Functional role of the Sperm Associated Antigen 11a (*Spag11a*) gene in epididymal oncogenesis and fertility: studies using transgenic models

A thesis submitted to University of Hyderabad for

the award of Ph.D. degree in Animal Biology

By

Sangeeta

Enrolment No: 14LAPH09

Department of Animal Biology

School of Life Sciences University

of Hyderabad Hyderabad-500 046,

India

July 2021

University of Hyderabad

(A Central University established in 1974 by an Act of Parliament)

Department of Animal Biology School of Life Sciences Hyderabad-500 046, INDIA

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This is to certify that this thesis entitled **"Functional role of the Sperm Associated Antigen 11a (*Spag11a*) gene in epididymal oncogenesis and fertility: studies using transgenic models"** is a record of bonafide work done by Miss. Sangeeta, a research scholar for Ph.D. programme in the Department of Animal Biology, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

22/7/21.

Dr. Suresh Yenugu

Supervisor

Dr. SURESH YENUGU
Associate Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, P.O. Central University
Hyderabad-500 046, T.S., India.

K. Sreenivasulu
22/7/2021

Head

Department of Animal Biology

अध्यक्ष / Head
जंतु जैविकी विभाग
Department of Animal Biology
जीव विज्ञान संकाय/School of Life Sciences
हैदराबाद विश्वविद्यालय/University of Hyderabad
हैदराबाद-५०० ०४६ / Hyderabad-500 046

Dean

School of Life Sciences

DEAN
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046

Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, Hyderabad - 500046, Telangana, India

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1. **Sangeeta K**, Yenugu S. Transgenesis and active immunization mediated reduction of sperm associated antigen 11A mRNA and protein levels affect fecundity in the rat. ***Theriogenology***. 2020 Nov; 157:517-524. doi: 10.1016/j.theriogenology.2020.08.027.
2. **Sangeeta K**, Yenugu S. siRNA-mediated knockdown of sperm-associated antigen 11a (Spag11a) mRNA in epididymal primary epithelial cells affects proliferation: a transcriptome analyses. ***Cell Tissue Research***. 2020 Mar; 379(3):601-612. doi: 10.1007/s00441-019-03107-6.
3. **Sangeeta K**, Yenugu S. Characterization of isolated rat caput epididymal primary epithelial cells: A molecular biology approach. ***Theriogenology***. 2019 Sep 1; 135:13-18. doi: 10.1016/j.theriogenology.2019.05.025.

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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

Dr. Suresh Yenugu

Supervisor

Dr. SURESH YENUGU
Associate Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, P.O. Central University
Hyderabad-500 046, T.S., India.

Head

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अध्यक्ष / HEAD
जंतु जैविकी विभाग
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Hyderabad - 500 046.

University of Hyderabad

(A Central University established in 1974 by an Act of Parliament)

Department of Animal Biology
School of Life Sciences Hyderabad-
500 046, INDIA

DECLARATION

I, Sangeeta, hereby declare that this thesis entitled "Functional role of the Sperm Associated Antigen 11a (*Spag11a*) gene in epididymal oncogenesis and fertility: studies using transgenic models" submitted by me under the guidance and supervision of Dr. Suresh Yenugu is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree ordiploma.

Name : **Sangeeta**

Regd. No : **14LAPH09**

Signature : Sangeeta

Date : 22-July-2021

Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, Hyderabad - 500046, Telangana, India

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Dr. SURESH YENUGU
Associate Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, P.O. Central University
Hyderabad-500 046, T.S., India.

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CONTENTS

Abbreviations

AR	Acrosome reacted
BSA	Bovine serum albumin
cDNA	complementary DNA
CRISP	Cysteine rich secretory protein
DAPI	4,6-diamidino-2-phenylindole
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
FITC	Fluorescein isothiocyanate
Fluo-3, AM	1- [Amino-5- (2,7-dichloro-6-acetomethoxy-3-oxo-3H-xanthen-9-yl) phenoxy]-2-(2'-amino-5'-methylphenoxy) ethane-N, N, N', N'-tetra acetic 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
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
SPAGE11A	Sperm associated antigen 11a
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline with tween
PNA-FITC	FITC conjugated Lectin from Arachis hypogaea
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcription-Polymerase chain reaction
MEF	Mouse Embryonic Fibroblasts
MCF7	Michigan Cancer Foundation-7
EPEC	Epididymal primary epithelial cells
DMEM	Dulbecco's Modified Eagle Medium
EMEM	Eagle's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DEN	Diethyl nitrosamine
IMM	Immunized
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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ABSTRACT

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ABSTRACT OF THE STUDY

Background

Epididymis is the site of sperm maturation during which the male gametes acquire motility and fertilizing ability. The lumen of this organ is lined by epithelial cells, which form the predominant cell type. A variety of proteins are secreted by the epithelial cells in a region specific manner and have been implicated in sperm function and innate immunity and as markers of many types of cancers. The rat SPAG11A, an epididymis specific protein was implicated in sperm maturation and is antimicrobial in nature because of its structural similarity with β-defensins. Recent evidence indicates that β-defensins play a role in tumor growth, monitoring and treatment. Further, human β-defensins are implicated in cervical, thyroid, prostate, colon, oral and lung cancer carcinogenesis. However, the role of defensin-like proteins belonging to the SPAG11 family in cell proliferation and their possible contribution to cancer is not yet reported. On the other hand, proteins encoded by *Spag11* genes are found to be localized on the sperm, thus implicating them in sperm function as well. Studies that implicate a direct role for SPAG11A in sperm function and fecundity are not yet understood.

Methodology

Primary epithelial cells of the caput (PEC) were isolated from rats as described earlier with slight modifications. The effect of depleting *Spag11a* mRNA on global gene expression pattern was studied by treating EPECs with siRNA. The effect of overexpressing *Spag11a* in RCE cells was analyzed by transfecting a *Spag11a* expression construct. Gene array analyses was carried out using Affymetrix GeneChip Scanner and GeneTitan Platform.

Immunization to generate auto antibodies against SPAG11A was carried out in adult (aged 90 days) male Wistar rats and the antibody titer in serum and tissue fluids was determined by ELISA. The susceptibility of epididymis to tumorigenesis was determined by administering SPAG11A immunized rats with 0.01% diethynitrosamine (DEN) for 12 weeks.

Transgenic rats that express shRNA against *Spag11a* mRNA were generated using testicular electroporation method. S*pag11a*$^{-/-}$ mice (**C57BL/6NJNcbs-*Spag11a*^{em2MGEF}**) were commercially obtained from Mouse Genome Engineering Facility, National Centre for Biological Sciences, Bangalore, India.

Histopathological analyses on H and E stained tissue sections, gene array analyses using Affymetrix GeneChip Scanner and GeneTitan Platform, fecundity by natural mating, capacitation and acrosome reaction by flow cytometry, gene expression by real-time PCR and protein expression by immunoblotting were carried out in samples obtained from in vitro and in vivo studies.

Results

Objective 1: Evaluate the role of *Spag11a* gene in epididymal epithelial cell proliferation

The methodology to isolate epididymal primary epithelial cells (EPECs) was standardized. isolated cells were adherent and could be cultured for 14 days after isolation. Abundant E-cadherin expression confirming their epithelial nature and CRISP1 expression confirming their epididymis specific nature were the hallmarks. Such a molecular characterization for EPECs was not reported earlier.

The relative percent proliferation increased in EPECs transfected with siRNA against *Spag11a* (siSPAG11A) and not with scrambled siRNA (SCR). 4182 genes were differentially expressed between the SCR vs siSPAG11A groups of which 1354 and 1075were up and down regulated respectively. Transfection of EPECs with siSPAG11A also caused differential expression pattern of genes involved in epithelial cell proliferation. In immortalized rat epididymal epithelial (RCE) cells, overexpression of *Spag11a* resulted in a significant decrease in proliferation.

Objective 2: Determine the effect of SPAG11A protein ablation on chemically induced oncogenesis in the epididymis

In the caput obtained from SPAG11A immunized or DEN only treated rats, normal tubular morphology and normal concentration of spermatozoa was observed. However, in the caput of SPAG11A immunized + DEN treated rats, foci of fibrosis and infiltration of inflammatory cells in between the tubules of epididymis along with mild mucosal degeneration of mucosal epithelial cells was evident. A high throughput transcriptome analyses revealed that among the 25866 transcripts analysed, 2760 and 3460 were found to be up and down regulated in the caput of SPAG11A immunized rats. Consequent to DEN treatment, the number of up and down regulated transcripts were 3241 and 2573 transcripts respectively. 3714 transcripts were differentially expressed in the caput of both SPAG11A immunized and DEN only treated rats. Among the 3714 transcripts, 3549 of them displayed similar pattern in differential expression pattern.

Ten signalling pathways (Hippo, Myc, Notch, Nrf2, PI-3-Kinase/Akt, RTK-RAS, TGFb, p53, cell cycle (Cdk mediated) and b-catenin/Wnt) that play a crucial role in oncogenesis were identified. Differential expression of the molecular components of ten signalling pathways in the caput of SPAG11A immunized only and DEN only treated groups was compared. In all the pathways, the differential expression pattern of the components was similar in both the groups.

Objective 3: Study the role of *Spag11a* in sperm function and fertility using transgenic and knock out animal models.

Transgenic rats were generated by testicular electroporation. Pups (F1 generation) born to electroporated rats had the integration of the transgene into the genome and the integration was also observed in F2, F3 and F4 generations. A significant decrease of *Spag11a* mRNA levels was observed in the *Spag11a*-shRNA transgenic rats. In the SPAG11A immunized rats, the antibody titer in the serum, testis and epididymis of was significantly increased and was maintained till the end of the study.

A significant decrease in the litter size was observed in F0, F1, F2 and F3 generation transgenic rats Litter size in SPAG11A immunized rats was also significantly lesser than the control. Sperm count was marginally decreased in the *Spag11a*-shRNA transgenic rats it was significant in SPAG11a immunized rats. Spermatozoa obtained from SPAG11A immunized or *Spag11a*-shRNA transgenic rats were able to undergo capacitation and acrosome reaction.

The deletion of exon 1 of *Spag11a* gene in the knockout mice was confirmed by genotyping. In the knockout mice, litter size and sperm count were significantly reduced characterized by sperm with abnormal morphology. Sperm obtained from knockout mice had the ability to undergo capacitation and acrosome reaction to a certain extent. In the caput of knockout mice, severe mucosal epithelial hyperplasia moderate degenerative changes in mucosal epithelial cells of tubules were observed, whereas in the testes, severe inflammatory changes in seminiferous tubules with infiltration of giant cells was observed.

Conclusions

The isolation protocol and molecular characterization of EPECs described in this study will provide a basis to standardize the establishment of epididymal cell model systems. Si-RNA mediated ablation of *Spag11a* mRNA and in EPECs and overexpression of *Spag11a* in RCE cells affected proliferative capacity in opposite directions indicating that *Spag11a* may have a role in governing the proliferative dynamics in the epididymis.

In rats actively immunized against SPAG11A protein, DEN administration enhanced the pathophysiological parameters of the caput which pointed out to the onset of tumorigenesis. It appears that ablation of SPAG11A protein induces the susceptibility to tumorigenesis caused by a low dose carcinogen. Similarity in the differential expression of genes related to ten cancer pathways between these two groups provides concrete evidence that absence of SPAG11A protein creates a microenvironment that is favourable to tumorigenesis and thus it is possible that the anti-tumorigenic action of SPAG11A is a potential reason for the non-occurrence of cancer in the epididymis.

Besides the role in tumorigenesis, results of this study indicate the functional aspects of SPAG11A in sperm function and male reproductive physiology as well. Studies with immunized, transgenic and knockout animal models described in this study indicated compromised sperm function and fecundity. In depth analyses are further required to determine the molecular aspects of SPAG11A associated control on germ cell function.

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Introduction

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Male reproductive system

The male reproductive system consists of a pair of testes, epididymis, vas deferens, prostate, seminal vesicle, bulbourethral glands and the penile urethra (**Figure 1**).

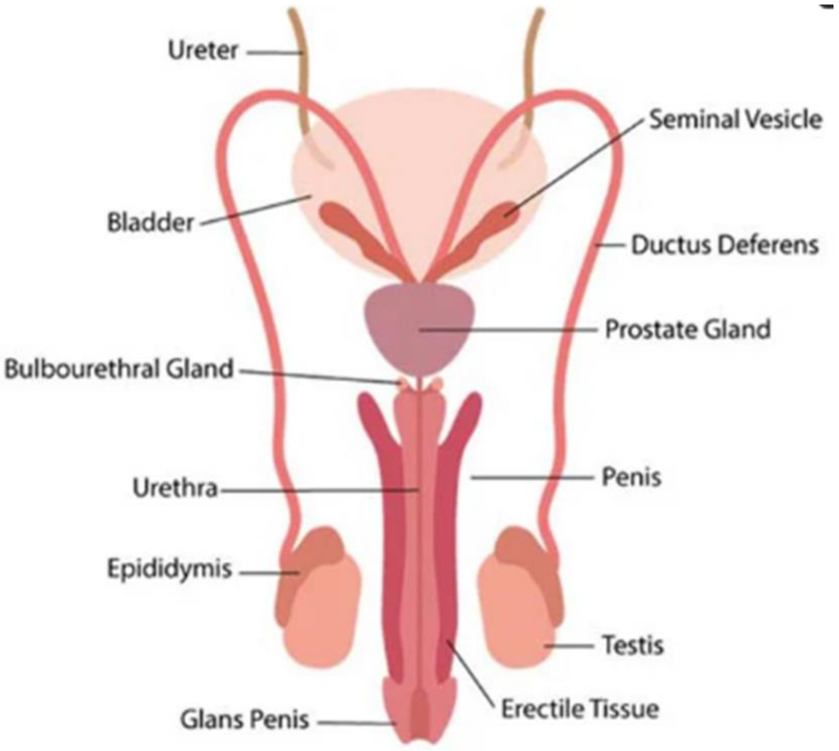

Figure 1*. Schematic representation of the male reproductive tract, (Adapted from L.* R. C. da Silva, 2018, *Pathogens)*

The two important functions of male reproductive system are production, maturation and protection of spermatozoa from infectious agents; and the production of hormones. Testis lie in the scrotum and produces the male sex hormone, testosterone, which has role in maintaining male secondary sexual characters and spermatogenesis (1). The spermatozoa produced in the testes are transported to the epididymis to undergo maturation. Male accessory glands namely prostate gland, seminal vesicles and bulbourethral glands secrete fluids that contribute to the major component of semen and also to provide milieu for the survival and protection spermatozoa.

Epididymis

The epididymis, one of the accessory organs, is a long and convoluted ductal structure of that is anatomically divided into three regions namely, caput (head), corpus (body) and cauda (tail) (2) (**Figure 2**). Spermatozoa produced in the testis are immotile and lack fertilizing capability (3). For attaining fertilization competency, they undergo epididymal maturation in the epididymis and capacitation/acrosome reaction in the female reproductive tract (4). Sperm maturation in the epididymis occurs during their epididymal transit that can range from 2 to 6 days. Each epididymal region is known to distinctly express a variety of maturation factors that are secreted into the lumen. During their transit, secretory proteins are added on to the surface of spermatozoa to facilitate maturation and acquisition of motility and fertilizing ability (5).

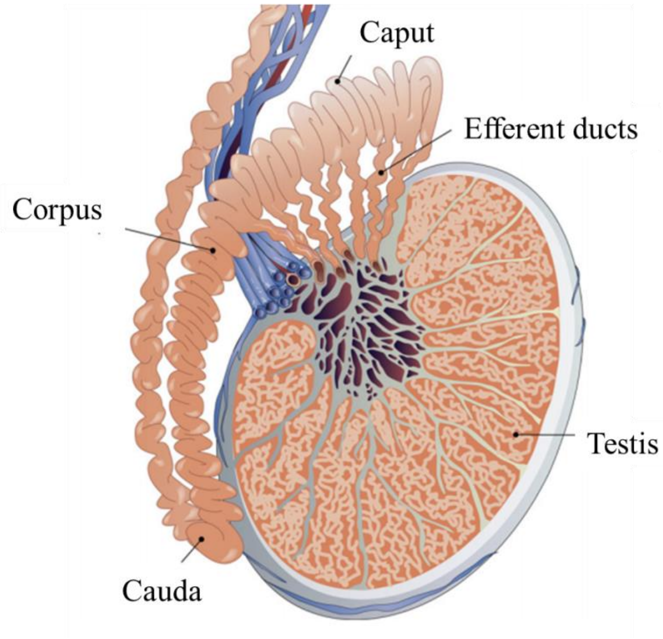

Figure 2. *Anatomical structure of epididymis. (Adapted from Encyclopaedia Britannica).*

Epididymal maturation also contributes the ability of spermatozoa to undergo capacitation in the female reproductive tract (6). Secretory activities in the epididymis are highly regulated in a region-dependent manner (7). There are several epididymis specific genes which are androgen regulated (8). Thus, sperm maturation in the epididymis is androgen dependent (9). While the sperm maturation primarily occurs in the caput and corpus, the functionally mature sperm are stored in the cauda region (10).

Epididymal cells and their function

The epididymis consists of six different cell types possessing distinct physiological functions. The principal cells, clear cells, basal cells and halo cells are present all along the organ, while narrow and apical cells are restricted to the proximal region (**Figure 3**) (11). Among the six different cell types in the epididymis, principal cells represent 80% of the total cells and play an essential role in the synthesis and secretion of epididymal proteins (**Figure 3**) (12). Epithelial cells provide a luminal microenvironment by forming a protective barrier and transport of ions, nutrients, solutes, proteins and water (13). The principal cells are responsible for the reabsorption of various components from the epididymal fluid (14). Clear cells are the second most abundant cell type and present in the caput and cauda. The clear cells play an essential role in endocytosis (14). Narrow cells and apical cells are present in the initial segment, but their function is not known (15, 16). Basal cells attached to the principal cells and a protective role in preserving the structural integrity of the lumen (17).

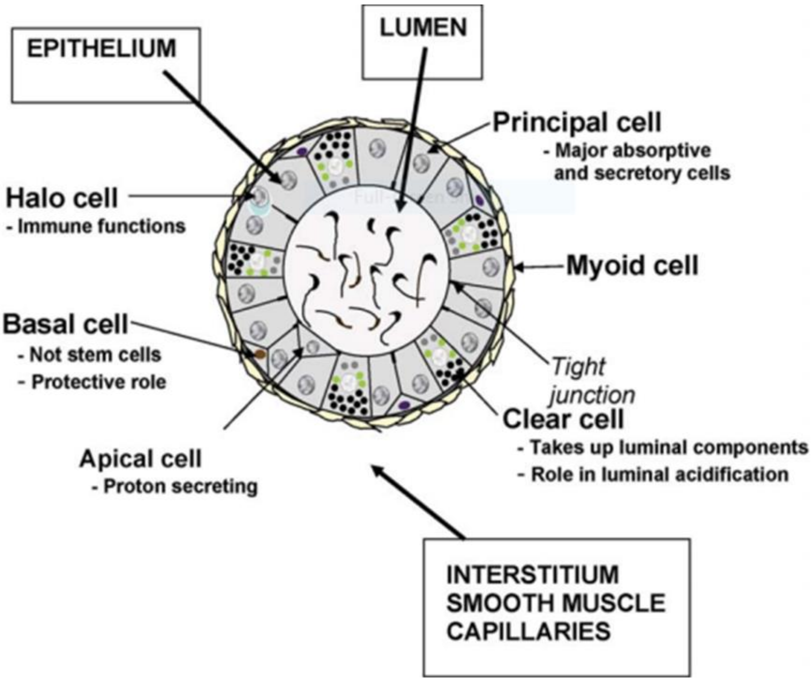

Figure 3. *Cellular organization in the epididymis, a cross sectional view (Adapted from: Robaire et al., 2003)*

Blood–epididymal barrier

Blood–epididymal barrier present in epididymal lumen is surrounded by different epididymal cell types that provide tight junctions at the apical pole of epididymis (18). It selectively excludes or concentrates ions and molecules between the epididymal luminal fluid and blood (19). The blood epididymal barrier participates in the confinement of autoantigenic spermatozoa in an immunoprivileged environment. Dendritic cells also surround the epididymal epithelium (20). Thus, blood epididymal barrier creates a microenvironment inside the epididymis for the protection of sperm. Damage to the epididymal-blood barrier can result in distorted luminal environment, which eventually affects sperm maturation and innate immunity thereby leading to poor sperm quality. The consequences of alterations in the epididymis-blood barrier are represented in **Figure 4**.

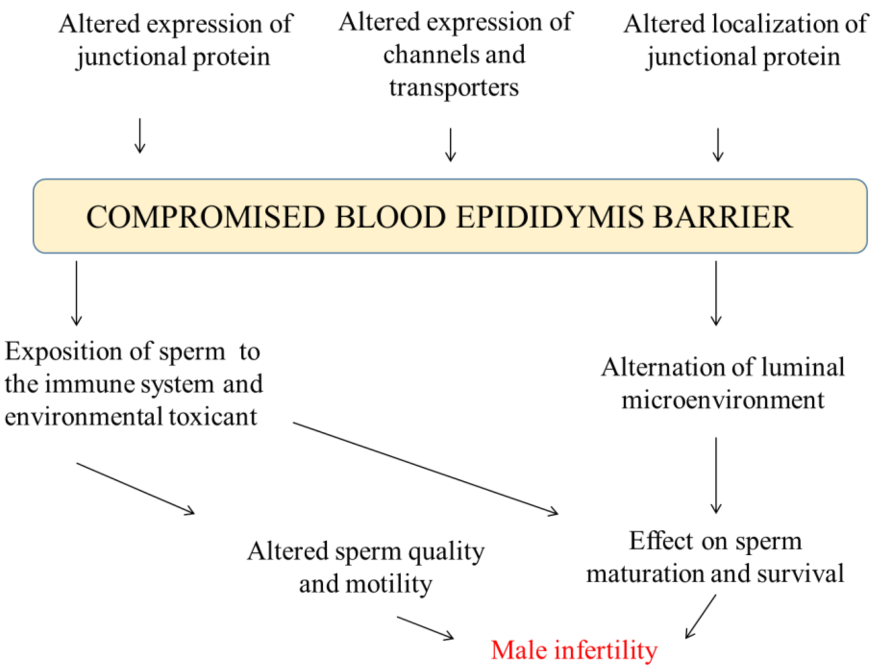

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Figure 4. *Consequences of damage to blood-epididymal barrier (Adapted from Cyr et al., 2010)*

Epididymal proteome

The epididymal lumen consists of different kinds of proteins, most of which are secretory in nature and are added on to the surface of spermatozoa to facilitate sperm maturation and innate immunity (21). Epididymal protein expression varies throughout the duct (22, 23). The caput region of epididymis exhibits the highest percentage of region-specific genes (24-26). A variety of them belong to different families (CRISP, defensin, SPAG11, Cathelicidin, Serine protease, Protease inhibitor) have been identified and characterized (5, 27, 28) (**Figure 5**). They contribute to diverse functions such as sperm maturation, stability of the sperm in female reproductive tract, innate immunity and also as markers for various cancers. For example, the human epididymis protein 4 (HE4) is known to be a marker for many types of cancer (29, 30). Further, epididymal proteins have a role in the protection and survival of sperm. Lactotransferrin is also known to have antioxidant, antibacterial and antiviral activities (31).

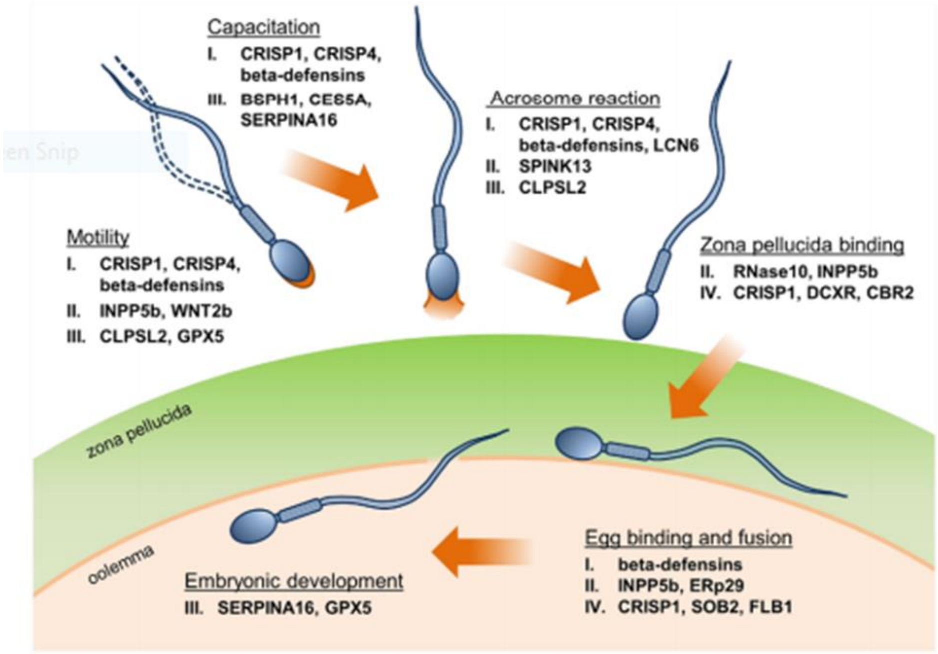

Figure 5. *Epididymal proteins affecting different sperm functions during fertilization (Adapted from Ida Björkgren et al., 2019)*

Defensins

Defensins are antimicrobial peptides that play an important role in innate immunity (32). In humans, three subtypes of defensins are identified, namely, α-defensins (human neutrophil peptides); β-defensins and θ-defensins depending on their secondary structure and disulphide bond pairing (32-34). β-defensins are small antimicrobial peptides that are conserved among species and mainly expressed in the testis and the epididymis (35, 36). There are 39 human, 52 mouse and 43 rat β-defensin genes identified (35). β-defensins exhibit antibacterial, antifungal and antiviral activities against a wide variety of microorganisms. Their mechanism of action involves permeabilization of cell membranes and interference with the basic metabolic processes (34). This protective role is suggested by *in vivo* and *in vitro* of their antimicrobial action. (37-41).

β-defensins not only have antimicrobial and host defence activities, they are also involved in sperm maturation, motility, capacitation and male fertility (42-45). β-defensins are expressed in the male reproductive tract (35, 46-48) and contribute to sperm maturation and capacitation (42, 49, 50). The localization and expression of *β-defensin* (*Defb*) genes in different species is studied (51, 52). The human *Defb* genes are clustered on chromosomes 8 and 20 (53) (**Figure. 5**). Similar to humans, *Defb* genes have been identified in rodents (54)

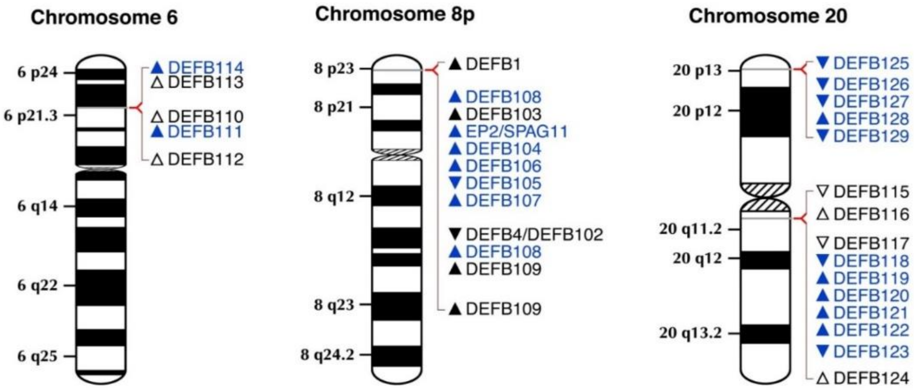

***Figure 6**. Major human β-defensin gene clusters. Gene names in black indicate widespread expression. Gene names in blue indicate expression predominantly in the male reproductive tract. Triangles point in the direction of transcription. The filled triangles indicate active genes, whereas no transcripts are known for the open triangle genes (Adapted from: Hall, Yenugu et al. 2007)*

Sperm Associated Antigen 11a (*Spag11a*) gene

The *SPAG11* gene (also known as *human epididymal protein 2 (HE2)* and *epididymal protein 2* (*EP2*)) in humans is located on chromosome 8p23 (55) (**Figure 6**). The fusion of two ancestral *Defb* genes resulted in the emergence of *SPAG11A* gene that contains two promoters. Presence of two promoters allows alternate splicing leads to translation of more than 20 protein isoforms (56). In the rat, three independent *Spag11* genes viz. *Spag11c, Spag11t*, and *Spag11a* (also referred to as *Spag11e* or *Bin1b*) are located on chromosome 16 and functionally characterized (57) (**Figure 7A**). The rat SPAG11A protein plays an important role in sperm maturation and innate immunity against microbes (42, 58). The proteins encoded by *Spag11c* and *Spag11a* genes contain a six-cysteine motif signature characteristic to β-defensins (**Figure 7B**) (53) and exhibit potent antimicrobial activity. Their mechanism of action is by membrane permeabilization and inhibition of macromolecular synthesis (40), which is similar to that exhibited by β-defensins.

