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Dynamic changes in the spatio-temporal expression of the β-defensin SPAG11C in the developing rat epididymis and its regulation by androgens

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ABSTRACT

Herein, we characterized the spatio-temporal expression, cellular distribution and regulation by androgens of the β-defensin SPAG11C, the rat ortholog of the human SPAG11B isoform C, in the developing epididymis by using RT-PCR, in situ hybridization and immunohistochemistry. We observed that *Spag11c* mRNA was ubiquitously expressed in rat fetuses, but preferentially detected in male reproductive tissues at adulthood. SPAG11C (mRNA and protein) was prenatally mainly detected in the mesenchyme of the Wolffian duct, switching gradually after birth to a predominant localization in the epididymis epithelium during postnatal development. In the adult epididymis, smooth muscle and interstitial cells were also identified as sources of SPAG11C. Furthermore, SPAG11C was differentially immunolocalized on spermatozoa surface during their transit from testis throughout caput and cauda epididymis. Developmental and surgical castration studies suggested that androgens contribute to the epididymal cell type- and region-specific modulation of SPAG11C mRNA levels and immunolocalization. Together our findings provide novel insights into the potential role of β-defensins in the epididymis.

1. Introduction

The epididymis is a highly convoluted duct that connects the efferent ducts to the vas deferens, and where the spermatozoa acquire forward motility and fertilizing ability. Besides sperm maturation, the epididymis is involved in sperm transport, protection against pathogenic and metabolic injuries, and storage prior to ejaculation. Gene expression and protein secretion by the epididymal tubule epithelium vary along the different epididymal regions (initial segment/caput, corpus and cauda epididymis), leading to highly specialized and region-specific luminal microenvironments that dynamically interact with the traveling spermatozoa (Belleannée et al., 2012; Robaire et al., 2006).

The development and maintenance of the structure and functions of the epididymis are regulated by a complex interplay of endocrine, lumicrine and neuronal systems, among which androgens are the primary regulatory factor. Plasma levels of androgens fluctuate throughout lifespan and their actions are mediated by the androgen receptor (AR), a steroid-hormone activated transcription factor, which spatio-temporal expression in the epididymis of several species is widely known (for review, see Patrão et al., 2009; Robaire and Hamzeh, 2011). During the late prenatal and early postnatal development in the male, androgens are essential for the stabilization and morphological differentiation of the Wolffian duct (WD), the anlagen of the epididymis. Disorders of androgens/AR signaling in this period, due to congenital causes or exposition to endocrine disruptors, impair reproductive tract masculinization and normal epididymal development, resulting in infertility later in life (Hannema and Hughes, 2007; Welsh et al., 2006, 2007). Despite the utmost importance of androgens for epididymal function, molecular aspects of their action are still poorly understood.

An interesting feature of the epididymis is the overrepresentation of genes related to innate immune response among epididymis-specialized genes encoding secreted proteins (Dean et al., 2008). Several of these gene products, such as antimicrobial proteins, are abundantly expressed in the epididymis of different species in a region-dependent fashion and present their expression regulated by androgens (Hall et al., 2002, 2007; Jelinsky et al., 2007; Johnston et al., 2005; Zhang et al., 2011). One group of these epididymal antimicrobial proteins belongs to the β-defensin family, which are small cysteine-rich cationic proteins that exhibit potent antimicrobial activity (Ganz, 2003). Constitutive and high expression levels of different β-defensins have been reported in the epididymal epithelial

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cells, sperm surface and in the ejaculate of different species, indicating their potential roles in sperm function and male fertility (Hall et al., 2002, 2007; Jelinsky et al., 2007; Yu et al., 2013; Zhao et al., 2011; Zhou et al., 2004). β-Defensins have been shown to participate during the response of the epididymis to an inflammatory stimulus, such as the lipopolysaccharide from Gram-negative bacteria (Cao et al., 2010; Li et al., 2001) and in the protection of the spermatozoa against immunorecognition (Yudin et al., 2005). Recently, Zhou et al. (2013) reported that knockout mice with partial deletion of a cluster of nine β-defensin genes in the chromosome 8 presented sperm dysfunction and male infertility, representing the first in vivo evidence for the importance of β-defensins to male fertility. Besides being antimicrobials, the literature indicates that many β-defensins have effects in a plethora of other actions including color coat pigmentation, as well as cancer and inflammatory disorders (Pazgier et al., 2006; Semple and Dorin, 2012). Thus, the understanding of the mechanism of action, regulation and biological significance of β-defensins in the epididymis is necessary to better understand the relevance of these multifaceted actions in human physiology and pathology that is not yet completely established.

