesothelial tumours that develop in the epididymal tail (Abdullah and Xing 2020). They only make up 0.03% of all reports, yet the majority

were benign. A benign cystic tumour of the gland called a cystadenoma is the most researched epididymal malignancy in humans (Toutziaris, Kampantais et al. 2013). There are no known cases of cancer cells spreading or invading the epididymis to cause secondary tumours. It's possible that the absence of tumorigenic stimuli or the presence of intrinsic neoplasia-inhibitory systems in the human epididymis prevent the development of cancer (Rizk, Scholes et al. 1990, Ganem, Jhaveri et al. 1998, Tilki, Kilic et al. 2008). The occurrence of epididymal 10 tumours is extremely rare, and nothing is known about it. The rarity of cancer is still a mystery. According to a theory, some epididymis-specific elements may be crucial in preventing the development of the tumour.

It is hypothesised that the epididymis's natural microenvironmental factors have a role in the absence of stemness in epithelial cells, strong antioxidative defences, active tumour suppression, and inactivation of oncogene products (Yeung, Wang et al. 2012). Strong immune surveillance in the epididymis keeps hyperplastic cells dormant. The rarity of cancer in this organ system is further influenced by the anti-angiogenic factors, existence of persistent tight junctions, ineffective immune escape, and misdirected pro-angiogenic factors (Mital, Hinton et al. 2011, Yeung, Wang et al. 2012). The threshold for tumour start is higher than it is for other organs due to the epididymal cells' reduced reactivity to tumor-causing stimuli (Yeung, Wang et al. 2012). Additionally, the epididymis possesses an immune-suppressive and anti-oxidative milieu that is required to keep large numbers of spermatozoa dormant and immunologically silent. Its role in sperm maturation and storage may directly contribute to the low incidence of cancer (Zhen, Li et al. 2009).

Role of defensins in cancer

The significance of -defensins in malignancies has grown in recent years (Droin, Hendra et al. 2009). Intriguingly, oral cancer cell lines and tumour tissues revealed that human -defensins are proto-oncogenes that contribute to the spread of the disease (Jin, Kawsar et al. 2010, DasGupta, Nweze et al. 2016, Xu, Zhang et al. 2016). A tumour 11 suppressor gene called human -defensin 1 (hBD-1) is also found on the short arm of chromosome 8 in the defensin cluster (Donald, Sun et al. 2003, Bullard, Gibson et al. 2008). Prostate malignancies and renal cell carcinomas both showed lack of hBD-1 expression (Sun, Arnold et al. 2006). However, it has been noted that hBD-2 is elevated in a number of malignancies, such as oesophageal (Shi, Jin et al. 2014), lung (Arimura, Ashitani et al. 2004, Shestakova, Zhuravel et al. 2008), cervical cancers (Markeeva, Lysovskiy et al. 2005), but downregulated in colon cancer (Gambichler, Skrygan et al. 2006, Semlali, Al Amri et al. 2015). In addition, it was discovered that cancer cell lines expressed more hBD3 than normal cells did (Shnitsar, Lisovskiy et al. 2004). Defensins destroy tumour cells through a mechanism involving DNA damage and membrane lysis (Barker and Reisfeld 1993, Selsted and Ouellette 2005, Mader and Hoskin 2006, McKeown, Lundy et al. 2006, Pazgier, Li et al. 2007). It has been determined that the human epididymal protein 4 (HE4) may serve as a biomarker for a variety of malignancies, renal fibrosis, and chronic

kidney disorders (Chen, Yang et al. 2017, Yuan and Li 2017, Mi and Zhang 2018, Mo and He 2018, Ferraro and Panteghini 2019, Li, Wang et al. 2019). Cancer cell gene expression, proliferation, invasion, and metastasis are all affected by the HE4 protein (Zhu, Guo et al. 2016, Zhu, Zhuang et al. 2016). These data revealed that β-defensins are essential for cell growth and carcinogenesis.

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Chapter 1

In silico, *in vivo* and functional characterization of rat *Spink2*, *Spaca7* and *Pdcl2*

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INTRODUCTION

Spermatogenesis is a complex process that involves an interplay between a wide variety of molecular factors (Eddy 2002), especially the crucial testis specific factors (Lu, Oura et al. 2019, Bellil, Ghieh et al. 2021). Disruption in the expression of genes that contribute to spermatogenesis caused fertility disorders (Linn, Ghanem et al. 2021). Identification and characterization of protein markers of spermatogenesis and their potential for the management of deregulated spermiation is an active area of investigation (Araujo and Bertolla 2021, Li, Wang et al. 2021). Some of the genes that have been recently characterized for their role in male gametogenesis are *Spats1, Rail4, Tex13a, Paz domain, Tcfl5*, etc (Capoano, Ortiz-Laquintana et al. 2021, Li, Mi et al. 2021, Qi-Xin, Lu et al. 2021, Wu, Wang et al. 2021, Xu, Zhang et al. 2021). Thus, identification and characterization of genes that may play a crucial role in spermatogenesis continues to be a vibrant area of research till date. In this study, we investigated the role of three rat genes, namely, serine protease inhibitor Kazal-type 2 (*Spink2*), sperm acrosomal membrane-associated protein 7 (*Spaca7*) and phosducin-like 2 (*Pdcl2*), that are predominantly expressed in the testis. The three genes characterized in this study do not belong to the same family or share similar structural or functional domains. However, analyses of a group of proteins belonging to different classes is essential to identify the importance of a diverse nature of proteins in male reproduction.

The SPINK protein family members contain a Kazal domain at the C-terminus of the serine protease inhibitor and have been implicated in a variety of biological processess, including male fertility. *Spink13*, an epididymis specific gene was found to be essential for acrosomal integrity and male fertility (Ma, Yu et al. 2013). Knockout of the *Spink2*, a gene predominantly expressed in the testis resulted in impaired spermatogenesis and fertility in mice (Lee, Park et al. 2011). SPINK3, localized on the acrosome, modulates sperm activity (Ou, Tang et al. 2012). A 4 fold decrease in *Spink2* expression was reported in azoospermic patients (Rockett, Patrizio et al. 2004). SPINK8, SPINK11 and SPINK12 are found to be epididymis specific and their roles in male fertility reported (Jeong, Lee et al. 2019).

Another set of proteins grouped under the SPACA family are known to contribute to the molecular aspects of male fertility. SPACA7 was identified as a novel male germ cell specific protein that is localized to the acrosome and implicated in fertilization in a mouse model (Korfanty, Toma et al. 2012, Nguyen, Westmuckett et al. 2014). In humans and murine models, loss of *Spaca1* gene resulted in autosomal recessive globozoospermia (Fujihara, Satouh et al. 2012, Chen, Saiyin et al. 2021). Regulation of sperm-egg membrane fusion machinery requires the cleavage of SPACA1 (Yamatoya, Kousaka et al. 2020). An association between SPACA1 distribution on sperm and the outcome of IVF was reported (Kishida, Harayama et al. 2016). Loss of SPACA4, a germ cell specific protein results in impaired fertilization in knockout mice (Fujihara, Herberg et al. 2021). Similarly, the essential role of SPACA6 in sperm-oocyte fusion was reported (Barbaux, Ialy-Radio et al. 2020, Noda, Lu et al. 2020).

The exclusive expression of SPACA5 in the elongated spermatids implicated its possible role in sperm function (Zhang, Yan et al. 2016). SPACA3, also known as SLLP1 or SPRASA, is a cancer-testis antigen (Wang, Zhang et al. 2004) and polymorphisms in *Spaca3* gene are associated with infertility (Prendergast, Woad et al. 2014). ACRV1, aka SPACA2 is a testis specific protein and is reported to be an ideal marker for stage specific identification of spermatogenesis (Osuru, Monroe et al. 2014).

PDCL2 family members, initially thought to be having a role in G-protein signaling were later found to act as molecular chaperones (Willardson and Howlett 2007, Krzemień-Ojak, Góral et al. 2017). PDCL3 regulates the expression of VEGF receptor to influence angiogenesis (Srinivasan, Chitalia et al. 2015). PDCL1 was found to be crucial for the assembly of G-protein receptor complex in retinal cone photoreceptors (Tracy, Kolesnikov et al. 2015). Microinjection of phosducin resulted in inhibition of second polar body emission and pronucleus formation (Moore, Ayabe et al. 1994). However, no information is available on the possible role of PDCL2 in reproductive function.

RATIONALE

Though quite a lot of evidence points out to the role of SPINK2 and SPACA7 in male reproductive function in mouse and humans, their role in the rats are not well characterized. The rat *Spink2* and *Spaca7* were indicated as predicted genes in the genome. It is to be noted that the functional role of a protein may vary among the species and could provide a different perspective. Further, earlier studies focused mostly on the role of SPINK2 and SPACA7 on spermatogenesis related aspects. Thus, we attempted to characterize and elucidate the functional role of SPINK2 and SPACA7 with specific reference to fecundity and sperm function. Our initial analyses indicated that *Pdcl2* is predominantly expressed in the rat testes; and in the absence of any evidence for the role of this gene in male reproductive function, we conducted studies in this direction. The role of a specific protein or a set of proteins in gametogenesis and maturation were studied by different approaches such as generation of knockout models (Lu, Oura et al. 2019, Capoano, Ortiz-Laquintana et al. 2021) and by active immunization, a classical method of ablation of a specific protein by generation of autoantibodies in animal models (Aponte, Gutierrez-Reinoso et al. 2018, Sangeeta and Yenugu 2020). As a prelude to studying the role of *Spink2*, *Spaca7* and *Pdcl2* in knockout animal models, we conducted initial studies on rats that were subjected to active immunization against these proteins. Further, the significance of this study lies in the fact that though these three proteins characterized in this study are not related, they all appear to play a crucial role in male reproduction. In the recent years, diagnosis of male infertility in patients is being done basing on the detection of an array of multiple markers that are crucial for sperm production, maturation and function (Bieniek, Drabovich et al. 2016). Thus, identification of markers of diverse nature will be of use to develop kits for the diagnosis of infertility.

MATERIALS AND METHODS

In silico analyses

The predicted gene sequences of *Spink2, Spaca7* and *Pdcl2* were derived from the rat genome available at National Center for Biological Information (NCBI). PCR primers were designed to amplify the predicted sequences (**Supplementary table 1**). The amplicons were sequenced and submitted at GENBank. The tools used to analyze the sequences for predicting the properties are listed in **supplementary table 2**. Genomic neighborhood analysis was performed based on the genome assemblies deposited in NCBI and Ensembl. Self-Optimized Prediction Method with Alignment (SOPMA) was used to predict the secondary structure (α-helix, β-turn, extended strand and random coil; in terms of percentage) (Frishman and Argos 1995, Geourjon and Deléage 1995).

Molecular modeling

I-TASSER based protein modelling was carried out to predict the three-dimensional models of proteins. Excluding the signal peptide sequence, the full-length sequence of the proteins that are retrieved from NCBI were submitted as an input for the program. Using LOMETS, a meta-server threading approach containing multiple threading programs, the best ten templates were identified from the PDB library. Basing on these templates, the three-dimensional structures of the proteins were generated by I-TASSER. The output was generated as a 3D model using nearest homolog as a template with higher sequence identity using BLOSUM 45, 62, 80 at different Pfam value (Arnold, Bordoli et al. 2006). The models generated were validated using PROCHECK (Ramachandran, Ramakrishnan et al. 1963). Proteins showing more than 90% in the core region and not more than 5% in the disallowed region are considered as good model. In cases where the models does not meet the requirements of quality structure, modeling was repeated after loop refinement and energy minimization and the structures were validated by analyzing the stereo chemical features using PROCHECK (Laskowski, Rullmannn et al. 1996). PYMOL was used to visualize the structure of the modelled rat LYZL proteins.

Recombinant protein production

The full length cDNA of rat *Spink2, Spaca7* and *Pdcl2* without the signal peptide were amplified using gene specific primers (**supplementary table 1**) and cloned into pQE80m vector such that there is a His-tag (MRGSHHHHHHGS) at the N-terminus. The cloned plasmid was transformed into *E.coli BL21* and the bacteria were grown to mid-log phase. Using 1 mM IPTG, the bacteria were induced to express the recombinant protein for 3 hr. The bacterial cells were then lysed and the recombinant protein was purified using a commercially available Ni-NTA agarose affinity protein purification kit (Qiagen, Valencia, CA, USA). The purified recombinant protein was confirmed by Western blotting using anti-His tag antibody (Santa Cruz Biotechnology, Dallas, USA; sc-57598 RRID:

AB_831408). Eluted fractions that contained the protein of interest were dialyzed extensively at 4°C against 10 mM sodium phosphate buffer, pH 7.4.

Secondary structure analyses

Circular dichroism was performed for the recombinant proteins using Jasco J-810 spectropolarimeter. 200 µl (0.1µg/µl) protein was placed in a Quartz cell with a path length of 0.2 cm and scanned in the far UV region (180-260 nm). Polarimetry data was collected for every 1 nm by accumulating the data collected from three scans at a scan speed of 50 nm/min. The mean residue ellipticity (MRE) was calculated using the polarimetry data and the spectrum was plotted (Sreerama and Woody 2004). Spectra of 10 mM phosphate buffer was subtracted from the spectrum of the protein and the percent of secondary structure elements in the protein were calculated using K2D3 tool of Dichroweb (Louis-Jeune, Andrade-Navarro et al. 2011). To determine the secondary structure at pH 3.0 and 10, citrate and Tris buffers respectively were used.

Gene expression

Total RNA (2ug) isolated from each of the different tissues of male Wistar rats (aged 90 days) was reverse transcribed and the expression pattern of *Spinkl-2*, *Spaca7* and *Pdcl2* was analysed using gene specific primers (**Supplementary table 1**) under standard PCR conditions (94°C for 1 min followed by 30 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, and with a final round of extension at 72°C for 10 min). The expression of glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*) was used for internal control purposes. PCR amplified gene products were analysed by electrophoresis on 2% agarose gels and their identity confirmed by sequencing.

The developmental regulation of *Spinkl-2*, *Spaca7* and *Pdcl2* expression was analyzed using RNA isolated from the testes and epididymides of 10- to 60-day old Wistar rats. All procedures involving animals were conducted using the guidelines for the care and use of laboratory animals and this study was specifically approved by the Institutional Animal Ethics Committee of University of Hyderabad (UH/IAEC/SY/2021-1/18).

Immunofluorescence localization

Testes collected from 90-day old Wistar rats were fixed by immersing in Bouin's solution and 4% paraformaldehyde solution respectively. Serial sections (five microns) were made and preheated to 60°C for 5 min followed by washings with xylene, gradient alcohol (70-100%) and PBS for 10 min each. Antigen retrieval was done by heating in 10 mM citrate buffer, pH 6.5 for 12 min; followed by permeabilization with PBS containing 1% triton X-100 (PBST) for 15 min and blocking with 10% goat serum for 45 min. The processed sections were incubated with polyclonal antibodies (immune serum generated in this study by active immunization) and Goat anti-Rat IgG tagged with FITC (Santa Cruz Biotechnology, Dallas, USA; sc-2091 RRID: AB_649008; sc-2012 RRID: AB_631744) for 1 h each

with washing with PBS in between incubations. The nucleus was stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, USA)) and images taken using fluorescence microscope. For immunolocalization on the spermatozoa, smears were prepared on glass slides and air dried. Permeabilization was done with PBST, followed by blocking with 10% goat serum and processed in a similar way as that of tissue sections.

Immunization to generate auto antibodies

Male Wistar rats (n = 6 per group) aged 90 days were immunized with SPINK2 or SPACA7 or PDCL2 recombinant protein. Pre-immune serum was prepared from blood obtained by snip tail method. For immunization, 200 µg of the protein in 100 µl of PBS mixed with equal volume of Freund's complete adjuvant was administered into the footpad and the remaining subcutaneously. Booster dose was given subcutaneously on 14th day after first immunization using 200 µg of recombinant protein mixed with Freund's incomplete adjuvant. Control animals received Freund's adjuvant without recombinant protein similar to the immunized animals.

Determining the antibody titer

Antibody titer in the serum of immunized animals was determined by ELISA as described earlier (Sangeeta and Yenugu 2020). Serum was prepared from blood collected through tail vein. Testicular fluids collected at the end of the experiment by making incisions and gentle squeezing were centrifuged at 10000 rpm for 10 min at 4 C. Protein concentration was determined in the supernatant and equal quantity was used for measuring the antibody titer using ELISA. Each of the recombinant protein (40 µg/ml) was coated on to the wells of the microtiter plate and incubated at 37˚C overnight and at 60˚C for 30 min. BSA (1 mg/ml) was added to each well and incubated for 2 hr to allow blocking. After thorough washing with TBS-T (PBS with 0.1% Tween-20), testicular fluid was added and further incubated for 3 hr followed by washing with TBS-T. HRP-conjugated anti-rat secondary antibody was then added to facilitate binding to the primary antibody. O-Phenylenediamine (OPD), the substrate for HRP was added to each well and the intensity of the color developed was measured at 490 nm. The intensity of the color will be directly proportional to antibody titer. Rats that had a significant serum titer were used for further studies.

Assessment of fecundity

The effect of ablation of testicular proteins on fecundity was analyzed by natural mating. A booster dose was administered (one week prior) to ensure high antibody titer before assessing the fecundity. Each of the control (unimmunized) or immunized male rat (with significant antibody titer) was placed along with two normal female rats for one week. The litter size was noted when the female rats delivered the pups.

Gene chip hybridization, data collection and enrichment analysis.

Total RNA was isolated from the testes of control and immunized rats using TRIzol. The quality of RNA was checked by the Agilent TapeStation system. Samples that had a RIN value of > 7.5 were further analyzed. RNA-seq was carried out using Illumina HiSeq platform. Then the data quality was checked using FastQC and MultiQC software (de Sena Brandine and Smith 2019). The data was checked for base call quality distribution, % bases above Q20, Q30, %GC, and sequencing adapter contamination. All the samples passed the QC threshold (Q30>90%). Raw sequence reads were processed to remove adapter sequences and low-quality bases using fastp (Chen, Zhou et al. 2018) QC passed reads were mapped onto Rattus norvegicus (assembly mRatBN7.2) reference genome using STAR v2 aligner (Dobin, Davis et al. 2013). On average 99.86% of the reads aligned onto the reference genome. Gene level expression values were obtained as read counts using feature Counts software 10.1093/bioinformatics/btt656). Differential expression analysis was carried out using the DESeq2 (Love, Huber et al. 2014). The read counts were normalized (variance stabilized normalized counts) using DESeq2 and differential enrichment analysis was performed. Genes with absolute log2 fold change ≥ 1 and p-value ≤ 0.05 were considered significant. The genes that showed significant differential expression were used for Gene Ontology (GO) and pathway enrichment analysis using DAVID software.

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 $p < 0.05$ compared to the respective control.

RESULTS

In silico characterization

The predicted sequences of rat *Spink2*, *Spaca7* and *Pdcl2* were obtained from the rat genome using the accession numbers XM_003751350.4, XM_006222297.3 and XM_573585.7. Multiple set of primers were designed to for each of the genes and PCR amplified. The amplicons were sequenced and obtained sequence was submitted to GenBank. Accession numbers MK279534, MK364792 and MK364795 were assigned for *Spink2*, *Spaca7* and *Pdcl2* respectively (**Table 1**). The mRNA sequences of *Spink2*, *Spaca7* and *Pdcl2* with the predicted protein sequence are presented in **figure 1**. General features of the characterized genes and their protein counterparts are presented in **table 1**. Gene neighborhood analyses in the rat, mouse and human indicate that all the three genes are located independently and not in a cluster with the other members of their respective families (**Figure 2A, 2B and 2C**). Interestingly, those genes that are in the vicinity of *Spink2* or *Spaca7* or *Pdcl2* of rat genome are also conserved in the mouse and human genomes. Hydropathy and amphipathicity analyses indicated a gravy index of -0.737, -1.001 and -0.552 for SPINK2, SPACA7 and PDCL2 respectively (**Figure 3**). The predicted secondary structure of the three proteins was determined by SOPMA (**Figure 4**). The percent alpha-helical content was found to be higher in PDCL2 (51.67) followed by SPACA7 (36.30) and SPINK2 (14.29). In SPINK2 and SPACA7, the beta-sheet and extended strand percent content was almost similar. However, the random coil percent is higher in SPINK2 (71.43) followed by SPACA7 (55.56) and PDCL2 (33.75). To gain further insight into the actual distribution of different secondary structures, the proteins were subjected to Circular Dichroism analyses (**Figure 4**). In all the three proteins it is evident that the percent of random coil is the highest followed by beta-sheet and alpha-helix. Since the structural stability of a protein depends on the microenvironment, we determined the effect of different ion concentrations and pH on the secondary structure of SPINK, SPACA7 and PDCL2. We observed that the percent of the three forms of secondary structure did not vary much with increasing salt concentrations (**Figure 5**). For SPINK2, the alpha helix and random coil was decreased at pH 3.0 and 7.0, while that of beta-sheet was increased (**Figure 6**). Alpha-helix content increased at pH 3.0 and 7.0 for SPACA7, which was compensated by a decrease in beta-sheet content. In the case of PDCL2, alpha helix content was decreased at pH 3.0, while it was increased at pH 7.0. A reverse trend was observed for beta-sheet content. Further insight into the higher order conformation of SPINK, SPACA7 and PDCL2 was predicted by three-dimensional modeling. The best protein models for these proteins were generated and their fitment into the Ramachandran plot determined (**Figure 7**). Only 5% and 3.2% of the amino acids are in the disallowed regions for SPACA7 and PDCL2, while it was 0% for SPINK2.