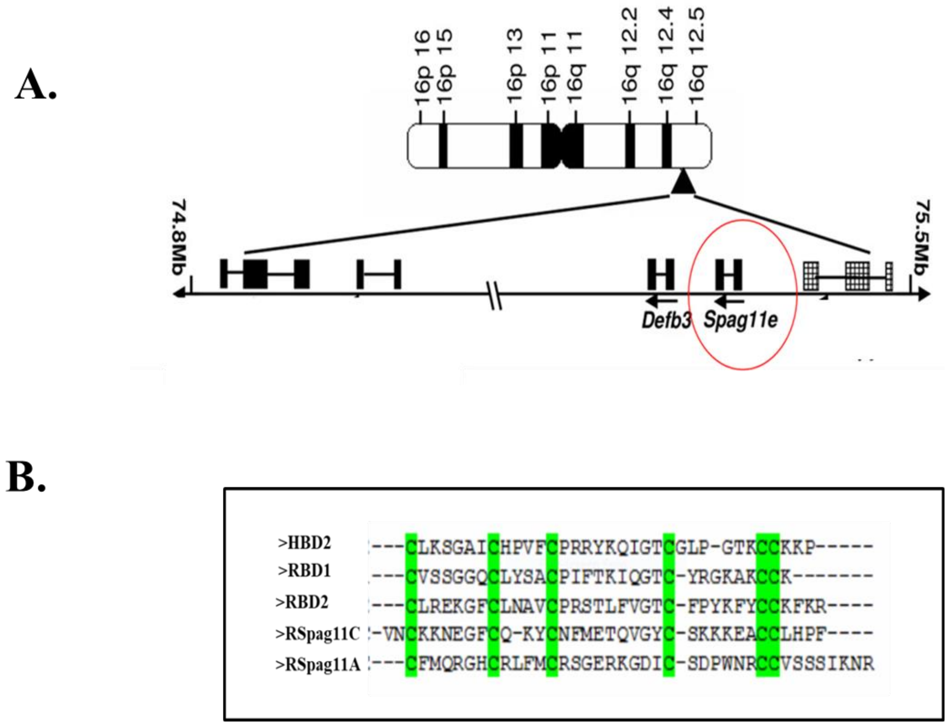

Figure 7. (A)*. Localization of rat Spag11 genes on chromosome 16.* ***(B)****. Alignment of human β-defensin signature motifs with similar arrays in rat β defensins and Spag11. The defensin and defensin like 6-cysteine motifs are highlighted in green (Adapted from: Yenugu, Hamil et al. 2006).*

Incidence of cancer in men

Cancer is the most common cause of the death in worldwide. Despite advances in the detection, diagnosis and treatment, this disease continues to remain as a serious threat. It is the second leading cause of mortality across the globe (59). Cancer can occur at any stage of life and males are more prone to cancer as compared to females (60). The higher risk of cancer observed in men could be due to mutations in the sex-determining Y chromosome, which leads to the loss of function of many male specific genes (61). The most common types of cancers in men are of prostate, lung, colorectal, melanoma and testicular (62). According to Globocan cancer statistics (2020), lung cancer is the most common cancer in men worldwide contributing to 14.3% of the total number of new cases diagnosed in 2020. The top three cancers namely lung, prostate and colorectal cancer contribute to 24.7 % of all cancer types while testicular cancers constitute 0.8% of total cancer cases (**Figure 8**).

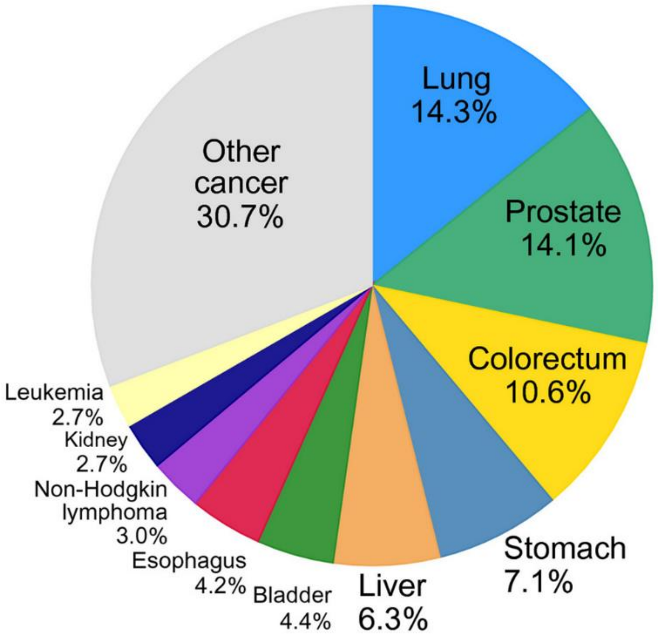

Figure 8. *Globocan cancer statistics in men for the year 2020 (Adapted from: https://www.uicc.org/).*

Epididymal cancer

Though majority of the organs in men are prone to cancer, the incidence of cancer in the epididymis is very rare (63). Testicular cancer is common in young men (64) and the incidence of prostate cancers are related to old aged men (65-68). As per available statistics, epididymal cancer incidence rate is only up to 0.03% of all male cancers; however, there are no reports of epididymal malignancy (63). The kidney and epididymis have same embryonic origin, but the kidney is 100 times more prone to develop cancer than epididymis (63). This makes the physiology of epididymis much more interesting.

Basing on the pathological parameters, epididymal tumours are classified as adenomatoid and cystamatoid type (68-70). The most frequent is the adenomatoid tumor (71). These are rare benign mesothelial neoplasia which occurs in the tail of epididymis (72). They account for only 0.03%, but mostly reported were benign. The most studied human epididymal cancer is cystadenoma, a benign cystic tumour of the gland (73). Metastasis and invasion of cancer cells to initiate secondary tumours in the epididymis are not reported. There is a possibility that the human epididymis lacks tumorigenic stimuli or has intrinsic inhibitory mechanisms against neoplasia that prevents the incidence of cancer (67, 74, 75). Limited information is available regarding the aspects of rare incidence of an epididymal tumors; and still the rarity of cancer is an enigma. It is hypothesised that certain factors in the epididymis might play important roles in the prevention of cancer in the epididymis (**Figure 9**).

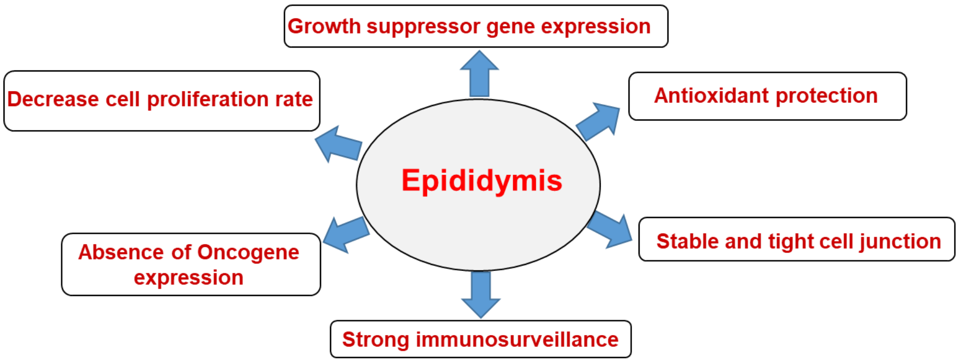

Figure 9. *The unique properties of epididymis. Factors that are expressed in the epididymis which may contribute to the rare occurrence of cancer in this organ system are proposed (Adapted from: Yeung et al; 2012)*

The inherent micro environmental factors expressed in the epididymis are proposed to contribute to lack of stemness character in the epithelial cells, robust anti-oxidative mechanisms, active tumour suppression and inactivation of oncogene products (63). Epididymis has strong immunosurveillance that restricts hyperplastic cells to dormancy. The presence of persistent tight junctions, anti-angiogenic factors, misplaced pro-angiogenic factors and inoperative immune escape also contribute to the rareness of cancer in this organ system (63, 76) (**Figure 8**). Further, the epididymal cells are less responsive to tumorigenic stimuli, thereby raising the threshold for tumor initiation above that of other organs (63). Moreover, epididymis has an anti-oxidative and immune-suppressive environment which is necessary to maintain high numbers of spermatozoa quiescent and immunologically silent. The low incidence of cancer may be a direct consequence of its role in sperm maturation and storage (77).

β-defensins and their role in cancer

In the recent years, the role of β-defensins in cancers is gaining importance (78). Interestingly, human β-defensins were identified to be proto-oncogenes and implicated in disease progression in oral cancer cell lines and tumour samples (79-81). Human β-defensin-1 (hBD-1), also located on short arm of chromosome 8 in the defensin cluster is a tumour suppressor gene (82, 83). The loss of *hBD-1* expression was demonstrated in renal cell carcinomas and prostate cancers (84). However, hBD-2 has been reported to be upregulated in several cancers, including oesophageal (85), lung (86, 87) cervical (88) and skin cancers (89), but downregulated in colon cancer (90, 91). Additionally, hBD3 expression was found to be increased in cancer cell lines compared to normal cells (92). The mechanism that involves killing of tumor cells by defensins is by membrane lysis and DNA damage (34, 93-96). The human epididymal protein 4 (HE4) is identified as a potential biomarker for many types of cancers (97-100), renal fibrosis (101) and chronic kidney diseases (102). HE4 protein modulates gene expression, proliferation, invasion, and metastasis of cancer cells (103, 104). These evidences suggested that β-defensins play a crucial role in tumorigenesis and cell proliferation.

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Chapter 1

Evaluate the role of *Spag11a* gene in epididymal cell proliferation

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1.1. INTRODUCTION

Epididymis is the site of sperm maturation during which the male gametes acquire motility and fertilizing ability. The lumen of this organ is lined by epithelial cells, which form the predominant cell type (1). A variety of proteins are secreted by the epithelial cells in a region specific manner (2) and have been implicated in sperm function and innate immunity (3-6). Early studies probing into the role of proteins in the epididymal lumen milieu involved micropuncture and cannulation, which are difficult to perform (7). To understand the molecular mechanisms of the role of epididymal proteins in sperm function, *in vitro* model systems have been developed. These include maintaining epididymal tissue explants (8,9), culturing of primary epididymal epithelial cells (10-12), generation of immortalized epididymal epithelial cells (13), isolation of epididymal epithelial cells from transgenic mice that express SV40 T antigen (14) and isolation of epididymal basal cells (15) and principal cells (16). However, each of the model system has its own advantages and disadvantages. Culturing of epididymal explants is feasible only for experiments with very shorter duration, whereas use of primary epididymal epithelial cells has limitations of slow growth and replication and limited life span. On the other hand, use of immortalized cell lines have been a matter of concern since the SV40 immortalization may cause endogenous changes at the molecular level and thus may not be similar to the epithelial cells *in vivo*. Considering the pros and cons of the different model systems available to study epididymal function, use of isolated primary cultures of epithelial cells appears to be dependable, since they closely reflect the *in vivo* epididymal epithelial cells. Thus, isolation and culturing of primary epididymal epithelial choice still remains the choice for investigators working in this area or research.

Isolation of primary epididymal epithelial cells and their culturing conditions is reported. The first isolation and culturing of rat epididymal epithelial cells was demonstrated by Kierszenbaum *et. al.,* (10). Further characterization of these cells by electron microscopy was reported (11,17). Isolation and culture of epididymal epithelial cells was successfully conducted in many species (9) (18-21). Though the structural and morphological characterization of the isolated rat epididymal primary epithelial cells (EPEC) was extensively carried out, a systematic molecular characterization and analyses of the purity of the cells is not yet reported. Hence in this study, we characterized the purity and molecular characterization of isolated rat EPECs using molecular techniques.

The luminal milieu established by epithelial layer of epididymis is very important for maturation, storage and functional aspects of sperm. Region specific gene and protein expression profile in epididymal epithelial cells contributes to sperm maturation (22,23). This is evident by the proteomic changes that occur on the sperm during the process of maturation (24). Further, diverse expression of miRNAs to regulate protein expression in epididymis is reported (25-27). In the last decade, the essentiality of epididymal proteins for fertility was reported using knock out animal models that lacked the expression of one or more of the proteins (28-32). Apart from their role in reproductive physiology (sperm function and fertility), epididymal proteins also contribute to entirely different physiological functions. The human epididymal protein 4 (HE4) is projected as a potential biomarker for many types of cancers (33-36), renal fibrosis (37) and chronic kidney diseases (38). HE4 protein modulates gene expression, proliferation, invasion and metastasis of cancer cells (39,40).

The *SPAG11* gene (also known as human epididymal protein 2 (HE2) and epididymal protein 2 (EP2) in humans is located on chromosome 8p23 (41). The transcriptional unit of *SPAG11* gene is a result of fusion of two ancestral β-defensin genes (42). Due to alternate splicing, about 20 protein isoforms are coded by this gene (42). In the rat, three independent *Spag11* genes viz *Spag11c*, *Spag11t* and *Spag11a* (also referred to as *Spag11e* or *Bin1b*) are characterized (43). The rat SPAG11A protein was implicated to be important in sperm maturation (44). The proteins encoded by *Spag11c* and *Spag11a* genes contain a six cysteine motif characteristic to β-defensins (43) and exhibit potent antimicrobial activity. Their mechanism of action is by membrane permeabilization and inhibition of macromolecular synthesis (45) , which is similar to that exhibited by β-defensins. Recent evidence indicates that β-defensins play a role in tumor growth, monitoring and treatment (46). Further, human β-defensins are implicated in cervical, thyroid, prostate, colon, oral and lung cancer carcinogenesis (47-52). Human β-defensin 3 exhibits anticancer properties (53). However, the role of defensin-like proteins belonging to the SPAG11 family in cell proliferation and their possible contribution to cancer is not yet reported.

Epididymis being a specialized organ for sperm maturation also exhibits another unique feature i.e., its ability to resist tumor formation. Several theories are proposed to explain the rarity of tumors in the epididymis (54). The non-responsiveness to tumor inducing signals may be due to constitutive expression of factors generally inducible in tumors and mechanisms that make the spermatozoa quiescent and immunologically inactive. However, the molecular mechanism that contributes to the low incidence of cancer in the epididymis is still not clear. Cell proliferation is one of the key regulatory mechanisms for the predisposition of cancer. During 1-14 days of postnatal development, the epididymal cells largely remain undifferentiated. The differentiation process that starts from day 15 is accompanied by decreased proliferation and by day 49 all the epididymal epithelial cells cease to proliferate (55). Epididymal cell proliferation is regulated by p-MAPK1/3 and DUSP6 (56) and microRNA (57). Experimental approaches wherein manipulation of expression of a specific gene to provide evidence on the factors that contribute to the proliferation of epididymal epithelial cells is gaining interest in the recent years. Depletion of hepatocyte nuclear factor 1 (*HNF1*) in human epididymal epithelial cells affected cell proliferation and migration (58). Similar observations were reported in motif for paired box 2 (*PAX2*) depleted immortalized human epididymal epithelial cells (59). Androgen receptor (*AR*) influenced transcriptional network that governs various cellular process in human epididymal epithelial cells is reported (60). Thus, these experimental strategies have proven to be very useful to elucidate the role of a particular factor in epididymal epithelial cell function.

In light of ample evidence that β-defensins play a crucial role in tumorigenesis, proteins that are β-defensin-like may also play a similar role. Rat SPAG11A exhibits structural and functional properties characteristic of β-defensins. Thus, SPAG11A may govern cell proliferation dynamics and contribute to the rare occurrence of cancer in the epididymis. In this study, we depleted the mRNA levels of *Spag11a* using siRNA to gain insight into the possible effects on rat epididymal epithelial cell transcriptional network and its relation to proliferation.

1.2. MATERIALS AND METHODS

1.2.1 Cell lines

Human breast cancer cell lines MCF-7, MDAMB231 and mouse embryonic fibroblast (MEF) cell lines obtained from National Centre for Cell Science, India, were cultured in DMEM (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) and maintained at 37ºC in the presence of 5% CO_2. Rat immortalized proximal caput cell line (RCE) was kind gift from Dr. Daniel Cyr, Department of Anatomy and Cell Biology McGill University, Montreal, Quebec, Canada. They were cultured in DMEM (Invitrogen) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 10 µg/ml insulin, 10 µg/ml transferrin, 80 ng/ml hydrocortisone, 1 µg/ml retinol, 10 ng/ml epidermal growth factor, 10 ng/ml cAMP, and 10% fetal bovine serum (FBS)] and maintained at 32ºC in the presence of 5% CO_2.

1.2.2 Isolation of caput epididymal primary cells

Primary epithelial cells of the caput (PEC) were isolated from rats as described earlier with slight modifications (10-12). Caput epididymides were collected and placed in sterile HBSS medium supplemented with antibiotics. Excess fat was removed and cut into small pieces. The tissue was then placed in 10 ml of 0.1% collagenase solution (1 ml of 1% collagenase and 9 ml of HBSS medium supplemented with antibiotics and incubated in a shaking water bath at 37 ºC and 80 rpm for 1.5 hr. The digested tissue was then centrifuged at 50 g for 5 min. Supernatant was discarded and the pellet resuspended in fresh 0.1% collagenase followed by incubation at 37 ºC and 70 rpm for 1.5 hr. The digested tissue is then centrifuged at 800 g for 2 min and the supernatant discarded, leaving behind 1 ml. The sample was then gently retro pipetted to obtain a homogenous suspension. The cells were then placed in a 100 mm cell culture dish containing 1% collagenase and 1% FBS in EMEM and incubated at 32 ºC for 5 - 8 hr. The supernatant was taken out and equally distributed in to two 60 mm culture dishes and supplemented with 5% FBS and 5 ng/ml EGF) and incubated for 48-72 h at 37 ºC in a 5% CO_2 incubator. Culture medium was replaced with fresh EMEM containing 10% FBS and the isolated cells allowed to attach. Culture medium was replaced every alternate day and the growth of the cells were monitored for confluence. The pictorial representation of the protocol is given in **figure 1**. All procedures involving animals were conducted using the guidelines for the care and use of laboratory animals to minimize suffering and this study was specifically approved by the Institutional Animal Ethics Committee of University of Hyderabad (IAEC/UH/2017/01/SY/P13).

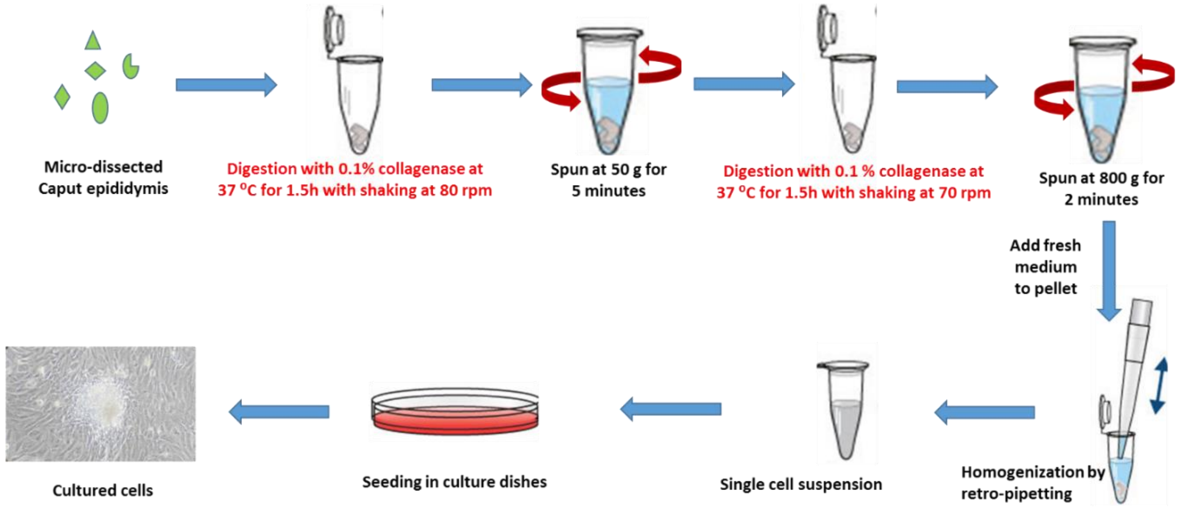

Figure 1. *Schematic representation of the procedure to isolate EPECs.*

1.2.3 Immunofluorescence

Antibodies to detect rat E-cadherin and CRISP1 were obtained commercially (Catalog Nos. sc-7870 and sc-21281 respectively; Santa Cruz Biotechnology, Dallas, USA). Isolated cells were fixed on glass slides and then treated with 1% Triton- X 100 to facilitate permeabilization. E-Cadherin and CRISP1 was detected by incubating the slides using FITC tagged polyclonal antibodies (1:200 dilution) For the control staining, antibodies were pre-incubated with antigen peptide. Photographs were taken using a fluorescence digital imaging system attached to a Leica Photomicroscope.

1.2.4 Western Blotting

Cells were lysed using RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS) supplemented with proteinase cocktail inhibitor. Rat caput and testes tissues were homogenated in RIPA buffer and centrifuged at 10,000 rpm for 10 min. The concentration of protein in lysates was determined by the Bradford’s method. 100 µg protein of each sample was resolved on SDS-polyacrylamide gel and protein was electrophoretically transferred onto a 0.2 µm pore size nitrocellulose membrane (Millipore). The nonspecific binding sites were blocked by incubating the membranes in 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween (TBS-T) for 1 h at room temperature. The membranes were probed with primary antibodies specific to rat E-Cadherin (Catalog Nos. sc-7870; Santa Cruz Biotechnology), β-Actin (Catalog No. sc-

1616; Santa Cruz Biotechnology), Vimentin (Catalog No. sc-6260; Santa Cruz Biotechnology) and CRISP-1 (Catalog Nos. sc-21281; Santa Cruz Biotechnology) in blocking solution overnight at 4 °C and then rinsed three times with TBS-T. Membranes were then incubated with anti-mouse or anti-rabbit secondary antibody conjugated with HRP for 1 h at room temperature and then rinsed three times with TBS-T for 10 min. Immunoreactive proteins were detected using an ECL prime Western blot detection kit (GE Healthcare Life Sciences, MA, USA), according to the manufacturer's instructions.

1.2.5 Flow cytometry

To determine the purity of the isolated epididymal epithelial cells, flow cytometry was employed. Cells were incubated with FITC labelled E-cadherin antibody and subjected to analyses. Fluorescence intensity was measured in a flow cytometer with excitation and emission at 495 and 515 nm respectively. The fluorescence intensity data and the forward scatter (FSC) were collected in logarithmic mode and the light scattering in the side scatter (SSC) was collected in linear mode. The fluorescence intensity was quantified for 10,000 individual cells in a flow cytometer ((BD Biosciences, San Jose, USA) and expressed as % of positive cells. Since the mean fluorescence intensity varies largely between individual experiments and makes it difficult to calculate the mean and standard deviation, data presented is a representative of multiple experiments.

1.2.6 Reverse transcription and polymerase chain reaction

Total RNA isolated from different tissues using commercially available kits (Qiagen) was subjected to DNase digestion to remove any contaminated DNA. 2 µg of total RNA was reverse transcribed and the expression of *Urp1a, Pate-F, Crisp1, Ar, Spag11a, Urp1b, Pate* and *Gapdh* in the isolated cells was carried out using exon spanning gene specific primers (**Table 1**) in a thermal cycler (cycling 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). No reverse transcriptase and no template controls were included to confirm that the amplification is non-specific. The PCR amplicons were electrophoresed on 2% agarose gels and the images captured using a gel documentation system. The identity of the PCR amplicons was confirmed by sequencing though a commercial source. For quantification of gene expression, real time PCR was carried out using SYBR master mix kit (Applied Biosystems, Warrington, UK) in a thermal cycler (Applied Biosystems) using standard conditions.

1.2.7. siRNA design, vector construction and transfection

The effect of depleting *Spag11a* mRNA on global gene expression pattern was studied by treating EPECs with siRNA (Catalog # 4390771; Ambion, ThermoFisher Scientific) that targets the two exons in this gene. Rat EPECs were transfected with 60 picomoles of siRNA mixed with lipofectamine RNAimax (Invitrogen) and incubated up to 24 hours. EPECs were also treated with an equal amount of nonspecific siRNA (scrambled; SCR, Catalog # 4390843; Ambion, ThermoFisher Scientific) to serve as negative control. Further, EPECs without any treatment (Untreated control; CON) and lipofectamine only treated (LIPO) were also included in the experimental setup. Following incubation, RNA was isolated and the changes in the transcriptome was analyzed by gene array analyses. The effect of siRNA on the mRNA and protein levels of *Spag11a* was analyzed by real time PCR and Western blotting.

The effect of overexpressing *Spag11a* in RCE cells was analyzed by transfecting a *Spag11a* expression construct generated by sub-cloning PCR-amplified full length cDNA into pcDNA vector using *BamHI* and *HindIII* restriction sites. 50 µg of the plasmid was transfected into 1 x 10^6 RCE cells and incubated up to 24 hours. Another set of cells were also transfected with empty vector (without the insert) to serve as a control (pcDNA). Transfections were conducted using lipofectamine 2000 as per the manufacturer's protocol (Invitrogen). Over expression was assessed by measuring the mRNA transcript and protein levels by real time PCR and Western blotting (using anti-HA primary antibody and FITC labelled secondary antibody) respectively.

1.2.8. Gene chip hybridization, data collection and enrichment analysis.

The experimental set up included three treatment conditions namely: 1) EPECs without any treatment (CON); 2) EPECs treated with nonspecific (scrambled) siRNA (SCR) and 3) EPECs treated with siRNA that targets *Spag11a* mRNA (si*SPAG11A*). The treatments were conducted in triplicates. RNA isolated from these cells was quality checked and assessed for pass criterion (A260/A280 ≥1.8; A260/A230 ≥1 and RNA integrity number (RIN) ≥6). RNA that passed the stringent criteria were subjected to gene array analyses using Affymetrix GeneChip Scanner and GeneTitan Platform (Imperial Life Sciences, Gurgaon, India). The array contained 68842 genes and the differentially expressed genes (DEGs) identified with the criteria that log2 |fold-change| is ≥1 and is $p<0.05$. Background subtraction and normalization was performed using Transcriptome analysis console (TAC) software.

1.2.9. Cell proliferation assay

MTT assay was used to assess proliferation. In brief, 5000 cells / well were seeded into 96 well micro titer plates and kept in CO_2 incubator for 24 h to allow adherence. Following treatment and incubation with siRNA or transfection with *Spag11e* expression vector, the medium was replaced and MTT (5µg/ml) was added into each well. The micro titer plates were then incubated for 4 h. The medium was replaced with 100µl DMSO and absorbance was measured at 570 nm in automated plate reader (Bio-Rad Laboratories, Hercules, USA). The relative percent proliferation was calculated using the formula: absorbance (siRNA treated) – absorbance (blank) / absorbance (scrambled) – absorbance (blank) x 100.

1.2.10. Statistical analyses

Changes in the parameters analyzed in this study were compared with the vehicle control. Statistical analyses were performed using one way ANOVA (multiple comparison; Holm-Sidak test) and Student’s t-test available in Sigma Plot software 12.5, Build 12.5.0.38 (SPSS Inc., Chicago, IL, USA). Values shown are Mean ± S.D. * denotes $p < 0.05$.

1.3. RESULTS

1.3.1 Isolation of primary cells

Isolated EPECs were observed for morphology and survival over a period of 3 weeks (**Figure 2**). EPECs appeared normal and healthy 3 days after isolation and were adherent to the surface of the culture dish (**Figure 2**). 7 days after plating, the density of the cells appeared increased. The cells were then sub cultured and observed for their growth and survival. 7 days after sub culturing (14 days after isolation), the morphology of the cells remained intact with so signs of stress. Similar observations were made when the cells were sub cultured for second time and observed after 7 days (21 days after isolation) (**Figure 2**).

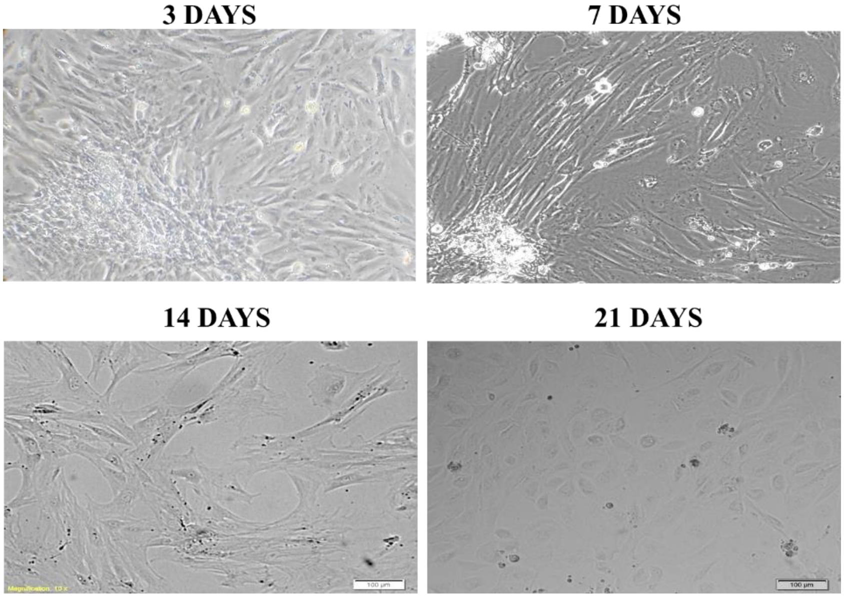

Figure 2. *Morphological features of isolated EPEC. Epididymal tissue was subjected to collagenase based digestion and the epithelial cell suspension placed in a culture dish. The cells were sub cultured and observed over a period of three weeks. Scale bar: 100 µm.*

1.3.2 Molecular characterization

To confirm whether the cells isolated were epithelial in nature, the expression of E-cadherin, an epithelial cell specific marker was analyzed by immunofluorescence microscopy and immunoblotting. We observed abundant E-cadherin in the isolated cells (**Figure 3**). Immunoblotting analyses confirmed the expression of E-cadherin in these cells and in the positive control cells (MCF-7). Such an expression was not evident in the E-cadherin negative MDMB231 cells (**Figure 4**). Further, to demonstrate that the EPECs isolated are not fibroblasts, we analyzed the expression of vimentin, a specific marker for fibroblasts.

Vimentin expression was not observed in EPECs. We also analyzed its expression in the rat immortalized epithelial cell line (RCE) and found it to be negative. Mouse embryonic fibroblasts (MEF) and MDA231 cells, used as positive controls showed abundant expression of vimentin (**Figure 3**).

