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Beta-defensins in the epididymis: clues to multifunctional roles

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Abstract

The epididymis is known to be exposed to a constant risk of inflammatory conditions resulting from bacterial infections, or from idiopathic and noninfectious causes. A common result of these conditions is epididymitis, a pathology that may lead to the destruction of the epididymal duct and transient or permanent sterility. The negative impact of epididymitis on semen quality and fertility justifies the need for a better understanding of cellular and molecular mechanisms by which innate immunity is modulated in the epididymis and the pathophysiology of this clinical condition. Our laboratory has been interested in the last years in the cellular and biochemical events involved in the epididymal defense response against a bacterial challenge, as well as in how these responses are integrated by glucocorticoids and the glucocorticoid receptor. We have been also interested in naturally occurring antimicrobial proteins secreted by the epididymis. Antimicrobial proteins are known to be part of the innate immune system, with potential biological role during a defense response against invasion by pathogens in the epididymis. Here we review the expression and regulation of antimicrobial proteins, particularly beta-defensins in the epididymis, highlighting isoforms of the beta-defensin-like SPAG11B gene. Aspects of their broad range of biological roles and potential use as targets to help to prevent or treat diseases, such as epididymitis, are also discussed.

Keywords: androgens, antimicrobial, epididymis, innate immunity, male reproductive tract.

Introduction

Immature testicular sperm are unable to fertilize an oocyte until their plasmatic membrane is subjected to sequential biochemical modifications that occur in the epididymis, as a result of interactions with the epididymal luminal milieu. Across the epididymis, a single and highly convoluted tubule, the composition of luminal fluid changes continuously due to the secretory and resorptive activities of the epithelial cells that line the epididymal tubule (for review, see Guyonnet *et al*., 2011). The epididymal epithelium contains different cell types (principal, basal, apical, narrow, clear and halo cells). Based on cell type distribution and gross morphology, this tissue can be divided in at least three regions: caput, corpus and cauda (Robaire *et al*., 2006). Further subdivisions of these regions into intraregional segments, limited by connective tissue septa, have been also identified in rodents (Johnston *et al*., 2005; Jelinsky *et al*., 2007). These discrete segments have been proved to present specific pattern of protein localization and gene expression, representing distinct regulatory subunits of the epididymis, with a potential role to direct the tightly regulated composition of the epididymal tubule fluid (Johnston *et al*., 2005; Jelinsky *et al*., 2007). Many factors such as androgens and other steroid hormones, paracrine and lumicrine factors and, more recently, microRNAs have been shown to regulate the segment-dependent gene expression in the epididymis (Robaire *et al*., 2006; Belleannee *et al*., 2012).

The epididymis, due to its anatomical position, is constantly exposed to infection by pathogens, such as *Chlamydia trachomatis*, *Neisseria gonorrhea*, and *Escherichia coli*, going upward in the urogenital tract. The microbial infections can lead to epididymitis, an inflammatory condition that may disrupt the structure of epididymal tubule resulting in infertility (Tracy *et al*., 2008). Epididymitis can be also a result of idiopathic and non-infectious conditions (Tracy *et al*., 2008). The understanding of this clinical condition depends on more information about the immunobiology of the epididymis.

Aspects of the innate and adaptive immune system in the epididymis have been recently reviewed by Hedger (2011). Among the effectors of the innate immunity in the epididymis are receptors structured to recognize unique microbial components, such as the Toll-like receptor 4 (TLR4) that, when activated by local or systemic lipopolysaccharide (LPS) from *Escherichia coli*, triggers an intracellular signaling cascade that results in the activation of the transcription factor nuclear factor kappa B (NFKB) and consequent modulation of pro- and anti-inflammatory genes (Rodrigues *et al*., 2008; Hedger *et al*., 2011; Silva *et al*., 2011). Moreover, the release of various humoral mediators, such as cytokines and chemokines, the recruitment and activation of phagocytes, and the production of endogenous antimicrobial proteins are also components of the innate immunity. Actually, more recently, a dense network of dendritic cells in the base of the epididymal epithelium projecting their dendrites toward the lumen and with the antigen-presenting capability *in vitro* was reported (Da Silva *et al*., 2011). Intraepithelial dendrites were most abundant in the proximal regions of the epididymis where they may be

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crucial for immune tolerance to sperm antigens. Their location in the epididymis also raises the possibility of additional region-specific non-immune functions for these cells (Da Silva *et al*., 2011). All these components play important roles in maintaining a delicate balance between effective defenses and potentially harmful inflammation responses (for review, see Hall *et al*., 2002, 2007). How these different cellular players affect the epididymal physiological functions and, consequently, the ability of this tissue to support and/or protect the sperm in normal and in infected/inflamed conditions, are still poorly understood.