Table 1. *General features of proteins characterized in this study.*

Attribute	**SPINK2-like**	**SPACA7**	**PDCL2**
Chromosome location	14	16	14
Gene accession number	LOC100910138	689077	498352
Gene length	6613	26972	20583
Total no. of exons	4	6	6
mRNA accession no.	MK279534	MK364792	MK364795
Length of mRNA	614	720	828
Coding sequence	105-365	70..549	129-851
Number of amino acids	86	159	240
Molecular Weight (kDa)	9.7	17.89	27.72
Localization	Secretory	Secretory	Cytoplasmic
Isoelectric point (pI)	4.93	4.67	4.72
GRAVY index	-0.279	-0.733	-0.521
Signal peptides	1-16	1-24	-
Disulfide bonds	38-68, 46-65, 54-86	17-18	125-152, 160-182
% homology with human	57.50	31.83	85.83
% homology with mice	82.56	75.47	96.67

Spink2

```
atg ctg aga ctg gtg ctg ttg ctc ctg gcc aca gac ttt gca gct tct gat gac tct ctg gac tct tcc gat tct
 M  L  R  L  V  L  L  L  L  A  T  D  F  A  A  S  D  D  S  L  D  S  S  D  S
caa ctc ata aag agg tca caa ttc aga aca cca gac tgt cat cgt ttt gac tac cca gta tgc tcc aag cac ctc
 Q  L  I  K  R  S  Q  F  R  T  P  D  C  H  R  F  D  Y  P  V  C  S  K  H  L
agc cct gtg tgc gga acg gat atg aac act tat ggg aat gaa tgc act ctg tgc atg aaa atc agg gag gat
 S  P  V  C  G  T  D  M  N  T  Y  G  N  E  C  T  L  C  M  K  I  R  E  D
ggt agc cat att aac atc atc aag gac gag cca tgc tga
 G  S  H  I  N  I  I  K  D  E  P  C  -
```

Spaca7

```
atg gca gcg aat agg gga gca agg acc ttc tta tcc gtt ttc ctg ctg tgc tgc tgg caa gtc act gag cta cat
 M  A  A  N  R  G  A  R  T  F  L  S  V  F  L  L  C  C  W  Q  V  T  E  L  H
cca gtc aag aca act tca ggt cca atc act gaa ggc gtt ttc aac tca aca act gaa aac ata cct gaa acc tta
 P  V  K  T  T  S  G  P  I  T  E  G  V  F  N  S  T  T  E  N  I  P  E  T  L
gat gaa att cta gcc caa gat att ttg gag cca aga act tca gca atg tct gca aca aca cca cga aca aga tcg
 D  E  I  L  A  Q  D  I  L  E  P  R  T  S  A  M  S  A  T  T  P  R  T  R  S
cca aca cag aca aca gta caa acc aag gaa cca aat gct ggt att gaa gaa aac tac caa gag gaa gcc ttt
 P  T  Q  T  T  V  Q  T  K  E  P  N  A  G  I  E  E  N  Y  Q  E  E  A  F
gag aac tac cac gag ttc ctg gag aat tta gaa cac tca tct agg aag aag gcg aaa agt gac aat aat gaa
 E  N  Y  H  E  F  L  E  N  L  E  H  S  S  R  K  K  A  K  S  D  N  N  E
aag aag agt tca aag gat gat gtg tat gag aaa ctg tct gtt ctg gac aga atg att gaa aac cca gga caa tct
 K  K  S  S  K  D  D  V  Y  E  K  L  S  V  L  D  R  M  I  E  N  P  G  Q  S
gaa ggc agc ctg gag cta aca gaa agc atc ttt tag
 E  G  S  L  E  L  T  E  S  I  F  -
```

Pdcl2

```
atg cag gat cca aat gaa gat aca gaa tgg aat gaa att tta agg aat ttt ggc att ctt cct cca aaa gaa gaa
 M  Q  D  P  N  E  D  T  E  W  N  E  I  L  R  N  F  G  I  L  P  P  K  E  E
cca aaa gat gaa att gaa gag atg gtg ttg cgc tta cag gag gaa gca atg gtt aag cca tat gag aag atg
 P  K  D  E  I  E  E  M  V  L  R  L  Q  E  E  A  M  V  K  P  Y  E  K  M
aca ctt gca cag ctg aaa gaa gca gaa gac gaa ttt gat gaa gaa gac ata aaa gct ata gaa ata tat aga
 T  L  A  Q  L  K  E  A  E  D  E  F  D  E  E  D  I  K  A  I  E  I  Y  R
gaa aag cgg tta cag gaa tgg aaa gca ctc acg aag aaa caa aaa ttt ggg gaa ttg aga gaa att tct gga
 E  K  R  L  Q  E  W  K  A  L  T  K  K  Q  K  F  G  E  L  R  E  I  S  G
aat cag tat gta aat gaa gtc aca aat gca gaa aaa gac ttg tgg gtt ata att cat cta tac aga tca agt gtc
 N  Q  Y  V  N  E  V  T  N  A  E  K  D  L  W  V  I  I  H  L  Y  R  S  S  V
cca atg tgt ttg gtg gtt aac cag cat ctg agt gtc cta gca aga aag ttt cca gaa acc aaa ttt gtt aaa gcc
 P  M  C  L  V  V  N  Q  H  L  S  V  L  A  R  K  F  P  E  T  K  F  V  K  A
atc gtg aat agc tgc att gaa cac tac cat gac aac tgt tta cca aca att ttt gtt tat aaa aat ggt cag ata gaa
 I  V  N  S  C  I  E  H  Y  H  D  N  C  L  P  T  I  F  V  Y  K  N  G  Q  I  E
ggc aaa ttc att gga gtt ata gaa tgt gga ggg ata aat ctc aag cta gaa gaa ctt gaa tgg aaa cta tca caa
 G  K  F  I  G  V  I  E  C  G  G  I  N  L  K  L  E  E  L  E  W  K  L  S  Q
gtt gga gca ata cag act gac ttg gaa gaa aac ccc aaa aag ggc att gca gat atg atg gtg tct tcc att
 V  G  A  I  Q  T  D  L  E  E  N  P  K  K  G  I  A  D  M  M  V  S  S  I
agg aac gtt tcc atc tat gac agt gac agc tct ggc agt gat gct gag gcc aag tag
 R  N  V  S  I  Y  D  S  D  S  S  G  S  D  A  E  A  K  -
```

Figure 1. *Sequence alignment of the characterized genes. mRNA sequence obtained by sequencing was translated and aligned with the corresponding protein sequence.*

FIGURE 2A

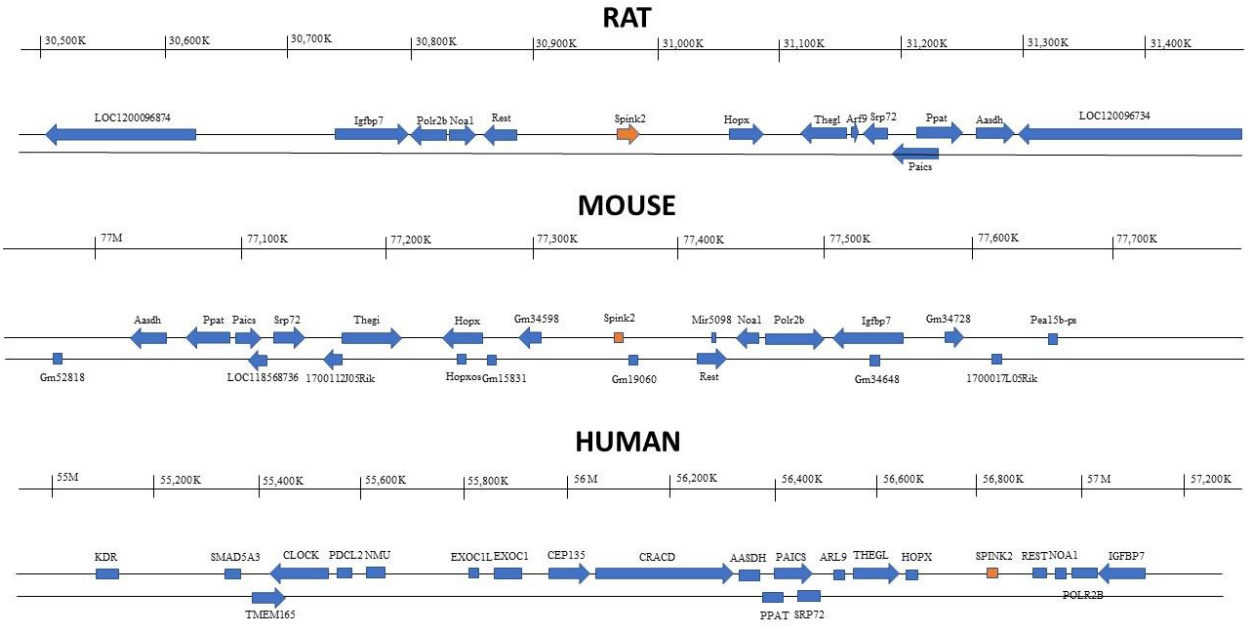


FIGURE 2B

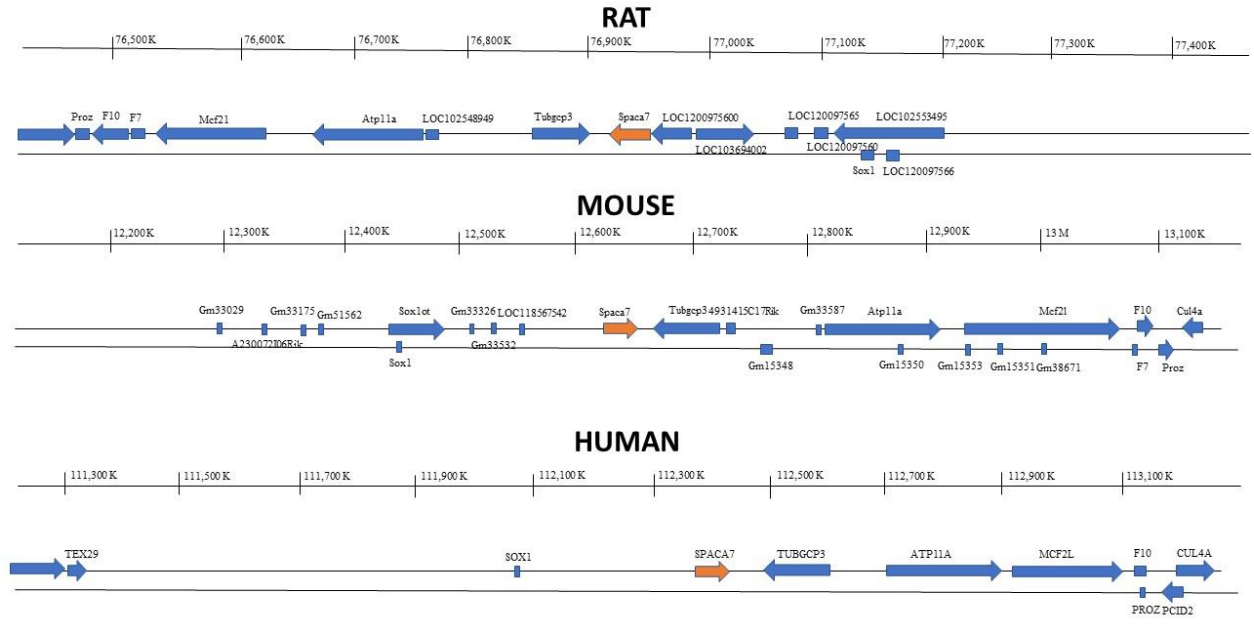


FIGURE 2C

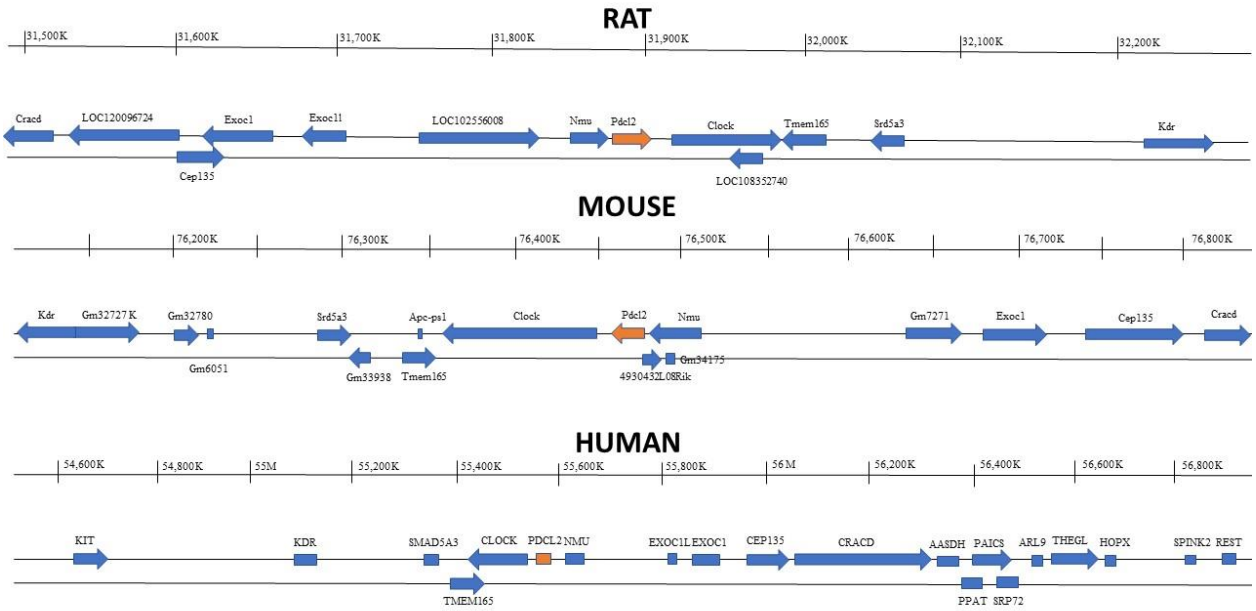


Figure 2. *Gene neighbourhood analyses. Rat, mouse and human Spink2, Spaca7 and Pdcl2 genes were identified in the respective genomes and the presence of other genes in the vicinity are indicated. Size of the arrow / bar is not to scale is only indicative and does not correspond to the gene length.*

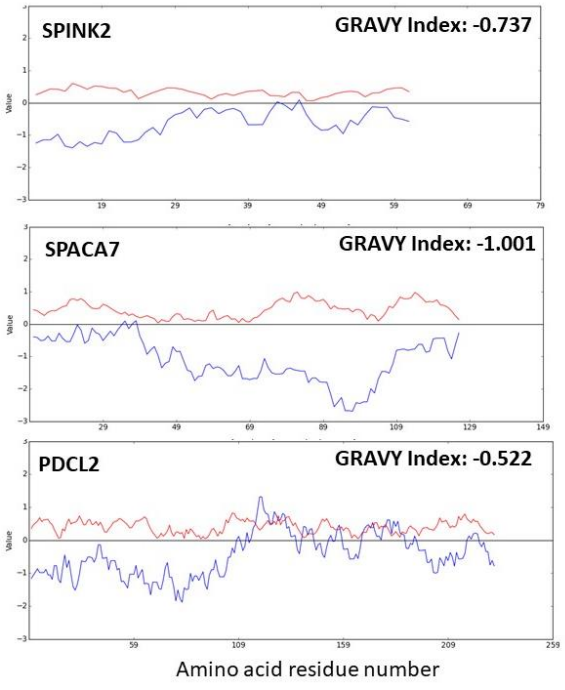
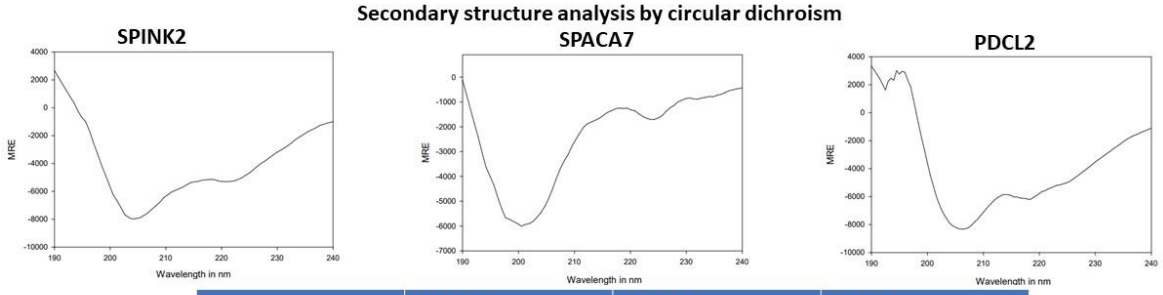


Figure 3. *Hydropathy and amphipathicity analyses. The protein sequences of rat SPINK2, SPACA7 and PDCL2 were subjected to in silico analyses. Blue and red lines indicate hydropathy and amphipathicity of the amino acids along the protein.*

Secondary structure prediction by SOPMA

	SPINK2	SPACA7	PDCL2
Alpha helix	14.29	36.30	51.67
Beta sheet	4.29	2.96	4.58
Extended strand	10.00	5.19	10.00
Random coils	71.43	55.56	33.75

Secondary structure analysis by circular dichroism



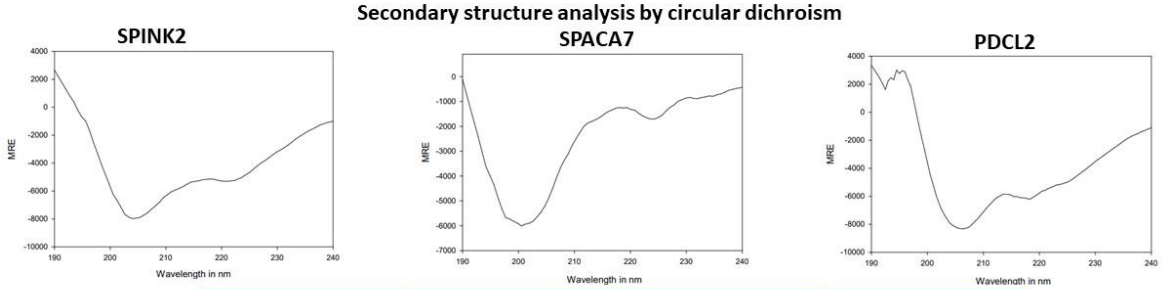
	SPINK2	SPACA7	PDCL2
Alpha helix	4.84	2.42	5.28
Beta sheet	21.85	32.12	35.65
Random coils	73.31	65.46	59.07

Figure 4. *Determination of secondary structure. SPINK2, SPACA7 and PDCL2 were subjected to SOPMA and the predicted secondary structural components determined. Soluble fractions of the proteins were analyzed in a spectropolarimeter and the secondary structure calculated using K2D3 tool of Dichroweb.*

Secondary structure prediction by SOPMA

	SPINK2	SPACA7	PDCL2
Alpha helix	14.29	36.30	51.67
Beta sheet	4.29	2.96	4.58
Extended strand	10.00	5.19	10.00
Random coils	71.43	55.56	33.75

Secondary structure analysis by circular dichroism



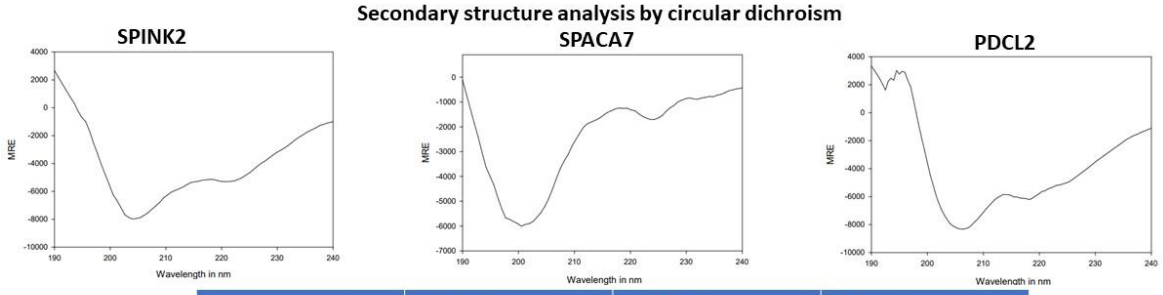
	SPINK2	SPACA7	PDCL2
Alpha helix	4.84	2.42	5.28
Beta sheet	21.85	32.12	35.65
Random coils	73.31	65.46	59.07

Figure 5. *Effect of ion strength on the secondary structure. SPINK2, SPACA7 and PDCL2 dissolved in PBS containing 0 to 300 mM NaCl were analyzed in a spectropolarimeter and the secondary structure calculated using K2D3 tool of Dichroweb.*