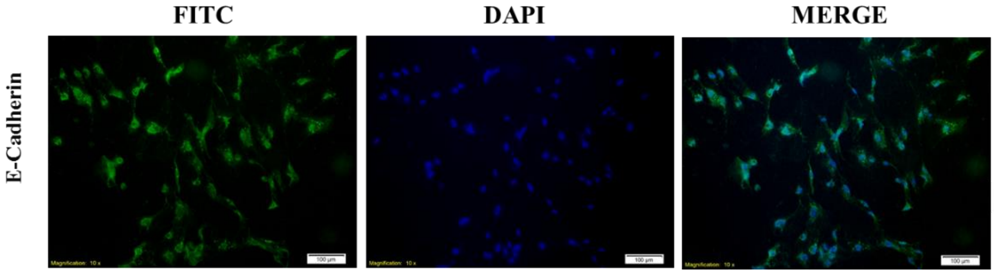

***Figure 3**. Immunofluorescence detection of epithelial marker. EPECs that are adhered to glass cover slips were stained with FITC tagged E-cadherin antibody and observed under a fluorescence microscope. Scale bar: 100 µm.*

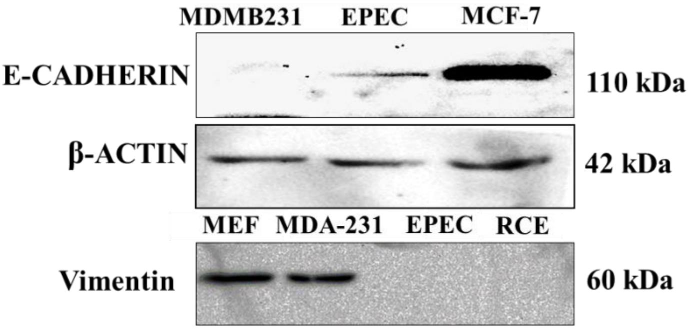

***Figure 4.** Detection of epithelial cell marker expression. Lysates of MDMB231, EPEC and MCF-7 cells were electrophoresed and subjected to immunoblotting with E-cadherin antibody. Lysates of MEF, MDA21, EPECs and RCE cells were electrophoresed and probed with Vimentin antibody.*

To determine the purity of the isolated cells flow cytometric analyses was conducted. Isolated EPECs were stained with FITC labelled E-cadherin and the percent of positive cells was calculated in a flow cytometer. 73 % of the cells isolated were E-cadherin positive (**Figure 5**), whereas 92% of MCF-7 cells (used as positive control) displayed fluorescence. Only 2.5% of the MEF cells (used as negative control) were positive for E-cadherin. Unstained cells in all the three groups did not display any positivity, indicating the absence of background fluorescence in the analyses (**Figure 5**). After confirming that the cells isolated were in fact epithelial cells, the cell specific marker expression was analyzed. The expression of CRISP1, an epididymis specific protein was abundant in EPECs as revealed by immunofluorescence microscopy (**figure 6**) and immunoblotting (**Figure 7A**). Based on previous studies we identified few genes whose expression is either positive (*Urp1a*, *Pate-F*, *Crisp1*, *Ar* and *Spag11e*) or negative (*Urp1b* and *Pate*) in the epididymis. *Urp1a*, *Pate-F*, *Crisp1*, *Ar* and *Spag11e* mRNA was expressed in the EPECs, whereas *Urp1b* and *Pate* were not detected, suggesting that the isolated cells are only epididymal in origin (**Figure 7B**).

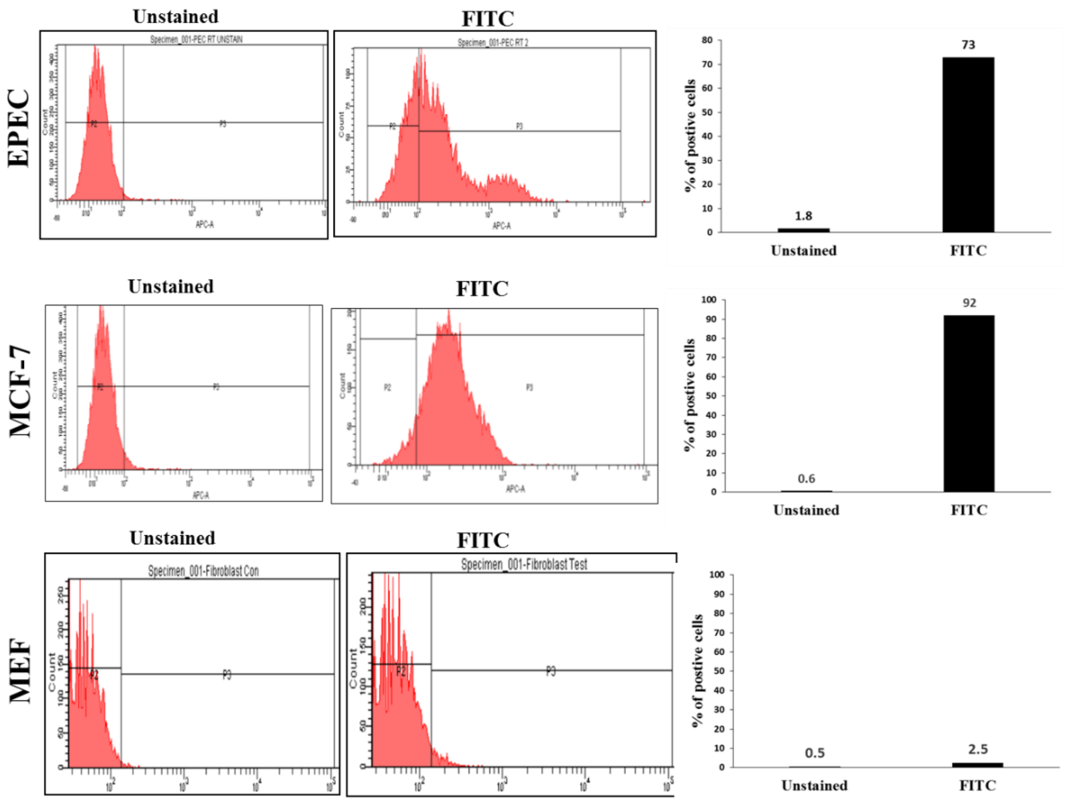

***Figure 5**. Flow cytometric analyses to determine purity. Representative flow cytometry output is shown. EPEC, MCF-7 and MEF cells were incubated with FITC labelled E-cadherin antibody. The cells were then subjected to flow cytometry. Percentage of cells that are positive to FITC signal are indicated in the adjacent histograms.*

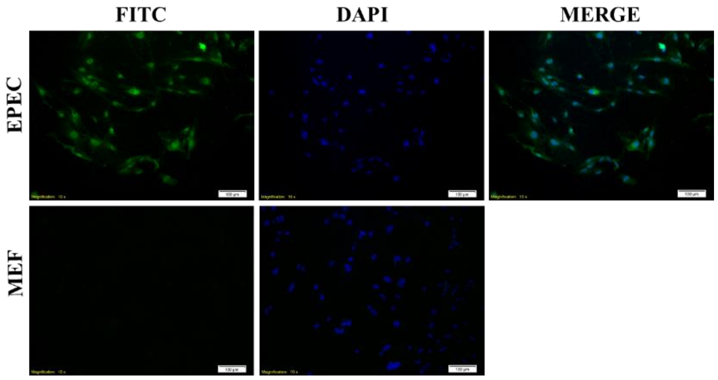

***Figure 6.** EPECs were stained with FITC tagged CRISP1 antibody and observed under a fluorescence microscope. The cells were counterstained with DAPI. Scale bar: 100 µm.*

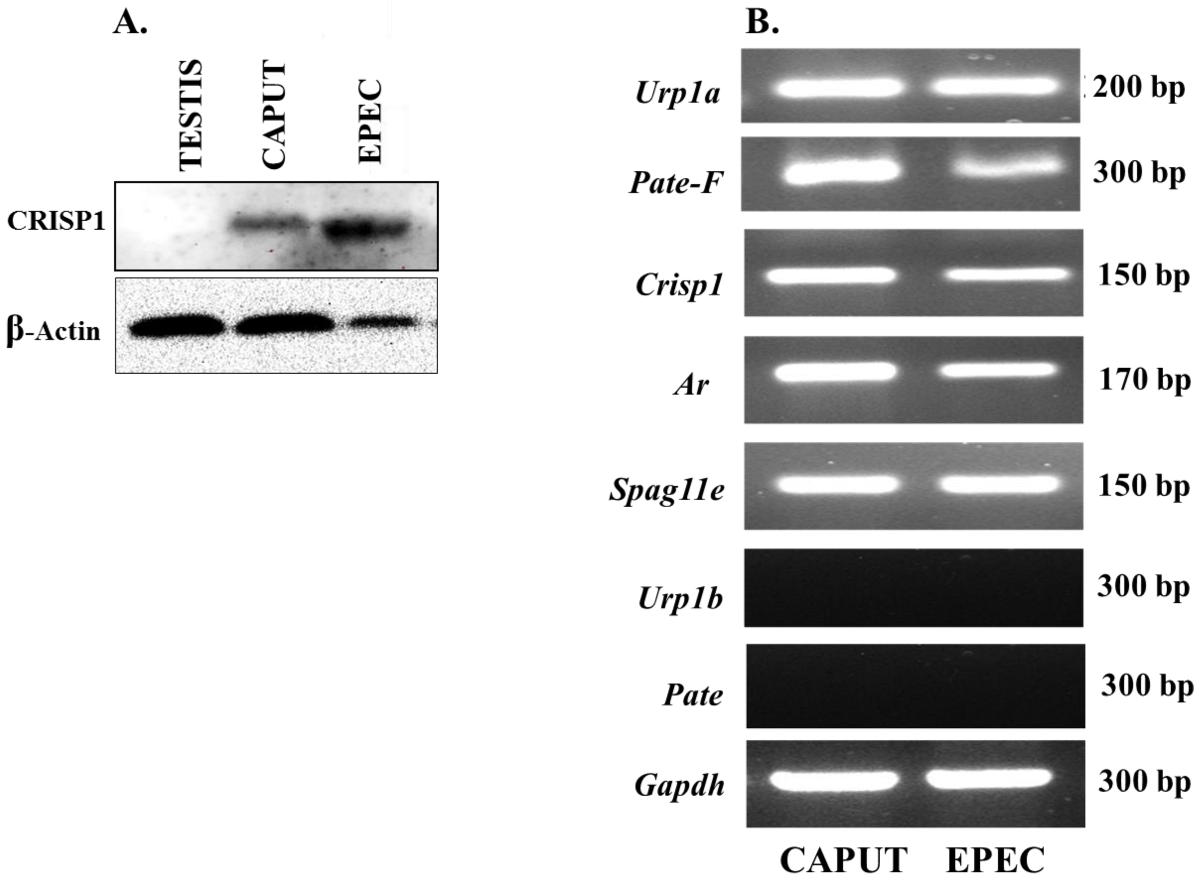

Figure 7. *Cell specific marker expression.* ***(A)*** *Detection of CRSP1 by immunoblotting. Lysates of EPEC were subjected to electrophoresis and CRISP-1 protein expression detected by probing with CRISP1 antibody.* ***(B)*** *PCR analyses to detect cell specific gene expression. RNA isolated from caput and EPECs was reverse transcribed and PCR performed with gene specific primers. Gapdh was used as the internal control.*

1.3.3. siRNA mediated suppression of *Spag11a* mRNA expression and the effect on proliferation

Spag11a mRNA levels in the EPECs obtained from the four experimental setups (CON, LIPO, SCR and siRNA) were analyzed by semi-quantitative PCR and real time PCR. EPECs transfected with siRNA showed decreased levels of *Spag11a* mRNA (**Figure 8A and 8B**). The mRNA levels were not affected in the LIPO or SCR groups, suggesting the specificity of the siRNA used. *Spag11a* mRNA levels were abundantly present in untreated control (CON) and scrambled siRNA (SCR) transfected EPECs. Reduced levels of *Spag11a* mRNA correlated with decreased amount of *SPAG11A* protein (**Figure 8C**). The effect of *Spag11a* knock down on the proliferative ability of EPECs was analyzed by MTT assay. The relative percent proliferation increased in EPECs transfected with siRNA against *Spag11a* at the 12 h time point. The increase was significant at 24 hours in the siSPAG11A group when compared to the SCR control (**Figure 8D**). These results clearly demonstrate that depletion of *Spag11a* mRNA affects cell proliferation.

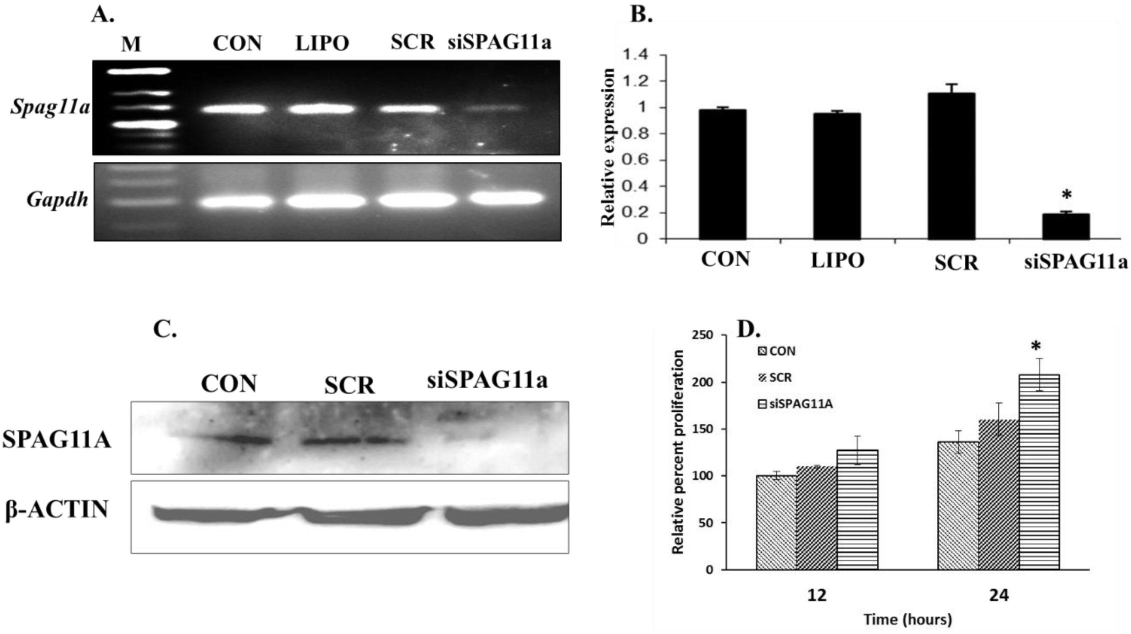

Figure 8. *Effect of siRNA mediated knock down of Spag11a on proliferation in EPECs. Epididymal primary epithelial cells were treated lipofectamine (LIPO) alone or non-specific siRNA (SCR) or siRNA that targets Spag11a mRNA (siSPAG11A). Cells without any treatment (CON) were also maintained. A) Semi-quantitative PCR for Spag11a and Gapdh (internal control) expression. B) Real time PCR analyses for Spag11a expression. C) Western blotting for the detection of SPAG11A and β-actin (internal control) proteins. D) Relative percent proliferation of EPECs. Values shown are mean ± S.D. *denotes $p<0.05$.*

1.3.4. Gene expression by gene array analysis

RNA isolated from EPECs of CON or SCR or siSPAG11A groups were subjected to gene array analyses. The data obtained was analyzed using DAVID software version 6.8 available online (61). Comparisons were made between CON vs SCR; SCR vs siSPAG11A groups. Scatter and Volcano plots revealing the distribution of differentially expressed genes (DEGs) between CON vs SCR and SCR vs siSPAG11A are shown (**figure 9)**. A total of 68842 genes were analyzed. The distribution of genes in CON vs SCR and SCR vs siSPAG11A groups are shown in **tables 2 and 3 respectively**. Only 98 genes were differentially expressed between the CON vs SCR groups (**Table 2**), indicating a minimal effect on global gene expression due to transfection protocol. A total of 4182 genes were differentially expressed between the SCR vs siSPAG11A groups (**Table 3**). Among the differentially expressed genes, 2154 were upregulated whereas 2028 were down regulated. Among the upregulated and down regulated groups, coding genes were 1354 and 1075 respectively. A complete list differentially expressed genes sorted first by the fold change value and then by the ANOVA p value for the CON vs SCR and SCR and siSPAG11A are available as supplementary information in our manuscript which can be accessed at https://doi.org/10.1007/s00441-019-03107-6. The top 20 upregulated and down regulated genes in the CON vs SCR and SCR vs siSPAG11A are listed in **tables 4 and 5 respectively**. The differentially expressed genes distributed according to their role in different cellular pathways between CON vs SCR and SCR vs siSPAG11A are available as supplementary information in our manuscript which can be accessed at https://doi.org/10.1007/s00441-019-03107-6. Further, using DAVID software, the differentially expressed genes were classified basing on the biological function (can be accessed at https://doi.org/10.1007/s00441-019-03107-6.). Since transfection with siRNA that targets *Spag11a* mRNA affected EPEC proliferation, we focused on the expression pattern of genes involved in epithelial cell proliferation. We observed that none of the differentially expressed genes in CON vs SCR were involved in epithelial cell proliferation. Differentially expressed genes that are associated with epithelial cell proliferation in SCR vs siSPAG11A are listed in **table 6**.

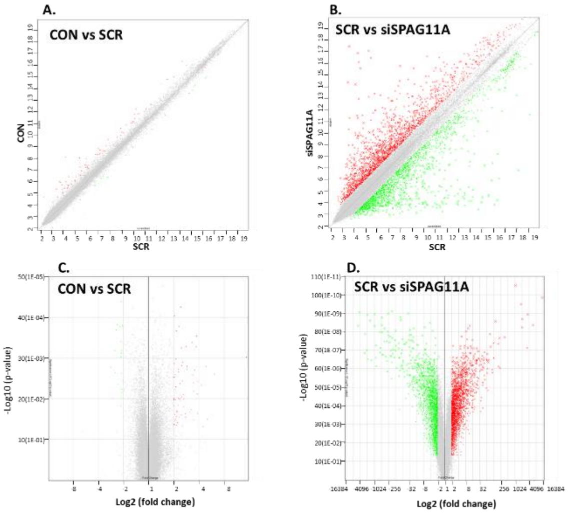

Figure 9. *Scatter and Volcano plots representing the distribution of differentially expressed genes. RNA isolated from untreated (CON) or non-specific siRNA (SCR) treated or siRNA that targets Spag11a mRNA (siSPAG11A) treated epididymal primary epithelial cells was subjected to gene array analyses. Scatter plots for gene expression between CON vs SCR* ***(A)*** *and SCR vs siSPAG11A* ***(B)****. Volcano plots showing the differential expression between CON vs SCR* ***(C)*** *and SCR vs siSPAG11A* ***(D)****. Green and red dots represent up regulated and down regulated genes respectively.*

Table 2. *Distribution of genes in the mock (CON) and nonspecific siRNA (SCR) transfected EPECs.*

	Coding	Precursor micro RNA	Non-coding	Unassigned	Multiple complex	Small RNA	Pseudo genes	Ribosomal	t-RNA	Total
Number of genes analyzed	24753	1626	25625	10838	2259	1324	2026	370	21	68842
Differentially expressed	67	1	9	18	1	2	0	0	0	98
Upregulated	49	1	7	17	1	2	0	0	0	77
Down regulated	18	0	2	1	0	0	0	0	0	21

Table 3. *Distribution of genes in the nonspecific siRNA (SCR) or SPAG11A siRNA transfected EPECs.*

	Coding	Precursor micro RNA	Non-coding	Unassigned	Multiple complex	Small RNA	Pseudo genes	Ribosomal	tRNA	Total
Number of genes analyzed	24753	1626	25625	10838	2259	1324	2026	370	21	68842
Differentially expressed	2429	66	503	970	89	33	69	11	12	4182
Upregulated	1354	20	220	441	66	0	52	1	0	2154
Down regulated	1075	46	283	529	23	33	17	10	12	2028

1.3.5. Validation of differential gene expression by real-time PCR

To confirm the observed differential expression of genes associated with epithelial cell proliferation between SCR vs siSPAG11A groups, real time PCR analyses was carried out. Genes listed in table 5 that exhibited a fold change of 3 and above were selected for the analyses. The expression level of all these gens in the SCR group is considered as 1. The expression pattern of all the up regulated and down regulated genes analyzed was similar to that observed in the gene array analyses (**Figure 10**).

Table 4. *Top 20 differentially expressed genes in mock (CON) vs nonspecific siRNA (siSCR) transfected EPECs.*

Gene Symbol	Fold Change	ANOVA p-value	Description	Group
DOWN REGULATED				
Rgs5	-2.84	0.001723	regulator of G-protein signaling 5;	Coding
Peg10	-2.75	0.009588	ENCODES a protein that exhibits poly(A) RNA binding	Coding
Col1a1	-2.54	0.000653	collagen, type I, alpha 1;	Coding
Cyr61	-2.52	0.002319	cysteine-rich, angiogenic inducer, 61;	Coding
Col1a2	-2.42	0.0002	collagen, type I, alpha 2	Coding
Col1a2	-2.37	0.000152	collagen, type I, alpha 2.	Coding
Fawaw	-2.33	0.004382	Unannotated AceView Transcript	Unassigned
Adam19	-2.2	0.000166	ADAM metallopeptidase domain 19;	Coding
Il1rl1	-2.15	0.014874	interleukin 1 receptor-like 1.	Coding
Flnc	-2.14	0.000931	filamin C, gamma.	Coding
Pgf	-2.13	0.000392	placental growth factor.	Coding
Cryab	-2.12	0.000041	crystallin, alpha B.	Coding
TID_00004648	-2.12	0.009605	Non-coding transcript	NonCoding
Fbn1	-2.11	0.000762	fibrillin 1	Coding
Actg2	-2.11	0.00239	actin, gamma 2	Coding
Vgll3	-2.1	0.008539	vestigial-like family member 3	Coding
Cnn1	-2.09	0.005891	calponin 1	Coding
uc029rcb.1	-2.09	0.007025	oocyte_clustered_small_RNA 4919	NonCoding
Fbln2	-2.08	0.000648	fibulin 2	Coding
Itga11	-2.04	0.000158	integrin, alpha 11	Coding
UPREGULATED				
Ddx58	2.89	0.001916	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Coding
Hmox1	3.11	0.020984	heme oxygenase 1	Coding
Meeshy	3.21	0.018905	Unannotated AceView Transcript	Unassigned
Gawfy	3.21	0.018905	Unannotated AceView Transcript	Unassigned
Plerry	3.24	0.018782	Unannotated AceView Transcript	Unassigned
Mx1	3.33	0.000414	myxovirus (influenza virus) resistance 1	Coding
Mmp12	3.38	0.0026	matrix metallopeptidase 12	Coding
RGD1563091	3.42	0.004071	INTERACTS WITH chloroprene	Coding
Olfm4	3.45	0.00663	olfactomedin 4	Coding
Slc6a14	3.57	0.001965	solute carrier family 6 (amino acid transporter), member 14	Coding
Usp18;	3.69	0.000528	ubiquitin specific peptidase 18	Coding
Oas1b	3.77	0.000287	2-5 oligoadenylate synthetase 1B	Coding
Skogler	3.86	0.023024	Unannotated AceView Transcript	Unassigned
Mx2	3.89	0.003839	myxovirus (influenza virus) resistance 2	Coding
Mmp13	4.3	0.004669	matrix metallopeptidase 13	Coding
uc029rsx.1	4.51	0.048696	non-coding RNA, oocyte_clustered_small_RNA7169	NonCoding
Oasl	4.87	0.001606	2-5-oligoadenylate synthetase-like	Coding
uc029wbf.1;	5.07	0.031753	oocyte_clustered_small_RNA12445	NonCoding
Cxcl10	6.12	0.002497	chemokine (C-X-C motif) ligand 10	Coding
Rsad2	14.8	0.000945	radical S-adenosyl methionine domain containing 2	Coding

Table 5. *Top 20 differentially expressed genes in nonspecific siRNA (siSCR) Spag11a targeting siRNA (siSPAG11A) transfected EPECs.*

Gene Symbol	Fold Change	ANOVA p value	Description	Group
DOWN REGULATED				
Cldn4	-5004.65	4.71E-09	claudin 4	Coding
Cdh1	-4406.64	8.82E-10	cadherin 1	Coding
Cxcl6	-2640.48	1.41E-07	chemokine (C-X-C motif) ligand 6	Coding
Krt18	-2518.12	1.32E-08	keratin 18, type I	Coding
Mmp9	-2124.85	9.90E-08	matrix metallopeptidase 9	Coding
Ass1	-1993.90	1.22E-09	argininosuccinate synthase 1	Coding
Perp	-1931.19	2.95E-07	PERP, TP53 apoptosis effector	Coding
Prom1	-1540.81	2.47E-09	prominin 1	Coding
C3	-1357.36	3.49E-07	complement component 3	Coding
Il1a	-996.08	1.09E-09	interleukin 1 alpha	Coding
Cdh6	-990.74	4.93E-09	cadherin 6, type 2, K-cadherin	Coding
Mmp7	-882.58	2.62E-07	matrix metallopeptidase 7	Coding
Mmp13	-868.11	7.31E-07	matrix metallopeptidase 13	Coding
Csf3	-866.72	5.53E-08	colony stimulating factor 3	Coding
Lamb3	-576.35	1.05E-08	laminin, beta 3	Coding
Prss22	-574.31	1.36E-07	protease, serine, 22	Coding
Epcam	-563.29	7.60E-08	epithelial cell adhesion	Coding
Itgb6	-485.34	1.72E-09	integrin, beta 6	Coding
Pdgfb	-484.97	4.35E-09	platelet-derived growth factor beta polypeptide	Coding
Slc6a14	-465.74	1.05E-08	solute carrier family 6	Coding
UPREGULATED				
A2m	108.09	0.000001	alpha-2-macroglobulin (A2m)	Coding
Fgf18	112.33	0.000002	fibroblast growth factor 18	Coding
Mmp23	124.24	4.13E-08	matrix metallopeptidase 23	Coding
Slit3	125.82	2.66E-07	slit guidance ligand 3	Coding
Abi3bp	149.00	2.75E-09	ABI family, member 3 (NESH) binding protein	Coding
Col14a1	195.44	4.53E-08	collagen, type XIV, alpha 1	Coding
A2m	203.48	7.68E-07	alpha-2-macroglobulin	Coding
Mfap5	223.11	2.16E-08	microfibrillar associated protein 5	Coding
LOC100910270	230.88	9.14E-07	uncharacterized	NonCoding
Aqp1	370.39	1.98E-08	aquaporin 1	Coding
Txnip	1074.63	3.17E-11	thioredoxin interacting protein	Coding
Klaker	1882.78	3.22E-10	Unannotated AceView Transcript	Unassigned
Sfrp1	2076.40	5.86E-09	secreted frizzled-related protein 1	Coding
Nov	2092.20	2.09E-09	nephroblastoma overexpressed	Coding
Klyker	3359.14	7.41E-08	Unannotated AceView Transcript	Unassigned
Figf	3627.32	1.15E-09	c-fos induced growth factor	Coding
Swaglor	6020.79	4.45E-09	Unannotated AceView Transcript	Unassigned
Stoyglor	14990.58	1.42E-10	Unannotated AceView Transcript	Unassigned

Table 6. *Differential expression of epithelial cell proliferation related genes in nonspecific siRNA (siSCR) or Spag11a siRNA (siSPAG11A) transfected EPECs.*

Gene	**Fold change**	**ANOVA p value**	**Gene**	**Fold change**	**ANOVA p value**	**Gene**	**Fold change**	**ANOVA p value**
POSITIVE REGULATION OF EPITHELIAL CELL PROLIFERATION			**EPITHELIAL CELL PROLIFERATION**			**REGULATION OF EPITHELIAL CELL PROLIFERATION**		
Il6	-112.32	8.73E-07	*Ehf*	-158.86	9.14E-08	*Serpinb5*	-335.06	2.14E-07
Esrp1	-77.93	1.67E-09	*Hnf1B*	-14.84	0.000014	*Tacstd2*	-21.86	0.00001
Mmp12	-15.86	0.000001	*Kit*	-7.38	0.000008	*Hes1*	-2.82	0.000121
Epgn	-12.21	0.000004	*Ncstn*	2.31	0.000148	*Smad3*	-2.07	0.000135
Egfr	-7.63	0.000007	*Fgf10*	2.39	0.013685	*Frs2*	2.37	0.000123
Esrp2	-4.56	0.000035	*Col8a2*	2.91	0.000143	*Fgf10*	2.39	0.013685
Pax2	-4.4	0.000037	*Col8a1*	5.67	0.000002	*Pgr*	9.14	0.000001
Fgf1	-4.24	0.00007				*Tgfb3*	13.81	0.000002
Wnt5a	-3.4	0.000034						
Cyp7b1	-2.73	0.000519						
Bnc1	-2.1	0.001964						
Grn	2.06	0.006464						
Fgf10	2.39	0.013685						
Hyal1	2.5	0.001001						
C5ar2	2.5	0.001001						
Tcf7l2	2.65	0.000307						
Fgf2	2.68	0.000355						
Tbx18	2.74	5.32E-07						
Osr2	2.89	0.001345						
Gas1	3.1	0.004586						
C5ar1	3.27	0.000888						
Foxp1	3.81	0.000101						
Vegfc	5.32	0.000001						
Fgf7	27.48	0.000012						
Igf1	40.94	2.04E-07						
Bmp6	67.23	1.57E-07						
Sfrp1	2076.4	5.86E-09						

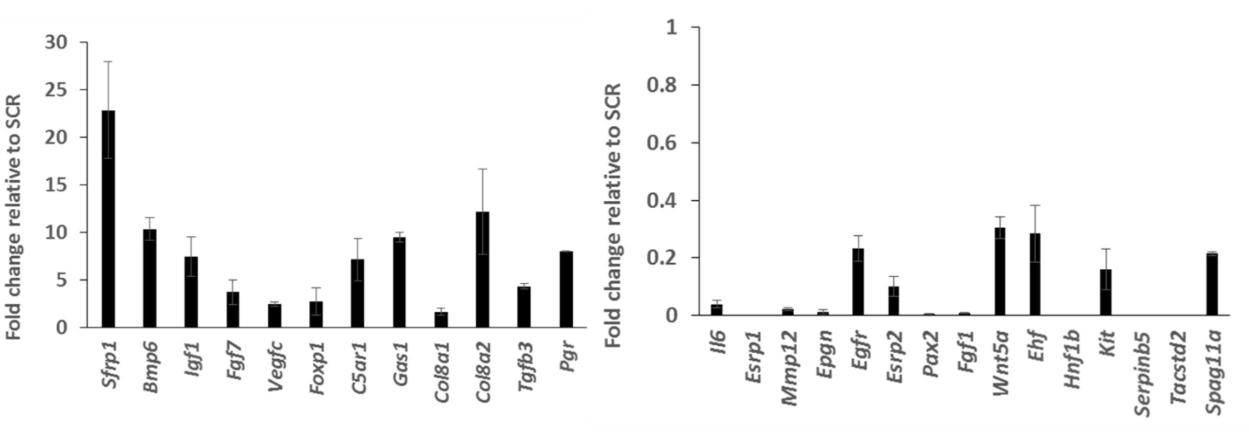

Figure 10. *Validation of the expression of differentially expressed genes. RNA isolated from EPECS treated with non-specific siRNA (SCR) or siRNA against Spag11a mRNA (siSPAG11A) was reverse transcribed and the resulting cDNA was subjected to real time PCR using gene specific primers. Gapdh expression was used as internal control. The expression level of all the genes in the SCR group is considered as 1. Bars represent the relative gene expression compared with the corresponding gene expression in SCR group. Values shown are mean ± S.D obtained from three different experiments. *denotes* $p<0.05$.