Antimicrobial proteins constitute the first line of defense against invading pathogens. In mammals, they are expressed and secreted by professional phagocytic cells, including macrophages and dendritic cells, and by all epithelial cells from tissues, including the epididymis, that come in contact with microbes. Some are constitutively expressed, and others are only expressed when the tissue has been injured or exposed to pathogens. They are structurally diverse and range from single, alpha-helical linear molecules to molecules with beta-sheet conformation and multiple disulphide linkages, presenting antimicrobial activity against a broad range of microorganisms (Ganz, 2003). Nowadays, there are increasing evidences indicating that antimicrobial proteins are endogenous weapons with multifunctional roles, displaying additional activities related to the modulation of the immune system response in different epithelial tissues, such as in the epididymis (see review by Hall *et al*., 2002, 2007; Yamaguchi and Ouchi, 2012). Therefore, the identification of the signaling mechanisms of these antimicrobials can be important to help in new strategies for the development of potential therapeutics for the prevention and/or treatment of infection- and/or inflammation-related diseases (see review by Yeung *et al*., 2011).

In mammals, defensins are one of the major families of antimicrobial proteins. Characteristically, they are 2-6 kDa cationic peptides with a beta-sheet rich fold and six cysteines that participate in three intramolecular disulphide bonds. Due to their size and spatial distribution of cysteine linkages, mammalian defensins are distinguishable in alpha-, beta- and theta-forms (for review see Ganz, 2003). In this review, the structure, expression and function of beta-defensins in the epididymis will be discussed. Aspects of one particular beta-defensin gene, the sperm associated antigen 11B (SPAG11B), will also be presented.

Beta-defensins and the epididymis

The beta-defensins, which present the three intramolecular disulphide bonds between Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6, are conserved across the vertebrate lineage. Since their first identification as antimicrobials in cattle airway epithelial cells, the expression of beta-defensins in epithelial cells from different tissues, including epididymis, has been reported in several species (Hall *et al*., 2002, 2007; Zhang *et al*., 2011). The observation that human beta-defensin 2 (DEFB102) expression is induced by LPS from *E. coli* and by various pro-inflammatory agents have suggested the potential of this protein family to be a therapeutic target for the prevention and/or treatment of inflammatory/infectious conditions (Ganz, 2003; Yamaguchi and Ouchi, 2012).

The analysis of the whole genome sequence of different species, as well as computational and experimental studies have indicated the existence of more than 30-40 beta-defensins genes in human and other species, organized in gene clusters localized in specific chromosomes (Hall *et al*., 2002, 2007; Yamaguchi and Ouchi, 2012). They all present a broad spectrum of natural microbicidal activity by forming multimeric pores and disrupting pathogen membrane (Ganz, 2003). They can also enhance the adaptive immunity by recruiting monocytes, T cells and dendritic cells through interaction with chemokine receptors (Yang *et al*., 1999). As a matter of fact, murine beta-defensin 2 was reported to also prime bone-marrow derived dendritic cell maturation through ligation to the TLR4 (Biragyn *et al*., 2002), and immunomodulatory function of beta-defensins is truly a current field of interest. Besides, defensins are known to signal through a melanocortin receptor that controls pigmentation, inflammation, and feeding behavior in domestic dogs (Candille *et al*., 2007) and to become venom in the platypus (Whittington *et al*., 2008), indicating the multifunctionality of this gene family. In fact, phenotypic traits such as behavior, blood composition, immune system, neuromuscular function and reproduction have been associated with beta-defensin expression in different inbred mouse strains, information that can be visualized in the Mouse Phenome Database (http://phenome.jax.org; Maddatu *et al*., 2012).