Secondary structure prediction by SOPMA

	SPINK2	SPACA7	PDCL2
Alpha helix	14.29	36.30	51.67
Beta sheet	4.29	2.96	4.58
Extended strand	10.00	5.19	10.00
Random coils	71.43	55.56	33.75

Secondary structure analysis by circular dichroism



	SPINK2	SPACA7	PDCL2
Alpha helix	4.84	2.42	5.28
Beta sheet	21.85	32.12	35.65
Random coils	73.31	65.46	59.07

Figure 6*. Effect of pH on the secondary structure. SPINK2, SPACA7 and PDCL2 dissolved in buffers of varying pH were analyzed in a spectropolarimeter and the secondary structure calculated using K2D3 tool of Dichroweb.*

Templates used for modelling

Rank	SPINK2 PDB Hit	SPACA7 PDB Hit	PDCL2 PDB Hit
1	2jxdA	5fvmA	1a0rP
2	2jxdA	6h02A	2dbcA
3	2jxdA	6gmhM	2trcP
4	2jxd	5w2mA	2trc
5	1bmo	1eaq	1a0r
6	2jxdA	5c6gB	2trcP
7	2jxd	5ijnG	2trc
8	2jxdA	1vt4A	2trcP
9	2jxdA	5fiaA	2trcP
10	2jxdA	2pff	2trcP

Three dimensional structure

Residues in most favoured regions	35	57.4%	70	58.3%	147	67.4%
Residues in additional allowed regions	22	36.1%	33	27.5%	54	24.8%
Residues in generously allowed regions	4	6.6%	11	9.2%	10	4.6%
Residues in disallowed regions	0	**0.0%**	6	**5.0%**	7	**3.2%**
Number of non-glycine and non-proline residues	61	100.0%	120	100.0%	218	100.0%
Number of end-residues (excl. Gly and Pro)	2		2		2	
Number of glycine residues (shown as triangles)	3		5		11	
Number of proline residues	4		8		9	
Total number of residues	70		135		240	

Figure 7*. Three-dimensional structure. The amino acid sequence of SPINK2, SPACA7 and PDCL2 and the three-dimensional structure derived using LOMETS, I-TASSER, BLOSUM AND PROCHECK in silico tools.*

Expression pattern of *Spink2*, *Spaca7* and *Pdcl2*

The mRNA expression of *Spink2*, *Spaca7* and *Pdcl2* were analyzed by PCR in different tissues obtained from adult rats (90 day old). All the three genes were found to be predominantly expressed in the testis **(Figure 8A**). A faint expression for *Pdcl2* was observed in the caput epididymis. The developmental expression pattern of these genes was analyzed in the epididymis and testis of 1-to-60-day old rats. The expression of *Spink2* and *Spaca7* was noticed starting from 20 days old, while that of *Pdcl2* was observed in all the age groups analyzed (**Figure 8B**). The faint expression of *Pdcl2* observed in the caput epididymis of adult rats was also reflected in this tissue obtained from all the age groups. Since the expression of *Spink2*, *Spaca7* and *Pdcl2* was found to be predominant in the testis, their protein expression was analyzed by immunofluorescence staining. SPINK2 protein expression appeared to be localized in the periphery of the seminiferous tubule, while that of SPACA7 and PDCL2 are on the periphery and on the spermatids in the lumen (**Figure 9**). Immunofluorescence detection on the spermatozoa revealed that all of these proteins were localized on the head midpiece and tail regions (**Figure 10**).

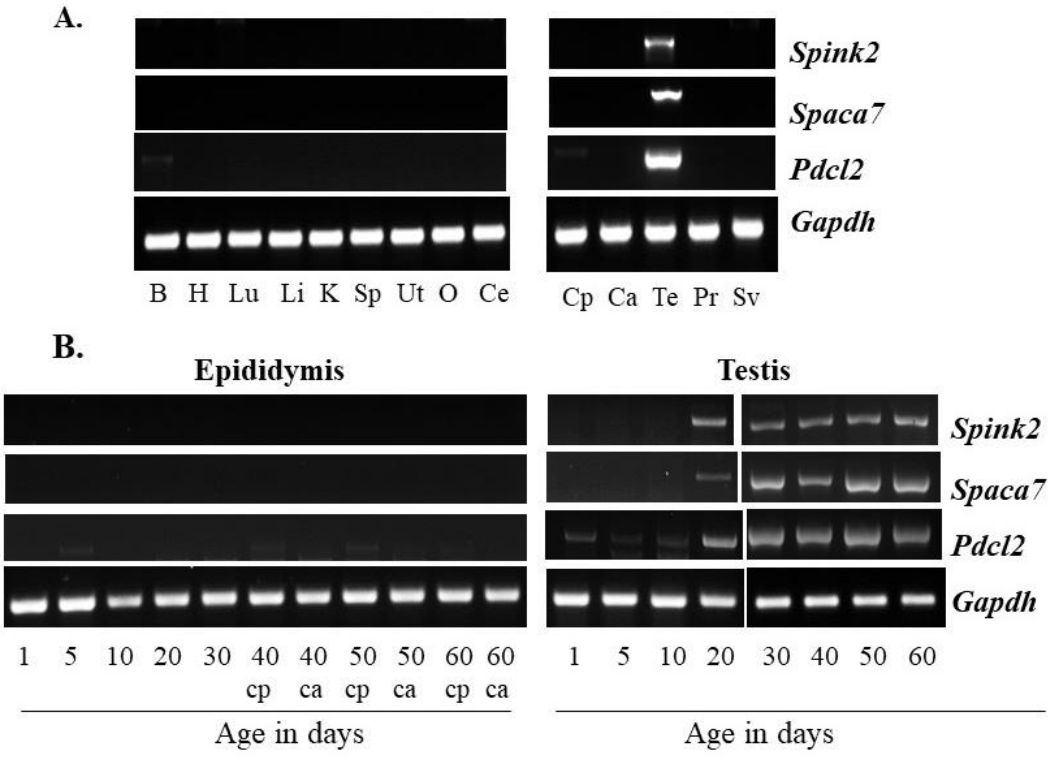


***Figure 8**. Tissue and developmental expression pattern of Spink2, Spaca 7 and Pdcl2. RNA isolated from different tissues was reverse transcribed and the expression pattern analyzed by PCR using gene specific primers. Cp and Ca denote caput and cauda respectively.*

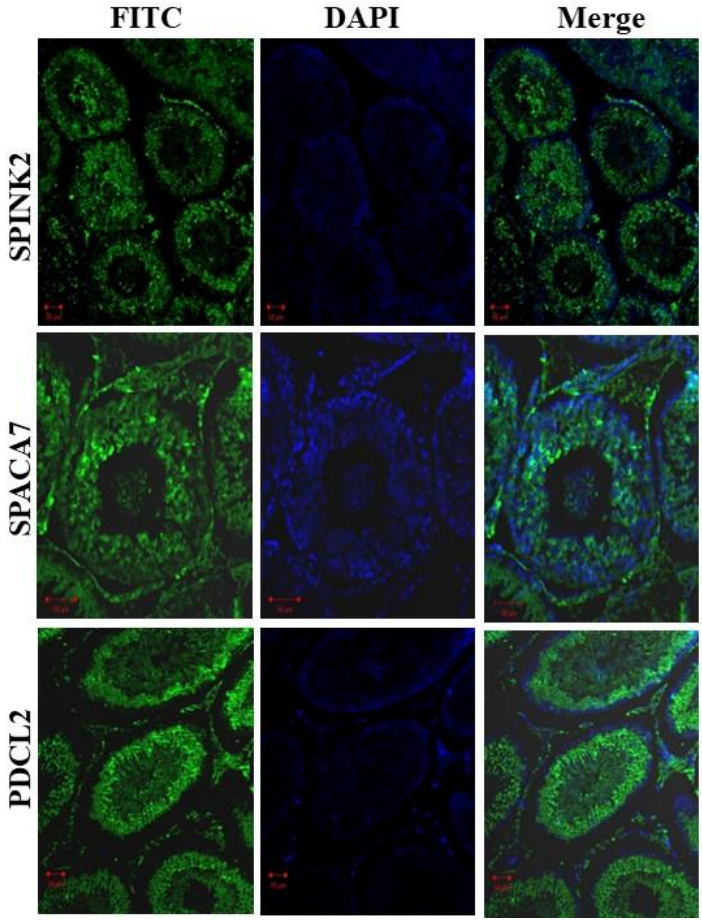


Figure 9. *Immunofluorescence localization of SPINK2, SPACA7 and PDCL2. Five-micron sections of testis of adult rats were probed with respective polyclonal antibody and followed by anti-rat secondary antibody tagged with FITC. The sections were also stained with the background nuclear stain DAPI.*

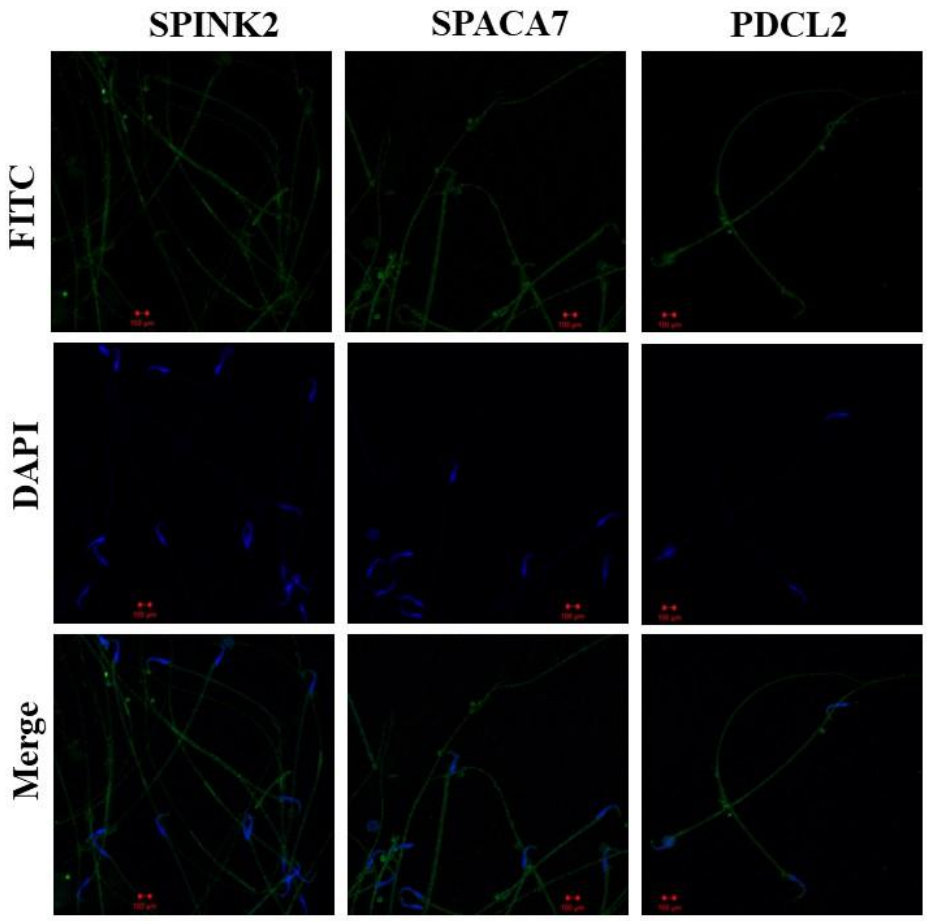


Figure 10. *Immunofluorescene localization of SPINK2, SPACA7 and PDCL2. Spermatozoa of adult rats smeared on glass slides were probed with respective polyclonal antibody and followed by anti-rat secondary antibody tagged with FITC. The sections were also stained with the background nuclear stain DAPI.*

Active immunization and antibody titer

Male rats when immunized with the recombinant SPINK2 or SPACA7 or PDCL2 displayed a significant antibody titer in the serum and the tissues fluids of caput, cauda and testis (**Figure 11**). Rats that had a high titer were used for further experimentation.

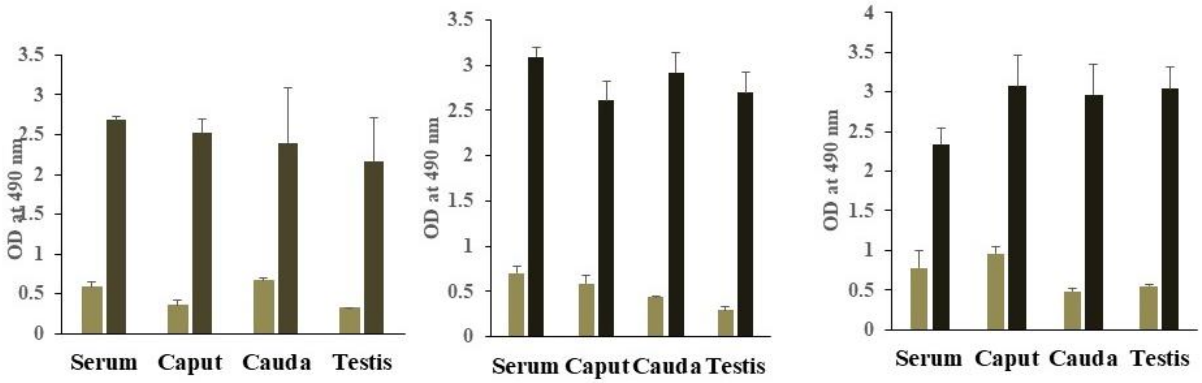


Figure 11*. Antibody titre in immunized rats. Recombinant SPINK2 or SPACA7 or PDCL2 mixed with complete Fruend's adjuvant was administered via the foot pad route. Booster dose was given two weeks after first immunization. Serum was collected and the antibody titre estimated by ELISA.*

Histopathological analyses

To assess the possible anatomical changes that may happen in the epididymis, testis and prostate of SPINK2 or SPACA7 or PDCL2 immunized rats, histopathological examination was carried out (**Figures 12 and 13**). Histopathological changes observed are presented briefly in **table 2**. In the caput epididymis of SPINK2 immunized rats, foci of inflammation and fibrosis in between tubules along with infiltration of inflammatory cells. Moderate degeneration of the epithelial cells of tubules was observed in SPACA7 immunized rats while mild mucosal degeneration and hyperplasia of epithelial cells is evident in the PDCL2 counterparts. Cauda epididymis obtained from SPINK2 immunized rats displayed multiple foci of necrosis, clumping of dead sperms and infiltration of plasma cells. Mild accumulation of fluids in few tubules of cauda epididymis was evident in SPACA7 immunized rats. Perivascular inflammation was predominant in the cauda of rats immunized with PDCL2. Cystic degenerative changes in between seminiferous tubules and infiltration of inflammatory cells was evident in the testes of SPINK2 immunized rats. SPACA7 immunization resulted in moderate vacuolar and cystic degeneration in between seminiferous tubules. Edema and accumulation of fluids in between seminiferous tubules was evident in testes of PDCL2 immunized rats. No histopathological changes were observed in the prostate of rats immunized with SPINK2 or SPACA7 or PDCL2.

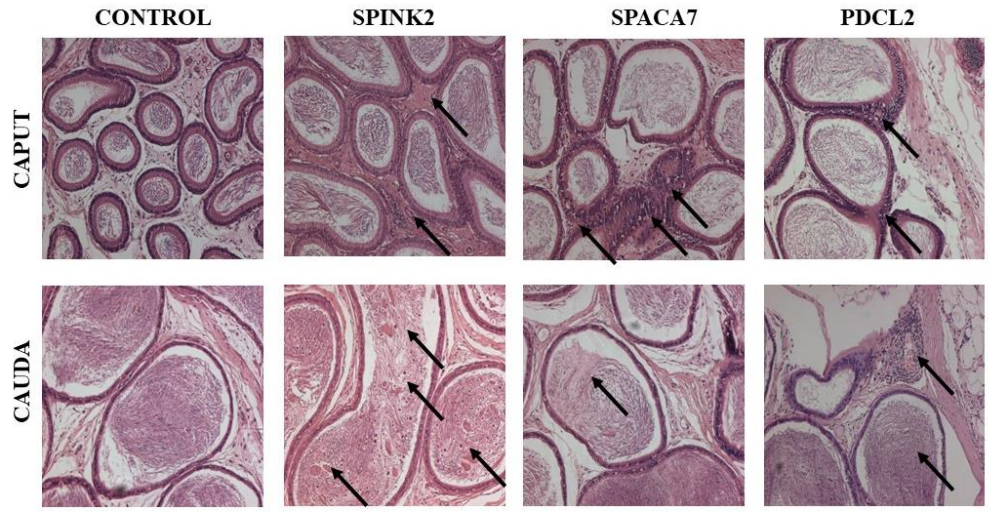


***Figure 12**. Histopathological evaluation of epididymis of immunized rats. Caput and cauda epididymis obtained from control and immunized rats were fixed and five-micron sections were prepared. The sections were stained with Hematoxylin and Eosin. Arrows indicate the anatomical change.*

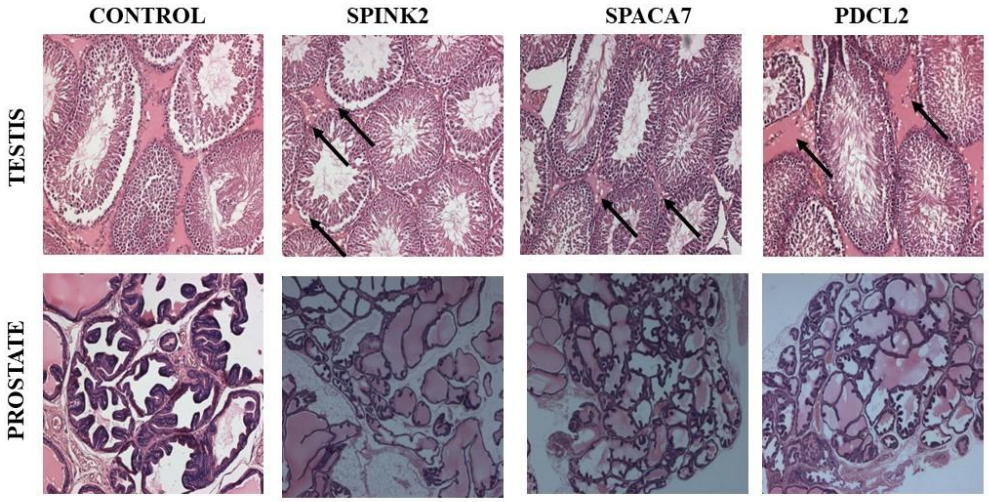


***Figure 13**. Histopathological evaluation of testis and prostate of immunized rats. Testis and prostate obtained from control and immunized rats were fixed and five-micron sections were prepared. The sections were stained with Hematoxylin and Eosin. Arrows indicate the anatomical change.*

Table 2. *Histological changes in the male reproductive tract tissues of immunized rats*.

	SPINK2	SPACA7	PDCL2
Caput	Foci of inflammation and fibrosis in between tubules along with infiltration of inflammatory cells	Moderate degeneration of the epithelial cells of tubules	Mild mucosal degeneration and hyperplasia of epithelial cells
Cauda	Multiple foci of necrosis, clumping of dead sperm and infiltration of plasma cells	Mild accumulation of fluids in few tubules	Perivascular inflammation
Testis	Cystic degenerative changes in between seminiferous tubules and infiltration of inflammatory cells	Moderate vacuolar and cystic degeneration in between seminiferous tubules	Edema and accumulation of fluids in between seminiferous tubules
Prostate	Normal morphology	Normal morphology	Normal morphology

Fecundity, sperm count and sperm function

The fecundity of SPINK2 or SPACA7 or PDCL2 immunized rats was assessed by the size of the litter produced when the immunized animals were subjected to natural mating with females of proven fertility. Litter size was significantly reduced in the females mated with immunized male rats (**Figure 14**). The average litter size in the SPINK2 or SPACA7 or PDCL2 immunized rats was 1.00 ± 2.00, 5.00 ± 1.32 and 4.66 ± 2.75 respectively. Sperm count was significantly reduced in rats immunized with SPINK2 or SPACA7 or PDCL2 immunized rats (**Figure 14**).