1.3.6. Over expression of *Spag11a* and its effect on proliferation in RCE cells

Since the relative proliferation index was found to be increased due to siRNA mediated *Spag11a* mRNA depletion in EPECs, we tested whether the cell proliferation would decrease if *Spag11a* is over expressed in highly proliferating immortalized rat epididymal epithelial cells. Real-time PCR and Western blotting analyses confirmed the abundant expression of *Spag11a* mRNA and SPAG11A protein respectively in RCE cells transfected with *Spag11a* expressing construct (**Figure 11A and 11B**). A significant decrease in proliferation was observed after 24 h in SPAG11A over expressing when compared to the respective empty vector (pcDNA) transfected cells (**Figure 11C**).

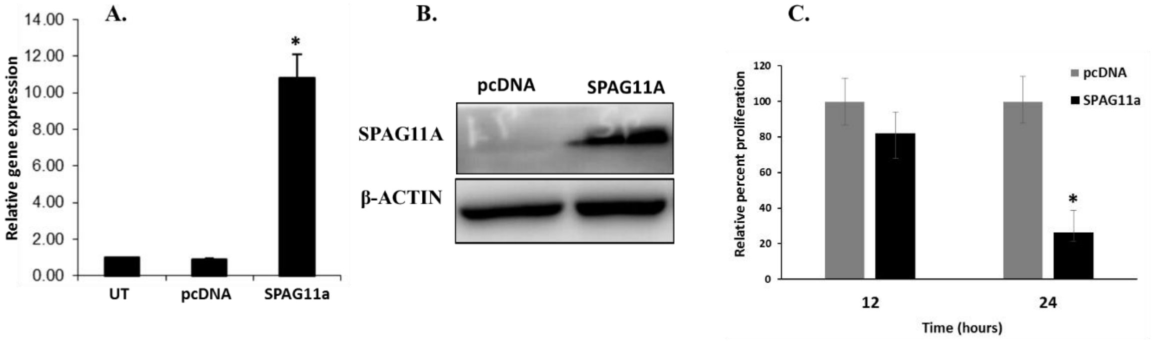

Figure 11. *Effect of Spag11a over expression on proliferation of RCE cells. Rat proximal caput immortalized cells (RCE) were transfected with empty vector (pcDNA) or pcDNA vector cloned with SPAG11A gene (SPAG11A). CON represents untransfected control. Cells were collected 12 and 24 hours after transfection to assess gene expression by real-time PCR* ***(A)****, protein expression by Western blotting* ***(B)*** *and relative percent proliferation by MTT assay* ***(C)****. Values shown are mean ± S.D obtained from three different experiments. *denotes p<0.05.*

1.4. DISCUSSION

Understanding epididymal architecture and the contribution of its different cell types to sperm function has been an active area of investigation for almost twenty years. Since sperm maturation takes place in this organ system, the *in vivo* role of proteins secreted by the epithelial cells of epididymis have been analyzed using a wide variety of molecular approaches. To understand these functions at the cellular level, *in vitro* model systems such as culturing the epididymal tissue for shorter durations (62) primary epididymal cells (10) and immortalized epididymal cells (13,14,63) have been used. However, use of epididymal explants and immortalized cell lines have limitations. The former system can be used only for short durations whereas the latter being an immortalized version does not exactly reflect the epididymal epithelial cells. Thus, isolation of epididymal epithelial cells from the epididymis and culturing these primary cells is still the preferred choice. Previous studies described the isolation of epididymal epithelial cells and immortalization of these cells from different species (10,13,14,63). However, characterization of these cells at the molecular level for their purity is not yet reported. Basing on previously reported protocols, we isolated rat caput epididymal epithelial cells and systematically characterized for their identity and purity using molecular biology approaches.

The morphological features observed in the isolated EPECs were in agreement with the previous studies (10,11). We observed that EPECs appeared as aggregates and upon plating and attached to the substrate over a period of time. They adopted a flat configuration, migrated and form a monolayer. When subcultured with trypsin, and transferred to new flasks, the cells readily attach to the substrate and eventually form a monolayer. These features were reported in a previous study which initially standardized the isolation of epididymal epithelial cells (10,11). Thus, the EPECs isolated in this study display characteristics that were specific to epididymal epithelial cells. Though previous studies have reported the immortalization of epididymal epithelial cells and isolation of immortalized epididymal cells from transgenic mice harbouring the tsSV40 large T-antigen and their characterization at the molecular level, (13,14) till date there are no reports on specific molecular marker based in depth characterization of isolated epididymal epithelial cells. We observed abundant expression of E-cadherin in EPECs, which indicated their epithelial nature. We observed that 73% of the cells isolated were epithelial in nature using flow cytometry. To the best of our knowledge, quantification of the purity of isolated or immortalized epididymal cells is not reported. Our results demonstrate that staining with epithelial marker and measuring the purity of isolated cells by flow cytometry will be an important step during isolation of epididymal epithelial cells. This procedure will give first-hand information on the purity of cells and avoid introduction of additional methods to eliminate contaminating cells like fibroblasts in situations wherein the yields are low.

We further characterized EPECs for the expression of cell specific markers. Immunofluorescence staining and immunoblotting revealed abundant expression of CRISP1, an epididymal epithelial cell marker in the EPECs isolated by the procedure adopted in this study. The mRNA expression of *Urp1a, Pate-F, Crisp1, Ar* and *Spag11e* and the absence of *Urp1b* and *Pate* mRNA further reinforced the epididymal epithelial nature of EPECs. CRISP1 was detected as a specific marker in isolated epididymal epithelial cells using immunoperoxidase localization (10). The expression of markers of the murine epididymal epithelium namely PEB-like protein (phosphatidyl ethanolamine binding protein), E-RABP (epididymal retinoic acid–binding protein), and EP17 (epididymal protein of 17 kd) was demonstrated in epididymal epithelial cells isolated from transgenic mice harbouring the tsSV40 large T-antigen (14). Further, androgen receptor, estrogen receptor, and 4-ene-steroid-5-alpha-reductase types 1 and 2 as well as epididymal-specific markers Crisp-1 and epididymal retinoic acid binding protein expression was detected in immortalized epididymal epithelial cells of rat (64). In the immortalized cells derived from different parts of the epididymis, nuclear androgen receptor (AR), polyoma enhancer activator (PEA3), and the epididymal mRNA encoding the canine counterparts of human HE1, HE4 and HE5/CD52 epididymal mRNA were analyzed for the specificity of the cells (13). Results of our study provide a systematic approach to characterize isolated primary epididymal epithelial cells, which will be useful for investigators conducting studies using epididymal primary cells as a model system.

Analyzing gene regulatory networks in male reproductive organs contributed immensely to the understanding of a variety of physiological and pathological processes. The region / segment specific mRNA expression in the epididymis (65,66), generated exciting interest to study correlations between the genome signature and function; and this area of research continues to be explored in much depth. A genome wide open chromatin analyses revealed the importance of regulatory elements that govern epididymal gene expression and function (67). Previous studies suggested a relation between region specific global miRNA and the cellular processes in the epididymis (26,27,68). Browne *et al* showed correlation between the region specific diversity of differentially expressed genes in the epididymis to the epithelial cell processes, luminal environment and sperm maturation (69).

An extension to genome wide studies is to analyze global gene expression profile under conditions of over expressing or down regulation of a specific gene both *in vitro* and *in vivo*. Genes important for epididymal function were investigated for their ability to influence global gene expression profile in different model systems. The effect of androgen receptor (*AR*) activation mediated transcriptional network was investigated in human epididymal epithelial cells (60). siRNA mediated depletion of hepatocyte nuclear factor 1 (*HNF1*) resulted in differential expression of many transcripts involved in critical cellular processes of the epididymal cells (58). The importance of *p-MAPK1/3* and *DUSP6* in epididymal epithelial cell proliferation was reported (56). Thus, studying the influence of epididymal genes on global gene expression provided novel information on cellular, molecular and physiological functions of this organ system. Except for the few studies mentioned above, extensive analyses on the influence of epididymis specific genes to affect the transcriptome are lacking. We previously characterized the antimicrobial functions of rat *SPAG11A*, a protein expressed by the epididymis (caput) specific *Spag11a* gene (43). However, the influence of this gene on epididymal function with specific emphasis on global mRNA profile is not yet reported. In this study, we characterized the influence of *Spag11a* gene on global gene expression using rat epididymal epithelial cells.

siRNA mediated suppression of *Spag11a* mRNA levels in the rat primary caput epididymal cells resulted in differential expression of a plethora of genes that are involved in many cellular processes. DAVID analyses revealed that majority of differentially expressed genes belong to the categories of cellular processes involved in transcription, proliferation, growth, migration, differentiation, apoptosis, signal transduction, phosphorylation and response to stress (hypoxia or lipopolysaccharide). On the same lines, genes associated with a wide variety of cellular pathways mediated by NF-kB, MAP kinases, EGFR, B cell receptor, TGF-β receptor and Wnt are differentially regulated. It appears that *Spag11e* may control cell proliferation by influencing the effectors of different pathways and molecular processes. Such an influence on the transcriptome of human epididymal epithelial cells under conditions of siRNA mediated depletion of *PAX2* and *HNF1* mRNA was reported (58,59). Though a variety of cellular processes are affected by the down regulation of *Spag11a* mRNA levels, we focused on the expression profile of genes involved in cell proliferation, because of the fact that cell proliferation in the epididymis is highly regulated, a potential reason for the rare incidence of epididymal cancers. We observed that genes involved in regulation of epithelial cell proliferation were differentially regulated upon siRNA mediated suppression of *Spag11a* mRNA levels. Further, cell proliferation was found to be increased in EPECs wherein *Spag11a* mRNA levels were suppressed and a reverse effect was observed under conditions of over expression of this gene in RCE cell lines. Decreased epithelial cell proliferation in the caput and corpus was demonstrated in dual specificity phosphatase 6 (*Dusp6*) knockout mice (56). A wide variety of molecular mechanisms that govern cell proliferation may be affected by *Spag11e* and this needs further investigation. Over expression of *Spag11e* could result in cell cycle arrest directly or affect the expression of proteins that control the cell cycle and the reverse may be true when this gene is knocked down. The reverse effects on cell proliferation observed under *Spag11a* knock out and overexpression conditions indicates its possible role in regulating cell proliferation and thereby to the rare occurrence of cancer in the epididymis.

The influence of epididymal genes on epithelial cell proliferation is reported to a limited extent. miR-29a mediated suppression of nuclear autoantigenic sperm protein (NASP) contributed to inhibition of cell proliferation (57). Similarly, siRNA mediated suppression of paired box gene family member 2 (*PAX2*) in human epididymal epithelial cells influenced the expression of genes involved in cellular motion, migration and proliferation (59). *In vivo* studies using *Dusp6* null mice revealed the critical role of this gene in cell proliferation in caput and corpus regions (56). Further, the importance of the components of the ERK pathway in the proliferation of cells of the initial segment was demonstrated (56). Inactivation of androgen receptor in the principal cells of the proximal epididymis caused epithelial cell hyperplasia and hypertrophy in a mouse model (70). Results of our study provides the first line of evidence to the role of *Spag11a* gene in governing epididymal epithelial cell proliferation. Further studies on the molecular mechanisms by which *Spag11a* influences expression of genes involved in epithelial cell proliferation will provide concrete on the ability of *Spag11a* in this cellular process.

In conclusion, for the first time we report the role of a caput specific gene in epididymal epithelial cell proliferation. siRNA mediated knockdown of *Spag11a* promoted proliferation of isolated EPECs. On the other hand overexpression of this gene inhibited cell proliferation. Knock down of *Spag11a* in EPECs influenced the expression of genes that may affect multiple cellular pathways, including cell proliferation. Thus, understanding the regulation of cell proliferation by *Spag11a* provides further information to the puzzling reasons for the rare occurrence of cancers in the epididymis.

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Chapter 2

Determine the effect of SPAG11A protein ablation (by active immunization) on chemically induced oncogenesis in the epididymis

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2.1. INTRODUCTION

Tumorigenesis is a complex phenomenon that is characterized by perturbations in a variety of physiological, cellular and molecular processes. While tumorigenesis is considered as an evolutionary process, it is also caused by environmental insults and induced genetic modifications. The risk of development of tumors is uneven throughout the whole body and the concept of relative susceptibility of various organs to cancer has been studied for decades. The lifelong capacity to regenerate mutated cells by the stem cells and the influence of extrinsic factors in an organ is a major factor that determines the cancer risk (1). In the year 2020, the most common forms of cancer documented around the world are of breast, lung, colon, rectum, prostate, skin and stomach. While lung and prostate cancers are the most common in men, in women, breast cancer topped the list followed by colorectal, lung, cervical and thyroid cancers (2). In the male reproductive tract, the number of cases reported in 2020 for prostate, testicular and penis cancers were 14,14,259, 74,458 and 36,068 respectively (3). Despite the fact that cancers in the male reproductive organs are common, the incidence of epididymal cancers are extremely rare (4, 5) and is not mentioned in the exhaustive list of a variety of cancers by the National Cancer Institute, USA (6). Basing on the histopathological assessment, human epididymal tumors are of rhabdomyosarcoma, leiomyosarcoma, liposarcoma, adenocarcinoma, and malignant fibrous histiocytoma in nature (1).

The epididymis is a unique organ that contributes to sperm maturation. During the epididymal transit, the immature spermatozoa acquire motility and fertilizing ability and this is facilitated by segmental expression of a variety of genes and proteins (7-10) that are secreted into the lumen and also added on to the surface of the maturing gametes. A variety of epididymis specific proteins belonging to different families have been implicated to contribute to sperm maturation (9, 11). The Sperm Associated Antigen 11A (SPAG11A) protein exclusively expressed in the epididymis (Caput) is encoded by the Spag11 gene located in the beta-defensin cluster on Chromosome 8 (12). The human *SPAG11* gene by virtue of having two promoters encodes a large number of transcript variants and some of them are similar to beta-defensins both in terms of structure and function (12, 13). In the rat, the *Spag11a* gene (previously referred to as Binb or *Spag11e*) is located on Chromosome16 and its expression is epididymis (Caput) specific (14). The SPAG11A protein is implicated to have roles in sperm maturation and epididymal innate immunity (antimicrobial properties) (14-18). In our previous study, we demonstrated that siRNA mediated ablation of Spag11a mRNA in rat primary epididymal epithelial cells resulted in increased cell proliferation and differential expression of genes associated with epithelial cell proliferation (19). Further, over expression of *Spag11a* in rat caput immortalized epithelial cells resulted in decreased cell proliferation, indicating that this gene may have a role in governing the proliferative dynamics of epididymal epithelial cells (19). It is predicted that epididymis specific factors may contribute to the low incidence of tumors in epididymis (20).

To further explore the possibility that the epididymis specific SPAG11A protein may contribute to the low incidence of epididymal tumors by modulating the proliferation of epithelial cells, this study was conducted to decipher the anatomical and molecular changes that occur under conditions of ablation of this protein. To accomplish this, we used active immunization protocol to generate autoantibodies against SPAG11A and tested the susceptibility of epididymis to a low dose of carcinogen (Diethyl nitrosamine; DEN). We report that administration of a low dose of carcinogen alone did not generate any tumorigenic signals, whereas the same dose elicited these signals in SPAG11A immunized rats. Transcriptome analyses of the key components in ten signaling pathways that are involved in tumorigenesis indicated that the differential expression observed in the caput of DEN only treated rats was also evident in the caput of SPAG11A immunized only rats. These results clearly indicate that ablation of SPAG11A protein initiates a microenvironment in the caput that is conducive for tumorigenesis. To the best of our knowledge, we for the first time report the role of a caput specific protein in contributing to the low incidence of tumours in the epididymis.

2.2 MATERIALS AND METHODS

2.2.1 Recombinant protein production

The rat *Spag11a* coding region was cloned into a bacterial expression plasmid and transformed into *E. coli* BL21. The transformed bacterial cells grown to mid-log phase were treated with 1 mM isopropyl-1-thio-β-D-galactoside for 1 h at 37 C to induce recombinant protein expression. As a precautionary measure, 1% glucose was maintained in the bacterial medium prior to induction to minimize the toxic effects of the protein on *E. coli*. (21). Using Ni-NTA agarose affinity purification system, the recombinant protein with the His tag at the N-terminus was purified as per the manufacturer's instructions (Qiagen, Valencia, CA, USA). The identity of the purified recombinant protein was confirmed by Western blotting using anti-His tag antibody (Santa Cruz Biotechnology, Dallas, USA. The fractions that contained the protein of interest were dialyzed extensively at 4°C against 10 mM sodium phosphate buffer, pH 7.4.

2.2.2 Immunization to generate auto antibodies

Immunization protocol to generate auto antibodies was carried out in adult (aged 90 days) male Wistar rats as described previously (22). A mixture of 100 µg SPAG11A recombinant protein in 300 µl of PBS and 300 µL of Freund's complete adjuvant was administered subcutaneously. Booster doses that contained 100 µg of the recombinant protein in 300 µl PBS mixed with 300 µl Freund's incomplete adjuvant were administered two weeks after first immunization. Control animals (unimmunized) received Freund's adjuvant without recombinant protein similar to the immunized animals.

2.2.3 Determining the antibody titer

The presence of antibodies against SPAG11A in the serum was carried out by ELISA as described earlier (22). Blood was collected through tail vein and serum separated. The luminal fluids of testis and caput epididymis were also collected. SPAG11A recombinant protein (40 µg/ml) was coated on to each well of the microtiter plate and incubated at 37˚C overnight and at 60˚C for 30 min. Following blocking with 1 mg/ml BSA for 2 hr, serum / luminal fluid was added and incubated for 3 hr and thoroughly washed four times with TBS-T (PBS with 0.1% Tween-20). HRP conjugated anti-rat secondary antibody (1:10000 dilution) was added to facilitate binding to the primary antibody. O-Phenylenediamine (OPD) was added to each well and the intensity of the colour developed was measured at 490 nm, which is a direct measure of the antibody titer. Rats (n=6) that had a significant serum titer were used for further studies.

2.2.4 Treatment with the carcinogen

SPAG11A immunized rats (n=10) that had good antibody titre were provided drinking water containing 0.01% diethynitrosamine (DEN) through drinking water for 12 weeks and this group is referred to as IMM+DEN. Booster doses of SPAG11A protein was given to maintain antibody titre throughout the DEN treatment period. The dose of the carcinogen was based on a previous study (23). Similarly, age matched male Wistar rats (n=10) were provided with drinking water containing 0.01% DEN for 90 days and this group is referred to as DEN only treated.

2.2.5 Histopathology

Caput, testis, prostate and liver obtained from control, immunized and DEN treated rats were soaked in Bouin's fluid for 24 h. Following this, they were extensively washed with 70% ethanol and subjected to step-wise dehydration with serially graded ethanol (80, 90 and 100%). They were processed with isopropanol overnight at 60°C, embedded in paraffin wax and five micron sections were made. Deparaffinization of the sections with xylene, rehydration in graded ethanol (100, 90, 80, 70 and 50 %) and washing with distilled water was carried out. They were then stained with Harris hematoxylin solution for 10 min, washed with distilled water and subjected to differentiation in 1% hydrochloric acid for 30 sec and immersion in 0.2% ammonia water for 30 sec to 1 min. After washing with distilled water, sections were counterstained with 0.2% eosin Y solution for 1 min and dehydrated serially with 50, 70, 80, 90 and 100% alcohol. After 3 washes with xylene they were mounted using xylene based mounting medium. Multiple fields (atleast 25) in the sections were examined, photographed and evaluated by a board-certified histopathologist who had no information on the experimental set up and treatment protocol. Histopathological changes were evaluated as per the established principles described earlier (24-26)

2.2.6 Gene chip hybridization, data collection and enrichment analysis.

The experimental set up included three treatment groups namely: 1) Rats without any treatment (CON); 2) Rats that were immunized with SPAG11A protein (IMM); and 3) Rats treated with the carcinogen (DEN). Each group had 6 rats. RNA isolated from the caput of rats from all these groups were quality checked and assessed for pass criterion (A260/A280 ≥1.8; A260/A230 ≥1 and RNA integrity number (RIN) ≥6). RNA that passed the stringent criteria were subjected to gene array analyses using Affymetrix GeneChip Scanner and GeneTitan Platform (Imperial Life Sciences, Gurgaon, India). The array contained 68842 genes and the differentially expressed genes (DEGs) identified with the criteria that log2 |fold-change| is ≥1 and is $p<0.05$. Background subtraction and normalization was performed using Transcriptome analysis console (TAC) software.

2.3 RESULTS

2.3.1 Antibody titre

To determine whether SPAG11A immunization resulted in the generation of antibodies, the titre was measured by ELISA in the serum. We observed a very good antibody in the serum titre at all the time points tested (**Figure 1**). Further, significant antibody titre was also observed in the serum and luminal fluids of testis and caput obtained from immunized rats and DEN+immunized rats at the end of the treatment period (**Figure 1**). These results demonstrate that antibodies to SPAG11A were constantly present throughout the DEN treatment period.

2.3.2 Histopathology

Anatomical damage in the caput, testis, prostate and liver obtained from control, SPAG11A immunized, DEN treated and SPAG11A immunized + DEN treated rats was assessed. Histological changes observed in these tissues are summarized in **table 1**. In the caput obtained from SPAG11A immunized or DEN treated rats, normal tubular morphology and normal concentration of spermatozoa was observed (**Figure 2**). However, in the caput of SPAG11A immunized + DEN treated rats, foci of fibrosis and infiltration of inflammatory cells in between the tubules of epididymis along with mild mucosal degeneration of mucosal epithelial cells was evident. Though SPAG11A immunization did not cause anatomical damage in prostate, moderate degenerative changes in mucosal epithelial cells and mild hyperplasia of mucosal epithelial cells was observed in the DEN treated rats (**Figure 2**). Similar damage was also observed in the prostate of SPAG11A + DEN treated rats. SPAG11A immunization alone caused mild degeneration of spermatogonial cells of seminiferous tubules of testes. In the testes of DEN alone or SPAG11A + DEN treated rats, moderate to severe degenerative changes in tubules along with multi nucleated cells and moderate degeneration of spermatogonial cells in seminiferous tubules along with multi focal necrosis and inflammation was observed (**Figure 2**). SPAG11A immunization alone did not cause any notable damage to the liver anatomical architecture. DEN treatment resulted in multi focal coagulative necrosis along with hypertrophy of hepatocytes [pre neoplastic stages], and dysplastic hepatocytes [pre neoplastic stages] (**Figure 2**). The liver of SPAG11A immunized + DEN treated rats exhibited mild peri billary fibrous tissue proliferation, foci of necrosis in hepatocytes of centri lobular region, vacular degeneration of hepatocytes in centri lobular region, foci of inflammation and fibrosis along with hypertrophy and dysplastic changes of hepatocytes (**Figure 2**).

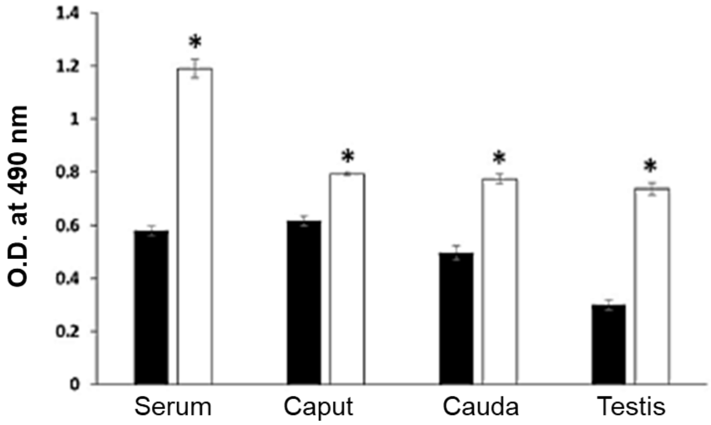

Figure 1. *Antibody titer in the serum and tissue fluids of* SPAG11A *immunized rats.*

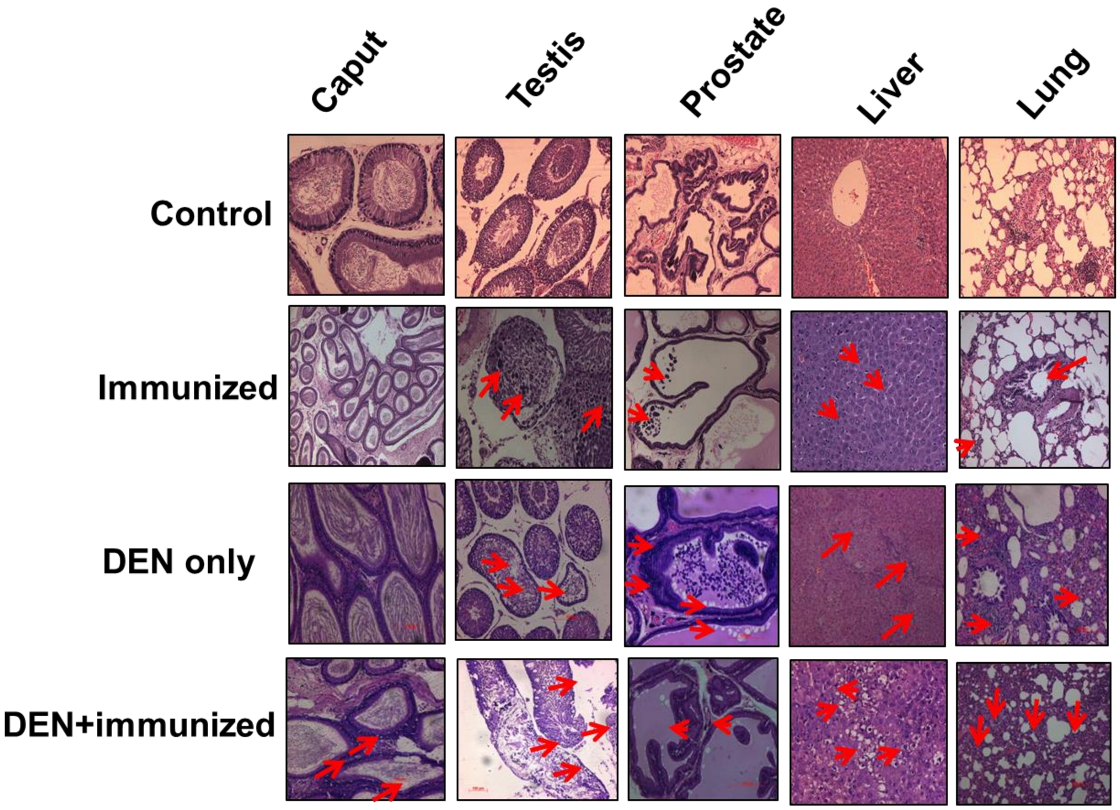

Figure 2: *Histological sections of caput, testis, prostate, liver and lung obtained from control,* SPAG11A *immunized, DEN only treated and* SPAG11A *immunized + DEN treated rats.*

Table 1. *Effect of* SPAG11A *immunization on the histopathological changes in different tissues.*

	Control	**SPAG11A Immunized**	**DEN only**	**SPAG11A Immunized + DEN**
CAPUT	Normal morphology of tubules with normal concentration of sperm in tubules	Normal tubular morphology with normal concentration of sperms in the tubules.	Normal tubular morphology with normal concentration of sperms.	Foci of fibrosis and infiltration of inflammatory cells in between the tubules of epididymis. Mild mucosal degeneration of mucosal epithelial cells.
PROSTATE	Normal morphology with no damage to mucosal and sub mucosal layers and tubular glands.	Tubules of prostate glands appeared normal; accumulation of inflammatory cells were observed.	Moderate degenerative changes in mucosal epithelial cells and mild hyperplasia of mucosal epithelial cells.	Infiltration of multi nucleated giant cells noticed in the tubules of prostate; accumulation of inflammatory cells; mild degenerative changes in mucosal epithelial layer of prostate.
TESTIS	Spermatogonial cells appeared normal in seminiferous tubules, with normal concentration of sperm / spermatid in tubules. Leydig cells and sertoli cells appeared normal	Mild degeneration of spermatogonial cells of seminiferous tubules of testes, with normal Leydig and sertoli cells	Moderate to severe degenerative changes in tubules along with multi nucleated cells. Moderate degeneration of spermatogonial cells in seminiferous tubules. Multi focal necrosis along with inflammatory changes.	Severe degenerative changes noticed in spermatogonial cells in the seminiferous tubules along with infiltration of multi nucleated giant cells noticed. Infiltration of multinucleated giant cells
LIVER	Normal Liver architecture; Hepatocytes appeared normal in portal, peri portal and centri lobular region. Normal bile duct, portal vein and portal triad contain portal vein, hepatic artery and bile duct.	Normal hepatic architecture. Normal portal traid with bile duct and hepatic artery and portal vein.	Multi focal coagulative necrosis along with hypertrophy of hepatocytes [pre neoplastic stages], and dysplastic hepatocytes [pre neoplastic stages] was observed.	Mild peri billary fibrous tissue proliferation; Foci of necrosis in hepatocytes of centri lobular region; vacoular degeneration of hepatocytes in centri lobular region; foci of inflammation and fibrosis along with hypertrophy and dysplastic changes of hepatocytes.

2.3.3 Transcriptome analyses

Histopathological analyses indicated that anatomical damage was not evident in the caput of DEN alone treated rats, while severed damage that is indicative to tumorigenesis was observed in the SPAG11A + DEN treated rats. In order to determine whether ablation of SPAG11A protein (due to immunization) contributes to tumorigenesis at the transcriptome level, RNA isolated from the caput of control, SPAG11A immunized and DEN treated rats was subjected to high throughput transcriptome analyses. Among the 25866 transcripts analyzed, 2760 and 3460 were found to be up and down regulated in the caput of SPAG11A immunized rats. Consequent to DEN treatment, the number of up and down regulated transcripts were 3241 and 2573 transcripts respectively. We then explored for the common transcripts that are differentially expressed in both these groups. 3714 transcripts were differentially expressed in the caput of both SPAG11A immunized and DEN only treated rats. Among the 3714 transcripts, 3549 of them displayed similar pattern in differential expression pattern.