An interesting aspect of this family of antimicrobial proteins is their high abundance and, in some cases, almost restricted expression in the male urogenital tract, particularly in the epididymis where beta-defensin region- and cell-specific expression is displayed (Hall *et al*., 2002, 2007; Avellar *et al*., 2004, 2007; Patil *et al*., 2005; Radhakrishnan *et al*., 2005, 2007; Zhang *et al*., 2011). Microbial signature molecules, developmental signals, androgens, and cytokines are among factors that have been shown to regulate beta-defensin expression in a tissue-specific manner and, in the epididymis, in a region- and cell-specific pattern (Hamil *et al*., 2000; Li *et al*., 2001; Palladino *et al*., 2003; Avellar *et al*., 2004, 2007; Jelinsky *et al*., 2007; Fei *et al*., 2012). As a result, beta-defensin expression profile may vary even when closely related species are compared, particularly in the epididymis, in which a complex pattern of gene regulation for beta-defensins is suggested, reinforcing the role of local factors in their expression and, possibly, in their functional repertoire. As an illustration, we presented in the Fig. 1 the segmental

expression pattern of several beta-defensins in the epididymis of rodents, as a result of the analysis of the public database available at the Mammalian Reproductive Genetics Database (http://mrg.genetics.washington.edu), as described by Johnston *et al*. (2005) and Jelinsky *et al*, (2007).