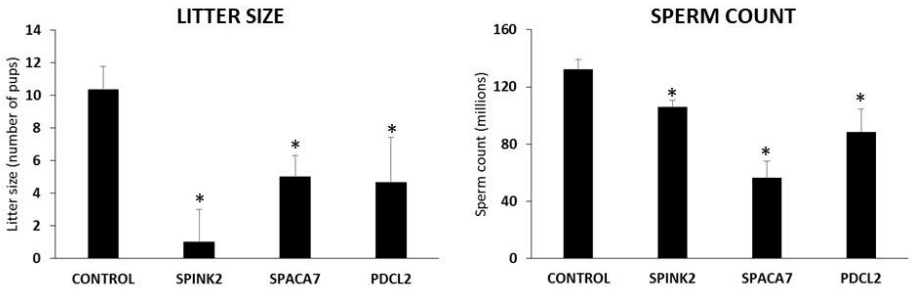


Figure 14. *Litter size and sperm count. Each male rat from the control and immunized groups were cohabited with two females. Following detection of pregnancy, they were separated and the number of pups born to each female was noted. For the sperm count, cauda from the control and immunized rats was cut open and the gametes collected in phosphate buffered saline. The number of sperm were*

*counted using a hemocytometer. Values presented are Mean ± S.D. *denotes p < 0.05 compared to control.*

Testicular transcriptome landscape in immunized rats

RNA isolated from the testes of control or SPINK2 or SPACA7 or PDCL2 immunized rats was subjected to transcriptome analyses to determine the overview of a biological processes that may be affected upon ablation of these proteins. The differentially expressed genes (DEGs) that belong various categories are presented in **table 3**. The highest number of DEGs (872) was observed in the testes of PDCL2 immunized rats, while it was 434 and 283 in SPACA7 and SPINK2 counterparts respectively. A comprehensive list of up and down regulated genes in the testes of SPINK2 or SPACA7 or PDCL2 immunized rats are compiled in **supplementary tables 3 to 8**. The top 20 up and down regulated genes for each of these categories are presented in **tables 4 to 9**. The influence of the differential gene expression on different biological processes and cellular pathways was determined by gene ontology and KEGG pathway analyses (**supplementary tables 9 to 14**). The gene ontologies in which at least 10 genes were differentially expressed are listed in **table 10**. In the testes of SPINK2 immunized rats, processes related to extracellular exosome formation, extracellular space and response to drugs had 10 or more of DEGs. In the case of SPACA7 immunization, gene ontologies related to extracellular space, oxidation-reduction processes, endoplasmic reticulum membrane and response to drugs displayed more than 10 differentially expressed genes. There were 30 gene ontologies for which at least 10 genes were differentially expressed in the testes of PDCL2 immunized rats. Among these, gene ontology related to nuclear function had 142 genes that are differentially expressed, while it was 56 and 45 for the gene ontologies of protein binding and positive regulation of transcription from RNA polymerase II promoter respectively.

***Table 3**. Distribution of genes in the testis of SPINK2 immunized vs control rat.*

SPINK2 IMMUNIZED								
	Coding	Noncoding (lncRNA, snoRNA, snRNA)	Unassigned	Small RNA	Ribosomal	tRNA	Others	TOTAL
Number of genes analyzed	19567	7480	0	109	11	103	174	27444
Differentially expressed	169	109	0	1	0	2	2	283
Upregulated	101	61	0	1	0	1	1	165

Down regulated	68	48	0	0	0	1	1	118
SPACA7 IMMUNIZED								
Number of genes analyzed	19538	7455	0	110	11	99	177	27390
Differentially expressed	268	163	0	0	0	2	1	434
Upregulated	172	63	0	0	0	0	0	235
Down regulated	96	100	0	0	0	2	1	199
PDCL2 IMMUNIZED								
Number of genes analyzed	19568	7480	0	113	11	100	176	27448
Differentially expressed	630	228	0	6	3	1	4	872
Upregulated	455	103	0	5	3	0	2	568
Down regulated	175	125	0	1	0	1	2	304

***Table 4**. Top 20 upregulated genes in the testes of SPINK2 immunized rats.*

GENE NAME	GENE BIOTYPE	FOLD CHANGE (log2)	p value	DESCRIPTION	PROTEIN NAME
Amer3	protein_coding	9.63	0.02	-	APC membrane recruitment protein 3
LOC120097076	lncRNA	8.55	0.03	-	-
LOC108350879	lncRNA	8.23	0.03	-	-
Igsf23	protein_coding	7.66	0.01	-	immunoglobulin superfamily member 23
LOC120093265	lncRNA	7.21	0.01	-	-
Gzmm	protein_coding	7.16	0.01	granzyme M	granzyme M isoform X1; granzyme M precursor; granzyme M isoform X2
LOC120093264	lncRNA	7.11	0.01	-	-
Rrad	protein_coding	6.89	0.01	RRAD, Ras related glycolysis inhibitor and calcium channel regulator	GTP-binding protein RAD; GTP-binding protein RAD isoform X1
LOC120099175	protein_coding	6.83	0.01	-	uncharacterized protein LOC120099175
LOC688459	protein_coding	6.83	0.02	hypothetical protein LOC688459	uncharacterized protein C6orf141 homolog
Zeb2-as1	lncRNA	6.76	0.01	ZEB2 antisense RNA 1	-
LOC120093161	C_region	6.76	0.03	-	-
Mir29c	miRNA	6.63	0.03	microRNA 29c	-
Rcvrn	protein_coding	6.47	0.04	recoverin	recoverin
Edn1	protein_coding	6.39	0.02	endothelin 1	endothelin-1 preproprotein
Tac4	protein_coding	6.39	0.03	tachykinin precursor 4	tachykinin-4 preproprotein
LOC120102259	lncRNA	6.39	0.03	-	-
Klhdc7b	protein_coding	6.30	0.03	-	kelch domain-containing protein 7B
Aicda	protein_coding	6.21	0.04	activation-induced cytidine deaminase	single-stranded DNA cytosine deaminase
Insyn1	protein_coding	6.11	0.03	inhibitory synaptic factor 1	inhibitory synaptic factor 1

***Table 5**. Top 20 down regulated genes in the testes of SPINK2 immunized rats*.

GENE NAME	GENE BIOTYPE	FOLD CHANGE (log2)	p value	DESCRIPTION	PROTEIN NAME
LOC102551538	V_segment	-7.67	0.03	-	-
Ces2a	protein_coding	-7.63	0.04	carboxylesterase 2A	pyrethroid hydrolase Ces2a precursor
Adamtsl2	protein_coding	-7.34	0.02	-	ADAMTS-like protein 2 isoform X1; ADAMTS-like protein 2 isoform X2; ADAMTS-like protein 2 isoform X3
Trnas-aga	tRNA	-7.28	0.01	-	-
LOC102548951	lncRNA	-7.23	0.01	-	-
Ifit1	protein_coding	-7.23	0.04	interferon-induced protein with tetratricopeptide repeats 1	interferon-induced protein with tetratricopeptide repeats 1-like protein
LOC120100998	protein_coding	-7.04	0.01	-	60S ribosomal protein L6-like
LOC120093847	lncRNA	-7.04	0.02	-	-
LOC103691619	lncRNA	-6.90	0.02	-	-
LOC120095558	lncRNA	-6.90	0.03	-	-
Wfdc9	protein_coding	-6.90	0.03	WAP four-disulfide core domain 9	protein WFDC9 isoform X1; protein WFDC9 precursor
LOC120100623	lncRNA	-6.90	0.02	-	-
LOC102552337	protein_coding	-6.75	0.03	-	oogenesin-3-like
LOC120094201	lncRNA	-6.67	0.02	-	-
LOC120099009	protein_coding	-6.58	0.03	-	LOW QUALITY PROTEIN: uncharacterized protein LOC120099009, partial
LOC102555382	lncRNA	-6.58	0.02	-	-
LOC120095907	lncRNA	-6.49	0.04	-	-
Lep	protein_coding	-6.28	0.05	leptin	leptin precursor; leptin isoform X1
Clrn1	protein_coding	-6.17	0.04	clarin 1	clarin-1

Ccdc190	protein_coding	-6.17	0.04	coiled-coil domain containing 190	coiled-coil domain-containing protein 190 isoform X1; coiled-coil domain-containing protein 190

***Table 6**. Top 20 up regulated genes in the testes of SPACA7 immunized rats.*

GENE NAME	GENE BIOTYPE	FOLD CHANGE (log2)	p value	DESCRIPTION	PROTEIN NAME
LOC103690754	protein_coding	8.43	0.05	-	Kruppel-like factor 18
LOC120097076	lncRNA	8.13	0.04	-	-
Gabrp	protein_coding	7.48	0.01	gamma-aminobutyric acid type A receptor subunit pi	gamma-aminobutyric acid receptor subunit pi precursor; gamma-aminobutyric acid receptor subunit pi isoform X1; gamma-aminobutyric acid receptor subunit pi isoform X2
LOC103692519	protein_coding	7.43	0.03	-	60S ribosomal protein L9-like
LOC120098480	protein_coding	7.13	0.02	-	keratin-associated protein 5-2-like
Afp	protein_coding	7.07	0.02	alpha-fetoprotein	alpha-fetoprotein precursor; alpha-fetoprotein isoform X1
Igsf23	protein_coding	7.07	0.01	-	immunoglobulin superfamily member 23
LOC120093265	lncRNA	6.92	0.01	-	-
LOC120093264	lncRNA	6.84	0.01	-	-
Edn1	protein_coding	6.67	0.02	endothelin 1	endothelin-1 preproprotein
LOC120102259	lncRNA	6.67	0.02	-	-
LOC120103009	lncRNA	6.58	0.03	-	-
LOC102546604	lncRNA	6.48	0.02	-	-
LOC120098271	lncRNA	6.48	0.02	-	-
LOC120099175	protein_coding	6.48	0.02	-	uncharacterized protein LOC120099175
LOC120096012	lncRNA	6.37	0.03	-	-
LOC103691283	lncRNA	6.37	0.02	-	-
Zeb2-as1	lncRNA	6.37	0.02	ZEB2 antisense RNA 1	-
Tnfrsf13c	protein_coding	6.26	0.04	-	tumor necrosis factor receptor superfamily member 13C
LOC120095890	lncRNA	6.26	0.03	-	-

***Table 7**. Top 20 down regulated genes in the testes of SPACA7 immunized rats.*

GENE NAME	GENE BIOTYPE	FOLD CHANGE (log2)	p value	DESCRIPTION	PROTEIN NAME
LOC108349942	lncRNA	-9.70	0.02	-	-
LOC108349630	lncRNA	-7.79	0.01	-	-
LOC108350487	lncRNA	-7.54	0.04	-	-
LOC103691682	V_segment	-7.53	0.00	-	-
LOC120097113	lncRNA	-7.34	0.04	-	-
Thrsp	protein_coding	-7.12	0.03	thyroid hormone responsive	thyroid hormone-inducible hepatic protein
LOC120100746	lncRNA	-7.12	0.01	-	-
LOC120097866	lncRNA	-6.91	0.02	-	-
LOC108348495	lncRNA	-6.60	0.02	-	-
LOC102547251	lncRNA	-6.60	0.02	-	-
Trnal-aag	tRNA	-6.53	0.03	-	-
Trnal-aag	tRNA	-6.46	0.03	-	-
LOC102550954	lncRNA	-6.46	0.03	-	-
Or51t1	protein_coding	-6.46	0.02	-	olfactory receptor 51T1
Ckmt2	protein_coding	-6.38	0.02	creatine kinase, mitochondrial 2	creatine kinase S-type, mitochondrial isoform X1; creatine kinase S-type, mitochondrial precursor
LOC120097471	lncRNA	-6.38	0.02	-	-
Rhox5	protein_coding	-6.30	0.02	Rhox homeobox family member 5	homeobox protein Rhox5 isoform X1; homeobox protein Rhox5; homeobox protein Rhox5 isoform X2
LOC120100091	lncRNA	-6.30	0.03	-	-
Hephl1	protein_coding	-6.21	0.03	hephaestin-like 1	ferroxidase HEPHL1; ferroxidase HEPHL1 isoform X1
Gucy2f	protein_coding	-6.21	0.04	guanylate cyclase 2F	retinal guanylyl cyclase 2 precursor

***Table 8**. Top 20 upregulated genes in the testes of PDCL2 immunized rats*.

GENE NAME	GENE BIOTYPE	FOLD CHANGE (log2)	p value	DESCRIPTION	PROTEIN NAME
LOC120096641	lncRNA	9.02	0.05	-	-
LOC120097076	lncRNA	7.84	0.04	-	-
LOC100912943	protein_coding	6.84	0.03	-	mucin-3A-like
Gpr143	protein_coding	6.78	0.01	G protein-coupled receptor 143	G-protein coupled receptor 143; G-protein coupled receptor 143 isoform X1
LOC108352834	protein_coding	6.78	0.02	-	acidic proline-rich protein PRP25-like
Insyn1	protein_coding	6.65	0.02	inhibitory synaptic factor 1	inhibitory synaptic factor 1
Loxl4	protein_coding	6.58	0.02	lysyl oxidase-like 4	lysyl oxidase homolog 4 isoform X1; lysyl oxidase homolog 4 precursor; lysyl oxidase homolog 4 isoform X2
Zeb2-as1	lncRNA	6.50	0.02	ZEB2 antisense RNA 1	-
LOC120099175	protein_coding	6.50	0.02	-	uncharacterized protein LOC120099175
LOC103691283	lncRNA	6.42	0.02	-	-
Galr1	protein_coding	6.34	0.03	galanin receptor 1	galanin receptor type 1
Fam156b	protein_coding	6.34	0.03	-	protein FAM156A/FAM156B-like
LOC120093264	lncRNA	6.34	0.02	-	-
LOC120103009	lncRNA	6.25	0.03	-	-
Klk13	protein_coding	6.25	0.03	kallikrein related-peptidase 13	kallikrein-13 precursor
LOC120095094	protein_coding	6.25	0.04	-	putative uncharacterized protein MSANTD5
Olr1394	protein_coding	6.25	0.03	olfactory receptor 1394	olfactory receptor Olr1394
Klhdc7b	protein_coding	6.25	0.03	-	kelch domain-containing protein 7B
Gzmm	protein_coding	6.25	0.03	granzyme M	granzyme M isoform X1; granzyme M precursor; granzyme M isoform X2
LOC108349679	lncRNA	6.16	0.04	-	-

***Table 9**. Top 20 down regulated genes in the testes of PDCL2 immunized rats.*

GENE NAME	GENE BIOTYPE	FOLD CHANGE (log2)	p value	DESCRIPTION	PROTEIN NAME
RT1-N2	protein_coding	-10.94	0.01	RT1 class Ib, locus N2	RT1 class Ib, locus N2 isoform X5; RT1 class Ib, locus N2 precursor; RT1 class Ib, locus N2 isoform X1; RT1 class Ib, locus N2 isoform X3; RT1 class Ib, locus N2 isoform X4; RT1 class Ib, locus N2 isoform X2
LOC102546775	lncRNA	-9.84	0.02	-	-
Nefl	protein_coding	-7.80	0.01	neurofilament light	neurofilament light polypeptide
LOC120093224	lncRNA	-7.33	0.01	-	-
LOC102549213	lncRNA	-6.95	0.04	-	-
RGD1566006	protein_coding	-6.85	0.03	-	paired immunoglobulin-like type 2 receptor beta isoform X1; paired immunoglobulin-like type 2 receptor beta isoform X2; paired immunoglobulin-like type 2 receptor beta isoform X3
RGD1561736	protein_coding	-6.63	0.05	-	60S ribosomal protein L10-like
Gng4	protein_coding	-6.63	0.02	-	guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-4
Clec4f	protein_coding	-6.63	0.02	C-type lectin domain family 4, member F	C-type lectin domain family 4 member F
Fzd10	protein_coding	-6.54	0.03	frizzled class receptor 10	frizzled-10 precursor
Has1	protein_coding	-6.44	0.03	hyaluronan synthase 1	hyaluronan synthase 1; hyaluronan synthase 1 isoform X1
LOC108350474	lncRNA	-6.44	0.02	-	-
LOC108352734	lncRNA	-6.44	0.04	-	-
LOC120098955	lncRNA	-6.33	0.03	-	-
LOC120102428	lncRNA	-6.33	0.04	-	-
LOC103690816	lncRNA	-6.33	0.02	-	-

LOC102551022	lncRNA	-6.22	0.04	-	-
Npy5r	protein_coding	-6.22	0.03	neuropeptide Y receptor Y5	neuropeptide Y receptor type 5 isoform X1; neuropeptide Y receptor type 5
Defb28	protein_coding	-6.22	0.03	-	beta-defensin 115
LOC120103273	snoRNA	-6.09	0.04	-	-

Table 10. *Gene ontology analyses in the testes of immunized rats*.

GO	Category	Description	Gene count
SPINK2 IMMUNIZED			
GO:0070062	CC	extracellular exosome	26
GO:0005615	CC	extracellular space	16
GO:0042493	BP	response to drug	10
SPACA7 IMMUNIZED			
GO:0005615	CC	extracellular space	26
GO:0055114	BP	oxidation-reduction process	18
GO:0005789	CC	endoplasmic reticulum membrane	13
GO:0042493	BP	response to drug	11
PDCL2 IMMUNIZED			
GO:0005634	CC	Nucleus	142
GO:0005515	MF	protein binding	56
GO:0045944	BP	positive regulation of transcription from RNA polymerase II promoter	45
GO:0000122	BP	negative regulation of transcription from RNA polymerase II promoter	39
GO:0006355	BP	regulation of transcription, DNA-templated	39
GO:0003677	MF	DNA binding	38
GO:0006351	BP	transcription, DNA-templated	30
GO:0043565	MF	sequence-specific DNA binding	29
GO:0003700	MF	transcription factor activity, sequence-specific DNA binding	28
GO:0007165	BP	signal transduction	24
GO:0006366	BP	transcription from RNA polymerase II promoter	22
GO:0030054	CC	cell junction	21
GO:0006357	BP	regulation of transcription from RNA polymerase II promoter	20
GO:0000978	MF	RNA polymerase II core promoter proximal region sequence-specific DNA binding	19
GO:0043547	BP	positive regulation of GTPase activity	19
GO:0003682	MF	chromatin binding	18
GO:0043005	CC	neuron projection	18

GO:0030154	BP	cell differentiation	17
GO:0035556	BP	intracellular signal transduction	16
GO:0001077	MF	transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	15
GO:0001701	BP	in utero embryonic development	15
GO:0008285	BP	negative regulation of cell proliferation	15
GO:0000981	MF	RNA polymerase II transcription factor activity, sequence-specific DNA binding	14
GO:0004674	MF	protein serine/threonine kinase activity	14
GO:0007420	BP	brain development	14
GO:0005765	CC	lysosomal membrane	13
GO:0005096	MF	GTPase activator activity	12
GO:0005578	CC	proteinaceous extracellular matrix	12
GO:0010629	BP	negative regulation of gene expression	10
GO:0015629	CC	actin cytoskeleton	10

DISCUSSION

Tissue specific expression of genes confer unique properties for an organ. The testis, because of its primary role in gametogenesis, evolved its own physiological process, some of which are general and while some are unique. The uniqueness arises out due the expression of a variety of genes and their protein products that are testis specific dictated by tight transcription control mechanisms (Kimmins, Kotaja et al. 2004, Djureinovic, Fagerberg et al. 2014, Brown 2019). Further, within the testis, the stage specific expression of genes during spermatogenesis is crucial (Johnston, Wright et al. 2008). Recent studies characterized the functional role of testis –specific or –predominantly expressed genes (Lin, Li et al. 2016, Kurihara, Otsuka et al. 2017, Pineau, Hikmet et al. 2019, Hong, Han et al. 2021). Such identification and characterization is an active area of research which contributes to in depth understanding of the molecular mechanisms that govern testicular function. Hence, in this study, we characterized the functional role of three rat genes, namely *Spink2*, *Spaca7* and *Pdcl2* that are predominantly expressed in the testis. It is a well-accepted concept that functional divergence exists between orthologs and paralogs (Altenhoff, Studer et al. 2012, Gabaldón and Koonin 2013). Though the functional role of Spink and *Spaca7* is reported in humans and mice, we attempted to characterize their functional role in the rat.

In silico analyses indicated that rat *Spink2*, *Spaca7* and *Pdcl2* exist independently on chromosomes 14, 16 and 14 respectively and not clustered with their homologs. As revealed by neighborhood analyses, it is interesting to note that for each of these rat genes, other genes that are present in their vicinity are same as that are found for mouse and humans, indicating the conserved evolution in the three species. The secondary structure derived by circular dichroism for SPINK2, SPACA7 and PDCL2 varied to a large extent to what was predicted by SOPMA analyses. Absence of significant changes in the secondary structure of these proteins under different ion concentrations and varying pH indicates their stable nature under different physiological conditions. Their stable structural features were also confirmed by Ramachandran plot analyses, wherein very few amino acids were indicated in the disallowed regions, indicating that the models generated may truly reflect the structural aspects at the biological level.

The mRNA expression of rat *Spink2*, *Spaca7* and *Pdcl2* was found to be predominant in the testis. In the humans and mice, *Spink2* expression was predominant in the testis (Fink, Hehlein-Fink et al. 1990, Möritz, Lilja et al. 1991, Lee, Park et al. 2011). Similarly, *Spaca7* expression was restricted to the testis in mice and humans (Korfanty, Toma et al. 2012). On the same lines, the expression of *Pdcl2* is predicted to be testis specific in mice and humans (*PDCL2*)(*PDCL2*)('PDCL2')(Anon). The predominant expression of the three genes in mice, rat and humans indicate a conserved evolution pattern and possibly the functional role. Developmental expression of organ specific genes provides vital information on the correlation between the onsets of a physiological process at specific stages of

ontogeny. Testicular and epididymal genes are developmentally regulated because of the variations in androgen levels during development (Rodriguez, Kirby et al. 2001). We observed the onset of expression of rat *Spink2*, *Spaca7* and *Pdcl2* in 20-day old rats and continued till adulthood. In the rat, testicular androgens begin to increase at around 30 days post-natal age (Harris and Bartke 1974, Harris and Bartke 1981) and this could be a triggering factor for the onset of expression of these genes. Besides testosterone, testicular factors are also known to control gene expression; and their influence on the expression of *Spink2*, *Spaca7* and *Pdcl2* needs further investigation. It appears that *Pdcl2* expression is at low levels until 10 days. This could be due to a variety of testicular factors that govern its expression. Alternatively, the expression of Pdcl2 is required for testicular development right from day 1 of post-natal age, while that of Spink2 and Spaca7 are important at later stages. Immunofluorescence staining SPINK2, SPACA7 and PDCL2 protein expression in the rat testes, thus corroborating the mRNA expression pattern. The distribution of these proteins in all cell types of the testes indicates their functional role in many cellular processes, including gametogenesis. Localization of these proteins on the sperm surface could be due to their addition to the maturing spermatids in the testis. However, the significance of binding of these proteins to the sperm surface should be studied in depth.