Carcinogens are known to induce oncogenesis by modulating multiple signaling pathways. A recent report identified that majority of the tumours in humans are associated with alterations in one or more of the crucial signalling pathways (27). The signaling pathways identified were Hippo, Myc, Notch, Nrf2, PI-3-Kinase/Akt, RTK-RAS, TGFb, p53, cell cycle (Cdk mediated) and b-catenin/Wnt. Basing on this background we analyzed the differential expression of the molecular components of these pathways in the caput of SPAG11A immunized only and DEN only treated groups when compared to the control. Two criteria were used. Criterion 1: A log2fold value => plus 1 and <= minus 1 indicates upregulation and down regulation respectively to an extent of two fold. Those genes which had such log2fold values in both SPAG11A and DEN only treated groups are indicated in grey shaded background. Criterion 2: Genes whose log2 fold values are in the range of > 0 to < plus1 and < 0 to > minus 1 to determine the trend in differential expression even though they are not above the recommended threshold log2fold values. Genes that had the same trend of differential expression in both the groups with set of criteria are indicated in yellow colour shaded background. The differential expression of the signaling components in tend pathways are presented (**Tables 1 to 10**). In the RTK-RAS pathway, among the 35 genes analyzed, 15 of them classified under criterion 1 were differentially expressed (10 and 5 up and down regulated respectively) in both the groups. 18 of the 35 genes that had a log2fold change as per criterion 2 also showed a similar trend in expression (**Table 1**). 61 genes were analyzed in the cell cycle pathway (Table 2). 8 genes with criteria 1 were differentially regulated (1 up regulated and 7 down regulated) in both the groups. 25 genes with differential expression as per criteria 2 showed the same trend between the groups (**Table 2)**. None of the genes in the Hippo pathway showed similar differential expression among the two groups (**Table 3)**. In the 32 genes of Myc pathway, 8 genes under criterion 1 (3 up and 5 down regulated), whereas 5 genes under criterion 2 showed displayed similar differential expression in both the groups (**Table 4)**. Among 23 genes of Notch pathway, 3 and 10 genes under criterion 1 and 2 respectively were differentially regulated in both the groups (**Table 5**). In the Nrf pathway, 1 gene that satisfies criterion 1 and 7 genes under criterion 2 showed same trend of differential expression in both the groups (**Table 6**). 40 genes in the PI3K pathway were analyzed. Similar trend in differential expression between the two groups was observed for 7 genes and 12 genes under criterion 1 and 2 respectively (**Table 7**). In the Tgf-b pathway, among the 32 genes analyzed, 7 (6 down regulated and 1 up regulated) classified under criterion 1; and 14 classifieds under criterion 2 were differentially expressed in a similar fashion between the two groups (**Table 8**). 4 genes each under criteria 1 and 2 displayed similar differential expression in the TP53 pathway (**Table 9**). In the WNT pathway, out of 27 genes analyzed, 28 genes under criterion 1 were differentially expressed (13 up regulated and 15 down regulated) similarly in both the groups. 8 genes under criterion 2 displayed similar differential gene expression in both groups (**Table 10**).

Table 1. *Differential expression of genes involved in RTK-RAS pathway*

GENE	**IMM vs CON**	**DEN vs CON**	**GENE**	**IMM vs CON**	**DEN vs CON**	**GENE**	**IMM Vs CON**	**DEN vs CON**
Mapk11	-7.44	-0.73	*Map2k1*	-0.57	-0.67	*Mapkapk5*	0.066	-0.396
Mapk15	-7.32	-1.34	*Mapkapk2*	-0.57	-0.37	*Mapkap1*	0.094	-0.007
Mapk8ip2	-7.19	-1.83	*Hras*	-0.50	-0.92	*Mapk3*	0.177	0.399
Rtkn2	-5.38	-0.27	*Mapkapk3*	-0.46	0.14	*Mapk8ip3*	0.204	0.114
Nf1	-3.79	3.43	*Kras*	-0.42	-1.44	*Mapk8*	0.315	0.806
Diras3	-2.93	-1.94	*Nras*	-0.40	-0.02	*Mapk14*	0.333	0.489
Mapk12	-2.88	-2.44	*Rras*	-0.29	0.22	*Mapk1ip1l*	0.415	-0.108
Mras	-2.66	-1.25	*Mapk13*	-0.29	-0.43	*Mapk1ip1*	0.504	-0.315
Mapk9	-2.31	-1.34	*Mapk1ip1l*	-0.27	1.00	*Mapk14*	0.658	0.410
Braf	-2.18	-0.96	*Mapkbp1*	-0.26	-0.26	*Mapk9*	0.691	0.152
Mapk15	-1.80	-7.86	*Mapk1*	-0.18	0.28	*Mapk8*	0.947	0.606
Rtkn2	-1.74	0.33	*Rras2*	-0.09	0.23	*Rras2*	1.643	-1.064
Mras	-1.53	-0.80	*Kras*	-0.01	-0.45	*Diras1*	1.731	1.566
Mapk9	-1.47	-4.52	*Mapk7*	-0.01	0.33	*Mapkapk5*	2.228	1.691
Mapk8ip1	-1.36	-1.00	*Diras2*	-0.01	0.35	*Eras*	2.566	2.076
Hras	-0.72	-1.17	*Mapk6*	0.06	0.63	*Mapk4*	2.738	1.776
Rtkn	-0.63	-0.70	*Mapk6*	0.06	0.11	*Nf1*	3.024	-1.942
						Mapk10	9.353	10.088

Table 2. *Differential expression of genes involved in cell cycle pathway*

GENE	**IMM vs CON**	**DEN vs CON**	**GENE**	**IMM vs CON**	**DEN Vs CON**	**GENE**	**IMM Vs CON**	**DEN vs CON**
Cdk1	-6.97	1.47	*Cdkn2c*	-0.15	0.00	*Cdk11b*	0.51	0.04
Cdkl5	-6.44	2.73	*Cdkn1a*	-0.12	-0.41	*Cdkn2b*	0.64	-9.80
Cdkn1c	-2.82	2.61	*Cdk12*	-0.12	0.35	*Cdkn1c*	0.70	0.41
Cdk10	-1.17	-1.34	*Cdk2ap1*	-0.11	-0.58	*Cdkn2a*	0.71	-0.01
Cdk19	-0.88	-0.62	*Cdkn2aip*	-0.11	-0.30	*Cdk18*	0.80	0.26
Cdkn1a	-0.86	-0.46	*Cdkn2aipnl*	-0.03	-0.20	*Cdk5rap2*	0.82	1.10
Cdkal1	-0.83	-0.65	*Cdk6*	-0.02	0.73	*Cdkn3*	0.84	0.35
Cdk16	-0.76	0.25	*Cdk9*	0.00	-0.40	*Cdk14*	0.93	1.53
Cdk10	-0.70	-0.71	*Cdkl3*	0.00	6.80	*Cdkn2b*	1.01	1.55
Cdk2	-0.56	-0.98	*Cdk12*	0.00	-0.44	*Cdk16*	1.17	-0.09
Cdk11b	-0.51	-0.95	*Cdkl3*	0.02	-0.34	*Cdkal1*	1.41	0.44
Ccnd2	-0.44	1.16	*Cdkl3*	0.12	-0.13	*Cdkl4*	1.43	1.22
Cdk13	-0.39	-0.28	*Cdkl2*	0.15	-0.29	*Cdkl5*	1.62	-6.98
Cdk17	-0.37	-0.28	*Ccndbp1*	0.18	0.02	*Cdk1*	1.88	-7.51
Ccnd3	-0.32	-0.18	*Cdk2ap2*	0.19	0.00	*Ccnd1*	1.88	1.59
Cdk5	-0.30	-0.44	*Cdkn1b*	0.19	0.14	*Ccnd1*	2.38	2.04
Cdk4	-0.29	-0.54	*Cdk5rap3*	0.23	-0.07	*Cdk5rap2*	2.38	-0.40
Cdk20	-0.27	-0.26	*Cdkl2*	0.37	0.36	*Cdkl1*	5.52	5.21
Cdk7	-0.27	-0.57	*Cdkn2a*	0.40	-0.67	*Cdk15*	5.67	0.00
Cdk5rap1	-0.21	-1.13	*Cdk5r1*	0.42	0.30	*Cdk5r2*	5.89	6.36
						Cdk1	7.17	0.00

Table 3. *Differential expression of genes involved in HIPPO pathway*

GENE	IMM vs CON	DEN vs CON
Yap1	-1.18	0.65
Lats2	-0.40	0.59
Yap1	-0.36	0.74
Yap1	-0.06	-0.20
Taz	0.04	-0.39
Lats1	0.14	0.23
Yap1	0.15	-0.89

Table 4. *Differential expression of genes involved in MYC pathway*

GENE	IMM vs CON	DEN vs CON	GENE	IMM vs CON	DEN vs CON
Mycbp2	-11.48	-0.99	*Mgat2*	-0.20	-0.13
Myct1	-6.12	1.03	*Mgat4a*	-0.16	1.86
Max	-3.16	-0.65	*Mga*	-0.09	0.28
Max	-2.97	-0.60	*Bmyc*	-0.08	0.06
Mycn	-2.53	-2.19	*Mgat4e*	0.00	0.27
Mgat5	-1.84	-0.07	*Mycbp2*	0.03	-1.16
Mgat5	-1.84	6.64	*Mgat4b*	0.09	0.63
Mycbp	-1.59	-1.65	*Mycbp2*	0.15	0.61
Hmga1	-1.32	-1.52	*Myc*	0.44	0.60
Mycl	-1.02	0.53	*Myc*	1.46	-0.71
Mgat3	-0.78	0.00	*Mgarp*	1.83	2.20
Mycbpap	-0.74	12.46	*Myc*	2.60	0.81
Mgat1	-0.35	0.47	*Mgam*	3.59	3.60
Max	-0.33	0.00	*Mgat5b*	5.09	5.55
Mycbp2	-0.29	0.32	*Mycbpap*	10.17	9.60
Mycbp2	-0.28	0.26	*Mycbpap*	12.98	-0.36

Table 5. *Differential expression of genes involved in NOTCH pathway*

GENE	IMM vs CON	DEN vs CON	GENE	IMM vs CON	DEN vs CON
Creb1	-10.22	0.36	*Crebbp*	0.22	0.11
Creb3	-1.68	-0.43	*Crebl2*	0.32	0.03
Creb3l2	-1.02	0.40	*Jagn1*	0.43	0.08
Notch3	-0.86	0.19	*Notch2*	0.52	0.41
Ep300	-0.71	0.88	*Crebrf*	0.64	-3.92
Crebl2	-0.69	0.53	*Creb3l4*	0.64	-4.34
Notch4	-0.68	-0.43	*Notch1*	0.66	0.89
Crebrf	-0.65	-0.21	*Creb5*	1.06	1.72
Creb3	-0.43	-2.02	*Jag2*	1.32	1.65
Crebzf	-0.37	-0.11	*Creb1*	1.50	0.55
Notch2	-0.31	0.54	*Creb3l1*	1.66	2.38
Jag1	0.22	0.52			

Table 6. *Differential expression of genes involved in NRF2 pathway*

GENE	IMM vs CON	DEN vs CON
Cul1	-13.26	-0.59
Nfe2l3	-3.51	-4.19
Cul2	-1.66	-0.66
Cacul1	-1.54	-0.48
Cul3	-0.62	-0.62
Cacul1	-0.59	0.25
Nfe2l1	-0.54	-0.20
Cul4a	-0.39	-0.44
Keap1	-0.33	-0.28
Cul2	-0.31	-0.14
Cul3	-0.20	0.11
Cul4a	-0.16	0.62
Nfe2l2	-0.12	-0.30
Cul5	-0.05	0.49
Cul4b	-0.04	-0.08
Cul1	0.01	-0.20
Nrf1	0.07	-9.71
Cul9	0.22	0.62
Nrf1	0.85	0.11
Nfe2	6.09	0.00

Table 7. *Differential expression of genes involved in PI3K pathway*

GENE	IMM vs CON	DEN vs CON	GENE	IMM vs CON	DEN vs CON
Akt3	-4.95	-1.53	*Mtor*	-0.18	-0.21
Pik3c2b	-3.97	0.90	*Aktip*	-0.15	-0.59
Pik3ip1	-3.10	-1.03	*Lamtor2*	-0.15	-0.29
Pik3cb	-2.20	-1.66	*Pik3r5*	-0.10	1.10
Pik3ip1	-2.19	-0.53	*Pik3c2g*	-0.04	0.17
Pik3c2b	-1.64	-0.33	*Akt2*	0.04	0.08
Akt3	-0.98	-1.03	*Pikfyve*	0.05	0.33
Pik3r3	-0.93	-0.16	*Pten*	0.09	0.53
Mtor	-0.86	-0.16	*Lamtor5*	0.20	-0.33
Pik3cd	-0.75	0.09	*Lamtor3*	0.26	-0.26
Pik3r2	-0.66	-0.51	*Lamtor2*	0.34	-0.27
Lamtor4	-0.56	-0.97	*Pik3r1*	0.48	1.07
Pik3ca	-0.53	-0.43	*Lamtor2*	0.48	-1.09
Pik3r4	-0.47	-0.19	*Pik3r1*	0.50	0.65
Akt1	-0.44	-0.36	*Lamtor2*	0.61	-0.25
Pik3cg	-0.33	0.84	*Lamtor2*	0.75	-0.05
Pik3c3	-0.32	-0.28	*Lamtor1*	0.76	0.54
Pik3c2a	-0.28	0.13	*Pik3r6*	1.98	1.55
Pik3r4	-0.28	-0.13	*Pik3ap1*	2.92	3.33
Akt1s1	-0.25	-0.37	*Pik3ap1*	2.95	3.49

Table 8. *Differential expression of genes involved in TGF-β pathway*

GENE	IMM vs CON	DEN vs CON	GENE	IMM vs CON	DEN vs CON
Acvr1	-11.60	-3.72	*Acvr1b*	-0.17	0.08
Acvr1c	-6.05	-3.95	*Smad4*	-0.07	0.28
Tgfbrap1	-5.23	-0.18	*Smad5*	-0.07	0.10
Smad7	-2.64	-1.62	*Acvrl1*	-0.02	-0.13
Smad6	-2.53	-0.75	*Acvr2a*	-0.01	0.09
Acvr1	-2.52	-0.95	*Smad9*	0.00	0.00
Tgfb1	-1.62	-0.23	*Tgfb1i1*	0.18	0.92
Tgfa	-1.54	-1.31	*Smad1*	0.29	0.12
Tgfbr3	-1.20	0.18	*Smad2*	0.40	2.24
Tgfb2	-0.98	0.33	*Smad2*	0.61	0.56
Tgfbr2	-0.91	1.23	*Acvr2b*	0.66	0.63
Acvrl1	-0.84	1.17	*Tgfb3*	0.89	1.33
Smad3	-0.60	-0.24	*Tgfbrap1*	0.91	0.92
Tgfbr1	-0.33	-0.21	*Tgfbr3l*	1.57	0.80
Tgfbr1	-0.22	0.56	*Tgfbi*	2.52	2.90
Tgfb2	-0.20	0.02	*Smad2*	2.89	0.31

Table 9. *Differential expression of genes involved in TP3 pathway*

GENE	IMM vs CON	DEN vs CON
Mdm1	-6.99	-0.39
Tp53i3	-5.71	2.09
Tp53bp1	-0.55	-0.26
Tp53	-0.18	1.73
Mdm2	-0.06	0.29
Tp53bp2	-0.03	0.12
Atm	0.08	0.22
Tp53	0.30	-0.16
Tp53inp1	0.34	1.52
Tp53i11	0.55	0.96
Tp53rk	0.74	0.80
Tp53i3	0.81	-6.24
Tp53	0.93	-2.48
Mdm2	1.06	-0.26
Tp53i13	1.14	1.44
Tp53	1.32	-0.80
Mdm4	1.37	1.01
Tp53inp2	1.64	1.45
Atm	9.98	5.13
Mdm1	12.19	0.00

Table 10. *Differential expression of genes involved in WNT pathway*

GENE	IMM vs CON	DEN vs CON	GENE	IMM vs CON	DEN vs CON
Rnf112	-5.50	-6.04	*Rnft2*	-1.11	-1.35
Rnf32	-4.80	-2.57	*Fzd7*	-1.09	0.92
Rnf225	-4.64	-10.43	*Rnf169*	-1.05	0.02
Rnf182	-4.61	-5.14	*Rnf2*	-0.98	-0.81
Apc	-4.44	2.32	*Rnf215*	0.90	0.78
Apc2	-4.12	0.36	*Rnf165*	0.97	0.51
Rnf186	-3.65	-3.76	*Rnf227*	1.06	1.04
Rnf5	-3.40	-0.77	*Ctnnal1*	1.19	0.75
Rnf149	-3.38	-3.06	*Rnf103*	1.30	3.88
Rnf208	-2.86	-2.16	*Rnf103*	1.30	0.95
Rnf150	-2.82	-1.10	*Rnf113a1*	1.63	1.57
Fzd3	-2.29	-1.13	*Rnf123*	1.94	-0.05
Rnf125	-2.06	-1.03	*Ctnnd2*	2.41	2.56
Fzd10	-1.99	-0.79	*Rnf144b*	2.45	0.00
Rnf212	-1.94	-0.38	*Rnf25*	2.45	-0.25
Rnf157	-1.84	-0.61	*Rnf128*	2.47	1.35
Rnf32	-1.78	-5.34	*Fzd1*	2.49	2.76
Rnf24	-1.57	-1.17	*Fzd4*	2.51	2.64
Rnf216	-1.32	-0.48	*Rnf144a*	2.77	3.19
Fzd9	-1.27	0.60	*Rnf157*	2.91	3.71
Rnf34	-1.16	-1.05	*Apc*	3.81	-0.30
Ctnnbip1	-1.14	-1.33	*Rnf183*	4.08	3.49
Rnf128	-1.11	2.30	*Ctnna2*	7.26	5.72
			Rnf222	8.38	7.76

2.4 DISCUSSION

The concept of active immunization to study the role of a particular protein in tumorigenesis was demonstrated previously. Immunological neutralization of hCG resulted in the reversal of tumorigenic effects caused by overexpression of this hormone (28). Increased levels of mRNA transcripts associated with hCG induced pituitary tumorigenesis were reversed by immunization mediated ablation of this hormone (28). Immunization of rats with IgG generated against soluble 53 KDa protein resulted in modified splenic responses to carcinogen and the same effect was observed in the offspring when immunized maternally (29, 30). Similar transplacental effects were observed for mammary tumorigenesis (31). Hence, use of active immunization protocols can be an effective tool to study the tumorigenic potential of specific proteins. In this study, we conducted active immunization in rats against SPAG11A protein to study the susceptibility of caput to low dose carcinogen. The absence of any histopathological changes in the caput of DEN only treated rats suggested the innate resistance of this organ for chemically induced tumorigenesis. However, such resistance was lost upon immunization with SPAG11A, indicating a potential role for this protein in contributing to the innate resistance towards tumorigenesis in the caput. Though studies that are directed at determining the tumour suppressor functions of epididymal proteins were not conducted till now, such reports for prostatic and testicular tumorigenesis are reported. Misexpression of cyclin D1 (*Ccnd1*) led to reduced testicular teratoma incidence and suppressed pluripotency abnormalities associated with tumour initiation (32). Similarly, knock down of testicular nuclear receptor 4 (*Tr4*) promoted prostate tumorigenesis by modulating DNA damage / repair system (33). Testicular dysgenesis and haemorrhagic and metastatic tumour formation was evident in conditional knockout mice that lacked *Smad4* or *Smad1* or *Smad5* in the Leydig and Sertoli cells (34, 35). Spontaneous testicular tumorigenesis was evident in xeroderma pigmentosum group A gene (*Xpa*)-deficient mice (36). Testicular atrophy and higher incidence of B-cell lymphomas were observed in WWOX hypomorphic mice (37). Tumour formation was increased in the testis in Nbn heterozygous mice (38).

Cell proliferation is tightly regulated by a variety of signaling pathways and any perturbation in the expression pattern of the components of these pathways leads to uncontrolled cellular growth. A strict balance has to be made between proliferation and cell death by apoptosis. Tumorigenesis arises due to disruption of cellular, biochemical and molecular processes induced by signals of diverse nature. Modifications in the genetic sequence to result in either over or under expression of the signaling components of cell proliferation pathways are considered to be the most important contributing factor for tumorigenesis. In this study, we observed that SPAG11A immunization promoted DEN induced pathological changes that are indicative of tumorigenesis. It is possible that ablation of SPAG11A could alter the microenvironment in the caput that is conducive for tumorigenesis. A recent land mark study indicated that almost 90 percent of the 33 cancer types in humans had at least one alteration in one or more of ten signaling pathways (Hippo, Myc, Notch, Nrf2, PI-3-Kinase/Akt, RTK-RAS, TGFb, p53, cell cycle (Cdk mediated) and b-catenin/Wnt). It is possible that SPAG11A ablation may affect the expression pattern of the signaling components of one or more these pathways to favour tumorigenesis. Hence, we analyzed the expression pattern of the signaling components in the caput of SPAG11A immunized and DEN only treated groups. We observed that the differential expression observed in the caput of SPAG11A for a number of genes that code for the signaling components in these pathways is similar to that observed in the DEN only treated rats. This significant observation indicates that absence of SPAG11A in the caput may derail the expression pattern of molecules that play a crucial role in cell proliferation. This observation is in line with our previous report that siRNA mediated ablation of Spag11a mRNA resulted in increased cell proliferation and alteration in the expression of genes that govern epithelial cell proliferation. Modulation of tumorigenesis and disruption in the associated gene expression due to ablation of a specific protein by active immunization was reported earlier. In hCG immunized mice, reversal of the heightened expression of transcripts associated with pituitary tumorigenesis was observed (28). Vaccination against soluble 53 kDa tumor-associated antigen (s53 TAA) resulted in suppression of p53 gene expression thereby resulting in reduced dimethylbenz(a)antracene induced mammary tumorigenesis (31). Maternal vaccination with anti s-53 IgG enhanced the expression of Fas and FasL to reduce splenic tumors in the offspring (29). Though previous studies have reported the effect of immunization on conferring resistance to tumorigenesis, there are no reports on the possibility of susceptibility due to ablation of a specific protein by active immunization. However, some reports have demonstrated the induction of tumorigenesis in the testis and prostate of knock out animal models (32-38), such studies on the susceptibility of epididymis to carcinogen induced tumorigenesis under conditions of transcript or protein ablation are not yet conducted. Thus, our study assumes significance in reporting the possibility of a caput specific protein to have a role in conferring resistance to tumorigenesis of the epididymis.

In conclusion, we report that low doses of DEN treatment did not induce any significant anatomical changes in the caput of control rats, while DEN induced tumorigenesis was favoured in the caput of SPAG11A immunized rats. The differential expression of signaling components (involved in ten pathways that of paramount importance in cancers) in the SPAG11A immunized rats was similar to that observed in the DEN only treated rats. These results suggest that ablation of SPAG11A protein favours tumorigenesis and this could be due to alterations in the expression pattern of factors that control cell proliferation. To the best of our knowledge, this is the first report to demonstrate a role for a caput specific protein in conferring resistance to cancers of the epididymis.

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Chapter 3

Study the role of Spag11a in sperm function and fertility using transgenic and knock out animal models

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3.1 INTRODUCTON

Sperm maturation occurs in the epididymis; a highly specialized convoluted structure divided into three regions namely caput, cauda and epididymis. In depth analyses using high resolution microscopic and genomic analyses, the epididymis is divided into 10 and 19 different regions in the mouse and rat respectively (1). Besides the anatomical differentiation, the epididymis is specialized in the fact that there exists a differential gene and protein expression pattern all along this organ, thus creating a varying epididymal luminal mileu throughout (2-4). Because of the distinct gene expression pattern in these three regions, a wide variety of proteins are produced into the lumen, which are then attached to sperm surface to aid in maturation (5). The nature of the proteins and the molecular mechanisms that underlie during sperm maturation has been an active area of research in the last two decades. It is well known that a wide variety of epididymis specific proteins secreted into the epididymal lumen contribute to sperm maturation (6). The influence of a wide variety of epididymal proteins belonging to different families on sperm function is reviewed by Björkgren et al (7). These include β-defensins (DEFB), cysteine rich secretory proteins (CRISP), lipocalins (LCN) and epididymal proteins modifying sperm proteins. We previously identified and characterized the role of lysozyme-like (LYZL) and prostate and testis expressed (PATE) proteins in sperm function and fecundity (8-11). Among the diverse variety of proteins that are implicated in sperm maturation, proteins / peptides belonging to the Sperm Associated Antigen 11 (SPAG11) family have been demonstrated to aid in this process (17). Further, localization of sperm associated antigen 11 (SPAG11) proteins in the male reproductive tract tissues and on sperm of different species provides evidence on the role of these proteins in male reproductive physiology (12-16).

The SPAG11 gene in humans is located on chromosome 8p23.1 and encodes many protein isoforms due to alternate splicing (12). On the contrary, the rat counterpart proteins are encoded by distinct genes. The mouse *Spag11a* gene is a caput specific gene that is primarily expressed in the principal cells in an androgen dependent manner (22). We and others have identified three rat *Spag11* genes, namely *Spag11c*, *Spag11t* and *Spag11a* (also known as Bin1b or Spag11e) (13, 15). Some of these proteins have structural and functional similarities to β-defensins. We previously demonstrated that the human and rat SPAG11 proteins are β-defensin-like and exhibit antimicrobial activities and that their expression in response to endotoxin or uropathogenic *E. coli* challenge involves NF-kB signaling and epigenetic modifications (18-20, 23-26). The expression of *Spag11a* was decreased in a lipopolysaccharide induced model of rat epididimytis, suggesting a role for this protein in epididymal innate immunity (27). On the same lines, the *Spag11a* transgenic mice showed enhanced resistance against bacterial infection in the epididymis (28). These studies have demonstrated the role of SPAG11 proteins in epididymal innate immunity as well.

The proteins encoded by *Spag11* genes are found to be localized on the sperm, thus implicating them in sperm function as well (13, 15). The binding of rat SPAG11A to the sperm surface during the epididymal transit to induce sperm motility was demonstrated *in vitro* (16). However, studies that implicate a direct role for SPAG11A in sperm function and fecundity are not yet understood. Hence, in this study, we generated transgenic and active immunized animal models to ablate *Spag11a* mRNA and SPAG11A protein respectively. Further, there are no reports on the role of this gene under conditions of complete absence of expression i.e. using knock out models. Thus, in this study we used *Spag11a*$^{-/-}$ mice to determine the importance of this gene in fecundity and sperm function.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Wistar rats (*Rattus norvegicus*) were obtained from National Animal Resource Facility for Biological Research, Hyderabad. Animals were kept in a hygienic condition with suitable humidity and had free access to food and water. The animals were housed in groups of maximum three animals in each cage. All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of University of Hyderabad (IAEC/UH/151/2017/01/SY/P12; IAEC/UH/151/2017/01/SY/P13).

3.2.2 Generation of transgenic rats

Transgenic rats that express shRNA against *Spag11a* mRNA were generated using testicular electroporation method as described previously (30, 31) with slight modifications. Four pGFP-VRS plasmids (A, B, C and D) that express shRNAs under the influence of an U6 promoter to target rat *Spag11a* mRNA were obtained commercially (OriGene technologies, Rockville, USA; Cat No: TG713093). Sequences of the four shRNAs are provided in **Table 1**. As a control, plasmid containing a sequence that expresses a non-specific shRNA (scrambled; SCR; Cat No: TR30013) was also included. Prior to use for animal transgenesis, the ability of the four plasmids to ablate *Spag11a* expression was assessed *in vitro*. Rat caput epididymal primary epithelial cells were transfected with a mixture of 2 µg of each of shRNA expressing plasmids or the SCR expressing plasmid and 2 µl reconstituted in 5 ml of transfection medium in a 60 mm culture plate and incubated for 48 h. RNA was isolated and reverse transcribed followed by real-time PCR to determine the mRNA levels of *Spag11a*.

Male Wistar rats (aged 40 days) were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (45 mg/kg) and xylazine hydrochloride (8 mg/kg). A mixture of all the four linearized plasmid DNAs in equal proportion reconstituted (30 µg in 30 µl) or the plasmid that expresses SCR shRNA (30 µg in 30 µl) was injected slowly into each testis at multiple sites. Trypan blue (0.04%) was used in the injection to monitor the accuracy of the injection and to ensure that the solution reached the tissue. The testes were then subjected to electroporation by applying electric pulses using BTX ECM 830 Electroporation System. Each pulse was of 0.05 second with an inter pulse interval of ~1 second delivered by an electric pulse generator while holding the injected testis in between tweezer-type electrodes. One month after subjecting to electroporation, the rats were allowed to mate with at least two wild type female rats.

To assess the presence of the integrated plasmid in the pups, genotyping was carried out. DNA was isolated from a tail piece of the pups and subjected to PCR using plasmid specific primers (**Table 2**). An amplicon of 300 bp is expected in the samples obtained from pups that carry the integrated plasmid in their genomes. Rats (n=3) that had integration of the plasmid were used for further studies. At the end of different experiments, the expression level of *Spag11a* mRNA and protein in the transgenic rats was analyzed in the caput epididymis using gene specific primers (**Table 2**) and anti-SPAG11A antibody (Cat No. SAB1305692; Sigma Aldrich, USA). Data analyses for different parameters was carried out only in rats that showed ablation in the *Spag11a* mRNA and protein expression levels.

Table 1. *Details of shRNAs used to generate transgenic rats and their target regions in Spag11a mRNA*

Name	5’ → 3’ sequence
Spag11a-shRNA-A	AACAGATGCTGCGTATCCAGTTCCATTAA
Spag11a-shRNA-B	CCTGGAATCAGAAACACCGTGTGCTTCAT
Spag11a-shRNA-C	GCCACTGTAGGCTCTTCATGTGCCGTTCT
Spag11a-shRNA-D	TCTGACCCCTGGAACAGATGCTGCGTATC

Table 2. *Details of the PCR primers used in this study.*

Gene	Direction	5’ → 3’ sequence
FOR REAL-TIME PCR		
Spag11a	Forward	GGGGACATACCACCTGGAATC
	Reverse	TGG AAC TGG ATA CGC AGC ATC TG
Gapdh	Forward	CCAATGTATCCGTTGTGGATCTG
	Reverse	GAGTTGCTGTTGAAGTCACAG
FOR GENOTYPING		
Spag11a-shRNA / SCR-shRNA	Forward	GAGGGCCTATTTCCCATGAT
	Reverse	CCTCGGCCTCTGCATAAATA

3.2.3 Recombinant protein production

Plasmid containing rat *Spag11a* coding region was transformed into *E. coli* BL21 and the transformed *E. coli* were grown to mid-log phase and fusion protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactoside for 1 h at 37 C. To avoid baseline expression of the protein prior to induction, 1% glucose was maintained in the bacterial medium and the induction time was kept to a minimum (1 h) to minimize the toxic effects of the peptides on *E. coli*. (20). Recombinant protein was purified using Ni-NTA agarose affinity purification system as per the manufacturer's instructions (Qiagen, Valencia, CA, USA). The purified recombinant protein with His tag at the N-terminus was confirmed by Western blotting using anti-His tag antibody (Santa Cruz Biotechnology, Dallas, USA. The fractions that contained the protein of interest were dialyzed extensively at 4°C against 10 mM sodium phosphate buffer, pH 7.4.