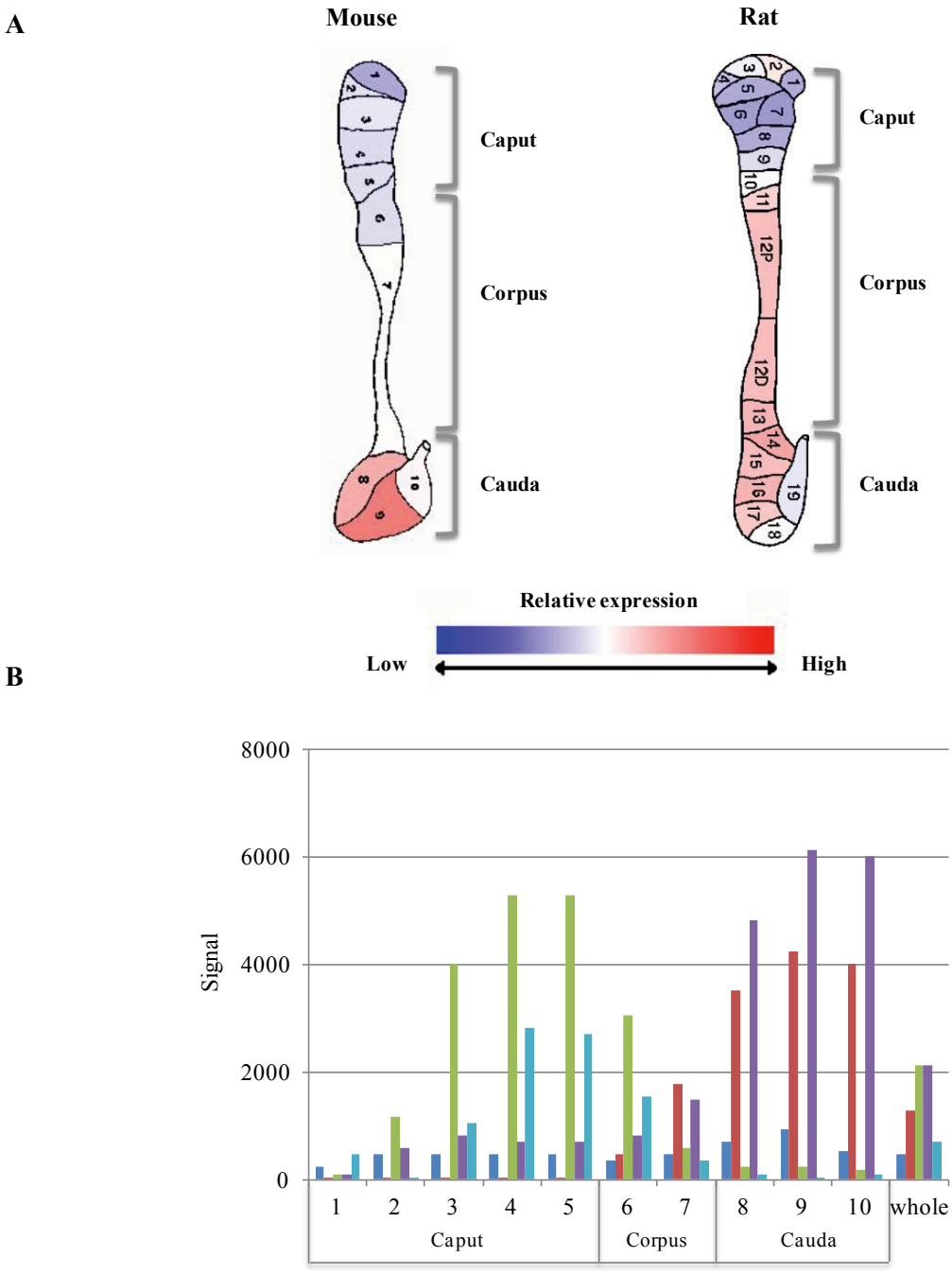

Figure 1. Segmental expression pattern of beta-defensin genes in the rodent epididymis obtained from the public database available at the Mammalian Reproductive Genetics Database (http://mrg.genetics.washington.edu), as described by Johnston *et al*. (2005) and Jelinsky *et al*. (2007). **Panel A**: Relative mRNA expression of beta-defensin 1 (Defb1) is highest in the segments 9 and 14 of the mouse and rat epididymis, respectively. Defb1 mRNA expression is high in the rat, but not in the mouse, corpus epididymis. A direct comparison of mRNA expression pattern within the data, without respect to absolute signal value of the microarray assay was performed. **Panel B**: Differential mRNA expression pattern for different beta-defensins genes (Defb1, light blue; Defb11, dark red; Defb15, green; Defb2, purple; Defb35, cyan) in the mouse epididymis. All genes are located in mouse chromosome 8. Microarray signal was obtained as described in Johnston *et al*. (2005). Average signal values of 50 or greater are expected to represent expression within the segment.

Preferential overexpression of several beta-defensins in the epididymal epithelia and their association with spermatozoa in the epididymal lumen and ejaculate also implicate them in fertility function (Hamil *et al*., 2000; von Horsten *et al*., 2002; Zhou *et al*., 2004; Avellar *et al*., 2007). For instance, the human beta-defensin 26 (DEFB126) has been shown to bind the glycocalyx of sperm and be involved in the attachment of the sperm to the oviduct epithelia (Yudin *et al*., 2003; Tollner *et al*., 2008), while a polymorphism of this gene has been recently linked to reduced fertility in men (Tollner *et al*., 2011). The knockdown of the beta-defensin 15 (rat homologue of human DEFB106) in the rat epididymis has been correlated with a decline in sperm motility and reduction in fertility (Zhao *et al*., 2011). The disruption of few beta-defensin genes has been reported, but the reproductive phenotype was not assessed (for example beta-defensin 1: Morrison *et al*., 2002; Moser *et al*., 2002; Ryan *et al*., 2011; beta-defensin 3: Augustin *et al*., 2011; beta-defensin 14: Navid *et al*., 2012).

The evolutionary forces that drive the selection and maintenance of a plethora of beta-defensins in mammals, the regulatory mechanisms that restrict the expression of defensins in the male reproductive tract and how immunity response and reproductive physiology functions are paired in beta-defensins function remain as questions to be investigated.

Most beta-defensin genes are composed of two exons separated by an intron of variable length, while some contain an additional one or two exons encoding an internal pro-sequence, a segment of carboxy-terminal mature sequences or untranslated regions (Patil *et al*., 2005). Alternatively spliced isoforms have also been found with some beta-defensins, including the atypical beta-defensin sperm associated antigen 11B (SPAG11B) gene (Patil *et al*., 2005; Hall *et al*., 2007), which is next discussed in more detail.