Basing on previous studies, we predicted that SPINK2 and SPACA7 may have a role in fertility (Lee, Park et al. 2011, Nguyen, Westmuckett et al. 2014). In the absence of any reports on the role of PDCL2 in fertility; and because of its predominant expression in the testis, we investigated its role in male reproductive function. In this study, active immunization of male rats against the three proteins resulted in significantly decreased fecundity. The reduced fecundity was associated with decreased sperm count in SPINK2 or SPACA7 or PDCL2 immunized rats. Reduction in fecundity was associated with decreased sperm count due to active immunization against testicular and epididymal proteins such as SPAG11A, GnRH, uPA, LYZLs, PATE proteins (Narmadha and Yenugu 2016, Rajesh and Yenugu 2017, Aponte, Gutierrez-Reinoso et al. 2018, Sangeeta and Yenugu 2020). Histopathological examination of caput, cauda and testis of immunized rats revealed damage to the anatomical architecture. Such a damage could affect spermatogenesis and sperm maturation as well. Ablation of SPINK2 or SPACA7 or PDCL2 could affect the male reproductive function at multiple targets. Further analyses on sperm parameters using computer assisted sperm analyzer would provide more information on the exact functions that are affected by these proteins.

The physiological, cellular, biochemical, transcriptional and translational processes governed by a protein is best characterized by eliminating the presence of that protein either by knocking out the gene responsible for its expression or by ablating the protein levels by active immunization. Since active immunization is relatively a simple technique than transgenesis, we chose this method for a preliminary investigation on the changes at the transcriptomics level under conditions of SPINK2 or SPACA7 or PDCL2 ablation. Differential expression of genes that cater to a variety of physiological and cellular processes was evident in the testis of immunized rats. There are only a few studies that reported on the

influence of ablation of a protein by active immunization on changes at the transcriptomics level. Active immunization against inhibin alpha-subunit resulted in the differential expression of genes involved in testicular development, testosterone secretion and testicular function (Akhtar, Wei et al. 2019). In our studies, we observed that ablation of the epididymis specific protein SPAG11A resulted in changes in the transcriptomic profile that favours oncogenesis akin to a condition caused by chemical carcinogen (Sangeeta and Yenugu 2022).

In the testis of SPINK2 and SPACA7 immunized rats, the topmost gene ontology that was affected was extracellular exosome, suggesting that these proteins may play a role in exosome mediated testicular function. The role of exosomes in sperm maturation was proposed earlier (Sullivan, Saez et al. 2005) and their importance in reproductive medicine is gaining importance in the recent years (Kharazi and Badalzadeh 2020). While the components of the exosomes derived from the endothelial cells of the testis play a vital role in spermatogenesis (Song, Gu et al. 2021), Sertoli cell derived exosomes protected the spermatogonial cells from the harmful effects of electromagnetic radiation (Salek, Baharara et al. 2021). The testicular extracellular vesicles from thy1-positive spermatogonial suppressed the proliferation of spermatogonial stem cells in the mouse (Lin, Fang et al. 2021). These lines of evidence indicate that SPINK2 and SPACA7, may regulate the function of factors involved in extracellular exosomes. Active immunization against PDCL2 caused differential expression of 142 genes that are involved in nuclear function and this topped the gene ontology analyses. Further, majority of the gene ontologies that were affected belonged to cellular processes that occur in the nucleus such as transcription, sequence specific DNA binding, chromatin binding, signal transduction, etc. Members of the phoscucin-like family are characterized to function as molecular chaperones regulators of expression of heteromeric G proteins and interact with the subunits of G proteins (Barhite, Thibault et al. 1998, Gao, Sinha et al. 2013, Krzemień-Ojak, Góral et al. 2017). A plethora of molecular mechanisms occur during spermatogenesis (Guerra-Carvalho, Carrageta et al. 2021). Phosducin-like family members, including PDCL2, being molecular chaperones may have critical role in the nuclear events of male gametogenesis, a domain of research that is not yet explored.

In conclusion, we report the predominant expression of *Spink2*, *Spaca7* and *Pdcl2* in the rat testis. Ablation of SPINK2, SPACA7 and PDCL2 by active immunization resulted in decreased fecundity that was associated with reduced sperm count and damage to the anatomical structure of the epididymis and testis. Further, differential gene expression of the components involved in extracellular exosomes and nuclear function were evident in the testis of SPINK2 / SPACA7 and PDCL2 immunized rats. Results of our study indicate the important role of these three proteins in male fertility and their absence may affect cellular and molecular process of gametogenesis.

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Chapter 2

Elucidating the role of *Spag11a* in epididymal oncogenesis and male fertility

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INTRODUCTION

Spermatozoa that are generated in the testis pass through a specialized organ called the epididymis, wherein they undergo maturation to gain motility and fertilizing ability. The tubules of the epididymis are primarily lined by epithelial cells (Johnston, Jelinsky et al. 2005). These cells produce proteins that are diverse in nature; and the proteome varies across the three regions (caput, corpus and cauda) (Dacheux, Belleannee et al. 2009). Multiple roles have been assigned to these proteins, especially in sperm function and innate immunity (Aitken, Nixon et al. 2007, Belleannee, Calvo et al. 2012, Li, Wang et al. 2012, Nixon, Stanger et al. 2015). Epididymal proteins are known to contribute to physiological processes that are not limited to male reproduction. For example, in many types of cancers, the human epididymal protein 4 (HE4) is considered as a diagnostic and prognostic marker (Ferraro and Panteghini 2018, Mi and Zhang 2018, Mo and He 2018, Li, Wang et al. 2019).

The human sperm associated antigen 11 (*SPAG11*) gene a fusion of two ancestral β-defensin genes, is located on chromosome 8p23 and is also referred to as human epididymal protein 2 (HE2) or epididymal protein 2 (EP2) (Osterhoff, Kirchhoff et al. 1994, Frohlich, Po et al. 2000). This gene encodes more than 20 isoforms due to alternate splicing (Frohlich, Po et al. 2000). In the rodents, three independent *Spag11* genes viz *Spag11t*, *Spag11c* and *Spag11a* (also referred to as *Bin1b or Spag11e*) are identified (Yenugu, Hamil et al. 2006). The important role of rodent SPAG11A in sperm function and maturation was demonstrated (Li, Chan et al. 2001). On the other hand, the rodent SPAG11A and SPAG11C proteins have been reported to participate in the first line of defense, because of the presence of a six cysteine motif, that is characteristic to β-defensins (Yenugu, Hamil et al. 2006). Similar to β-defensins, these proteins possess antimicrobial activity, that is mediated by their ability to permeabilize bacterial membranes and inhibition of macromolecular synthesis (Yenugu, Hamil et al. 2004). Human β-defensins, besides contributing to the innate immunity, are also implicated in tumor growth and have been considered as markers for prognosis (Droin, Hendra et al. 2009). Their functional role in lung, oral, colon, prostate, thyroid and cervical carcinogenesis is well documented (Bullard, Gibson et al. 2008, Han, Wang et al. 2014, Uraki, Sugimoto et al. 2014, Zhuravel, Gerashchenko et al. 2014, Cun Gao, Weiming Yue et al. 2016, Xu, Zhang et al. 2016). The anticancer properties of human β-defensin 3 is recently reported (Hanaoka, Yamaguchi et al. 2016).

Epididymis – tumour incidence

While the incidence of tumours is reported in most of the organ systems, the occurrence of epididymal cancers is very rare. Hence, epididymis is considered to be a specialized organ because of its special ability to resist tumour formation. Multiple hypothesis have been proposed on this special property of epididymis, that confers the rarity of tumour incidence (Yeung, Wang et al. 2012). One of the more compelling theories holds that the non-responsiveness of this organ to signals that induce tumours is due in part to processes that make the spermatozoa quiescent and immunologically inert as well as constitutive expression of proteins often induced in tumours. We recently reported that

overexpression of SPAG11A in immortalized epididymal epithelial cell lines resulted in inhibition of proliferation (Sangeeta and Yenugu 2020). On the other hand, siRNA mediated silencing of *Spag11a* mRNA in isolated epididymal primary epithelial cells resulted in promotion of proliferation (Sangeeta and Yenugu 2020). In our animal studies, the ablation of SPAG11A protein by active immunization resulted in the vulnerability of epididymis to a low dose carcinogen (Sangeeta and Yenugu 2022). These studies clearly indicated that SPAG11A plays a crucial part in regulating the cell proliferation and thus could be one of the mechanisms that contribute to the rarity of cancers in the epididymis. The functional importance of a protein can best studied by using transgenic or knock out animal models. To further decipher the functional role of *Spag11a* in the epididymis, especially to the prevention of tumour formation, we used *Spag11a* knock out mice and determined their susceptibility to carcinogenesis under conditions of low dose exposure to a carcinogen.

Epididymis – sperm function

Spermatozoa undergoes maturation while passing through the lumen of the epididymis and during this process a large number of proteins secreted by the epithelial cells are added on to their surface. Segment specific production of proteins in the epididymis is reported (Johnston, Jelinsky et al. 2005). The *Spag11a* gene expression is caput epididymis specific and its protein counterpart localized on the mature spermatozoa contributes to sperm function (Li, Chan et al. 2001, Yenugu, Hamil et al. 2006). Induction of motility in sperm due to the binding of rat SPAG11A to the sperm membrane was demonstrated *in vitro* (Zhou, Zhang et al. 2004). In our recent studies, we demonstrated that male rats immunized against SPAG11A protein or male transgenic rats that over expressed shRNA against *Spag11a* mRNA, displayed lower fecundity, sperm function and damage to the anatomical architecture of the male reproductive organs (Sangeeta and Yenugu 2020). To further explore the role of *Spag11a* in male reproduction, knock out animals were generated (unpublished). These *Spag11a* knock out mice displayed reduced fecundity and impaired sperm function (capacitation and acrosome reaction). Our preliminary results suggested that *Spag11a* has a pivotal role in sperm function. To take this further, we analysed the differential expression of sperm proteome between wild type and *Spag11a* knock out mice.

Gene polymorphisms and male fertility

Genetic changes and their relation to male infertility in the Indian context are reported only in the last ten years. Chromosomal abnormalities involving the heterochromatic regions of Y, chromosome 9 and the acrocentric chromosomes are implicated in infertility in Indian men (Suganya, Kujur et al. 2015). The 677C/T substitution polymorphism in MTHFR gene resulted in serious implications in spermatogenesis of Indian infertile men (Singh, Singh et al. 2005). Y-chromosome micro deletions are reported in Indian infertile males (Mitra, Dada et al. 2008). Further, the following genes were analyzed for polymorphisms in relation to infertility in the Indian context: CFTR, methylenetetrahydrofolate reductase (MTHFR), testicular RNA helicase, interleukin-1beta, TNFα and IL-6, FSHR, progesterone

receptor, mitochondrial DNA polymerase gamma, GSTT1 and GSTM1, APOB, ER-beta, UBE2B, androgen receptor, SRY and AFLP (Janeja, Banga et al. 2003, Shahid, Dhillion et al. 2004, Premi, Srivastava et al. 2006, Rajender, Singh et al. 2007, Suryavathi, Khattri et al. 2008, Khattri, Pandey et al. 2009, Khattri, Pandey et al. 2009, Jaiswal, Sah et al. 2012, Desai, Achrekar et al. 2013, Jaiswal, Trivedi et al. 2013, Poongothai 2013, Sen, Dixit et al. 2013, Shukla, Agnihotri et al. 2013, Naqvi, Hussain et al. 2014, Sharma, Mavuduru et al. 2014, Singh, Koner et al. 2016). It is important to note that the genes analyzed are more related to general physiology. Genetic analyses that directly influence male reproductive function, sperm maturation and function and fertilization are not analyzed in the Indian context.

RATIONALE

I. *Spag11a* and its role in epididymis tumorigenesis

Experimental proof provided by our preliminary *in vitro* and *in vivo* studies indicated that *Spag11a* has a potential role in governing epididymal cell proliferation (Sangeeta and Yenugu 2020, Sangeeta and Yenugu 2022). On the other hand, it is well known that β-defensins play a crucial role in tumorigenesis by controlling cell proliferation. SPAG11A protein contains the 6-cysteine domain that is characteristic to β-defensins. It appears that SPAG11A protein is functionally similar to the β-defensins. However, there is no concrete evidence to demonstrate these functional roles. Use of transgenic / knock out animal is the best system to study the functional role of a protein. To decipher, the role of *Spag11a* in epididymal tumorigenesis, we used *Spag11a* knock out mice that were treated with a low dose of carcinogen. It is hypothesised that the epididymis of *Spag11a* knock out mice will be more susceptible to the carcinogen than the wild type counterparts.

II. *Spag11a* and sperm function

In two animal model systems, namely, active immunization against SPAG11A and transgenic animals expressing shRNA against *Spag11a* mRNA, we showed that there is a reduction in fecundity and sperm function (Sangeeta and Yenugu 2020). Similarly, in the *Spag11a* knock out mice, the fecundity and sperm function was compromised (unpublished). It is possible that the expression of sperm proteins that are important for fecundity and sperm function are under the control or influence of SPAG11A. This can be best studied by analysing the sperm proteome of wild type and SPAG11A knock out mice. Thus, extensive proteome analyses was carried out to identify the differential expression of proteins that may have been affected due to the absence of SPAG11A protein.

III. Spag11a polymorphism and male infertility

Infertility is on the rise and it is attributed to a variety of reasons. Among them, genetic changes that affect the genes important in reproductive function are implicated. Till date, genetic changes such as mutations, single nucleotide polymorphisms and deletions are reported in genes involved in spermatogenesis. It is important to note that a lot of evidence suggests that proteins that are secreted in the epididymis aid in sperm maturation, sperm function and fertilization. Genetic changes in genes that encode epididymal proteins are not analysed. This is more evident in the Indian context. Hence, analyzing genetic changes in genes that encode epididymal proteins will help to identify factors that are to be supplemented for effective in vitro fertilization techniques. In this study, we propose to identify the common gene polymorphisms in human *SPAG11A* gene which may play an important role in sperm function.

METHODOLOGY

Generation of *Spag11a* knockout mice

Heterozygous *Spag11a* knockout mice (C57BL/6NJ) were commercially obtained from Mouse Genome Engineering Facility, National Centre for Biological Sciences, Bangalore, India. They were produced by CRISPR-CPF1 (Cas12a/sgRNA) system. The strategy and methodology utilized to produce these mice is described in **figure 1**. Briefly, the nucleotides between 712 and 1002 (291 bp), which includes a portion of exon 1 and intron 1, was excised from the mouse *Spag11a* gene (NM 153115.1) with the help of specific guide RNAs (**figure 1)**. The guide RNAs designed had the following sequences (5' to 3'): sgRNA#20 (on the plus strand): TTT(PAM) ACAGCACAAGACATAGAGGCCTCT; and sgRNA#18 (on minus strand): TTT(PAM) CATGTTTTACGGCTCGCTCTCTGT. The sgRNAs #18 and #20 target the genomic sequences 5'-ACAGAGAGCGAGCCGTAAAACAT-3' and 5'-ACAGCACAAGACATAGAGGCCTCT-3' respectively. B6/NJNcbs mice zygotes were injected with a pronuclear microinjection solution containing CPF1 (*Cas12a* protein; (40 ng/ul final)), sgRNA#18 (10 ng/ul final), and sgRNA#20 (10 ng/ul final). Two backcrosses between B6/NJNcbs wild-type mice and the founders to generate heterozygous mice. Heterozygote *Spag11a* N2 mice were produced by the two subsequent B6/NJNcbs backcrosses. Male heterozygous *Spag11a* N2 was crossed with female heterozygous *Spag11a* N2 to produce *Spag11a* exon1 deleted homozygote *Spag11a* knockout mice. The DNA extracted from the tail pieces of male and female mice was used for genotyping. The *Spag11a* knockout mice used in this study were age-matched and were derived from the same founder. *Spag11a* knockout mice were produced as part of project #AJ-1/2015, which was approved by NCBS-IAEC (R1-E) and the University of Hyderabad Institutional Ethics Committee accepted the use of animals in the study's experiments (UH/IAEC/SY/2021-22/09).

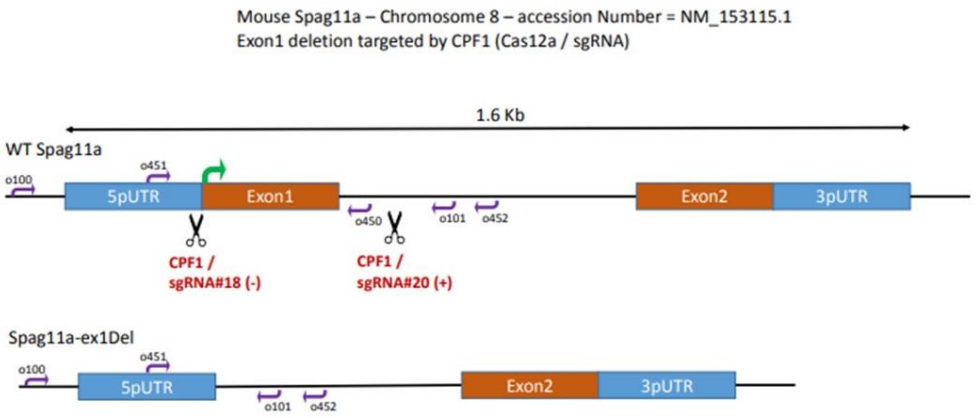

Figure 1. *Shows the method used to create mice lacking the Spag11a gene. The mouse Spag11a gene's structure is displayed in the upper panel. The locations that will be the focus of the knockout mice are indicated by scissors. The minus and plus strands were targeted by sgRNAs #18 and #20. Primer sets for genotyping are FP and RP. The exon1 deleted Spag11a gene structure is depicted in the lower panel.*

Genotyping

PCR was employed to genotype the mice using the DNA isolated from the tails. Briefly, the tail pieces were placed in 1 ml of lysis buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 0.1mg/ml Gelatin, 2.5mM MgCl2-6H_20, 0.45% v/v Nonidet P40 (NP40) and 0.45% v/v Tween20) and digested with 10 µl of Proteinase K (20mg/ml glycerol stock). The homogenates were mixed well and heated kept at 56°C overnight for complete lysis. The digested homogenates were centrifuged at 4,000 rpm for 3 minutes. The supernatant was diluted in the ratio of 1:5 and 1 µl of it was used as a template in the PCR reaction containing primers that flank on either side of the targeted region (**Figure 2)**. The PCR amplicons expected for the wild-type and *Spag11a* knockout mice are 392 and 101 bp respectively. In the heterozygote mice, two PCR amplicons of sizes 392 bp and 101 bp are expected (**Figure 2**) The PCR amplicons were sequenced and the deletion in the *Spag11a* gene was confirmed.

Total RNA isolation

Total RNA was extracted from tissues by TRIzol® reagent (Gibco). Tissues were crushed and made into powder in liquid nitrogen. 1 ml of TRIzol was added per tissue (100 mg). and vortexed for 1 minute, followed by incubation at room temperature for 5 minutes. Following a 10-minute centrifugation of the homogenate at 10,000 rpm, the supernatant was well mixed by pipetting it up and down several times. Phase separation was accomplished by adding 200 µl of chloroform and centrifuging for 10 minutes at 4 °C at 13,000 rpm. The sample was incubated for 10 minutes at room temperature after the aqueous upper layer was gently scraped off and placed into a new tube. Next, an equal volume of isopropanol was added for precipitation. Samples were centrifuged at 13,000 rpm for 10 minutes at 4 ℃ and the supernatant was discarded. The pellet was made in 70% ethanol (prepared in DEPC water), dissolved in it, and centrifuged at 10,000 g for 5 minutes before the ethanol was pipetted out completely. The pellet was entirely free of the ethanol by pipetting and air drying after being dissolved in 70% ethanol (made in DEPC water) and centrifuged at 10,000 g for 5 minutes. The pellet was then dissolved in 20 µl of water that had been treated with DEPC. RNA extraction quality was confirmed by electrophoresis of 2 µl of the sample on a 1% agarose gel. With the aid of a reverse transcription reaction kit, cDNA was created from 2 µg of total RNA in order to ascertain the mRNA expression pattern of the genes examined in this work (Promega, cDNA synthesis kit). Gene-specific primer pairs and a PCR 2X master mixture were used for the PCR, which was carried out in a 10 µl reaction solution. The amplified band was then detected using ethidium bromide.