3.2.4 Active immunization rat model

Generation of auto antibodies by immunization was carried out as described previously (11). Adult (aged 90 days) male rats were administered subcutaneously with a mixture of 100 µg of SPAG11A recombinant protein in 300 µl of PBS and 300 µL of Freund's complete adjuvant. Subsequent booster doses containing 100 µg of the recombinant protein in 300 µl PBS mixed with 300 µl Freund's incomplete adjuvant were administered every two weeks after first immunization. Control animals (unimmunized) received Freund's adjuvant without recombinant protein similar to the immunized animals.

The antibody titer in serum and fluids of caput, cauda and testis obtained from control (unimmunized) and SPAG11A immunized rats were determined by ELISA as previously described (11).

3.2.5 Generation of *Spag11a*$^{-/-}$ mice

Spag11a$^{-/-}$ mice (**C57BL/6NJNcbs-*Spag11a*^{em2MGEF}**) were commercially obtained from Mouse Genome Engineering Facility, National Centre for Biological Sciences, Bangalore, India. They were generated using CPF1 (Cas12a / sgRNA) system. The strategy used to generate these mice is provided in **figure 1**. Briefly, the mouse *Spag11a* gene (NM_153115.1) was targeted by specific guide RNAs to facilitate the excision of the region between 712 to 1002 (291 bp) that encompasses a part of exon 1 and intron 1 **(figure 1)**. The 5' → 3'sequence of the guide RNAs used are: sgRNA#18 (on minus strand): TTT(PAM) CATGTTTTACGGCTCGCTCTCTGT; and sgRNA#20 (on plus strand): TTT(PAM) ACAGCACAAGACATAGAGGCCTCT. The 5' → 3' genomic sequences targeted by sgRNA#18 and sgRNA#20 are ACAGAGAGCGAGCCGTAAAACAT and ACAGCACAAGACATAGAGGCCTCT respectively. Pronuclear microinjection solution containing CPF1 (Cas12a protein; (40 ng/ul final)), sgRNA#18 (10 ng/ul final) and sgRNA#20 (10 ng/ul final) were injected into B6/NJNcbs mice zygotes. Resulting founders were back crossed twice with B6/NJNcbs wild type mice. The animals resulting from the two successive B6/NJNcbs backcrosses were heterozygote *Spag11a*$^{+/-}$ N2 mice. *Spag11a* exon1 deleted homozygote (Spag11a$^{-/-}$) KO animals were obtained by crossing *Spag11a*$^{+/-}$ N2 with *Spag11a*$^{+/-}$ N2. Genotyping was performed with the DNA isolated from the tail regions of both male and female mice. PCR primers that flank on either sides of the targeted region were designed for genotyping (**figure 2**). The PCR amplicon sizes for the wild type and *Spag11a*$^{-/-}$ mice using these primers were 392 and 101 bp respectively. The PCR amplicons were sequenced to confirm the sequence that was deleted in the *Spag11a* gene. *Spag11a*$^{-/-}$ mice used in this study were age matched and were obtained from the same founder. The *Spag11a*$^{-/-}$ mice were generated under the NCBS-IAEC approved project #AJ-1/2015(R1-E). Animal experiments conducted for this study were approved by the University of Hyderabad Institutional Ethics Committee (UH/IAEC/SY/2021-1/20).

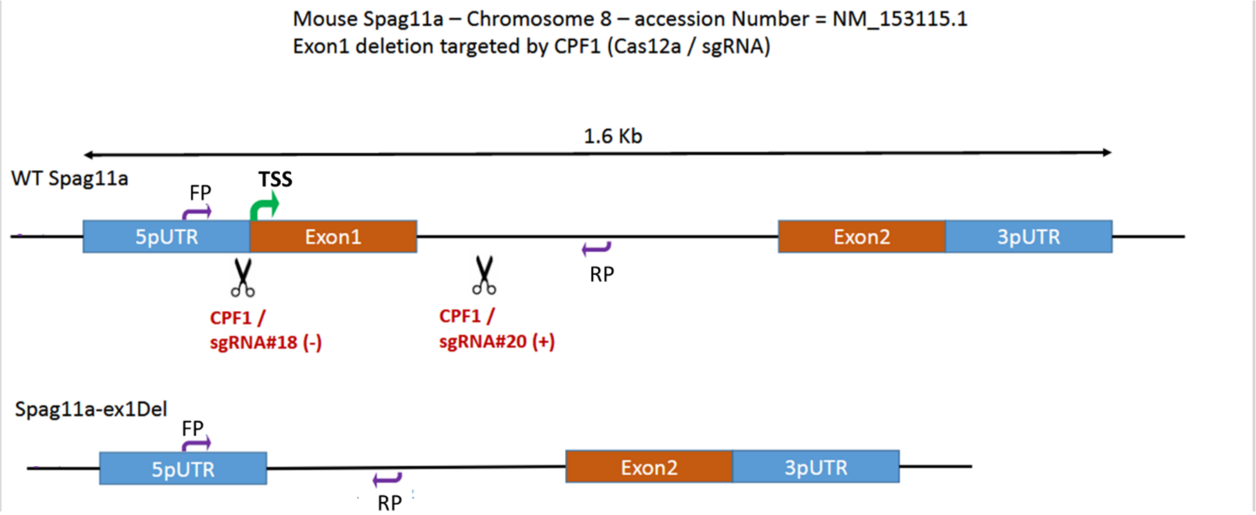

Figure 1. *Strategy to generate Spag11a*$^{-/-}$ *mice. Upper panel shows the gene structure of mouse Spag11a (5’UTR, exon1, intron, exon2 and 3’UTR). Scissors indicate the targeted regions to generate the knockout mice. sgRNA#18 and sgRNA20 were used to target the minus and plus strands. FP and RP are genotyping primers. Lower panel indicates the structure of exon1 deleted Spag11a gene mediated by CPF1-sgRNA#18-sgRNA20.*

Figure 2

AGACGTGCCTTCCTCTTCCATTGTGGACAACATTCTGGCTTCTGACATGTTGGTTTTGCAAATCTGTGAG
TTTGGCGTGTTCTAGCTGGCCTTCTGCCGACTTTCCATTCCTGGGGTGTGGCAGTCTTCGTCATGTTTTC
CTGTTCTCATTGCTGTGATGGAGATTACTGGTTTGTTTCATGGTGTTGTAATGTGCCACTCACATTTTGA
CTAGAGACTATGATGAGTAACACTACCCTGTAGGTTCTTTTACACCCTTTTAGGTTCACACGGGACTTTG
TTTCTTTGGGCAGATATCTGGGAGTGGCTTCTGGTCACAGGTGTTCTCAACTGAGGTGGATCCTGGCCAG
TTGAATACTTTGAAAGCCAAGGATGCATCTCTACTGAGTTGAGTCTCTGTCACCTACCATCTTACCACAG
CACCAGGGCTTGGTGCAATGTAGCCAGTTGCCAATGTCCCCAGTGGCAATTTCTTTCTTTTCAGGTTTCA
CATGCTACAGTGGCCAATTTCTGCCTCTGGGGAATGTCAGAATGCCTGTCTATTTTTAGGTAAAAAAGAG
AGAGTAGCTGGTACCATCTGCCCACAGCCTAACTCAGTAGAGCAGATAAATATACTCATTCCACAGTAGC
FP for genotyping
ATTCTCCAAAACTATAAAGGTCTCCTGTTTGTCCTTTCAAACCTGGATTAACTCAGTCATCAGTCACACC
sgRNA#18 target
TGCTTTCCTGC**ACAGAGAGCGAGCCGTAAAACAT**GAAAGTCTTGTTACTCTTTGCTGTCTTCTTCTGCTT
TGTCCAAGGAAACTCAGGTAAATGTCTTCTGAGCAGCCCTGGAGAAGGCAGGACCCTTTTTCAGTTTGTA
GACAGCACTGAGGTGTGTCCAGGTATAAGCGTCTGGCACACAGAGTTGGACCACTCTGAGGCTCAGGGCT
GGACCACTTTCATAATGAAGAGGTTTTTTGTTTGTTTGTTTGTTTGTTTGGTGAGAGTTGTCAATTTT**AC**
AGCACAAGACATAGAGGCCTCTTTTCTCCAAGACAAATAGAAAGACCTGATTGCAATCTGAGAGTTTATT
sgRNA#20 target

CAGGACAGAATTACAGCCACACATGTGTCAGAGCGGTAGCTGTGGACAAAGGGTAAGATTACTGCCACGC
RP for genotyping
TGTGAGCAGAGAGGGTATTCACATCTAACGGCACTTCCCTAGTTTTTGCATATAGCCTATTATATCCACA
AAACCCCAGATGTACCCTTTAAATGAATTTTCAGATGAGAGAAAATGGGTTACCTTAAGAAAGCTAGAGT
CCAGATTCTGAAACCTTTGAATTATTGGCTCTTCGGTCAAGGTGGACTACCCCCACATCGCCATGTCCTC
TCCAAACCATGGTAGACGTGATGAACACAGTATCATAAATCAGTCCCTGTCTGAAGTCTGGCTATTGCAG
GAGACTATTTTTCTAAACATGTCAGGCATACATTATGTTGCTGTTGCTTTTAAACACAGAGTGTGTTTAA
AAAGCCTCTTTGAGGAAGCTCCCCCACCCATCCACCCACTCCCAGCCTAGCCTACTGCCCTGGTATTACC
CTCTACTGGGCCTTCTTAGAAGGGTGAAAGGCCTCTTTTTTTCACCGTCTCTTTCCTACAGTGCTTTCTT
TCTGGAGACTCTAGTTGGTTATATTTTTACCTAGAAGGCAATACAAGAGACATTTTAATTTTTCTTCTTT
TTAATTGTAGTAAGAGTGAAAAATCTGGCCACAAGCTGAGAGGCCTACCCAGTCCTGATTTTTTTACAAC
CTGAGACCTTCCTCCTGTTATTCTGTTTTCTTAAATGCCATGCTCCCATCCCCACAGTATACTTCTGTCT
CCAGGCAGCTGAAGGACTCTGCTGGACCAGGGCCTACAGTGGGCCAGGTGATTCCACACACAGCCTGTCA
TGCCCTCCTGGAAACACCACGAGATATAAATGATTGCTATTGAAAGCTAAGGACTAAAAACTCAGTGGCA
CTGAGACCAAGCTTTGTTAAGAGTGGAGCAGATTCTTCACTGAGGCCTGGAGTCTCTGTTTATCTCCCTC
TGTTGTATCCACAGGGGACATCCCACCCGGAATCAGAAACACCGTGTGCCTCATGCAACAGGGCCACTGC
AGGCTCTTCATGTGCCGTTCTGGTGAGAGAAAAGGGGATATCTGCTCCGACCCCTGGAACAGATGCTGCG
TCCCCTATTCTGTTAAAGACAGAAGATAGAAGACTGAGGTTGGGAGATCTGGAATGTGGGGCGCAAGCAA
CTGAAGGGTGGAGACTTGGAAATGCCCCAAAGGCTTTGAGTATGTGTGGCTGACGGTGCATACTCAATAA
ACATTTGCTAAATAAATCTATCACCGAGGCAAAGGTCCGGTGAAAGGATTTTATGGAAAAAGGTGTTAAT
TTGCTATTTATCTACTTTGTCTTTTCTCTGTCACAGGTAATGTCTCTTACATACTCAGGAATAGGGTAAG
CAGAAACCAAGCTCGGCTGTAAACGCTGTTTGCAAAGCGACAGGAATGGCTGACTCACTGGCTGTGTGTC
CTCTATGGCGTTCAGCACATCAAAGCGGACATTGGCTTCTCTCCTCTTGCTGAGGCCTCCAGGGCTCCTG
CTCCATTTCCAGAGCCTTCTCACACTGTCACACCAAAACCTGAGTAGAACTTTGTAGCCTTTCAGCATGG
TGACCTTGTTGTCTTATAGAGAATATAGTAAAGGTTCAAACATGGCCA

Features in this sequence: INTRON; EXON; CDS; **Guide RNA targeted sequence**; **Genotyping primers**

Region targeted 712 to 1002 = 291 bp; Wild type amplicon size: 392; Knockout amplicon size: 101 bp

Figure 2. *Gene sequence of mouse Spag11a. Exon 1 and Exon 2 sequences are indicated in red colored font, while the red colored and underlined sequence is the CDS. Intronic, 5'UTR and 3'UTR sequences are in black colored font. Yellow highlighted sequences are the targets for the guide RNAs. The 291 bp region targeted for deletion (712 to 1002) lies within the yellow highlighted sequences. Grey highlighted sequences are identified to design forward primer (FP) and reverse primer (RP) for genotyping. The expected amplicon size for wild type and knockout is 302 and 101 bp respectively.*

3.2.6 Assessment of fecundity

Fecundity was analyzed by natural mating. Each male rat was cohabited for one week with two normal female rats and the litter size noted. Rats that had serum antibody titer were used for mating. One week prior to mating, a booster dose of the antigen was given. Unimmunized rats were used as control. In the case of transgenic rats, animals that were subjected to electroporation (F0 generation) were subjected to mating. Pups (F1 generation) born to rats of F0 generation were screened and those which displayed the integration of the plasmid were subjected to mating. The same was repeated for pups of F2 and F3 generations. However, litter size was taken for data analyses only in those rats wherein ablation of *Spagl1a* expression was observed.

3.2.7 Assessment of capacitation, acrosome reaction and intracellular calcium

Capacitation and acrosome reaction was measured by flow cytometry as described previously (9, 10). Cauda epididymides were minced in 10 ml of M2 medium (Sigma Aldrich, USA) and the spermatozoa collected by centrifugation at 850 g. Capacitation was assessed by measuring the fluorescence intensity of filipin, a cholesterol specific binding dye (Cat no. F9765, Sigma Aldrich, USA). Filipin binds to the cholesterol moieties of spermatozoa and its fluorescence intensity decreases during capacitation due to loss of cholesterol from the membrane (32). Spermatozoa were incubated with 25 µM filipin for 1 hr and thoroughly washed to remove the unbound dye. The filipin bound spermatozoa were then allowed to undergo capacitation in M2 medium for 4 hr. Fluorescence intensity was measured in the capacitated sperm in a flow cytometer using the excitation and emission wavelengths at 340 and 425 nm respectively.

The binding of FITC labelled PNA (PNA-FITC; Sigma Aldrich, USA) to the glycosylated proteins of acrosomal region was quantitated by flow cytometry to assess acrosome reaction. Decreased binding of PNA-FITC due to loss of acrosomal proteins during acrosome reaction results in decreased fluorescence intensity of FITC. Spermatozoa were treated with ionophore A23187 (10 μM) followed by washing with PBS and incubation with PNA-FITC (50 μg/ml) to stain the acrosomal region. Fluorescence intensity was measured in a flow cytometer with excitation and emission at 495 and 515 nm respectively. The intracellular calcium concentration ([Ca^{2+}]i) during capacitation and acrosome reaction was measured by monitoring the fluorescence intensity of the calcium binding dye Fluo-3-AM. Spermatozoa were loaded with Fluo 3-AM (10 mM) at 37°C for 20 min and allowed to capacitate in M2 medium. They were then washed twice with PBS at 500 g for 5 min to remove any free Fluo 3-AM. The fluorescence intensity of Fluo 3-AM, which is a direct function of [Ca^{2+}]i content was analyzed by flow cytometry with excitation and emission wavelengths at 488 nm and 515–540 nm respectively. For flow cytrometric analyses, the fluorescence intensity data and the forward scatter (FSC) were collected in logarithmic mode and the light scattering in the side scatter (SSC) was collected in linear mode. The fluorescence intensity was quantified for 10,000 individual sperm cells in a flow cytometer (BD Biosciences, San Jose, USA) and expressed as mean fluorescence intensity.

3.2.8 RT-PCR

Using commercially available kits (Qiagen), RNA was isolated to generate cDNA. *Spag11a* gene specific primers were designed and the PCR was conducted with the following conditions: 94°C for 1 min followed by 30-32 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. PCR amplicons were analyzed by electrophoresing them on 2% agarose gels. Real-time PCR was carried out using SYBR master mix kit (Applied Biosystems, Warrington, UK) in a thermal cycler (Applied Biosystems) using standard conditions. *Gapdh* expression was also determined to serve as internal control.

3.2.9 Western blotting

Tissues were homogenized on ice with RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS) supplemented with proteinase cocktail inhibitors. Protein content was estimated using commercially available kits. One hundred micrograms of each of the protein samples were electrophoresed on SDS-polyacrylamide gel and transferred onto a 0.2 μm pore size nitrocellulose membrane (Millipore). 5% non-fat milk prepared in 0.1 % Tween containing Tris-buffered saline (TBS-T) was used to block the nonspecific binding sites. To detect SPAG11A, the membranes were incubated with primary antibodies specific to rat SPAG11A (Sigma Aldrich, Catalog # SAB4200676) at 4 °C overnight and washed three times with TBS-T. Incubation with anti-mouse secondary antibody conjugated with HRP was carried out for 1 h at room temperature and washed three times with TBS-T for 10 min. ECL prime Western blot detection kit (GE Healthcare Life Sciences, MA, USA) was used to detect the Immunoreactive proteins. Beta-actin expression was used as the internal control.

3.2.10 Histopathology

Caput, cauda, testes and prostate from wild type and *Spag11a*$^{-/-}$ mice were soaked in Bouin's fluid for 24 h and extensively washed with 70% ethanol to remove the yellow color. Dehydration was carried out by placing them sequentially in serially graded ethanol (80, 90 and 100%) and final processing in isopropanol overnight at 60°C. Tissues were then embedded in paraffin wax and five micron sections were made for histological staining. Deparaffinization of sections was carried out by placing them in xylene followed by rehydration in graded ethanol (100, 90, 80, 70 and 50 %). Harris hematoxylin solution was added to the sections for 10 min to enable staining. They were further processed by distilled water washing, differentiation in 1% hydrochloric acid for 30 sec and immersion in 0.2% ammonia water for 1 min. After thoroughly washing with distilled water, counterstaining was carried out with 0.2% eosin Y solution for 1 min. Dehydration by placing them serially in 50, 70, 80, 90 and 100% alcohol with a final wash with 3 changes of xylene was carried out. They were then mounted using xylene based mounting medium. Capturing of the images and evaluation was conducted by a board-certified histopathologist who had no information on the sample background.

3.3 RESULTS

PART – I. Effect of ablation of Spag11a mRNA and SPAG11A protein by transgenesis and active immunization respectively.

3.3.1 Generation of transgenic rats

As an initial step, the ability of the four pGFP-VRS plasmids to ablate *Spag11a* mRNA levels was analyzed *in vitro*. Rat caput epididymal epithelial cells were transfected with a mixture of the four plasmids that express Spag11a mRNA targeting shRNA. *Spag11a* mRNA levels were significantly ablated in cells transfected with the plasmids that express shRNA that targets *Spag11a* mRNA (**Figure 3A**). These observations provide evidence that the shRNA expressed by the plasmids can target *Spag11a* mRNA and thereby cause a decrease in its levels.

After confirming the potency and specificity of the expression plasmids, we generated transgenic rats by testicular electroporation. To determine the integration of the plasmid into the genome, genotyping was carried by amplifying the region of the plasmid (300 bp) which encompasses the sequence that encodes for shRNA using DNA isolated from the tail piece of the pups. Genomic PCR with the samples obtained from pups (F1 generation) born to rats injected with a mixture of the plasmids (F0 generation) revealed the amplification of a 300 bp amplicon (**Figure 3B**), suggesting the integration of the plasmid into the genome. Similar observations were made in pups born to F0 generation rats injected with the plasmid encoding the SCR shRNA (**Figure 3B**). Genotyping of the pups born to F1 generation rats revealed the integration of the plasmid in some of the pups in F2 generation and similar results were observed in the F3 and F4 generation rats. Transgenic rats that had an integration of the plasmid encoding *Spag11a* mRNA targeting shRNA are designated as *Spag11a*-shRNA and those that had an integration for non-specific SCR shRNA are designated as SCR-shRNA. The effect of integration on the mRNA levels of *Spag11a* was analyzed in the caput epididymis by real-time PCR. A significant decrease was observed in the some of the *Spag11a*-shRNA transgenic rats when compared to the wild type control (**Figure 3C**). The mRNA levels of *Spag11a* was similar to the wild type rats in the SCR-shRNA transgenic rats, indicating that ablation of *Spag11a* mRNA was not non-specific (**Figure 3C**). Concomitant with mRNA levels, SPAG11A protein levels were also decreased in the *Spag11a*-shRNA transgenic rats and not in SCR-shRNA rats (Figure 1D). Since the SCR-shRNA transgenic rats did not show any significant changes in the *Spag11a* mRNA levels, they were not included in further studies to reduce animal usage. All comparisons were made with the wild type group. *Spag11a* expression is caput specific and access to this tissue was possible only after sacrificing the animals. Hence, we included all animals that had integration of the plasmid in our experiments. The expression level of *Spag11a* mRNA and protein was analyzed in all the rats at the end of each experiment. However, only those *Spag11a*-shRNA transgenic rats that displayed a significant reduction in the mRNA levels of *Spag11a* were considered for data analyses.

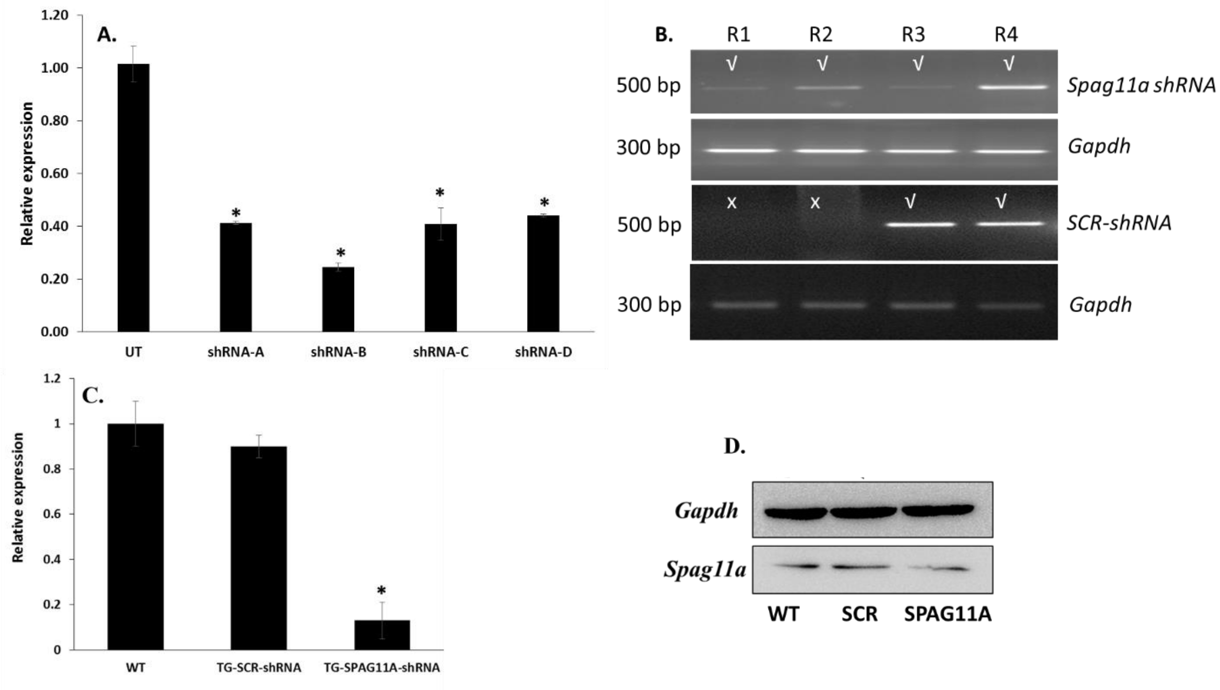

Figure 3. *Validation of the transgenic animal model.* ***(A)*** *Expression plasmid validation in vitro. Real time PCR was carried out to determine Spag11a mRNA levels using RNA obtained from rat primary caput epididymal cells transfected with 2 µg expression plasmid that encode shRNA to target Spag11a mRNA.* ***(B)*** *Genotyping of transgenic rats. DNA isolated from the tail piece of transgenic rats was PCR amplified using plasmid specific primers that span the shRNA coding region. R1 to R4 lanes represent the genomic PCR for four representative transgenic rats. Gapdh mRNA expression was used as internal control. √ - indicates the integration of the plasmid into the genome; X – indicates the absence of integration.* ***(C)*** *Spag11a mRNA expression in transgenic rats. Real time PCR was carried out with RNA isolated from the caput epididymis of transgenic rats that had the integration of the plasmid that encodes shRNA to target Spag11a mRNA (TG-SPAG11A-shRNA or the scrambled shRNA (TG-SCR-shRNA).* ***(D)*** *SPAG11A protein expression in transgenic rats. Whole protein lysates from the epididymides were subjected to Western blotting.*

3.3.2 Generation of SPAG11A immunized rats

Male rats were immunized with recombinant SPAG11A protein to generate auto antibodies and to study the effect of ablation of this protein on reproductive parameters. The antibody titer in the serum of immunized rats was significantly increased and was maintained till the end of the study (**Figure 4).** Since we are interested to determine the effect of ablation of SPAG11A on spermatogenesis and sperm maturation, the presence of antibodies in the epididymis (caput and cauda) and testis was also determined. The antibody titer was found to be significantly higher in all the three tissues analyzed (**Figure 4**).

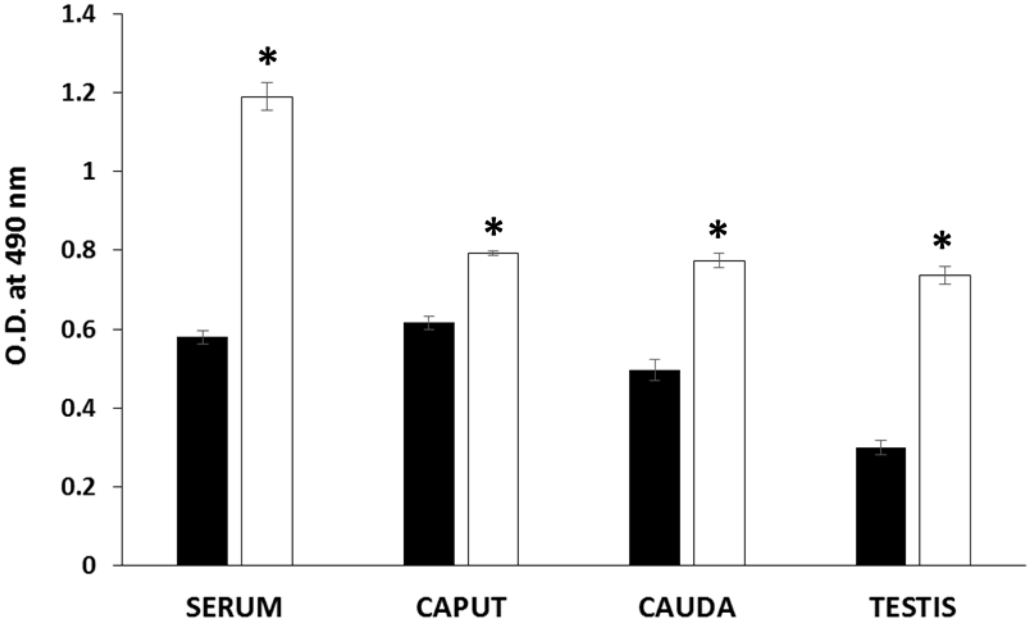

Figure 4. *Validation of active immunization model. Serum, epididymal (caput and cauda) and testicular fluids were collected and the titer of the antibodies against SPAG11A was determined by ELISA. ■ – unimmunized; □- immunized. Values expressed are Mean ± S.E. * indicates p < 0.05 compared to the respective control.*

3.3.3 Effect on fecundity

To determine the role of SPAG11A in reproductive physiology, especially on fecundity, each transgenic or immunized rat was cohabited for a week with at least two females to facilitate mating. For *Spag11a*-shRNA transgenic rats, the litter size was monitored for four generations (F0, F1, F2 and F3). A significant decrease in the litter size was observed in F0 generation rats which were subjected to electroporation (**Figure 5A**). The litter size was also significantly decreased in F1, F2 and F3 generation transgenic rats compared to the corresponding wild-type control (**Figure 5A**). On the other hand, the litter size in SPAG11A immunized rats was also significantly lesser than the control (**Figure 5B**).