Sperm associated antigen 11 B (SPAG11B)

Different from other beta-defensin-encoding genes, the sperm associated antigen 11b (*SPAG11B*) has evolved from the fusion of two ancestral beta-defensins genes, arranged in tandem, designed as components A and B (Fröhlich *et al*., 2001; Patil *et al*., 2005). Differential exons recruitment through alternative splicing results in at least twenty transcripts in human, non-human primates, rodents and bovine (SPAG11B isoforms A to W, designated SPAG11B/A to SPAG11B/W). The splicing pattern differ significantly among species, with transcripts arising from exons controlled by promoters A and B (e.g. SPAG11B/A and SPAG11B/D), only A (e.g. SPAG11B/C, SPAG11B/T) or only B (e.g. SPAG11B/E, also called Bin-1b in mouse; for review, see Hall *et al*., 2007; Table 1) that encode proteins that may contain or not a complete consensus beta-defensin motif (Table 1; Fig. 2). Different SPAG11B isoforms have been immunolocalized in epithelial cells along the epididymis, predominantly in the proximal regions, and in association with spermatozoa located in the epididymal lumen and in the ejaculate of human and other species, confirming the secretion of these proteins by the epididymal epithelia (Hamil *et al*., 2000; von Horsten *et al*., 2002; Avellar *et al*., 2004; 2007; Yenugu *et al*., 2006; Hall *et al*., 2007).

Recombinant full length SPAG11B/C, SPAG11B/E and SPAG11B/D, which are known to contain the typical beta-defensin motif in their C-terminal region, have been shown to present the expected antimicrobial activity against gram-negative bacteria (Hall *et al*., 2002, 2007). *In vitro* antibacterial activity against *E. coli* has been also detected for the N-terminal common region of SPAG11B isoforms derived from promoter A, and other full length recombinant SPAG11B variants (SPAG11B/K, SPAG11B/L; Avellar *et al*., 2004; Yenegu *et al*., 2006). The mechanism by which bacteria is killed by these SPAG11B protein products involves the binding of this positively charged proteins around the negatively charged bacteria surface, disruption of the outer and inner membrane barriers forming pores that inhibit the bacterial macromolecular synthesis (Yenegu *et al*., 2004). Interestingly, the variants K and L of rhesus *SPAG11B* do not present the beta-defensin motif, and the antibacterial mechanism of these variants is still unknown.

More recently, the association of full length human SPAG11B/D with three interacting proteins, named tryptase alpha/beta 1 (TPSAB1), tetraspanin 7 (TSPAN7) and attractin (ATRN) was reported, indicating additional actions of this specific SPAG11B isoform besides its antibacterial actions in host defense (Radhakrishnan *et al*., 2009). All these three proteins, which are implicated in host defense and reproductive function pathways, were also found to be co-expressed with SPAG11B/D in epithelial cells from human proximal epididymis. SPAG11B/D competitively inhibited TPSAB1 activity *in vitro*, what could address the modulation of TPSAB1-dependent suppression of sperm motility as a SPAG11B function (Radhakrishnan *et al*., 2009). The association of TSPAN7 and ATRN with spermatozoa was also demonstrated. The physiological relevance of these protein-protein interactions, however, has not yet been established.

SPAG11B splicing transcripts are differentially detected in tissues of the male reproductive tract, with higher abundance in the epididymis (Hamil *et al*., 2000; Li *et al*., 2001; Avellar *et al*., 2004, 2007; Yenugu *et al*., 2006). Mechanisms by which alternative splicing of the SPAG11B gene is regulated are still unknown. The comparison of the SPAG11B mRNA expression pattern between reproductive (testis) and non-reproductive (adrenal, kidney, intestine and liver) tissues from fetal and adult bulls has suggested that transcriptional and post-transcriptional mechanisms during pre- and post-natal development may be important to drive SPAG11B tissue expression (Avellar *et al.*, 2007). The presence of androgen response consensus sequence (ARE) in

SPAG11B promoter regions from human (Fröhlich *et al*., 2001) and rat (Romano *et al.*, 2012, Universidade Federal de São Paulo, Brazil; unpublished data), the down-regulation following surgical orchiectomy (Hamil *et al*., 2000; Ibrahim *et al*., 2001; Fei *et al*., 2012) and increased expression with sexual development (Li *et al*., 2001; Yenugu *et al*., 2006) of SPAG11B transcripts in epididymis from different species, have indicated the importance of androgens for the regulation of SPAG11B mRNA expression.