RT-PCR

RNA was extracted to produce cDNA using commercially available kits (Qiagen). Primer sets specific to the *Spag11a* mRNA were created, and the following conditions were used for the RT-PCR: initial denaturation at 94 °C for 10 minutes, 40 cycles of 94 °C for 15 seconds, 60 °C for 1 minutes. PCR amplicons were examined using 2% agarose gel electrophoresis. Real-time PCR was performed

under controlled conditions in an Applied Biosystems thermal cycler using a SYBR master mix kit (Applied Biosystems, Warrington, UK). β-actin expression was used to function as an internal control.

Gene chip hybridization, data collection, and enrichment analysis

Using the Agilent TapeStation system and the Illumina HiSeq platform, the RNA-seq procedure was carried out on the caput epididymis of wild-type and knockout mice (aged 90 days). The data quality was then examined utilising FastQC and MultiQC programmes (de Sena Brandine and Smith 2019). STAR v2 aligner was used to map QC-passed reads onto the reference genome for Mus musculus (GCF 000001635.27 GRCm39 genomic.fna) (Dobin, Davis et al. 2013). Utilizing DESeq2, differential expression analysis was performed (Love, Huber et al. 2014). DESeq2 was used to normalise the read counts (variance stabilised normalised counts), and differential enrichment analysis was carried out. Significant genes were those that displayed an absolute log2 fold change ≥ 0.9 and ≤ -0.8 with a p-value ≤ 0.050. Using the DAVID software, Gene Ontology (GO) was performed on the genes that displayed significantly differential expression levels.

Carcinogen treatment

The effect of a low dose of carcinogen on the susceptibility of various tissues in the *Spag11a* knockout mice was determined by treating the mice with low doses of diethylnitrosamine (DEN) (Thuy le, Morita et al. 2011). Briefly, wild type, heterozygous and *Spag11a* knockout mice were treated with drinking water containing 0.05 ppm DEN for 12 weeks. The animals had free access to drinking water. A Control group without any carcinogen treatment was also maintained. All mice were sacrificed and the tissues dissected after the completion of the treatment period. All the treatments and the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) at University of Hyderabad (UH/IAEC/SY/2021-22/09).

Histopathological examination

Histopathological analyses were carried out by hematoxylin and eosin staining. The tissues were removed from the mice, fixed in 4% paraformaldehyde for 24 hours, completely washed in 70% ethanol, and then gradually dehydrated in progressively higher concentrations of ethanol (80, 90, and 100%). Five-micron slices of the tissues, which had been encased in paraffin wax, were created. The sections were next subjected to an isopropanol treatment for an overnight period at 60°C, followed by xylene deparaffinization, rehydration in graded ethanol (100, 90, 80, 70, and 50%), and finally washing with distilled water. Following this, the sections were differentiated in 1% hydrochloric acid for 30 seconds before being submerged in 0.2% Harris hematoxylin solution for 10 minutes. Sections were cleaned with distilled water, counterstained for 1 minute with 0.2% eosin Y solution and serially dehydrated with 50, 70, 80, 90, and 100% alcohol. They were mounted using xylene-based mounting media after three xylene washes. The sections were viewed, photographed, and evaluated by a board-

certified histopathologist who was unaware of the experimental setup or treatment regimen in several fields (at least 25) of each section.

Tandem liquid chromatography and mass spectrometry (LC-MS-MS)

Caudal sperm protein samples were prepared from wild-type and *Spag11a* knockout mice. Sperm protein was extracted by lysis buffer (7 M urea, 2% (w/v) dithiothreitol (DTT), 2 M thiourea, 4% (w/v) CHAPS, 50 mM NaF, 2mM Na3VO4, containing a protease inhibitor (Sigma- Aldrich, USA). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the protein quality. Trypsin in-solution digestion of a 100µg protein sample was carried out, followed by an overnight incubation at 37 °C. The final sample was vacuum dried, dissolved in 50 µl of water with 0.1% formic acid, spun at 10,000 g, and the supernatant was collected into a different tube. For the purpose of peptide separation, 10 µl of the supernatant sample was loaded onto the BEH C18 UPLC column (UPLC systems, Waters), and three runs per sample were carried out for label-free quantification (LFQ). For MS and MSMS analysis, the peptides separated on the column were sent to a Waters Synapt G2 Q-TOF apparatus. MassLynx 4.1 WATERS processed the raw data. Protein Lynx Global Server (PLGS, WATERS) software was used to match the individual peptides of the MSMS spectra to the sequence of the mouse protein database in order to identify the proteins. The data obtained were further processed for bioinformatics analysis. We next carried out Gene Ontology (GO) based on the differentially expressed proteins and assigned them to their GO categories (biological process, cellular component, and molecular function).

Genomic DNA isolation to analyse SNPs

Blood (2-10 ml) was collected from fertile and infertile men in tubes containing and centrifuged at 3,000 rpm for 10 min. The plasma was collected, equal amount of 2X lysis mix was added and incubated for 10 min to lyse RBC. The samples were then centrifuged at 3,000 rpm for 10 mins. To the pellet of white cells formed at the bottom of the tube, 450 µl of WBC lysing solution and 200 µl of proteinase K (10 mg/ml) were added, votexed and incubated over night at 37^{o}C. Equal amounts of phenol (saturated with 0.1 M Tris HCl, pH 8.0) and the sample were combined, vortexed, and then centrifuged at 3,000 rpm for five minutes. After transferring the top aqueous layer into a clean tube, another equal volume of phenol was added. After vortexing, centrifugation, and removing the aqueous layer, the sample was transferred to a new tube with an equal amount of chloroform and centrifuged at 3,000 rpm for 5 minutes. The supernatant was removed and mixed with 1 µl of 4 M ammonium acetate and 2X volume of 100% ethanol for DNA precipitation. It was mixed and centrifuged for 20 minutes at 5000 rpm at 4^{o}C. After decanting the ethanol, 500 µl of 70% ethanol were used to wash the pellets. The pellet was dissolved in distilled water and left at 4^{o}C for 4-5 hours to allow the DNA to dissolve. NanoDrop provided estimates for the DNA's amount and quality (Thermo

Scientific NanoDrop2000 UV visible spectrophotometer). The Institutional Ethics Committee at the University of Hyderabad granted approval for this part of the research (UH/IEC/2021/07).

RESULTS

Generation of *Spag11a* knock out mice

Primers that are flanked on each side of the targeted sequence of the *Spag11a* gene were used for genotyping. Genomic PCR using DNA from wild-type animals produced an amplicon of 400 bp, whereas *Spag11a* knockout mice produced an amplicon of 100 bp (**Figure 2**). Two PCR amplicons of 400 bp and 100 bp size were seen in the heterozygote mice (**Figure 2**). PCR analyses showed that the knockout animals had no *Spag11a* mRNA expression (**Figure 2**).

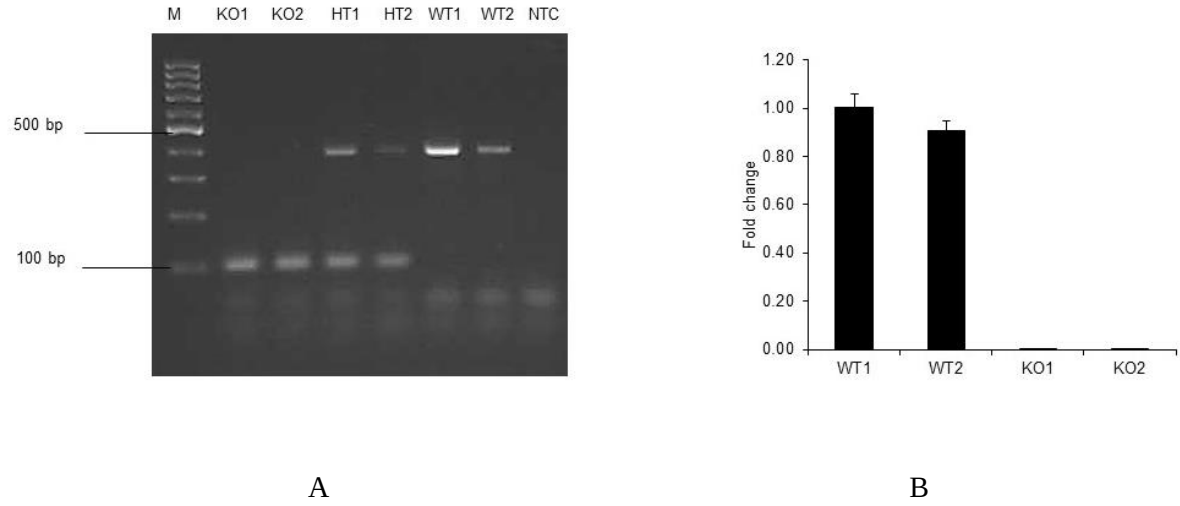

Figure 2. (A)*. Genotyping of knock out mice. DNA was isolated from the tail of mice and subjected to PCR using primers that flanked the deleted portion of the Spag11a gene.* ***(B)****. Spag11a mRNA expression. RT-PCR was done to confirm the absence of Spag11a in knock out mice. WT-Wildtype, HT-Heterozygous and KO-Knockout mice.*

Transcriptome analysis

We examined the differences in the caput's global transcriptome modifications between *Spag11a* knockout mice and their wild-type counterparts. We observed that 601 genes were differentially expressed of which 280 were up-regulated and 321 were down-regulated (**Supplementary table 1 and 2**). The fold change in the up regulated genes ranged from 1.0 to 9.26, whereas the fold change in the down regulated genes ranged from -9.7 to -1. **Tables 1** and **2** show the top 20 up- and down-regulated genes, respectively. To comprehend the potential physiological processes that the differentially expressed genes may affect, gene ontology was assessed using the DAVID software. Gene ontology using DAVID software was evaluated to understand the possible physiological processes that could be affected by the differentially expressed genes. A comprehensive list of gene ontologies that involve the up and down regulated genes are listed in **supplementary tables 3 and 4** respectively. The

top 10 gene ontologies affected by the up regulated genes are presented in **table 3**. Biological processes related to cell cycle, cell division and other cellular processes that are involved basic cellular function were found to be affected by the up regulated genes. Gene ontologies in which at least 10 down regulated genes are involved are listed in **table 4**. Cellular processes that contribute to membrane integrity, signal transduction and inflammatory responses are some of the significant ones affected.

Table 1. *Top 20 up regulated genes in the caput of Spag11a knockout mice.*

SNo.	Gene Symbol	Log FC	PValue	FDR
1.	Retn	9.265235328	9.71979E-13	3.34555E-09
2.	Zfp42	7.390159423	3.04594E-05	0.013343439
3.	F830010H11Rik	7.230706199	7.55368E-05	0.028888618
4.	Gm17745	7.14384357	0.000120499	0.038710749
5.	Lhfpl3	7.051414104	0.000193974	0.056995302
6.	Hhla1	6.952655111	0.000315208	0.079180087
7.	Gm52343	6.952655111	0.000315208	0.079180087
8.	Gm39604	6.846635558	0.000517265	0.109163668
9.	Sun5	6.732202564	0.00085757	0.1424986
10.	Gm33162	6.732202564	0.00085757	0.1424986
11.	Usp17lb	6.732202564	0.00085757	0.1424986
12.	Gm51538	6.732202564	0.00085757	0.1424986
13.	Gm30871	6.732202564	0.00085757	0.1424986
14.	Gm33230	6.732202564	0.00085757	0.1424986
15.	Gm12052	6.732202564	0.00085757	0.1424986
16.	n-TVcac7	6.732202564	0.00085757	0.1424986
17.	Lypd8l	6.732202564	0.00085757	0.1424986
18.	LOC118567349	6.60790479	0.00143701	0.187036315
19.	LOC100504180	6.60790479	0.00143701	0.187036315
20.	Gm29835	6.60790479	0.00143701	0.187036315

***Table 2**. Top 20 down regulated genes in the caput of Spag11a knockout mice.*

S No.	Gene Symbol	Log FC	P Value	FDR
1.	1700003M07Rik	-9.699640221	3.76863E-15	1.81603E-11
2.	Gm14275	-9.16564178	2.40226E-12	6.43113E-09
3.	Gm40008	-8.662649418	5.21239E-10	1.1417E-06
4.	Fcrl5	-7.970949731	2.40483E-07	0.0002146
5.	Icos	-7.88384623	5.05952E-07	0.000420359
6.	Gm15987	-7.692073463	2.40377E-06	0.001608788
7.	1700112D23Rik	-7.279359549	3.04594E-05	0.013343439
8.	4933402N22Rik	-7.279359549	3.04594E-05	0.013343439
9.	H2ac15	-7.135946873	7.55368E-05	0.028888618
10.	Pnoc	-7.058514108	0.000120499	0.038710749
11.	LOC118568174	-7.058514108	0.000120499	0.038710749
12.	Gm4788	-6.976688458	0.000193974	0.056995302
13.	Gm32666	-6.976688458	0.000193974	0.056995302
14.	Gm30120	-6.889941355	0.000315208	0.079180087
15.	Slc27a5	-6.889941355	0.000315208	0.079180087
16.	Gm31986	-6.699033022	0.00085757	0.1424986
17.	1700061I17Rik	-6.699033022	0.00085757	0.1424986
18.	Ephx4	-6.699033022	0.00085757	0.1424986
19.	Gm46958	-6.699033022	0.00085757	0.1424986
20.	Gm38930	-6.699033022	0.00085757	0.1424986

***Table 3**. Gene ontologies affected by the up regulated genes.*

Category	**GO Id and description**	**Gene count**
CC	GO:0005615~extracellular space	22
CC	GO:0005576~extracellular region	19
BP	GO:0007049~cell cycle	13
BP	GO:0009617~response to bacterium	11
CC	GO:0009986~cell surface	11
BP	GO:0051301~cell division	10
CC	GO:0005694~chromosome	9
CC	GO:0000775~chromosome, centromeric region	8
CC	GO:0016324~apical plasma membrane	7
BP	GO:0007059~chromosome segregation	6

Table 4. *Gene ontologies affected by the down regulated genes.*

Category	GO Id and description	Gene count
CC	GO:0016020~membrane	92
CC	GO:0016021~integral component of membrane	83
CC	GO:0005886~plasma membrane	78
BP	GO:0007165~signal transduction	26
CC	GO:0009897~external side of plasma membrane	24
CC	GO:0005887~integral component of plasma membrane	24
CC	GO:0005615~extracellular space	22
BP	GO:0002376~immune system process	21
MF	GO:0004888~transmembrane signaling receptor activity	18
BP	GO:0006954~inflammatory response	15
CC	GO:0009986~cell surface	15
BP	GO:0006955~immune response	14
BP	GO:0045087~innate immune response	13
MF	GO:0046982~protein heterodimerization activity	13
MF	GO:0005102~receptor binding	11
MF	GO:0005509~calcium ion binding	11
BP	GO:0007166~cell surface receptor signaling pathway	10
BP	GO:0002250~adaptive immune response	10
BP	GO:0007155~cell adhesion	10
BP	GO:0043066~negative regulation of apoptotic process	10

Surprisingly, we found that the knockout animals did not express 480 genes that were shown to be expressed in the caput of wild-type mice (**Supplementary table 5**). However, 581 genes were discovered to be expressed solely in the caput of knockout mice and undetectable in wild type mice (**Supplementary table 6**). To understand the physiological processes that the distinct genes in the caput of wild-type or knock out mice potentially affect, gene ontology was assessed using the DAVID software (**Supplementary tables 7 and 8**). Most noteworthy are those involved in the cell cycle, cell division-related genes and microRNAs related to cancer (**Tables 5 and 6**). Genes in pathways related to chemical carcinogenesis-receptor activation and chemical carcinogenesis-DNA adduct were differentially regulated were also identified (**Tables 5 and 6)**. The non-occurrence of cancer / tumours in the epididymis could be partly due to the pivotal role of *Spag11a* in modulating the expression of genes involved in the above-mentioned processes. Pathways for steroid hormone synthesis, olfactory

transduction and cytokine-cytokine receptor interaction are predicted to be affected due to differential expression of genes in *Spag11a* knockout mice. The role of odorant receptors in sperm chemotaxis is becoming more significant. Olfactory receptors and *Spag11a* might interact with one other (**Tables 5 and 6**).

***Table 5**. Top 20 gene ontologies for genes that are absent in the caput epididymis of Spag11a knockout mice (expressed only in the wild type mice).*

Category	GO Id and description
CC	GO:0016021~integral component of membrane
CC	GO:0005886~plasma membrane
BP	GO:0007186~G-protein coupled receptor signaling pathway
CC	GO:0005576~extracellular region
MF	GO:0004930~G-protein coupled receptor activity
CC	GO:0005615~extracellular space
BP	GO:0007165~signal transduction
CC	GO:0005887~integral component of plasma membrane
BP	GO:0007608~sensory perception of smell
MF	GO:0004984~olfactory receptor activity
MF	GO:0004871~signal transducer activity
MF	GO:0043565~sequence-specific DNA binding
BP	GO:0006811~ion transport
BP	GO:0006955~immune response
CC	GO:0009897~external side of plasma membrane
BP	GO:0045087~innate immune response
MF	GO:0004252~serine-type endopeptidase activity
MF	GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding
MF	GO:0005179~hormone activity
MF	GO:0005125~cytokine activity

***Table 6**. Top 20 gene ontologies for genes that are uniquely expressed in the caput caput epididymis of Spag11a knockout mice (absent in the wild type mice).*

Category	GO Id and description
CC	GO:0016021~integral component of membrane
CC	GO:0005886~plasma membrane
CC	GO:0005576~extracellular region
BP	GO:0007186~G-protein coupled receptor signaling pathway
MF	GO:0004930~G-protein coupled receptor activity
CC	GO:0005615~extracellular space
BP	GO:0007165~signal transduction
BP	GO:0007608~sensory perception of smell
MF	GO:0004984~olfactory receptor activity
CC	GO:0005887~integral component of plasma membrane
BP	GO:0006508~proteolysis
MF	GO:0004871~signal transducer activity
MF	GO:0005509~calcium ion binding
MF	GO:0008233~peptidase activity
BP	GO:0006811~ion transport
BP	GO:0007155~cell adhesion
CC	GO:0009897~external side of plasma membrane
MF	GO:0005125~cytokine activity
BP	GO:0055085~transmembrane transport
MF	GO:0004252~serine-type endopeptidase activity

Effect of *Spag11a* knockout on chemically induced carcinogenesis

There were no differences observed in the average body weight and relative organ weight of DEN treated wild-type and *Spag11a* knockout male mice. Histopathological analyses was conducted in different tissues of DEN treated wild type, heterozygous and knockout mice. While the anatomical structure remained unaffected in the caput of DEN treated wild type mice, mild to moderate mucosal epithelial cell hyperplasia (red arrow) as well as dysplastic alterations in a few mucosal epithelial cells (green arrow) was evident in the caput of DEN treated *Spag11a* KO mice (**Figure 3**). Moderate proliferation of connective tissue, anaplastic nature and hyperplasia were evident in the cauda of *Spag11a* knockout mice (**Figure 4**). The mucosal glands of seminal vesicles of *Spag11a* KO showed mild fibrosis and connective tissue growth (**Figure 5**). In the testes of *Spag11a* KO mice, considerable spermatogonial cell degeneration and infiltration of multinucleated cells were evident (**Figure 6**).

Among the tissues analysed, severe effects were observed in the prostate. In the prostate of *Spag11a* heterozygous mice, early-stage prostate cancer characterized by dysplastic and anaplastic changes in basal and mucosal cells of prostate epithelium [red arrow] (**Figure 7**). Further, basal cell tumour of prostate with signs of aggressive neoplastic basal cells invading entire mucosal folds and muscular region were observed. Dysplastic and anaplastic / basophilic changes in basal and mucosal cells of prostate epithelium and invasion of sub mucosal layer indicative of early stage prostate cancer is evident. In the *Spag11a* knockout mice, DEN treatment resulted in prostate tumour (**Figure 7**). Atypical basal cells of prostate formed an intra epithelial neoplasia in mucosal folds. The neoplastic cells are pleomorphic, basophilic and had variations in cytoplasm and nucleus ratio. Neoplastic basal cells of prostate invading into adjacent muscular layer and mild inflammatory reaction in mucosal epithelial layer with infiltration of plasma cells and few anaplastic / basophilic epithelial cells are noticed. Spread of dysplastic epithelial cells [no anaplastic] observed in mucosal glands. Gleason's score was assigned to the prostate obtained from wild type, heterozygous and knockout mice treated with DEN. The score was 1, 3.6 and 7.0 for wild type, heterozygous and knockout mice respectively (**Table 7**). Based on the Gleason's score, the prostate of wild type mice was found to be well differentiated with normal mucosal epithelial cells. The prostate of heterozygous mice with a Gleason's score of 3.6 were classified to are well differentiated with moderate anaplasticity. In the prostate of Spag11a knockout mice, which had a Gleason's score of 7, the tissue was poorly differentiated and severely anaplastic (**Table 7**).