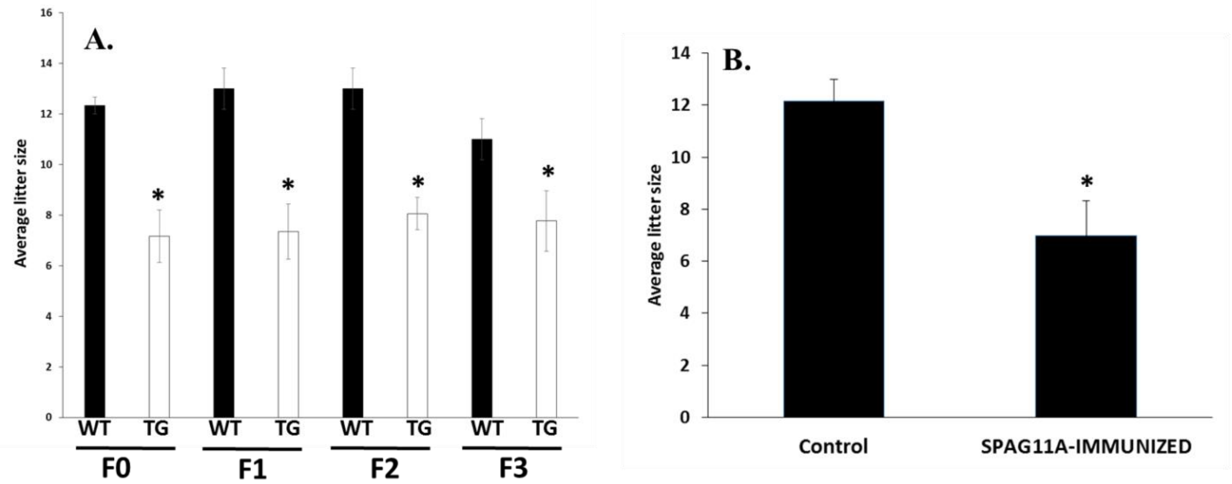

Figure 5. *Litter size in transgenic and immunized rats (n=3). Each rat (WT – wild type; TG – transgenic; Control (unimmunized); SPAG11A-immunized) was cohabited for one week with two female rats to allow mating.* ***(A)*** *Litter size in wild type (WT) and Spag11a-shRNA transgenic (TG) rats.* ***(B)*** *Litter size in control and SPAG11A-immunized rats. Values expressed are Mean ± S.E. * indicates $p < 0.05$ compared to the respective control.*

3.3.4 Effect on sperm count, capacitation and acrosome reaction

Sperm count was marginally decreased in the *Spag11a*-shRNA transgenic rats when compared to the wild type control (**Figure 6A**). However, a significant decrease in the sperm count was observed in SPAG11a immunized rats (**Figure 6B**). The ability of spermatozoa obtained from SPAG11A immunized or *Spag11a*-shRNA transgenic rats to undergo capacitation and acrosome reaction was analyzed *in vitro* using flow cytometry. The fluorescence intensity of cholesterol bound filipin was decreased significantly in the capacitated spermatozoa obtained from wild type / unimmunized rats compared to their uncapacitated counterparts, indicating the occurrence of capacitation (Figure 5A). Spermatozoa obtained from SPAG11A immunized or *Spag11a*-shRNA transgenic rats also displayed a significant decrease in the fluorescence intensity of membrane bound filipin (**Figure 7A**). Acrosome reaction was monitored by measuring the fluorescence intensity of FITC tagged PNA bound to the acrosomal membrane. Decreased fluorescence intensity was evident in acrosome reacted spermatozoa obtained from wild type / unimmunized rats, a trend observed when spermatozoa undergo acrosome reaction (**Figure 7B**). Such a decrease was also observed in the spermatozoa of SPAG11A immunized or

Spag11a-shRNA transgenic rats (**Figure 7B**). Intracellular calcium content ($[Ca^+]i$) in the spermatozoa was estimated by measuring the fluorescence intensity of the calcium binding dye Fluo 3-AM. In the capacitated / acrosome reacted spermatozoa of wild type / control rats, a significant increase in the fluorescence intensity of Fluo 3-AM was observed, suggesting the release of calcium from intracellular stores (**Figure 7C**). Such an increase was also observed in the spermatozoa of SPA11A immunized and *Spag11a*-shRNA transgenic rats (**Figure 7C**). These results provide evidence that capacitation, acrosome reaction and $[Ca^+]i$ release are not affected due to ablation of *Spag11a* mRNA or its protein counterpart.

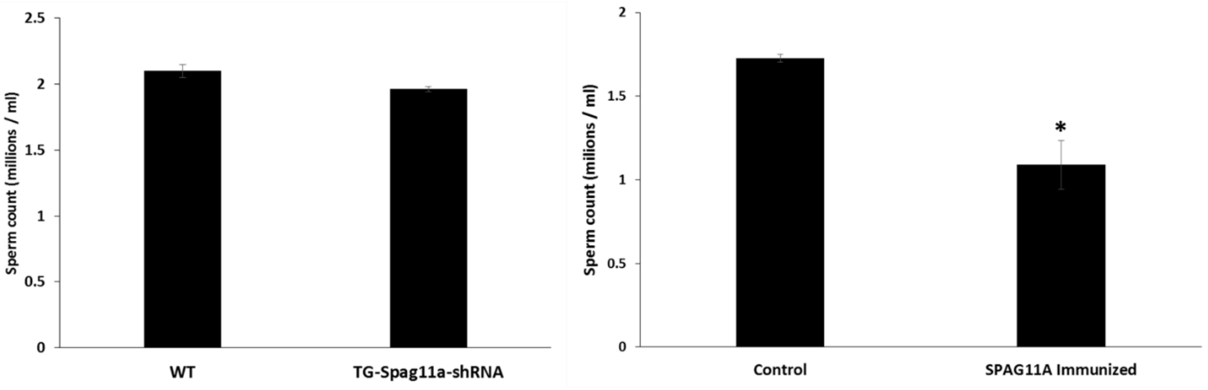

Figure 6. *Sperm count in transgenic and immunized rats (n=3). Spermatozoa obtained from the cauda were counted in a hemocytometer and the average sperm count was determined.* ***(A)*** *Sperm count in wild type (WT) and Spag11a-shRNA transgenic (TG-Spag11a-shRNA) rats.* ***(B)*** *Sperm count in control and SPAG11A-immunized rats. Values expressed are Mean ± S.E. * indicates $p < 0.05$ compared to the respective control.*

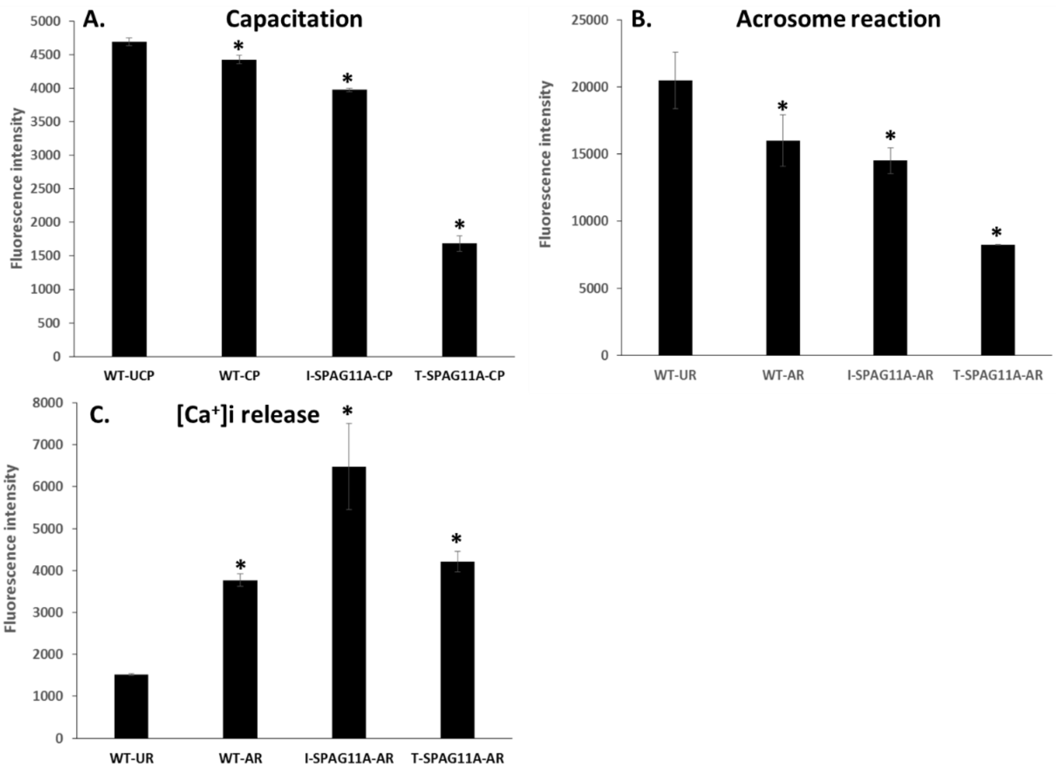

Figure 7. *Evaluation of capacitation, acrosome reaction and intracellular calcium content. Spermatozoa obtained from different groups of rats (n=3) were stained with filipin* ***(A)*** *or PNA-FITC* ***(B)*** *or Fluo 3-AM* ***(C)*** *in vitro to measure the extent of capacitation, acrosome reaction and intracellular calcium release respectively. Fluorescence intensity of the dyes was measured in a flow cytometer. WT-UCP – uncapacitated sperm of wild type; WT-CP – capacitated sperm of wild type; I-SPAG11A-CP – capacitated sperm of SPAG11A immunized rats; T-SPAG11A-CP - capacitated sperm of Spag11a-shRNA transgenic rats. WT-UR – acrosome unreacted sperm of wild type; WT-AR – acrosome reacted sperm of wild type; I-SPAG11A-AR – acrosome reacted sperm of SPAG11A immunized rats; T-SPAG11A-AR – acrosome reacted sperm of Spag11a-shRNA transgenic rats. Values expressed are Mean ± S.E. * indicates $p < 0.05$ compared to the respective control.*

PART – II. Fecundity and sperm function in *Spag11a* knockout mice.

3.3.5 Genotyping and *Spag11a* expression in knockout mice

Using *Spag11a* gene specific primers (designed on either side of the flanking regions of the targeting sequence), genotyping was performed. As shown in **figure 8A**, genomic PCR with DNA from wild type animals resulted in the generation of 392 bp amplicon, while the same was 101 bp in the *Spag11a*$^{-/-}$ mice. Two PCR amplicons with sizes 392 bp and 101 bp were observed with DNA isolated from the heterozygous mice, i.e. *Spag11a*$^{+/-}$ mice. To further confirm whether the expression of *Spag11a* is absent in *Spag11a*$^{-/-}$ mice, RT-PCR was performed with RNA isolated from caput of six sets of wild type (WT1-6) and *Spag11a*$^{-/-}$ (KO1 to 6) mice that were age matched and born from a single founder. *Spag11a* mRNA expression was completely absent in all the six *Spag11a*$^{-/-}$ mice as demonstrated by semi quantitative and real-time PCR analyses (**figure 8B and 8C**). Concomitant with this, SPAG11A protein was also not detected in the caput of *Spag11a*$^{-/-}$ mice (**figure 8D**).

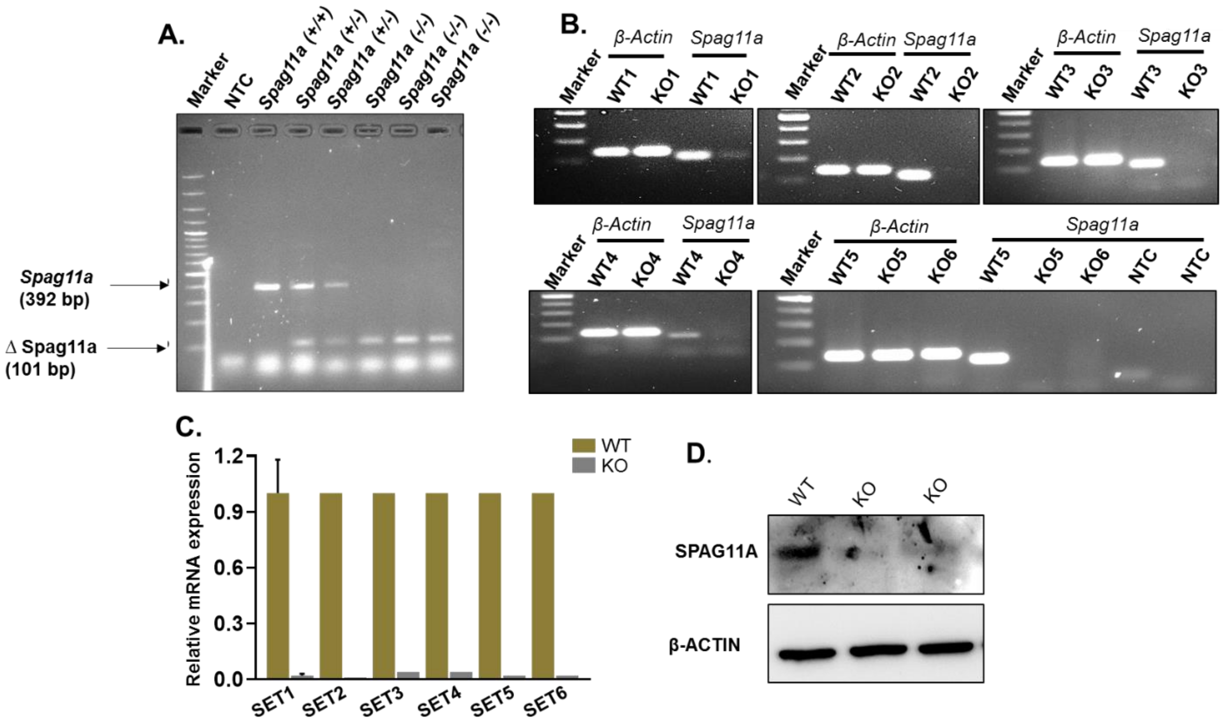

Figure 8**. Validation of the knockout mice.* ***(A) *Genotyping was carried out by PCR using DNA isolated from the tails regions of the pups. A 392 bp amplicon is evident in the wild type (Spag11a*$^{+/+}$*) mice, whereas in the heterozygous mice (Spag11a*$^{+/-}$*) two amplicons of 392 and 101*

bp are amplified. In the homozygous knockouts (Spag11a$^{-/-}$), the amplicon size observed is 101 bp. ***(B)*** *RT-PCR for Spag11a mRNA expression. RNA isolated from the caput of six sets of wild type (WT1-6) and Spag11a$^{-/-}$ (KO1-6) mice were reverse transcribed and PCR performed to detect Spag11a mRNA using gene specific primers. Gapdh expression was used as internal control.* ***(C)*** *Real-time PCR quantification for Spag11a mRNA in six sets of WT and KO mice.* ***(D)*** *Immunoblotting for the detection of SPAG11A protein in WT and KO mice. β-actin was used as internal control.*

3.3.6 Body and organ weights

No significant change in the body weight was observed in the *Spag11a*$^{-/-}$ mice compared to the wild type. Similarly, the relative organ weight ratio of most of the organs did not vary in the *Spag11a*$^{-/-}$ mice (**table 3**).

Table 3. *Relative organ weights in wild type and Spag11a$^{-/-}$ mice.*

	WILD TYPE	KNOCKOUT
Caput	0.887 ± 0.152	0.870 ± 0.130
Cauda	0.661 ± 0.065	0.827 ± 0.070
Testis	4.13 ± 0.423	4.05 ± 0.400
Prostate	0.678 ± 0.277	0.648 ± 0.253
Seminal Vesicle	10.79 ± 0.87	9.04 ± 0.813
Vas deferens	0.660 ± 0.286	0.671 ± 0.231
Liver	55.29 ± 3.84	58.30 ± 2.34
Lung	7.41 ± 1.72	7.38 ± 1.42
Kidney	7.24 ± 0.357	8.32 ± 0.65
Spleen	3.27 ± 0.44	2.89 ± 0.24
Heart	7.34 ± 0.78	7.39 ± 0.84
Bladder	1.02 ± 0.51	1.08 ± 0.56
Body weight	26.90 ± 2.08	26.69 ± 0.76

3.3.7 Fecundity and sperm function

The effect of *Spag11a* knock out on male fecundity was assessed by mating the male *Spag11a*$^{-/-}$ mice with wild type females of proven fertility. Litter size was significantly reduced in the *Spag11a*$^{-/-}$ mice when compared to the wild type mice (**figure 9A**). Concomitant to this, decreased sperm count was also observed in the *Spag11a*$^{-/-}$ mice (**figure 9B**). Spermatozoa obtained from *Spag11a*$^{-/-}$ mice displayed abnormalities in the head and tail structures (**figure 9C**). The tails appeared to be bent and rounded, whereas the head portion was tilted.

Sperm function was further explored by measuring the ability to undergo capacitation and acrosome reaction. Spermatozoa obtained from *Spag11a*$^{-/-}$ mice were subjected to capacitation and the fluorescence intensity of the cholesterol binding dye fillipin was measured. In the spermatozoa of wild type mice, fluorescence intensity was decreased upon capacitation (CP), when compared to their uncapacitated (UCP) counterparts (**figure 10A**). Decreased fluorescence intensity was also observed in the spermatozoa of *Spag11a*$^{-/-}$ mice, indicating that this molecular process is not affected due to *Spag11a* knock down (**figure 10A**). The fluorescence intensity of PNA-FITC was measured to determine the ability of spermatozoa to undergo acrosome reaction. The fluorescence intensity of PNA-FITC was significantly reduced in the acrosome reacted (AR) spermatozoa of wild type mice when compared to their unreacted counterparts (UR) (**figure 10B**). However, fluorescence intensity was not decreased significantly in the acrosome reacted spermatozoa of *Spag11a*$^{-/-}$ mice (**figure 10B**).

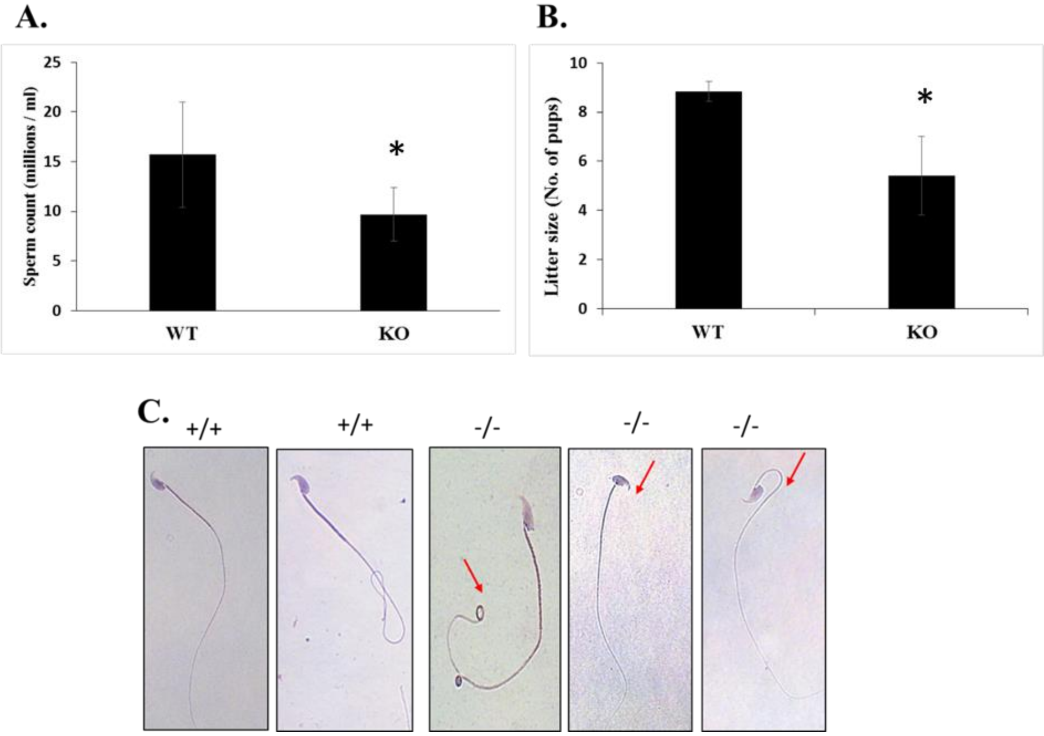

Figure 9. *Evaluation of fecundity. (A) Litter size. Wild type and Spag11a*$^{-/-}$ *mice (n = 6 from each group) were cohabited for one week with two wild type female rats to allow mating. The number of pups born were noted. (B). Sperm count. Spermatozoa collected from the cauda of wild type and Spag11a*$^{-/-}$ *mice (n = 6 from each group) were counted in a hemocytometer and the average sperm count was determined. Values expressed are Mean ± S.D. * indicates* $p < 0.05$ *compared to the wild type control. (C) Sperm morphology. Spermatozoa obtained from wild type and (+/+) and Spag11a*$^{-/-}$ *(-/-) mice were smeared on a glass slide, stained with hematoxylin and eosin and observed under a light microscope. Arrows indicate the structural abnormality. Images shown are representative of multiple fields observed.*

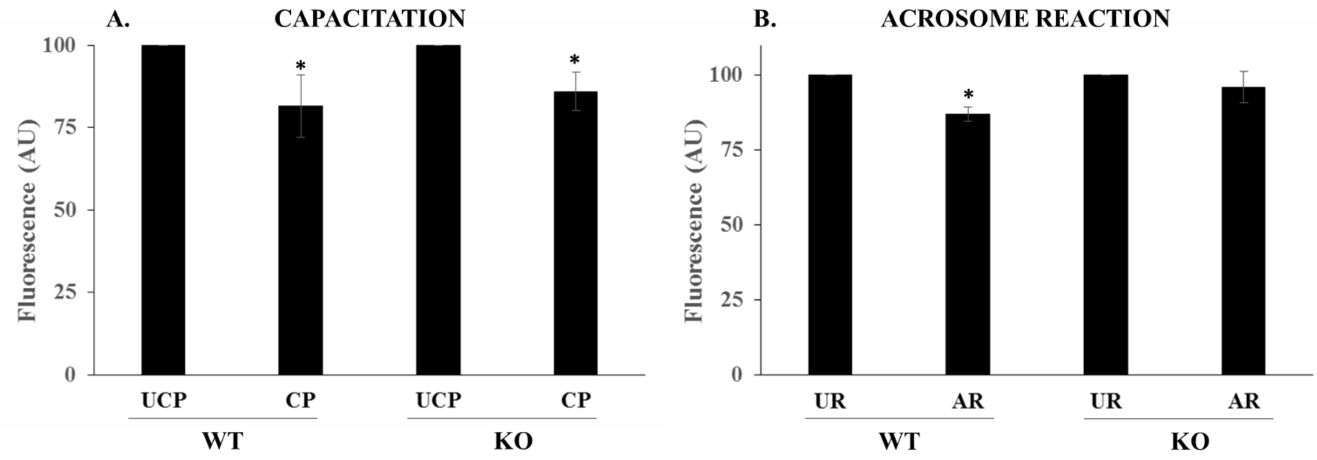

Figure 3. *Determination of capacitation and acrosome reaction.* ***(A)*** *Spermatozoa obtained from wild type (WT) and Spag11a$^{-/-}$ (KO) mice (n=6) were allowed to capacitate in M2 medium and stained with the cholesterol binding dye, filipin (25 μM).* ***(B)*** *Spermatozoa obtained from six sets (n = 6) of wild type (WT) and Spag11a$^{-/-}$ (KO) mice were incubated with the ionophore A23187 (10 μM) and stained with PNA-FITC. Fluorescence intensity of was measured in a flow cytometer. Values expressed are Mean ± S.D. * indicates $p < 0.05$ compared to the respective control.*

3.3.8 Histopathology

To evaluate the possible effect on anatomical changes in the male reproductive tract tissues, caput, cauda, testis and prostate from *Spag11a*$^{-/-}$ mice were subjected to thorough histopathological examination. In the caput, severe mucosal epithelial hyperplasia moderate degenerative changes in mucosal epithelial cells of tubules were observed, whereas the cauda appeared to be normal (**figure 11**). However, in the testes, severe inflammatory changes in seminiferous tubules with infiltration of giant cells was observed. Further, moderate apoptosis of Leydig cells was evident (**figure 11**). No changes in the anatomical features of the prostate was observed in the *Spag11a*$^{-/-}$mice (**figure 11**).

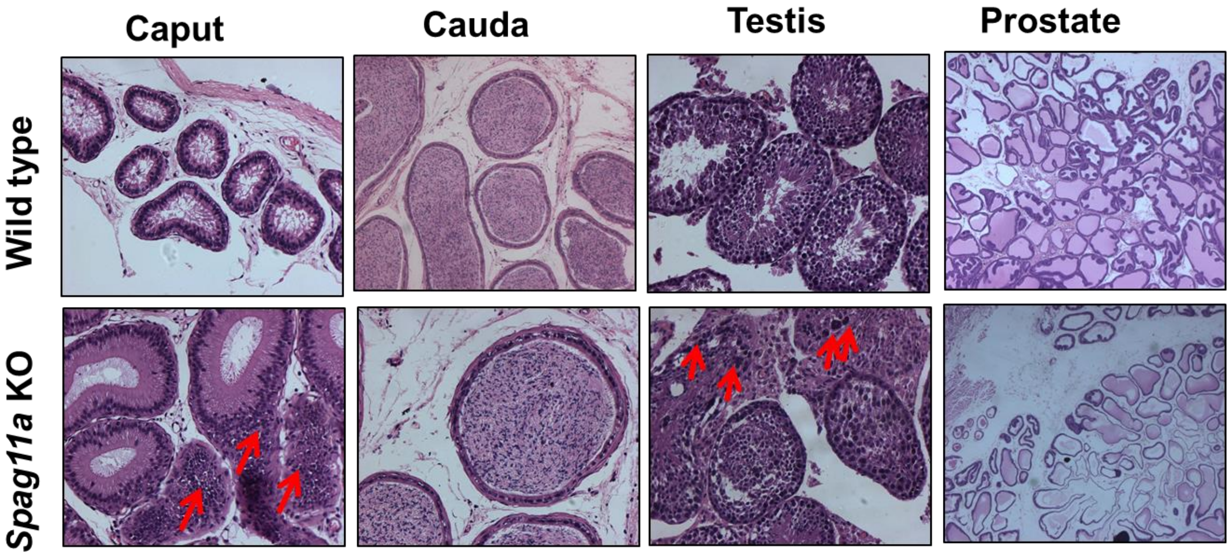

Figure 11. *Histopathological analyses. Caput, cauda, testis and prostate obtained from wild type and Spag11a*$^{-/-}$ *mice were stained with hematoxylin and eosin. Arrows indicate the anatomical damages. All images are with a scale bar of 100 µm.*

3.4 DISCUSSION

The role of epididymal and testicular proteins in spermatogenesis, sperm function and fertilization have been studied for many decades to understand the crucial steps that govern male gamete physiology. The importance of sperm transit through epididymis to acquire forward motility and fertilizing ability was first described five decades ago (33, 34). The functional characterization of epididymal proteins for post testicular male contraception and the methods developed are summarized in the review by Sipila et al (35). In this study, we adopted two model systems to down regulate the mRNA levels of epididymis specific *Spag11a* mRNA and its encoded protein by testicular electroporation mediated transgenesis and active immunization respectively. Such a method of generating genetically modified organisms has been practiced and found to be acceptable to study the function of a particular gene or protein (30, 31, 36-38). We observed a significant decrease in the *Spag11a* mRNA levels in the caput epididymis of transgenic rats that had integration of a plasmid which encodes for *Spag11a* mRNA targeting shRNA. *In vivo* testicular electroporation mediated gene suppression was reported using plasmid-based expression vectors that expresses siRNA targeting *Tex101* and *Sp3111* genes in mouse models (39, 40). Overexpression of *Cirp* gene by *in vivo* testicular electroporation in the cryptorchid testes was reported in mice (41). Thus, the transgenic rats generated in this study by testicular electroporation can be used as a model to study the role of *Spag11a* in sperm function and fecundity.

Another important method to study the functional role of testis / epididymis specific proteins is by generating animal models by active immunization and these models continue to be a good choice till date. Studies conducted in the last three years evaluated the effects of active immunization against gonadotrophin hormone (GnRH), B-cell epitopes and IZUMO3 in male reproductive function (42-44). We observed a very high antibody titer in the serum, caput, cauda and testis of SPAG11A immunized rats, which is a hall mark of the active immunization animal model. We previously used such animal models to study the role of LYZL1, LYZL4, PATE and PATE-F proteins in sperm function and fecundity (9, 11). Hence, in this study, using the active immunization concept to evaluate the role of SPAG11A in sperm function and fecundity is on par with the current methods in this field of research.

Litter size generated by *Spag11a*-shRNA transgenic rats was significantly reduced in all the generations analyzed. The effect of suppression of mRNA levels by testicular electroporation on litter size has not been reported. To the best of our knowledge, for the first time, we have reported reduction in the litter size of *Spag11a*-shRNA expressing transgenic rats. The decrease in litter size over three generations indicated the inheritance of this integration. Litter size was also decreased in the SPAG11A immunized rats. Active immunization mediated reduction in litter size is reported in recent studies. Immunization with recombinant *Salmonella typhimurium* flagellin fljB (STF2)-GnRH resulted in decreased fecundity in rats (45). The B-cell epitope vaccine inhibited fertility in mice (43). Active immunization with LYZL or PATE proteins affected litter size in rats (9, 11). Reduction in the litter size observed in both the experimental models used in this study provide concrete evidence on the role of SPAG11A in fecundity.

We observed decreased sperm count in both the animal models. The decrease in the litter size could be due to the reduced sperm count. SPAG11A might play a crucial role in spermatogenesis or sperm maturation and thereby contributing to the lowered sperm count and litter size. SPAG11A binding to the sperm during epididymal transit and contributing to the maturation was demonstrated *in vitro* (16). Mice immunized with pSG.SS.C3d3.YL.Bin1b recombinant vaccine exhibited reduced fertility (46). Neutralizing INSL3 protein by passive immunization resulted in reduced sperm concentration (47). In this study, ablation of SPAG11A could have influenced sperm maturation. It is possible that spermatozoa that could not undergo maturation have been degraded which resulted in reduced sperm count. We also observed that the reduction in sperm count was higher in the immunized rats than in the transgenic rats. Though integration of the *Spag11a* targeting shRNA plasmid into the genome and a significant reduction in *Spag11a* mRNA was evident in the transgenic rats, a complete ablation may not have been achieved. This could contribute to the translation of the residual *Spag11a* mRNA into the functional protein. Thus, the presence of SPAG11A protein could have contributed to the higher sperm count in the transgenic animals. Whereas in the case of immunized rats, presence of antibodies against SPAG11A in the serum, caput, cauda and testis could have had much higher impact on sperm maturation.

To gain further insight into the molecular mechanisms associated with sperm function that may be affected due to ablation of *Spag11a* mRNA or its protein, capacitation, acrosome reaction and calcium dynamics was assessed *in vitro*. Spermatozoa obtained from the transgenic or immunized rats underwent capacitation and acrosome reaction and displayed increased calcium influx, indicating that SPAG11A may not have a role in these molecular processes.

Previous studies demonstrated that epididymal or testicular proteins though abundantly expressed in these organs, do not influence sperm function. For example, spermatozoa obtained from LYZL4 or PATE immunized rats did not display impairment in capacitation or acrosome reaction (9, 11). Capacitation and acrosome reaction were independent of estradiol receptor, though these molecular machinery was abundantly localized on the equine spermatozoa (48). It is possible that SPAG11A may act at a different target to affect fecundity and sperm count.