Table 1. Variant transcripts of sperm-associated antigen 11B (SPAG11B) gene in different species

Species	Gene	Transcript variant	GenBank Acession Number	References
Human (*Homo sapiens*)	SPAG11B	A	AF168616	Kirchhoff *et al*., 1990, Hamil *et al*., 2000, Avellar *et al*., 2004.
		B	NM_058206	
		C*	NM_058203.1	
		D*	AF168617	
		E*	NM_058207.1	
		F	AF170797	
		G	AF168618	
		H	AF168619	
		I	AF168620	
Rhesus (*Macaca mulatta*)	SPAG11B	B	AF566346.1	Avellar *et al*., 2004
		C*	AF466347.1	
		E*	AF466348.1	
		J	AF466349.1	
		K	AF466350.1	
		L	AF466351.1	
		M	AF466352.1	
		N	AF466353	
		O	AF466354.1	
		P	AF466355.1	
		Q*	AF466356.1	
Chimpanzee (*Pan troglotides*)	SPAG11B	A*	NM_001110235.1	Young *et al*., 1998
		B	NM_001110236.1	
		C*	NM_001110237.1	
		D*	NM_001110238.1	
		E*	NM_001110239.1	
Bull (*Bos taurus*)	SPAG11B	C*	DQ838981	Avellar *et al*., 2007
		D*	DQ838982	
		E*	DQ838983	
		U	DQ838984	
		V	DQ838985	
		W	DQ838986	
Rat (*Rattus norvegicus*)	Spag11c	C - v1*	NM_001037852.1	Patil *et al*., 2005 Li *et al*., 2001 Yenugu *et al*., 2004
	Spag11c	C - v2*	NM_001037850.1	
	Spag11b	E*	NM_145087.1	
	Spag11t	T	AY600144.1	
Mouse (*Mus musculus*)	Spag11b	C*	NM_001039563.2	Patil *et al*., 2005 Ibrahim *et al*., 2001
	Spag11e (Bin1b)	E*	NM_153115	
	Spag11b	Q (H)	NM_001034905.2	
Dog (*Canis familiaris*)	SPAG11B	C*	NM_001171544.1	Patil *et al*., 2005
Pig (*Sus scrofa*)	SPAG11B	C*	BK005522	Sang *et al*., 2006
	SPAG11B	E*	BK005523	

*Protein isoforms contain the characteristic beta-defensin antibacterial mofit containing six cysteines arranged in three intramolecular disulphide bonds.