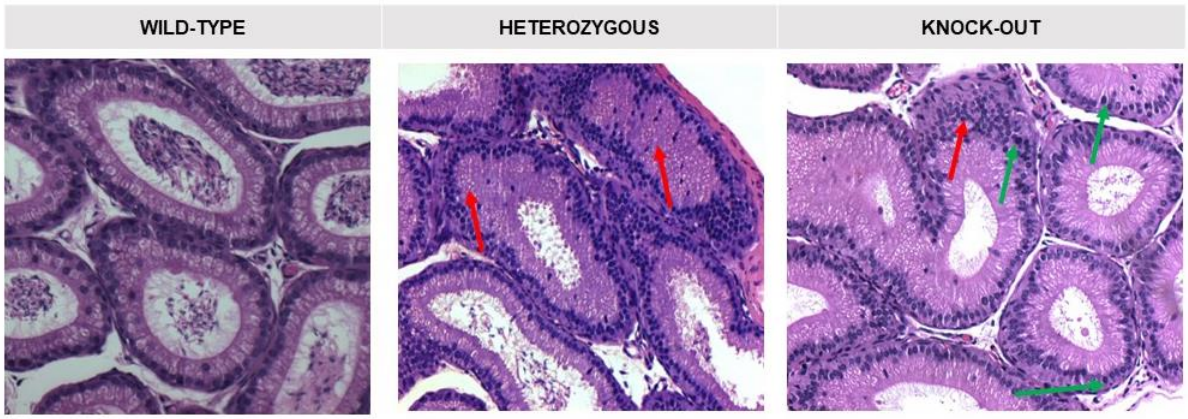

***Figure 3**. Histopathological changes in the caput epididymis of DEN treated wild type, heterozygous and knock out mice. Caput epididymis from mice treated with DEN mice were fixed and 5-micron sections taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope.*

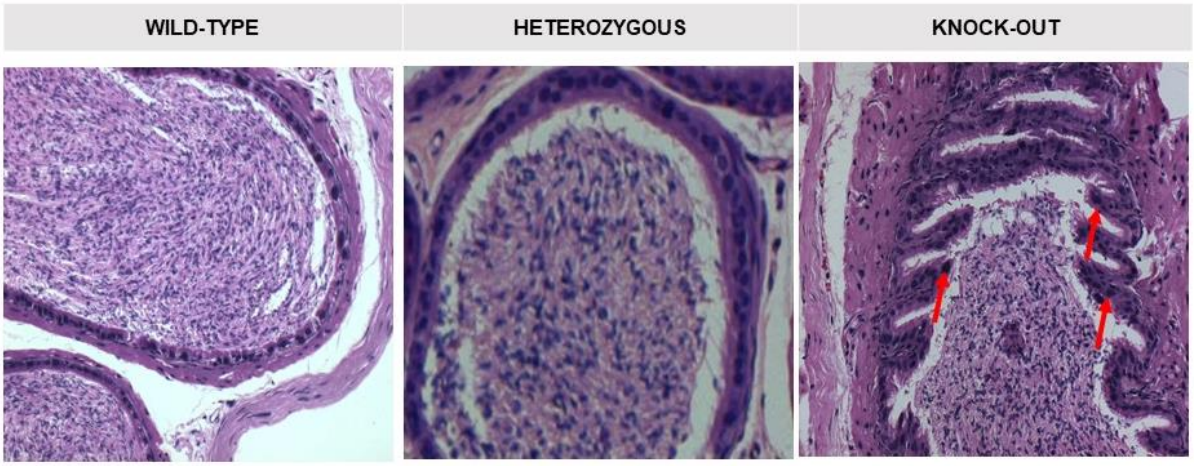

Figure 4. *Histopathological changes in the cauda epididymis of DEN treated wild type, heterozygous and knock out mice. Cauda epididymis from mice treated with DEN mice were fixed and 5-micron sections taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope.*

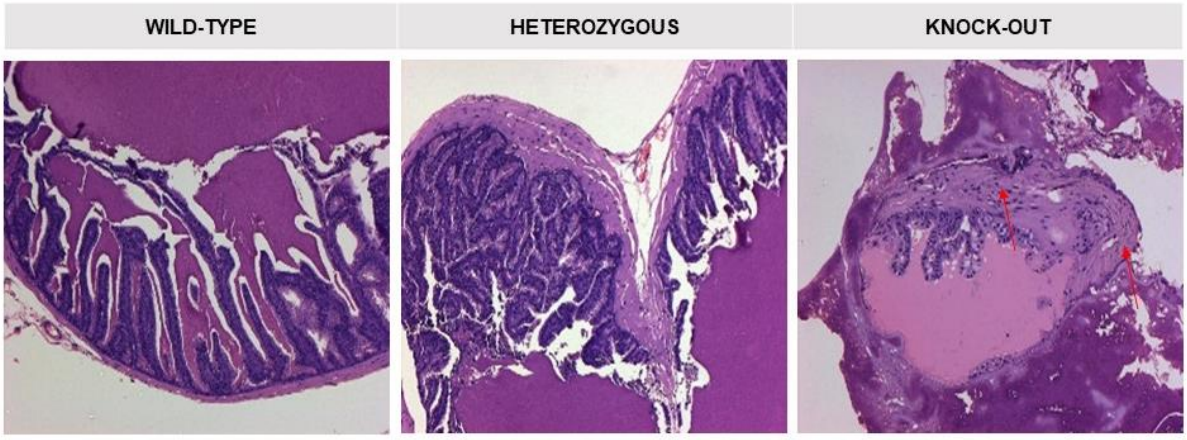

Figure 5. *Histopathological changes in the seminal veicles of DEN treated wild type, heterozygous and knock out mice. Seminal vesicles from mice treated with DEN mice were fixed and 5-micron sections taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope.*

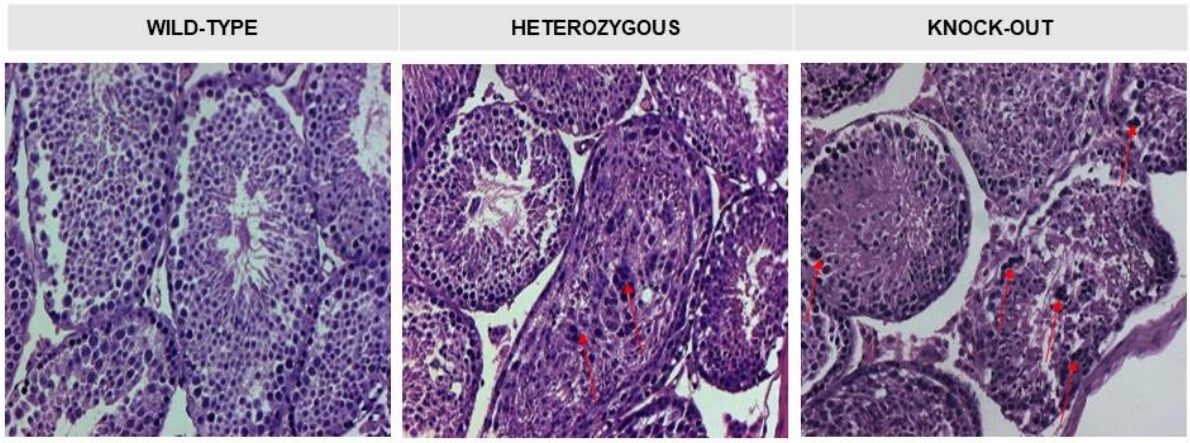

Figure 6. *Histopathological changes in the testes of DEN treated wild type, heterozygous and knock out mice. Testes from mice treated with DEN mice were fixed and 5-micron sections taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope.*

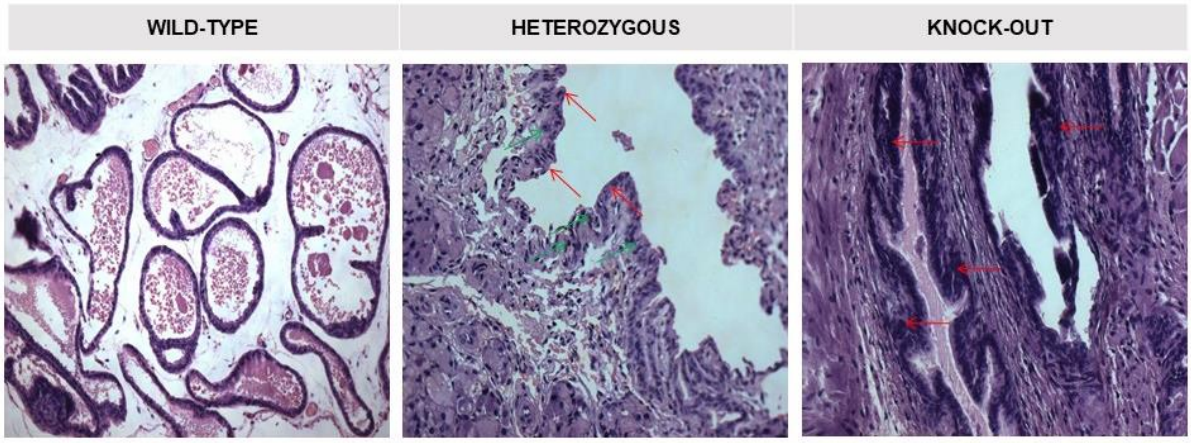

Figure 7. *Histopathological changes in the prostate of DEN treated wild type, heterozygous and knock out mice. Prostate from mice treated with DEN mice were fixed and 5-micron sections taken and stained with Hematoxylin and Eosin. Photomicrographs were taken in a phase contrast microscope.*

Table 7. *Gleason score for the prostate obtained from DEN treated mice.*

Genotype	**Gleason's score**	**Histological changes**
Wild-type	1	Well differentiated, normal mucosal epithelial cells
Heterozygous	3.6	Well differentiated, moderate anaplastic
Knock-out	7	Marked anaplastic, poorly differentiated

Sperm proteome analysis

We conducted quantitative proteomic research utilising LC-MS/MS analysis to look into the global proteome profile of spermatozoa in *Spag11a* knockout and wild type mice. These sperm samples yielded a total of 1825 proteins, 91 of which were determined to have significantly varied levels of expression. Of these, 91 proteins, 40 proteins were up regulated and 51 proteins were down regulated (**Tables 8 and 9**). The physiological and cellular processes in which the differentially expressed proteins are involved was determined by DAVID analyses. Proteins that were up regulated in the sperm of knock out mice are involved in spermatogenesis, macromolecular complex formation, protein phosphorylation and microtubule formation (**supplementary table 9**). On the other hand, proteins that were down regulated in the sperm of knock out mice are involved in spliceosomal complex formation, maintenance of cytoskeleton and cell differentiation (**supplementary table 10**). The cellular processes in which differential expression of sperm proteins was observed contribute to the male gametogenesis in a direct or indirect way.

Table 8*. Up regulated sperm proteins in wild type versus Spag11a knockout mice.*

ID	RATIO	PROTEIN IDENTITY
Q2TV84	1.950558	Transient receptor potential cation channel subfamily M member 1
Q5SQM0	1.953575	Echinoderm microtubule-associated protein-like 6
Q80Z25	1.963358	Oral-facial-digital syndrome 1 protein homolog
Q9R269	1.973866	Periplakin
Q8C9B9	2.003752	Death-inducer obliterator 1
Q9WV02	2.006024	RNA-binding motif protein, X chromosome
Q9D2H5	2.050518	Tripartite motif-containing protein 42
P13542	2.127619	Myosin-8
Q6PH08	2.160618	ERC protein 2
Q9JM99	2.205307	Proteoglycan 4
E9Q3S4	2.290803	Mitogen-activated protein kinase kinase kinase 19
Q8BGV5	2.321679	Zinc finger protein 8
A2AF47	2.379754	Dedicator of cytokinesis protein 11
Q99PG2	2.391928	Opioid growth factor receptor
Q99P68	2.433163	Sclerostin
P97820	2.459133	Mitogen-activated protein kinase kinase kinase kinase 4
P59672	2.490063	Ankyrin repeat and SAM domain-containing protein 1A
Q924N4	2.49095	Solute carrier family 12 member 6
Q9WVM6	2.54533	Tolloid-like protein 2
P51954	2.546261	Serine/threonine-protein kinase Nek1

Q3UZV7	2.716506	UPF0577 protein KIAA1324-like homolog
B2RY56	2.726874	RNA-binding protein 25
Q61235	2.797643	Beta-2-syntrophin
Q9QY23	3.018117	Plakophilin-3
Q497V5	3.022813	S1 RNA-binding domain-containing protein 1
Q9JIS8	3.375356	Solute carrier family 12 member 4
Q64511	3.460825	DNA topoisomerase 2-beta
E5FYH1	3.682227	Protein TOPAZ1
Q3UHD3	3.768917	Microtubule-associated tumor suppressor candidate 2 homolog
Q8BPI1	4.20783	Protein kintoun
Q6PAJ1	4.533588	Breakpoint cluster region protein
Q920G9	4.763753	Germ cell-less protein-like 1
Q6DYE8	4.925315	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3
Q5SX79	5.010236	Protein Shroom1
O09000	6.310371	Nuclear receptor coactivator 3
E9PVD1	6.404218	Coiled-coil domain-containing protein 62
Q9R0K7	8.79002	Plasma membrane calcium-transporting ATPase 2
O35126	9.313592	Atrophin-1
Q99PL5	10.26087	Ribosome-binding protein 1
Q8R422	13.99462	CD109 antigen

***Table 9**. Down regulated sperm proteins in wild type vs Spag11a knockout mice.*

ID	RATIO	PROTEIN IDENTITY
Q80UG8	0.013756	Tubulin polyglutamylase TTLL4
B1AS29	0.050709	Glutamate receptor ionotropic, kainate 3
Q04592	0.088382	Proprotein convertase subtilisin/kexin type 5
Q3TCX3	0.104994	KH homology domain-containing protein 4
Q69ZU6	0.109891	Thrombospondin type-1 domain-containing protein 7A
Q9Z2K1	0.127844	Keratin, type I cytoskeletal 16
A2AM05	0.132678	Centlein
B2RXC1	0.141631	Trafficking protein particle complex subunit 11
A8C756	0.157444	Thyroid adenoma-associated protein homolog
Q8BMG7	0.168603	Rab3 GTPase-activating protein non-catalytic subunit
Q8BW10	0.179062	RNA-binding protein NOB1
Q62273	0.182429	Sulfate transporter
P14211	0.190812	Calreticulin
Q6ZPZ3	0.192141	Zinc finger CCCH domain-containing protein 4
A2ARA8	0.194214	Integrin alpha-8
Q6NZJ6	0.194226	Eukaryotic translation initiation factor 4 gamma 1
A2RSQ0	0.220325	DENN domain-containing protein 5B
Q920R0	0.247562	Alsin
Q7M6Y6	0.247937	Maestro heat-like repeat-containing protein family member 2B
Q8K4L3	0.249211	Supervillin
Q9CUU3	0.253797	Synaptonemal complex protein 2
Q9JMH9	0.260775	Unconventional myosin-XVIIIa
P97500	0.267728	Myelin transcription factor 1-like protein
Q99NE5	0.270857	Regulating synaptic membrane exocytosis protein 1
Q3U3D7	0.275625	Transmembrane protein 131-like
O08999	0.27977	Latent-transforming growth factor beta-binding protein 2
O88573	0.286471	AF4/FMR2 family member 1
Q02357	0.293993	Ankyrin-1
Q6A068	0.29442	Cell division cycle 5-like protein
Q62018	0.294977	RNA polymerase-associated protein CTR9 homolog
Q64324	0.313077	Syntaxin-binding protein 2
O08648	0.31499	Mitogen-activated protein kinase kinase kinase 4
Q6Y685	0.315953	Transforming acidic coiled-coil-containing protein 1

O35927	0.322679	Catenin delta-2
P01027	0.323783	Complement C3
Q91YE5	0.324717	Bromodomain adjacent to zinc finger domain protein 2A
O35149	0.338733	Zinc transporter 4
P15208	0.350556	Insulin receptor
Q3U492	0.3518	Kielin/chordin-like protein
Q8CGB3	0.381808	Uveal autoantigen with coiled-coil domains and ankyrin repeats
P35951	0.386506	Low-density lipoprotein receptor
Q810J8	0.388335	Zinc finger FYVE domain-containing protein 1
Q3U3C9	0.397378	Genetic suppressor element 1
Q8VHD8	0.400177	Hornerin
A2A4P0	0.404481	ATP-dependent RNA helicase DHX8
Q63ZW7	0.417298	InaD-like protein
Q7TSC1	0.41768	Protein PRRC2A
Q80TJ7	0.450204	Histone lysine demethylase PHF8
Q80TZ9	0.477127	Arginine-glutamic acid dipeptide repeats protein
Q62141	0.47837	Paired amphipathic helix protein Sin3b
Q5XJV7	0.491078	SET domain-containing protein 5

We found it interesting that 983 proteins seen in the sperm of mice with wild types were not found in the sperm of mice with the *Spag11a* deletion (**supplementary table 11**). Contrarily, 751 proteins that are solely produced in the sperm of *Spag11a* knockout mice were not found in sperm from wild-type mice (**supplementary table 12**)**.** Detailed assessments of gene ontologies linked to proteins absent in the sperm of knockout mice are shown in **supplementary table 13**. Similar to this, gene ontologies related to proteins that are only found in the sperm of knockout mice are presented in **supplementary table 14**. A closer analyses on the effect of *Spag11a* knockout on processes involved in male gametogenesis was performed. Gene ontology analyses reveal that proteins involved in spermatogenesis, protein phosphorylation, male meiosis – I, spermatid development, flagellated sperm motility, etc were affected in the *Spag11a* knock out sperm (**Table 10**). We also identified 43 proteins that were completely absent in the sperm of *Spag11a* knock out mice (**Table 11**). The information revealed that sperm motility and spermatogenesis, in particular, were disrupted in *Spag11a* knockout sperm.

***Table 10**. Gene ontologies associated with male gametogenesis in which the differentially expressed proteins were involved.*

Category	GO Id and description	Protein count
BP	**GO:0007283~spermatogenesis**	**4**
BP	**GO:0007283~spermatogenesis**	**25**
BP	GO:0006468~protein phosphorylation	4
BP	**GO:0007141~male meiosis I**	**5**
BP	**GO:0007283~spermatogenesis**	**43**
BP	**GO:0007286~spermatid development**	**16**
BP	**GO:0030317~flagellated sperm motility**	**12**
CC	GO:0005874~microtubule	4
BP	GO:0071392~cellular response to estradiol stimulus	2
MF	GO:0005524~ATP binding	91
CC	GO:0005856~cytoskeleton	85
MF	GO:0005509~calcium ion binding	50
BP	GO:0016310~phosphorylation	39
BP	GO:0006468~protein phosphorylation	38
CC	GO:0005874~microtubule	24
MF	GO:0003779~actin binding	24
MF	GO:0051015~actin filament binding	20
MF	GO:0008017~microtubule binding	20
BP	GO:0030036~actin cytoskeleton organization	19
BP	GO:0070588~calcium ion transmembrane transport	13
BP	GO:0051321~meiotic cell cycle	13
MF	GO:0004714~transmembrane receptor protein tyrosine kinase activity	12
MF	GO:0008236~serine-type peptidase activity	12
BP	GO:0018108~peptidyl-tyrosine phosphorylation	8
BP	GO:0042632~cholesterol homeostasis	8
MF	GO:0005509~calcium ion binding	53

***Table 11**. Proteins related to the GO term "spermatogenesis" that are not detected in proteome of caudal sperm of Spag11a ko mice.*

	PROTEIN ID	**GENE NAME**
1	Q9DBR1	**5'-3' exoribonuclease 2(Xrn2)**
2	A2CG63	**AT rich interactive domain 4B (RBP1-like)(Arid4b)**
3	Q9WV27	**ATPase, Na+/K+ transporting, alpha 4 polypeptide(Atp1a4)**
4	Q5SXJ3	**BRCA1 interacting protein C-terminal helicase 1(Brip1)**
5	Q8VHK9	**DEAH (Asp-Glu-Ala-His) box polypeptide 36(Dhx36)**
6	P16381	**DNA segment, Chr 1, Pasteur Institute 1(D1Pas1)**
7	P05532	**KIT proto-oncogene receptor tyrosine kinase(Kit)**
8	O54785	**LIM motif-containing protein kinase 2(Limk2)**
9	Q8VCB1	**NDC1 transmembrane nucleoporin(Ndc1)**
10	Q9WVM1	**Rac GTPase-activating protein 1(Racgap1)**
11	Q6ZPE2	**SET binding factor 1(Sbf1)**
12	G5E8Z2	**TATA-box binding protein associated factor 4b(Taf4b)**
13	B2RR83	**YTH domain containing 2(Ythdc2)**
14	Q9R159	**a disintegrin and metallopeptidase domain 25 (testase 2)(Adam25)**
15	P27038	**activin receptor IIA(Acvr2a)**
16	P09470	**angiotensin I converting enzyme (peptidyl-dipeptidase A) 1(Ace)**
17	P34821	**bone morphogenetic protein 8a(Bmp8a)**
18	Q8C633	**calcium binding protein, spermatid specific 1(Cabs1)**
19	O54833	**casein kinase 2, alpha prime polypeptide(Csnk2a2)**
20	Q8BRC6	**cilia and flagella associated protein 91(Cfap91)**
21	Q8BLA1	**deleted in lung and esophageal cancer 1(Dlec1)**
22	Q80XI3	**eukaryotic translation initiation factor 4 gamma, 3(Eif4g3)**
23	Q5SV77	**gametogenetin binding protein 2(Ggnbp2)**
24	Q8K337	**inositol polyphosphate-5-phosphatase B(Inpp5b)**
25	Q8K0T4	**katanin p60 subunit A-like 1(Katnal1)**
26	A2AG06	**meiosis specific with coiled-coil domain(Meioc)**
27	Q61884	**meiosis-specific nuclear structural protein 1(Mns1)**