The functional significance of any gene is best studied using transgenic or knockout model systems. In the last one year, studies using a number of knockout animal models that lack expression of epididymis or testis specific genes have been reported to decipher their role in male reproduction. While some of the genes have been found to be indispensable for male reproductive function (fertility), a few have been found to either partially dispensable or completely dispensable. Very recently it is reported in *Lcn8* knockout mice, though the spermatogenesis and fertility were normal, defective sperm were evident (49). Deletion of calcium-binding protein spermatid-associated 1 (*Cabs1*), a spermatid specific protein, did not affect fertility, but was a crucial component of the sperm annulus essential for proper sperm tail assembly and motility (50). Similarly, the structural differentiation and energy metabolism of sperm were dependent on serine protease 55 (PRSS55), the deletion of which affected fertility in mice (51). Motile ciliopathy featuring situs inversus totalis and asthenospermia was evident in cilia and flagella associated protein 45 (CFAP45) deficient spermatozoa of humans and mice (52). Dysregulation of the expression pattern of a large number of genes involved in sperm maturation, capacitation and acrosome reaction was observed in *Plag1*$^{-/-}$ mice (53). The indispensable role of *Cfap97d1* in flagellar axonemal maintenance (54), adenylate kinase 1 for sperm motility (55), autophagy core protein ATG5 in sperm individualization and elongation (56), *Fam170a* in subfertility (57), *Tmprss12* in sperm motility and uterotubal junction migration (58), NELL2-mediated lumicrine signaling through OVCH2 for fertility (59), intraflagellar transport protein IFT81 (60), RNA capping enzyme DCP2 (61) and *Prss55* (62) in fertility were reported in the last one year. On the other hand, recent reports also suggest the dispensability of many genes in male fertility. Examples include the predicted gene 31453 (*Gm31453*), carboxypeptidase A5 (*Cpa5*) (63), exonuclease 5 (64), testis-specific peroxiredoxin 4 (65), *Tex33* (66), the testis-specific gene, *4930524B15Rik* (67), *Fam170b* (57), 13 testis- or epididymis-enriched evolutionarily conserved genes (68), PDZ-binding kinase (*Pbk*) (69), Epididymal proteins Binder of SPerm Homologs 1 and 2 (*Bsph1/2*) (70), *lncRNA5512* (71) and *Prss51* (62). Thus, deciphering the role of epididymal and testis genes using transgenic / knockout models in male fertility continues to be an active area of investigation. Hence, we studied the role of *Spag11a* gene in male reproductive function using knockout mice.

In this study, we report that the body weight and relative organ weights of many tissues including that of the male reproductive tract in the *Spag11a*$^{-/-}$ mice did not differ when compared to the wild type mice, indicating that *Spag11a* gene may not be involved in the developmental process. *Spag11a*$^{-/-}$ mice displayed reduced fecundity (measured in terms of litter size) with a concomitant reduction in the sperm count. Further, morphological abnormalities (curved tail and bent head) were evident in the spermatozoa of *Spag11a*$^{-/-}$ mice. Decreased fertility associated with perturbations in sperm count and structure are reported for other epididymal and testicular genes. Knockout mediated ablation of the calcium-binding protein spermatid-associated 1 (*Cabs1*) resulted in significantly impaired sperm tail structure and subfertility (50). Similarly, under conditions of serine protease 55 knockout, spermatozoa exhibit ultra-structural abnormalities and a concomitant reduction in fertility (51). Reduced fertility associated with lower sperm count was observed in autophagy core protein, *Atg5* knockout mice (56). Abnormal head morphology and reduced progressive sperm motility was evident in *Fam170a*$^{-/-}$ subfertile mice (57). Results of our study indicate that absence of SPAG11A protein may affect fertility due to defects in spermatogenesis and thereby reduced sperm count. Determining the exact events in the spermiation pathways and the ultra-structural features of spermatozoa of *Spag11a*$^{-/-}$ are warranted to pin point the role of this protein in sperm function and fertility.

Male fertility is primarily a manifestation of the ability of the sperm to fuse with the ovum to produce the offspring. Though the deletion of a certain genes allows normal spermatogenesis, defects in sperm function, especially in capacitation, acrosome reaction and the fusigenic ability also determine the outcome of fertility. The spontaneous acrosome reaction (AR) frequency was affected in the spermatozoa of *Lcn8*$^{-/-}$ mice (49). Perturbations in the gene expression related to sperm maturation, motility, capacitation and the acrosome reaction were observed in *Plag1*$^{-/-}$ mice (53). Spermatogenesis was severely impaired in *Kiss1, Ift81* and *Prss5* knockout male mice (60, 62, 72). On the other hand, no abnormalities in sperm parameters were noted in *Bsph1* and *Bsph2* double knockout mice (70). Normal spermatogenesis and sperm function was observed in *Gm31453* and *Cpa5* null mice (63). In this study, we observed that the spermatozoa obtained from *Spag11a*$^{-/-}$ had the ability to undergo capacitation. On the other hand, ionophore induced acrosome reaction was did not occur in the spermatozoa obtained from knockout mice. In our previous study, spermatozoa from transgenic rats that over express shRNA against *Spag11a* underwent capacitation. Similar results were observed in rats that were subjected to SPAG11A active immunization (29). Results obtained from the *Spag11a*$^{-/-}$ mice provide further strength on the role of this gene in sperm function (acrosome reaction) that the inability of the sperm from *Spag11a*$^{-/-}$ mice to undergo acrosome reaction could be one of the reasons for subfertility. Further, the subfertility could also be due to reduced sperm count that may have arisen due to hampered spermatogenesis. The histopathological alterations observed in testis and caput of *Spag11a*$^{-/-}$ mice also lends evidence to the possible disruption in spermatogenesis and sperm maturation. Reduced fertility and spermatogenesis associated with damage to the testicular / epididymal architecture is reported. Histological perturbations such as presence of sloughed germ cells and symblasts in the intraflagellar transport protein (*Ift81*) null mice is reported (60). Abnormal seminiferous tubules were evident in in ubiquitin-specific protease 26 (*Usp26*) knockout mice (73). It is possible that damage to the testicular and caput regions could contribute to the subfertility in *Spag11a*$^{-/-}$ mice.

In conclusion we report the generation of a transgenic and active immunized rat models to ablate *Spag11a* mRNA and SPAG11A protein expression respectively. Decreased litter size and sperm count was evident in both these animal models suggesting a definite role for SPAG11A in fecundity and sperm production. Because of the ability of spermatozoa obtained from the transgenic or immunized rats to undergo capacitation and acrosome reaction, it may be proposed that the role of SPAG11A is limited to spermatogenesis or sperm maturation. Further studies to pinpoint its role in the molecular mechanism that affect fecundity and sperm count are warranted. *Spag11a*$^{-/-}$ mice display subfertility, reduced sperm count, structurally and functionally (acrosome reaction) defective sperm and anatomical damage to the testis and caput. The subfertility could be due to impaired sperm function and decreased sperm count caused by damage in the testis and caput epididymis. However, the role of other factors such as the hormonal status, expression profile of molecular machinery that dictate spermatogenesis and sperm maturation, ultra-structural architecture of the sperm and effect on other organ systems that may indirectly affect fertility in *Spag11a*$^{-/-}$ mice needs further in depth investigations.

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SUMMARY

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SUMMARY OF THE STUDY

- **OBJECTIVE-WISE**

Objective 1: Evaluate the role of *Spag11a* gene in epididymal epithelial cell proliferation

The methodology to isolate epididymal primary epithelial cells (EPECs) was standardized. EPECs appeared normal and healthy 3 days after isolation and were adherent to the surface of the culture dish. Sub-cultured cells were healthy and could be cultured for 14 days after isolation. Abundant E-cadherin was detected in the isolated cells by immunofluorescence microscopy and immunoblotting which confirmed their epithelial nature. (Figure 3). Further, they were found to be negative for vimentin, a specific marker for fibroblasts. Flow cytometry analyses indicated that 73 % of the cells isolated were E-cadherin positive. The expression of CRISP1, an epididymis specific protein was abundant in EPECs as revealed by immunofluorescence microscopy and immunoblotting. Similarly, EPECs were positive for the epididymis specific markers Urp1a, Pate-F, Crisp1, Ar and Spag11e genes, while they were negative for Urp1b and Pate, which are known to be absent in the epididymis. Thus, the molecular characterization revealed that the isolated EPECs are epididymis specific with high degree of purity. Such a molecular characterization for EPECs was not reported earlier.

The effect of siRNA mediated suppression of *Spag11a* mRNA expression and the possible effect on proliferation in EPECs was analysed. The relative percent proliferation increased in EPECs transfected with siRNA against *Spag11a* (siSPAG11A) at the 12 h time point. The increase was significant at 24 hours when compared to the scrambled siRNA (SCR) control. These results clearly demonstrate that depletion of *Spag11a* mRNA increases proliferation of EPECs. RNA isolated from EPECs of SCR or siSPAG11A groups were subjected to gene array analyses. A total of 4182 genes were differentially expressed between the SCR vs siSPAG11A groups (Table 3). Among the differentially expressed genes, 2154 were upregulated whereas 2028 were down regulated. Among the upregulated and down regulated groups, coding genes were 1354 and 1075 respectively. An in depth analyses revealed that transfection of EPECs with siSPAG11A caused differential expression pattern of genes involved in epithelial cell proliferation. The differential expression pattern was confirmed by real time PCR analyses. Thus, the increased proliferation observed due to suppression of *Spag11a* mRNA levels could be a result of the differential expression of genes that govern epithelial cell proliferation. We also tested whether the cell proliferation would decrease if *Spag11a* is over expressed in highly proliferating immortalized rat epididymal epithelial (RCE) cells. A significant decrease in proliferation was observed after 24 h in SPAG11A over expressing RCE cells. This is an additional layer of evidence to demonstrate the role of *Spag11a* gene in regulating cell proliferation in the epididymis.

Objective 2: Determine the effect of SPAG11A protein ablation on chemically induced oncogenesis in the epididymis

In this part of the study, we investigated the effect of ablation of SPAG11A by active immunization on the susceptibility of epididymis to a low dose of carcinogen. Immunized rats had a high antibody titre in the serum, caput and testis when administered with recombinant SPAG11A protein. Some of the immunized rats were administered diethyl nitrosamine (DEN) for a period of 90 days. In the caput obtained from SPAG11A immunized or DEN only treated rats, normal tubular morphology and normal concentration of spermatozoa was observed. However, in the caput of SPAG11A immunized + DEN treated rats, foci of fibrosis and infiltration of inflammatory cells in between the tubules of epididymis along with mild mucosal degeneration of mucosal epithelial cells was evident. In order to determine whether ablation of SPAG11A protein (due to immunization) contributes to tumorigenesis at the transcriptome level, RNA isolated from the caput of control, SPAG11A immunized and DEN treated rats was subjected to high throughput transcriptome analyses. Among the 25866 transcripts analyzed, 2760 and 3460 were found to be up and down regulated in the caput of SPAG11A immunized rats. Consequent to DEN treatment, the number of up and down regulated transcripts were 3241 and 2573 transcripts respectively. We then explored for the common transcripts that are differentially expressed in both these groups. 3714 transcripts were differentially expressed in the caput of both SPAG11A immunized and DEN only treated rats. Among the 3714 transcripts, 3549 of them displayed similar pattern in differential expression pattern.

A recent report identified that majority of the tumours in humans are associated with alterations in one or more of the crucial signalling pathways (reference). The signaling pathways identified were Hippo, Myc, Notch, Nrf2, PI-3-Kinase/Akt, RTK-RAS, TGFb, p53, cell cycle (Cdk mediated) and b-catenin/Wnt. Basing on this background we analyzed the differential expression of the molecular components of these pathways in the caput of SPAG11A immunized only and DEN only treated groups when compared to the control. In all the pathways, the differential expression pattern of the components was similar in both the groups. These results strongly suggest that a tumorigenic milieu that is caused by a carcinogen is also reflected in the caput under conditions of SPAG11A ablation, thus further reinforcing the role of this protein in preventing tumorigenesis in the epididymis.

Objective 3: Study the role of *Spag11a* in sperm function and fertility using transgenic and knock out animal models.

Transgenic rats were generated by testicular electroporation. The integration of the plasmid containing the transgene (300 bp) into the genome was assessed by genotyping using DNA isolated from the tail piece of the pups. Genomic PCR with the samples obtained from pups (F1 generation) born to rats injected with a mixture of the plasmids (F0 generation) revealed the amplification of a 300 bp amplicon, suggesting the integration of the plasmid into the genome. Genotyping of the pups born to F1 generation rats revealed the integration of the plasmid in some of the pups in F2 generation and similar results were observed in the F3 and F4 generation rats. A significant decrease of *Spag11a* mRNA levels was observed in the some of the *Spag11a*-shRNA transgenic rats when compared to the wild type control. Concomitant with mRNA levels, SPAG11A protein levels were also decreased in the *Spag11a*-shRNA transgenic rats. In the SPAG11A immunized rats, the antibody titer in the serum, testis and epididymis of was significantly increased and was maintained till the end of the study.

Litter size for the *Spag11a*-shRNA transgenic rats, was monitored for four generations (F0, F1, F2 and F3). A significant decrease in the litter size was observed in F0 generation rats which were subjected to electroporation. The litter size was also significantly decreased in F1, F2 and F3 generation transgenic rats compared to the corresponding wild-type control. On the other hand, the litter size in SPAG11A immunized rats was also significantly lesser than the control. Sperm count was marginally decreased in the *Spag11a*-shRNA transgenic rats when compared to the wild type control. However, a significant decrease in the sperm count was observed in SPAG11a immunized rats. Spermatozoa obtained from SPAG11A immunized or *Spag11a*-shRNA transgenic rats displayed a significant decrease in the fluorescence intensity of membrane bound filipin indicating the occurrence of capacitation. Such a decrease was also observed in the spermatozoa of SPAG11A immunized or *Spag11a*-shRNA transgenic rats stained with PNA-FITC, indicating the occurrence of acrosome reaction. An increase in the fluorescence intensity of Fluo-3AM (a calcium binding dye) was also observed in the spermatozoa of SPA11A immunized and *Spag11a*-shRNA transgenic rats indicating the occurrence of acrosome reaction. These results provide evidence that capacitation, acrosome reaction and [Ca+]i release are not affected due to ablation of *Spag11a* mRNA or its protein counterpart.

The deletion of exon 1 of *Spag11a* gene in the knockout mice was confirmed by genotyping. In the knockout mice, *Spag11a* mRNA expression was completely absent as demonstrated by semi quantitative and real-time PCR analyses, which also coincided with the absence of the protein. No significant change in the body weight was observed in the *Spag11a*$^{-/-}$ mice compared to the wild type. Similarly, the relative organ weight ratio of most of the organs did not vary in the *Spag11a*$^{-/-}$ mice. Litter size was significantly reduced in the *Spag11a*$^{-/-}$ mice when compared to the wild type mice. Concomitant to this, decreased sperm count was also observed in the *Spag11a*$^{-/-}$ mice. Spermatozoa obtained from *Spag11a*$^{-/-}$ mice displayed abnormalities in the head and tail structures. The tails appeared to be bent and rounded, whereas the head portion was tilted. The fluorescence intensity of fillipin was also decreased in the spermatozoa of *Spag11a*$^{-/-}$ mice, indicating that this molecular process is not affected due to *Spag11a* knock down. The fluorescence intensity of PNA-FITC was significantly reduced in the acrosome reacted spermatozoa of wild type mice when compared to their unreacted counterparts. However, fluorescence intensity was not decreased significantly in the acrosome reacted spermatozoa of *Spag11a*$^{-/-}$ mice. Knockout mice tissues were subjected to histopathological analyses. In the caput, severe mucosal epithelial hyperplasia moderate degenerative changes in mucosal epithelial cells of tubules were observed, whereas the cauda appeared to be normal. However, in the testes, severe inflammatory changes in seminiferous tubules with infiltration of giant cells was observed. Further, moderate apoptosis of Leydig cells was evident. No changes in the anatomical features of the prostate was observed in the *Spag11a*$^{-/-}$mice.

- **OVERALL SUMMARY**

In vitro models to study epididymal functions are not fully available. The isolation protocol and molecular characterization of EPECs described in this study will provide a basis to standardize the establishment of epididymal cell model systems. Si-RNA mediated ablation of Spag11a mRNA in EPECs led to increased proliferation and differential expression of genes involved in epithelial cell proliferation. On the contrary, overexpression of *Spag11a* in immortalized epididymal cells caused decreased proliferative capacity. These results indicate that *Spag11a* may have a role in governing the proliferative dynamics in the epididymis.

In rats actively immunized against SPAG11A protein, DEN administration enhanced the pathophysiological parameters of the caput which pointed out to the onset of tumorigenesis. Such a tumorigenic process was not witnessed in the caput of DEN only treated rats. It appears that ablation of SPAG11A protein induces the susceptibility to tumorigenesis caused by a low dose carcinogen. Further, the differential expression pattern of many genes in ten cancer related pathways were similar in the caput obtained from SPAG11A immunized or DEN alone treated rats. Such a similarity in the differential expression provides concrete evidence that absence of SPAG11A protein creates a microenvironment that is favourable to tumorigenesis and thus it is possible that the anti-tumorigenic action of SPAG11A is a potential reason for the non-occurrence of cancer in the epididymis.

Besides the role in tumorigenesis, results of this study indicate the functional aspects of SPAG11A in sperm function and male reproductive physiology as well. Studies with immunized, transgenic and knockout animal models described in this study indicated compromised sperm function and fecundity. In depth analyses are further required to determine the molecular aspects of SPAG11A associated control on germ cell function.

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Publications

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Characterization of isolated rat caput epididymal primary epithelial cells: A molecular biology approach

Kumari Sangeeta, Suresh Yenugu*

Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, 500046, India

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ABSTRACT

Sperm maturation in the epididymis is a tightly regulated process which involves secretion and addition of a variety of proteins onto the sperm surface. The molecular mechanisms governing these processes has gained interest in the last decade. In vitro model systems to study the role of epididymal proteins in sperm maturation and other physiological process are very important. Isolation of epididymal cells, culture of epididymal explants and generation of immortalized cells were standardized to be used as *in vitro* models to study epididymal function. However, isolation and maintenance of primary cultures of epididymal epithelial cells seems to be the best option because of its closeness to the *in vivo* conditions. Though structural and morphological characterization of primary cultures of epididymal epithelial cells were carried out, the same were not conducted at the molecular level. In this study, we isolated adult rat epididymal primary epithelial cells (EPECs) and characterized them for their purity and cell specific expression of molecular markers. Isolated EPECs exhibited normal cell morphology and were sub cultured and maintained up to 3 weeks. EPECs expressed the epithelial marker, E-cadherin and their purity was estimated to be 73% using flow cytometry. EPECs abundantly expressed CRISP1, *Urp1a*, *Pate-F*, *Crisp1*, *Ar* and *Spag11e*, markers of epididymal cells and were negative for *Urp1b* and *Pate*, markers negative for epididymis. Results of our study provide a systematic characterization of EPECs at the molecular level and thus a refinement to the previously reported characterization methods.

1. Introduction

Epididymis is the site of sperm maturation during which the male gametes acquire motility and fertilizing ability. The lumen of this organ is lined by epithelial cells, which form the predominant cell type [1]. A variety of proteins are secreted by the epithelial cells in a region specific manner [2] and have been implicated in sperm function and innate immunity [3–6]. Early studies probing into the role of proteins in the epididymal lumen mileu involved micropuncture and cannulation, which are difficult to perform [7]. To understand the molecular mechanisms of the role of epididymal proteins in sperm function, *in vitro* model systems have been developed. These include maintaining epididymal tissue explants [8,9], culturing of primary epididymal epithelial cells [10–12], generation of immortalized epididymal epithelial cells [13], isolation of epididymal epithelial cells from transgenic mice that express SV40 T antigen [14] and isolation of epididymal basal cells [15] and principal cells [16]. However, each of the model system has its own advantages and disadvantages. Culturing of epididymal explants is feasible only for experiments with very shorter duration, whereas use of primary epididymal epithelial cells has limitations of slow growth and replication and limited life span. On the other hand, use of immortalized cell lines have been a matter of concern since the SV40 immortalization may cause endogenous changes at the molecular level and thus may not be similar to the epithelial cells *in vivo*. Considering the pros and cons of the different model systems available to study epididymal function, use of isolated primary cultures of epithelial cells appears to be dependable, since they closely reflect the *in vivo* epididymal epithelial cells. Thus, isolation and culturing of primary epididymal epithelial choice still remains the choice for investigators working in this area or research.

Isolation of primary epididymal epithelial cells and their culturing conditions is reported. The first isolation and culturing of rat epididymal epithelial cells was demonstrated by Kierszenbaum et al., [10]. Further characterization of these cells by electron microscopy was reported [11,17]. Isolation and culture of epididymal

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* Corresponding author.
E-mail addresses: ysnaidu@yahoo.com, sureshsl@uohyd.ernet.in (S. Yenugu).

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REGULAR ARTICLE

siRNA-mediated knockdown of sperm-associated antigen 11a (*Spag11a*) mRNA in epididymal primary epithelial cells affects proliferation: a transcriptome analyses

Kumari Sangeeta[1] · **Suresh Yenugu**[1]

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Abstract

Differential expression of a variety of proteins in the four major regions of the epididymis contributes to maturation of spermatozoa and region-specific cellular functions as well. Proliferation of epithelial cells of the epididymis is highly controlled and thus is one of the major reasons for the nonoccurrence of cancers in this organ system. The molecular mechanisms and the contribution of region-specific genes in epithelial cell proliferation are not yet fully understood. In this study, for the first time, we analyzed the role of sperm-associated antigen 11a (*Spag11a*), a caput-specific beta-defensin–like antimicrobial gene in governing epididymal cell proliferation and global gene expression. siRNA-mediated knockdown of *Spag11a* mRNA in epididymal primary epithelial cells resulted in increased cell proliferation. Out of the 68,842 genes analyzed, 4182 genes were differentially expressed (2154 upregulated and 2028 downregulated). A variety of genes that participate in different cellular processes and pathways were differentially regulated. Genes that are important for epithelial cell proliferation were found to be differentially regulated and these changes were confirmed by real-time PCR. Overexpression of *Spag11a* in immortalized rat caput epididymal cells resulted in decreased proliferation capacity. Results of this study indicate that *Spag11a* plays a crucial role in governing epididymal epithelial cell proliferation.

Keywords Epididymis · Cell proliferation · siRNA · Transcriptome · Sperm-associted antigen 11A

Introduction

The luminal milieu established by epithelial layer of epididymis is very important for maturation, storage, and functional aspects of sperm. Region-specific gene and protein expression profile in epididymal epithelial cells contribute to sperm maturation (Dacheux et al. 2009; Johnston et al. 2005). This is evident by the proteomic changes that occur on the sperm during the process of maturation (Aitken et al. 2007). Further, diverse expression of miRNAs to regulate protein expression in epididymis is reported (Belleannee et al. 2012a; Li et al. 2012; Nixon et al. 2015). In the last decade, the essentiality of epididymal proteins for fertility was reported using knockout animal models that lacked the expression of one or more of the proteins (Bjorkgren et al. 2016; Carvajal et al. 2018; Juma et al. 2017; Meng et al. 2015; Zhang et al. 2018). Apart from their role in reproductive physiology (sperm function and fertility), epididymal proteins also contribute to entirely different physiological functions. The human epididymal protein 4 (HE4) is projected as a potential biomarker for many types of cancers (Ferraro and Panteghini 2018; Li et al. 2019; Mi and Zhang 2018; Mo and He 2018), renal fibrosis (Chen et al. 2017), and chronic kidney diseases (Yuan and Li 2017). HE4 protein modulates gene expression, proliferation, invasion, and metastasis of cancer cells (Zhu et al. 2016a; Zhu et al. 2016b). Members of the sperm-associated antigen 11 (SPAG11), WFDC domain containing, lysozyme-like (LYZL), prostate and testis expressed (PATE), cystatin, and beta-defensin families exhibit antimicrobial activities and are implicated in innate immune responses of the epididymis (Ribeiro et al. 2016; Wang et al. 2012).

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✉ Suresh Yenugu
ysnaidu@yahoo.com; sureshsl@uohyd.ernet.in

[1] Department of Animal Biology, University of Hyderabad, Gachibowli, Hyderabad 500046, India

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Research paper

Male reproductive tract antimicrobial expression in the extremes of ages of rats

Kumari Sangeeta, Suresh Yenugu*

Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

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Lipopolysaccharide

ABSTRACT

Alterations in the global gene expression profile are considered to contribute to the various physiological and pathological changes during the course of ageing. Genes that code for the molecular components of the innate system are alter markedly as ageing occurs; and this may define the susceptibility of very young and very old individuals to reproductive tract infections. The expression pattern of genes that code for beta-defensins (effectors of innate immune response) in male reproductive tract tissues of different stages of ageing is not yet reported. Further, the induction of beta-defensins during endotoxin challenge and whether epigenetic modulators can influence the expression of these genes in different stages of ageing are not reported. We analyzed the basal mRNA levels of beta-defensins and defensin-like proteins (Sperm Associated Antigen 11 (SPAG11) family members), their induction during endotoxin challenge and modulation by epigenetic modifiers (Trichostatin A and Azacytidine) in the caput, cauda, testis, prostate and seminal vesicle of rats that represent early stage to late stages of life (20 day to 730 day old). We observed differential basal gene expression pattern in the male reproductive tract tissues and the induction by LPS was not consistent neither among the age groups not the tissues analyzed. Trichostatin A and Azacytidine also influenced antimicrobial gene expression and the pattern was not consistent in different tissues obtained from different age groups. Results of this study demonstrate that antimicrobial gene expression varies to a great extent during ageing and is strongly influenced by endotoxins and epigenetic modulators.

Abbreviations

TSA	Trichostatin A
AZA	5-azacytidine
LPS	lipopolysaccharide
PCR	polymerase chain reaction
UPEC	uropathogenic *E. coli*
SPAG	sperm associated antigen
PBS	phosphate buffered saline

1. Introduction

Ageing is characterized by changes at the molecular, cellular and tissue level (Kenyon, 2010). Though a number of gene associations with diseases are characterized, APOE, FOXO3 and 5q33.3 loci have been proposed to be related to longevity (Sebastiani et al., 2012). In a recent study, the co-regulation of age related genes in multiple tissues was demonstrated (Yang et al., 2015). The Age Associated Gene Co-expression Identifier (AAGCI) was developed to identify age related co-expression of genes that span a variety of biological processes in human tissues (Yang et al., 2018). Chronological ageing is identified as a major risk factor for many diseases such as stroke and cancer. A variety of model organisms were studied to understand age related changes in genes involved in physiological and pathological processes (Jin et al., 2001; Jones et al., 2001; Weindruch et al., 2001; Glass et al., 2013). Age dependent variations in the expression of genes involved in metabolism, immune responses, Alzheimer's disease and cancer are reported (Shaw et al., 2010; Brink et al., 2009; Vinuela et al., 2018). Changes in transcriptional landscape in human peripheral blood (Peters et al., 2015) and loss of innate immune antimicrobial function of dermal fat (Zhang et al., 2019) are associated with ageing. Further, significant changes in the genes involved in immune system and defense response were observed in ageing populations (Harris et al., 2017). The compromised immune related gene expression appears to be a major risk factor for infections during extremes of ages (very young and very old).

Reproductive tract infections (RTIs) are very common and a majority of them are sexually transmitted. However, RTIs that occur in early and late stages of life are not linked to sexual activity and their

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* Corresponding author at: Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India.
E-mail addresses: sureshsl.uoh@nic.in, ysnaidu@yahoo.com (S. Yenugu).

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Transgenesis and active immunization mediated reduction of sperm associated antigen 11A mRNA and protein levels affect fecundity in the rat

Kumari Sangeeta, Suresh Yenugu*

Department of Animal Biology, University of Hyderabad, Hyderabad, 500 046, India

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ABSTRACT

Spermatozoa acquire motility and fertilizing ability during their transit through the epididymis. A wide variety of proteins secreted into the epididymal lumen are added on to the sperm surface to allow morphological and molecular changes involved in sperm maturation. Proteins of the Sperm Associated Antigen 11 (SPAG11) family are known to be localized on the sperm surface. The rat SPAG11A protein was implicated in sperm maturation during epididymal transit *in vitro*. However, systematic analyses on the significance of SPAG11A in fertility and sperm function is not yet reported in vivo. In this study, using testicular electroporation, we generated transgenic rats that express shRNA to ablate endogenous Spag11a mRNA. Genotyping revealed the integration of the plasmid that expresses shRNA against Spag11a mRNA. Significant decrease in the mRNA levels of Spag11a and its encoded protein was observed in the caput epididymis of transgenic rats. We also generated an active immunization rat model to ablate endogenous SPAG11A protein by administering recombinant SPAG11A protein. Immunized rats had a high antibody titer in the serum and the tissue fluids of caput, cauda and testis. In both these model systems, the litter size and sperm count was significantly reduced. However, spermatozoa obtained from the transgenic or immunized rats underwent capacitation and acrosome reaction and the associated calcium release. Results of this study indicate the role of SPAG11A in fecundity and sperm production and not in sperm function, especially capacitation and acrosome reaction.

1. Introduction

Sperm maturation occurs in the epididymis, a highly specialized convoluted structure divided into three regions namely caput, cauda and epididymis. Because of the distinct gene expression pattern in these three regions, a wide variety of proteins are produced into the lumen, which are then attached to sperm surface to aid in maturation [1]. The nature of the proteins and the molecular mechanisms that underlie during sperm maturation has been an active area of research in the last two decades. The influence of a wide variety of epididymal proteins belonging to different families on sperm function is reviewed by Björkgren et al. [2]. These include β-defensins (DEFB), cysteine rich secretory proteins (CRISP), lipocalins (LCN) and epididymal proteins modifying sperm proteins. We previously identified and characterized the role of lysozyme-like (LYZL) and prostate and testis expressed (PATE) proteins in sperm function and fecundity [3–6]. Further, localization of sperm associated antigen 11 (SPAG11) proteins in the male reproductive tract tissues and on sperm of different species provides evidence on the role of these proteins in male reproductive physiology [7–11].

The SPAG11 gene in humans is located on chromosome 8p23 and encodes many protein isoforms due to alternate splicing [7]. Some of these proteins have structural and functional similarities to β-defensins. They exhibit potent antimicrobial activity and are also implicated in fertility because of their secretion by the epididymis and localization on the sperm surface [12–15]. On the contrary, the rat counterpart proteins are encoded by distinct genes. We and others have identified three rat *Spag11* genes, namely *Spag11c*, *Spag11t* and *Spag11a* (also known as Bin1b or Spag11e) [8,10]. The proteins encoded by these genes are antimicrobial and implicated to contribute to the innate immune responses of the male reproductive tract [8]. Further, these proteins are found to be localized on the sperm, thus implicating them in sperm function as well [8,10].

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* Corresponding author. Department of Animal Biology, University of Hyderabad, Hyderabad, 500046, India.
E-mail address: ysnaidu@yahoo.com (S. Yenugu).

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Associate Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, P.O. Central University
Hyderabad-500 046, T.S., India.

Student's publication

Dr. SURESH YENUGU
Associate Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, P.O. Central University
Hyderabad-500 046, T.S., India.

Student's publication

Dr. SURESH YENUGU
Associate Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
Gachibowli, P.O. Central University
Hyderabad-500 046, T.S., India.

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