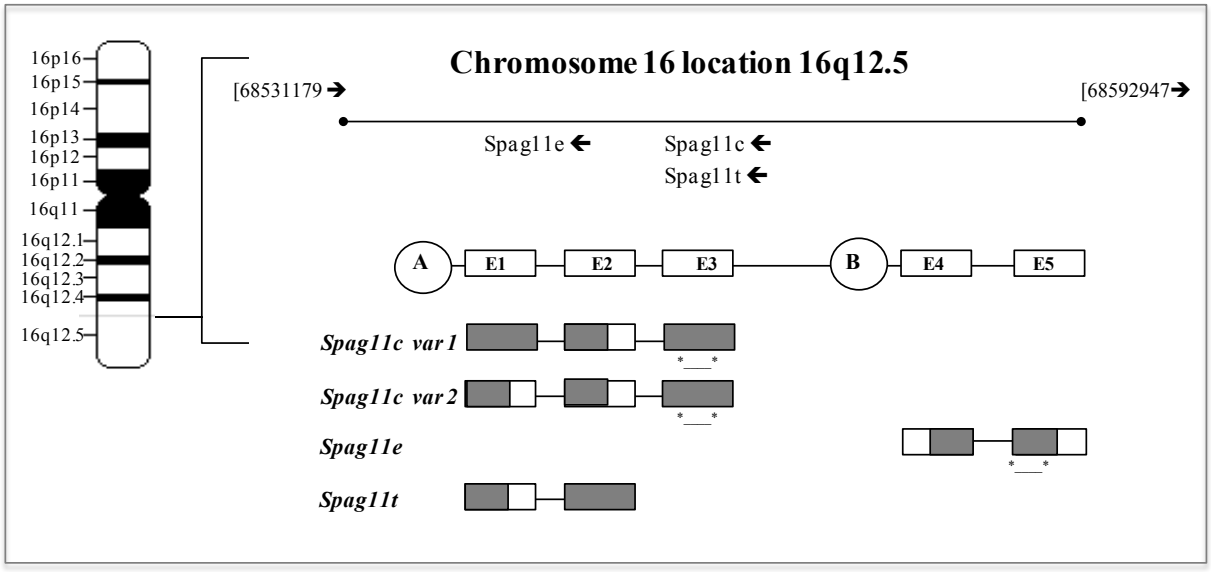

Figure 2. Genomic organization and alternative splicing transcripts of the rat Spag11B gene. Open circles indicate promoters A and B. Filled rectangles indicate exons and connecting lines indicate introns. The six-cysteine motif homologous to the active domain of beta-defensins in the isoforms is also shown (*–*).

The regulation and involvement of SPAG11B gene products during an inflammatory response is still controversial. Li *et al*. (2001) observed that SPAG11B/E (Bin1b) binds to the head of sperm in different regions of the epididymis to promote sperm motility and efficiently suppresses *E. coli* colony growth in primary-cultured rat caput epididymal epithelia, indicating its potential *in vivo* antimicrobial activity. The authors also showed an upregulation of Spag11e mRNA levels in inflamed epididymis caused by the ligation of vas deferens in rats. More recently, a transgenic mouse overexpressing SPAG11B/E in the caput epididymis showed an enhanced resistance to local epididymal infection by *E. coli* and *C. albicans*, confirming the potential *in vivo* role of this SPAG11B product in the regulation of an inflammatory response in the epididymis (Fei *et al*., 2012). Cao *et al*. (2010), however, reported a decrease in the expression of Spag11e (mRNA and protein), as well as in nine other beta-defensins, in the caput of epididymis challenged with local administration of LPS from *E. coli*. This LPS treatment also significantly reduced both sperm binding to SPAG11B/E and sperm motility, effects reversed *in vitro* by incubation of spermatozoa with recombinant SPAG11B/E, suggesting that disruption of the function of this SPAG11B variant may be involved in the impairment of sperm motility. Functionally, participation of mouse SPAG11B/E has been reported to participate in motility acquisition during sperm maturation (Zhou *et al*., 2004).

The correlation of SPAG11B gene expression and the clinical diagnosis of male fertility has been also explored. Transcriptional profiling of epididymis from normal and vasectomized men indicated no change in the mRNA abundance of SPAG11B/C (Legare *et al*., 2004). On the other hand, SPAG11B/B has been proposed by Drabovich *et al*. (2011) as one of the biomarkers that can be used to discriminate between normal, nonobstructive azoospermia, and post-vasectomy (simulated obstructive azoospermia) seminal plasma samples from patients undergoing clinical assays for differential diagnosis of azoospermia. Moreover, the correlation of single-nucleotide polymorphisms (SNP) detected in exon or intron regions with gene expression of the SPAG11B gene and seminal parameters in bulls revealed alterations in fresh and post-thaw sperm motilities and in fresh sperm concentrations (Liu *et al*., 2011).

Despite the advances in the knowledge of the SPAG11B gene in the last years, further research is needed to characterize and elucidate specific biological functions and signaling pathways of individual SPAG11B proteins. The better understanding of the complex roles and functions, as innate immunity players in the epididymis infection and inflammation, will benefit our understanding of epididymal biology and these pathological conditions.

Concluding remarks

In the last decades, epididymal transcriptome studies have revealed an overrepresentation of genes related to immune response among epididymis-specialized secreted genes, such as beta-defensins. Among these genes, SPAG11B arises as a beta-defensin like gene with a complex genomic structure and mRNA splicing pattern, which is constitutively expressed in the epididymis. The mechanisms underlying SPAG11B gene expression and yet-to-be discovered physiological

roles remain to be elucidated. The better understanding of these effectors of the innate immunity in the epididymis, and their correlation with sperm maturation and fertility, will help to gain insights into epididymis immunobiology and pharmacological tools that can be used for the prevention/treatment of inflammatory/infectious diseases in the epididymis and identification of new therapeutic targets for male fertility.

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