28	O88735	**microtubule-associated protein 7(Map7)**
29	Q64249	**nuclear receptor subfamily 6, group A, member 1(Nr6a1)**
30	Q9JMB7	**piwi-like RNA-mediated gene silencing 1(Piwil1)**
31	Q8CGT6	**piwi-like RNA-mediated gene silencing 4(Piwil4)**
32	Q402U7	**protease, serine 44(Prss44)**
33	Q5SSW2	**proteasome (prosome, macropain) activator subunit 4(Psme4)**
34	Q99MV7	**ring finger protein 17(Rnf17)**
35	P13808	**solute carrier family 4 (anion exchanger), member 2(Slc4a2)**
36	Q6IUP1	**spermatogenesis and oogenesis specific basic helix-loop-helix 1(Sohlh1)**
37	Q3UMC0	**spermatogenesis associated 5(Spata5)**
38	Q9D411	**testis-specific serine kinase 4(Tssk4)**
39	Q8C0S4	**tetratricopeptide repeat domain 21A(Ttc21a)**
40	O55047	**tousled-like kinase 2 (Arabidopsis)(Tlk2)**
41	Q8CHB8	**tubulin tyrosine ligase-like family, member 5(Ttll5)**
42	Q5VCS6	**tudor domain containing 5(Tdrd5)**
43	Q8C0R7	**zinc finger, MYND-type containing 15(Zmynd15)**

***SPAG11A* gene polymorphisms in infertile men**

Blood samples were collected from fertile and infertile men as per the inclusion and exclusion criteria mentioned in the methodology section. Genomic DNA was isolated from samples and PCR was performed using exon-specific primers. Amplicons were sequenced and multi-aligned with the MultAlin tool. *Spag11a* gene polymorphism analyses indicated that the occurrence of four SNPs each in exon 1 and exon 3. Details of the SNPs and their occurrence in infertile and fertile men are presented (**Table 12**). In exon 1, the SNPs were found at positions 88, 100, 135 and 211 and the mutations observed was T/C, G/A, C/G and A/C respectively. There was no statistical difference in the frequency of the SNPs at positions 88, 135 and 211 between the fertile and infertile groups. The frequency of SNP at position 100 was 6% in the infertile men, while it was 13 % in the fertile men. The position of the SNPs in exon 3 were found to be at 25, 30, 37 and 61 and the mutations associated were T/C, A/G, C/T and C/T respectively. The frequency of SNPs at positions 25, 30, 37 and 61 was 14%, 19%, 19% and 14% respectively in infertile men. In the fertile men frequency of SNPs at positions 25, 30, 37 and 61 was 7%, 7%, 7% and 7% respectively. These results indicate that the SNPs found on exon 3 occur at a

significantly higher frequency in infertile men. The association of polymorphisms between an epididymis-specific gene and male infertility is demonstrated for the first time. Analyses of polymorphisms in larger sample numbers will provide further evidence.

***Table 12**. Frequency of SNPs in the Spag11a gene in fertile and infertile men.*

Exon	Position	SNP	Genotype frequency Infertile (n=48)	Genotype frequency Control (n=15)
Exon1	88	T/C	85 %	80 %
Exon1	135	C/G	62 %	60 %
Exon1	211	A/C	97 %	93 %
Exon1	100	G/A	6 %	13 %*
Exon3	25	T/C	14 %	7 %*
Exon3	30	A/G	19 %	7 %*
Exon3	37	C/T	19 %	7 %*
Exon3	61	C/T	14 %	7 %*

DISCUSSION

Spag11a gene and epididymal carcinogenesis

Incidence of cancers is dependent on a variety of factors. At the genetic level, mutations in the DNA sequence that may result in overexpression or downregulation of certain genes are known to contribute to carcinogenesis. Cancers of the male reproductive tract caused by genetic alterations are very common, especially of the testis and prostate. For example, testicular teratoma was evident in animals where in the expression of cyclin D1 (*Ccnd1*) was perturbed (Lanza, Dawson et al. 2016). Prostate tumorigenesis associated with DNA damage was promoted under conditions of testicular nuclear receptor 4 (*Tr4*) knock down (Lin, Lee et al. 2014). In the Leydig and Sertoli cell specific conditional knock out mice for *Smad4* or *Smad1* or *Smad5*, metastatic tumour formation associated with testicular dysgenesis and haemorrhagic was evident (Pangas, Li et al. 2008, Archambeault and Yao 2014). In knock out mice that lacked xeroderma pigmentosum group A gene (*Xpa*), testicular tumorigenesis was developed (Nakane, Hirota et al. 2008). In the WWOX hypomorphic mice, occurrence of B-cell lymphomas and testicular atrophy was observed (Ludes-Meyers, Kil et al. 2007). *Nbn* heterozygous mice displayed testicular tumour incidence compared to their wild type counter parts. (Dumon-Jones, Frappart et al. 2003). However, incidence of cancer in the epididymis is very rare. It is hypothesised that a variety of factors that are epididymis specific contribute to this unique property (Yeung, Wang et al. 2012). Our previous studies demonstrated that the incidence of chemically induced epididymal hyperplasia was higher in rats immunized against the epididymis specific protein SPAG11A (Sangeeta and Yenugu 2022). This indicated that absence of SPAG11A protein renders this organ susceptible to tumorigenesis. To further study the role of SPAG11A in contributing to the resistance towards cancers in the epididymis, in this part of the study, we used *Spag11a* knock out mice and subjected them to low dose carcinogen treatment.

Tumour initiation is a result of uncontrolled cell proliferation, a tightly regulated process mediated by complex signalling pathways. In majority of the cancers, disturbances in the expression pattern of the components of cell signalling pathways leads to the dysregulation of the cellular processess and thereby leading to imbalance in cell proliferation. A recent study reported that perturbations in gene expression of the components of one or more of the most common ten signalling pathways (Hippo, Myc, Notch, Nrf2, PI-3-Kinase/Akt, RTK-RAS, TGFb, p53, cell cycle (Cdk mediated) and b-catenin/Wnt) are associated with majority of the 33 types of human cancers analysed (Sanchez-Vega, Mina et al. 2018). In this study, as a first step, to determine whether knock out of *Spag11a* will result in disturbances in the cellular signalling pathways, especially related to cell proliferation, whole transcriptome analyses of the caput epididymis was carried out in wild type and *Spag11a* knock out mice. Differentially regulated genes were associated with a wide variety of cellular processes. The top 10 gene ontologies affected by the up regulated genes are were related to cell cycle, cell division and other cellular processes that are involved basic cellular function. On the other hand down regulated genes were in

involved in gene that contribute to membrane integrity, signal transduction and inflammatory responses are some of the significant ones affected. These preliminary results indicated that absence of *Spag11a* could affect the cellular cycle pathways in the caput epididymis. Our previous in vitro studies also indicated that siRNA mediated knock down of *Spag11a* resulted in increased proliferation of isolated epididymal primary epithelial cells (Sangeeta and Yenugu 2020).

In the DEN treated *Spag11a* knock out mice, the incidence of pathological features akin to tumorigenesis was more evident than their wild type counterparts. Features that reflect hyperplasia, inflammation and tumorigenesis were observed in the caput and cauda epididymis, testis and seminal vesicles. Among all the tissues analysed severe pathological changes that indicate tumorigenesis was observed in the prostate. Previous studies have also reported the onset of tumorigenesis in testis, prostate and other endocrine tissues under conditions of ablation of a specific gene using knock out models (Dumon-Jones, Frappart et al. 2003, Ludes-Meyers, Kil et al. 2007, Nakane, Hirota et al. 2008, Pangas, Li et al. 2008, Sachdeva, Bhardwaj et al. 2012, Archambeault and Yao 2014, Lin, Lee et al. 2014, Lanza, Dawson et al. 2016). Promotion of testicular teratoma initiation in mice that misexpresses cyclin D1, the tumour suppressor activity of TR4 nuclear receptor in the prostate, testicular dysgenesis in Smad4 and Smad5 knock out mice, spontaneous tumorigenesis in xeroderma pigmentosum group A gene (Xpa)-deficient mice, testicular atrophy in WWOX hypomorphic mice and increased susceptibility to tumour formation in Nbn heterozygous mice are some of the latest findings that demonstrate that loss of a specific gene can induce tumorigenesis in the male reproductive tract models (Dumon-Jones, Frappart et al. 2003, Ludes-Meyers, Kil et al. 2007, Nakane, Hirota et al. 2008, Pangas, Li et al. 2008, Archambeault and Yao 2014, Lin, Lee et al. 2014, Lanza, Dawson et al. 2016). However, the incidence of tumorigenesis in the epididymis was not demonstrated earlier. This study, for the first-time reports that knock out of *Spag11a* contributes to susceptibility of caput epididymis to chemically induced carcinogenesis.

Spag11a **gene and sperm function**

Transgenic or knockout animals are the best models to study gene function. Such model systems have been extensively used to study the role of a gene or multiple genes in male reproductive physiology. Epididymal or testicular genes have been analysed, some of which were dispensable for fertility, while the rest were crucial for male gametogenesis. For example, the *Lcn8* knockout mice displayed normal spermatogenesis and fertility, but the animals produced defective sperm (Wen, Liu et al. 2021). While the fertility was not affected, the crucial role of calcium-binding protein spermatid-associated 1 (*Cabs1*) in the formation of sperm annulus, tail assembly and motility was demonstrated in a knockout animal model (Zhang, Zhou et al. 2021). Deletion of serine protease 55 (PRSS55) affected energy metabolism of spermatozoa, which ultimately resulted in infertility in mice (Zhu, Li et al. 2021). Cilia and flagella associated protein 45 (CFAP45) knockout mice were characterized with asthenospermia associated with

motile ciliopathy (Dougherty, Mizuno et al. 2020). Deficiencies in sperm function (capacitation and acrosome reaction) and sperm maturation were reported in *Plag1*$^{-/-}$ mice (Wong, Damdimopoulos et al. 2020). On the same lines, the importance of *Cfap97d1*, adenylate kinase 1, autophagy core protein ATG5, *Fam170a*, *Tmprss12*, NELL2, IFT81, DCP2 and *Prss55* are some of the epididymal and testicular genes whose indispensability has been demonstrated (Devlin, Nozawa et al. 2020, Huang, Liu et al. 2020, Kiyozumi, Noda et al. 2020, Kobayashi, Endo et al. 2020, Larasati, Noda et al. 2020, Li, Zhang et al. 2020, Qu, Yuan et al. 2020, Xie, Zhang et al. 2020). We previously demonstrated that active immunization against SPAG11A or shRNA mediated ablation of *Spag11a* mRNA or knock out of *Spag11a* resulted in decreased fecundity and sperm function (Sangeeta and Yenugu 2020). However, there is no evidence on the specific processes that are affected under conditions of Spag11a knock out. Hence, we analysed the sperm proteome of wild type and *Spag11a* knock out mice to determine whether any proteins that are crucial for spermatogenesis and sperm maturation are affected.

Analysis of the sperm proteome in the *Spag11a* knock out mice revealed differential expression of proteins. Proteins that were up regulated in the sperm of knock out mice are involved in spermatogenesis, macromolecular complex formation, protein phosphorylation and microtubule formation. Proteins that were down regulated in the sperm of knock out mice are involved in spliceosomal complex formation, maintenance of cytoskeleton and cell differentiation. Interestingly, a large number of proteins that were detected in the sperm of wild type mice were not detected in the sperm of *Spag11a* knockout mice. These proteins have roles in spermatogenesis, protein phosphorylation, male meiosis – I, spermatid development, flagellated sperm motility. Changes in proteome of male reproductive organs, including the sperm under conditions of a gene knockout were demonstrated earlier. Drastic changes in the testicular proteome was reported in *Akap-4* knockout mice (Fang, Huang et al. 2019). In the testis of *Sun5* knockout mice, differential expression of proteins was evident, which play a key role in spermatogenesis (Zhang, Yang et al. 2021). Homozygous loss-of-function mutations of *QRICH2* gene in humans resulted in altered sperm proteome (Shen, Zhang et al. 2019). Loss of LY6/PLAUR domain containing 4 (LYPD4) gene in knockout mice caused perturbations in the expression of proteins in the spermatozoa (Wang, Cheng et al. 2020). It is very clear from out study that the cellular processes in which differential expression of sperm proteins was observed contribute to the male gametogenesis in a direct or indirect way.

Spag11a **polymorphism and male infertility**

Infertility is a serious problem and affects about 15% of couples throughout the world (de Kretser 1997). Male factor infertility is one of the most complex disorders in reproductive medicine and understanding of the factors responsible for these conditions remained an active area of research in the last three decades. It is estimated that genetic abnormalities are responsible for nearly 30% of male factor infertility (Ferlin, Raicu et al. 2007). A variety of factors such as environmental, hormonal, life

style and genetic abnormalities are responsible for the increase in male infertility rates. Genetic changes affect hormonal homeostasis, spermatogenesis and sperm quality. Majority of the genetic studies focused on genes that are involved in spermatogenesis or factors that aid spermatogenesis. It is important to note that a lot of evidence suggests that proteins that are secreted in the epididymis aid in sperm maturation, sperm function and fertilization. Genetic changes in genes that encode epididymal proteins are not analysed. This is more evident in the Indian context. Hence, analyzing genetic changes in genes that encode testicular and epididymal proteins will help to identify factors that are to be supplemented for effective in vitro fertilization techniques.

In this study, we identified the possible *Spag11a* gene polymorphisms in fertile and infertile men. In exon 1, the SNPs were found at positions 88, 100, 135 and 211 and the mutations observed was T/C, G/A, C/G and A/C respectively. There was no statistical difference in the frequency of the SNPs at positions 88, 135 and 211 between the fertile and infertile groups. The frequency of SNP at position 100 was 6% in the infertile men, while it was 13 % in the fertile men. The position of the SNPs in exon 3 were found to be at 25, 30, 37 and 61 and the mutations associated were T/C, A/G, C/T and C/T respectively. The frequency of SNPs at positions 25, 30, 37 and 61 was 14%, 19%, 19% and 14% respectively in infertile men. In the fertile men frequency of SNPs at positions 25, 30, 37 and 61 was 7%, 7%, 7% and 7% respectively. These results indicate that the SNPs found on exon 3 occur at a significantly higher frequency in infertile men. Screening for SNPs using microarrays in infertile men is a recent development. Using microarray technology, SNPs and deletions in the *SPATA16* and *DPY19L2* genes was found to be associated with globozoospermia (Dam, Koscinski et al. 2007, Harbuz, Zouari et al. 2011, Koscinski, Elinati et al. 2011). Besides these, a number of SNPs have been identified using microarray technologies (Aston 2014). Genome wide studies to identify SNPs and their relation to male infertility were reported. SNPs in *TGFBR3* and *BMP7*, human leukocyte antigen (*HLA*) region were significantly associated with non-obstructive azoospermia. Copy number variations were identified in infertile men using genome wide studies (Dalgaard, Weinhold et al. 2012, Tuttelmann, Laan et al. 2012, Zhao, Xu et al. 2012, Lopes, Aston et al. 2013). In this study, the association of polymorphisms between an epididymis-specific gene and male infertility is demonstrated for the first time. Analyses of polymorphisms in larger sample numbers will provide further evidence.

CONCLUSIONS

This part of the study presents the role of *Spag11a*, an epididymis specific gene in multiple physiological processes. Loss of *Spag11a* gene (by knockout) in mice caused differential expression of genes involved in a variety of cellular processes. The KEGG pathway analyses suggested that the absence of *Spag11a* may activate microRNAs associated with cancer, chemical carcinogenesis-receptor activation and chemical carcinogenesis-DNA adducts pathways, which may contribute to the promotion of oncogenesis in the epididymis. Further, the epididymis and prostate of *Spag11a* knockout mice appeared to be more susceptible to DEN-induced carcinogenesis compared to wild type mice, which is evident by histopathological examination. Hyperplasia, anaplasia, dysplasia, neoplasia, and inflammation in the epididymis and prostate of *Spag11a* knockout mice, while that of wild type mice displayed normal anatomical structure. Our results provide concrete evidence that the loss of *Spag11a* makes the epididymis and other tissues more susceptible to chemical carcinogenesis. The involvement of an epididymal gene in carcinogenesis is being demonstrated for the first time and also provides a possible answer to the complex question of why epididymal cancers are rare. On the other hand, the sperm proteome analyses revealed that the loss of *Spag11a* affected resulted in differential expression of proteins that are crucial for spermatogenesis process and sperm motility. SNPs were detected in the *SPAG11A* gene in infertile patients for the first time, which indicates that polymorphisms in genes that contribute to sperm maturations can also be a factor for male infertility. Results of this part of the study indicate the role of Spag11a gene in fertility and prevention of tumorigenesis. In depth analyses on the molecular mechanisms through which SPAG11A protein modulates cell proliferation are essential.

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Characterization of SPINK2, SPACA7 and PDCL2: Effect of immunization on fecundity, sperm function and testicular transcriptome

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ABSTRACT

Testicular factors play a vital role in spermatogenesis. We characterized the functional role of rat *Spink2, Spaca7* and *Pdcl2* genes. Their primary, secondary and tertiary structure were deduced *in silico*. The genes of rat *Spink2, Spaca7* and *Pdcl2* mRNA were predominantly expressed in the testis. SPINK2, SPACA7 and PDCL2 protein expression was evident in all the cell types of testis and on spermatozoa. Ablation of each of these proteins by active immunization resulted in reduced fecundity and sperm count. Damage to the anatomical architecture of testis and epididymis was evident. In SPINK2 immunized rats, 283 genes were differentially regulated while it was 434 and 872 genes for SPACA7 and PDCL2 respectively. Genes that were differentially regulated in the testis of SPINK2 immunized rats primarily belonged to extracellular exosome formation, extracellular space and response to drugs. SPACA7 ablation affected genes related to extracellular space, oxidation-reduction processes, endoplasmic reticulum membrane and response to drugs. Differential gene expression was observed for nuclear function, protein binding and positive regulation of transcription from RNA polymerase II promoter in testis of PDCL2 immunized rats. Results of our study demonstrate the role of SPINK2, SPACA7 and PDCL2 in spermatogenesis and in important molecular processes that may dictate testicular function and other physiological responses as well.

1. Introduction

Spermatogenesis is a complex process that involves an interplay of a wide variety of molecular factors [1], especially the testis specific factors [2,3]. Disruption in the expression of genes that contribute to spermatogenesis contribute to fertility disorders [4]. Identification and functional characterization of proteins involved in spermatogenesis is an active area of investigation [5,6]. Some of the genes that have been recently characterized for their role in male gametogenesis are *Spats1, Rail4, Tex13a, Paz domain, Tcfl5*, etc [7–11]. In this study, we investigated the role of three rat genes, namely, serine protease inhibitor Kazal-type 2 (*Spink2*), sperm acrosomal membrane-associated protein 7 (*Spaca7*) and phosducin-like 2 (*Pdcl2*), that are predominantly expressed in the testis.

The SPINK protein family members contain a Kazal domain at the C-terminus of the serine protease inhibitor and have been implicated in a variety of biological processes, including male fertility. Kazal domain is an evolutionary protein domain predominantly present in serine proteases. They occur in tandem arrays which contain small alpha and beta fold and three disulfide bonds. *Spink13*, an epididymis specific gene was found to be essential for acrosomal integrity and male fertility [12]. Knockout of the *Spink2*, resulted in impaired spermatogenesis and fertility in mice [13]. SPINK3, localized on the acrosome, modulates sperm activity [14]. A 4 fold decrease in *Spink2* expression was reported in azoospermic patients [15]. SPINK8, SPINK11 and SPINK12 are found to be epididymis specific and their roles in male fertility reported [16].

Members of the SPACA family contribute to the molecular aspects of male fertility. SPACA7, localized on the acrosome, a novel male germ cell specific protein is implicated in mouse fertilization [17]. In humans and murine models, loss of *Spaca1* gene resulted in autosomal recessive globozoospermia [18,19]. Regulation of sperm-egg membrane fusion machinery requires the cleavage of SPACA1 [20]. An association

Abbreviations: SPINK2, serine protease inhibitor Kazal-type 2; SPACA7, sperm acrosomal membrane-associated protein 7; PDCL2, phosducin-like 2; SOPMA, Self-Optimized Prediction Method with Alignment; NCBI, National Center for Biological Information; MRE, mean residue ellipticity; ELISA, enzyme linked immunosorbant assay.

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