Among the epididymal proteins with structural characteristics similar to β-defensins, there are several alternatively spliced products encoded by the *SPAG11B* (sperm-associated antigen 11B) gene in human and several other species. This gene is unique among other β-defensin genes due to its complex genomic structure and mRNA splicing pattern that gives rise to protein isoforms that contains (e.g. SPAG11B/C, D, and E) or not (e.g. SPAG11 A, D and G) the characteristic β-defensin signature in their C-terminal region (for review, see Hall et al., 2007; Ribeiro et al., 2012). As other β-defensins, *SPAG11B* gene products were abundantly found in the epididymal epithelial cells, sperm surface, epididymal fluid and in the ejaculate of human and other species and were also found to be androgen dependent (Avellar et al., 2004, 2007; Hamil et al., 2000; Li et al., 2001; Osterhoff et al., 1994; Pujianto et al., 2013; von Horsten et al., 2002, 2004; Yenugu et al., 2006; Zhou et al., 2004). The recombinant SPAG11B isoforms tested so far displayed in vitro antimicrobial activity (Avellar et al., 2004, 2007; Hamil et al., 2000; Li et al., 2001; Yenugu et al., 2004). Additionally, SPAG11B/E (also known as BIN1B) was shown to affect rat sperm motility (Zhou et al., 2004) and to prevent mouse epididymal infection by *Escherichia coli* (Fei et al., 2012). Little is known, however, about the physiological significance of other SPAG11B products besides SPAG11B/E, and if the existence of many isoforms is related to functional redundancy or isoform-specific specialized epididymal roles.

Herein, we characterized the ontogeny of the expression and cellular distribution of SPAG11C, the rat ortholog of the human SPAG11B isoform C, throughout the prenatal and postnatal development of the epididymis. Then, we studied the in vivo effects of androgens on the regulation of SPAG11C expression, at mRNA and protein levels, during ontogeny and by using surgical castration of adult rats as experimental model. Our results indicated dynamic changes in the spatio-temporal expression of SPAG11C in the developing epididymis and revealed the participation of transcriptional and post-transcriptional mechanisms in the regulation of the β-defensin SPAG11C by androgens in this tissue. Our data contribute to broaden the view of the potential biological role of a β-defensin in the epididymis.

2. Materials and methods

2.1. Animals

Wistar rats were bred and housed in the Animal Facility at Instituto Nacional de Farmacologia/Universidade Federal de São Paulo – Escola Paulista de Medicina (UNIFESP-EPM) under controlled light (12 h light:dark cycle) and temperature (22–24 °C), with free access to food and water. Matings were established overnight and the copulation was confirmed in the following morning by the presence of spermatozoa in the vagina (time defined as embryonic day e0.5). Experiments were conducted using male embryos (e14.5, e16.5, e17.5, e18.5, and e20.5) and neonatal [postnatal day (pnd) 1, pnd5 and pnd10], prepubertal (pnd20 and pnd40) and mature (pnd90 and pnd120) male rats. All experimental procedures were conducted in compliance with the guidelines for the care and use of laboratory animal and were approved by the Research Ethical Committee from UNIFESP-EPM (protocols 01254/05 and 1563/09).

2.2. Surgical castration and testosterone replacement

Male rats were randomly distributed into sham-operated (control) and surgical castrated groups at pnd90. Animal treatments (surgical castration following or not testosterone replacement) were performed as previously described in our laboratory (Mendes et al., 2004). Briefly, rats were sacrificed 7 (7-day CA) and 15 days (15-day CA) after surgery. An additional group of rats was castrated, and 7 days after the surgery, treated daily with testosterone propionate (10 mg/kg body mass, s.c.) for 6 days (7-day CA + T). Treatment effectiveness was confirmed by monitoring the expected changes in the relative mass of the male reproductive tract tissues and testosterone plasma levels as previously reported (Maróstica et al., 2005; Mendes et al., 2004; Silva et al., 2010).

2.3. Fetuses collection, tissue and sperm isolation

Dams were sacrificed by inhalation of an overdose of isoflurane (Cristália, Itapira, SP, Brazil) followed by bilateral pneumothorax and whole fetuses were harvested at the appropriate ages. WDs (including both the duct epithelium and the surrounding mesenchyme) were microdissected from fetuses at e17.5 and e20.5 as described previously (Staack et al., 2003). Male rats were sacrificed by decapitation. Their epididymides were harvested as a whole at pnd1–pnd20 or divided into caput (including initial segment), corpus and cauda epididymis at pnd40 and all later ages. An array of reproductive and non-reproductive tissues was also collected from male rats at pnd120. Testes and epididymides harvested from adult rats at pnd120 were also immediately processed for the isolation of spermatozoa as described previously (Silva et al., 2012) and then used in immunofluorescence studies.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen WDs (RNeasy Mini Kit, Qiagen, Valencia, CA, USA) and postnatal epididymis (Trizol, Invitrogen) and used in RT reactions containing oligo(dT) (Thermoscript RT-PCR system, Invitrogen). Resulting cDNA samples (250 ng) were amplified by PCR (final volume of 20 µl) in 20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 U Taq polymerase (Invitrogen) and 0.4 µM of each forward and reverse primers (Table 1). For each set of primers the number of cycles to amplify each cDNA in the linear range (21–30 cycles) was determined under the following PCR conditions: 95 °C (2 min), 60 °C (1 min), 72 °C (1.5 min), and a final extension at 72 °C (3 min). Negative controls were routinely performed to assess genomic or assay contamination. PCR products were loaded onto agarose gels 2% containing ethidium bromide, visualized with UV illumination and photographed. Amplicons were gel purified and their sequences were confirmed by automated DNA sequencing performed at the INFAR DNA Core Facility (UNIFESP-EPM, São Paulo, Brazil).