Reproductive Biology and Endocrinology

Research

SPAM1 (PH-20) protein and mRNA expression in the epididymides of humans and macaques: utilizing laser microdissection/RT-PCR

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Abstract

Background: The Sperm Adhesion Molecule 1 (SPAM1) is an important sperm surface hyaluronidase with at least three functions in mammalian fertilization. Previously our laboratory reported that in the mouse, in addition to its expression in the testis, Spam1 is synthesized in the epididymis where it is found in membranous vesicles in the principal cells of the epithelium in all three regions. Since *SPAM1* is widely conserved among mammals the aim of the study was to determine if its expression pattern in the epididymis is conserved in rodents and primates.

Methods: We used laser microdissection (LM)/RT-PCR on frozen and paraffin-embedded epididymal sections of humans (n = 3) and macaques (n = 2) as well as *in situ* transcript hybridization to determine if transcripts are present in the epididymal epithelium. Western analysis and immunohistochemistry were used to detect and confirm the protein expression, and hyaluronic acid substrate gel electrophoresis analyzed its hyaluronidase activity. An *in silico* analysis of the proximal promoter of *SPAM1* was also performed to identify relevant putative transcription binding sites for the androgen receptor.

Results: We demonstrate that mRNA unique to *SPAM1* is present in the principal cells of the epididymal epithelium in all individuals of both species studied. SPAM1 protein is present in all three regions of the epididymis, as well as the vas deferens, and is localized similarly to the transcripts. SPAM1 was shown to have hyaluronidase activity at pH 7.0. In the proximal promoter of *SPAM1* were uncovered putative epididymal transcription factor binding sites including androgen receptor elements (AREs), consistent with epididymal expression.

Conclusions: These findings allow us to conclude that epididymal SPAM1 is conserved in at least two mammalian classes, rodents and primates. This conservation of expression suggests that the protein is likely to play an important function, possibly in sperm maturation.

Introduction

The Sperm Adhesion Molecule 1 (SPAM1 or PH-20) is a glycosyl-phosphatidylinositol (GPI)-linked protein found on all mammalian spermatozoa that have been examined, and has been reported to play multiple roles in fertilization [1]. Although a recent gene targeting study indicates that in mice, which have four active non-somatic hyaluronidases, Spam1 is not essential for fertilization [2]; this is

unlikely to be the case in humans where SPAM1 is the only such hyaluronidase present. In primates SPAM1 has been shown to play a role in three functions during fertilization: (1) hyaluronidase enzyme activity necessary for penetrating the cumulus [3] and for which it is best known [4], (2) zona pellucida binding [3], and (3) Ca^{2+} signaling-associated acrosomal exocytosis [5–7].

Unlike somatic ubiquitous hyaluronidases that are active only at acidic pH [8], SPAM1 has hyaluronidase activity at both neutral and acidic pHs, which arise from two different regions within the hyaluronidase domain [4]. The neutral enzyme activity, which is predominant in insoluble membrane-bound SPAM1, is necessary for sperm to penetrate the hyaluronic acid (HA)-rich extracellular matrix of the cumulus cells surrounding the oocyte. The acidic enzyme activity is present in soluble SPAM1 that is generated during the acrosome reaction (AR), after cleavage at its carboxy terminus [9]. In primate sperm where SPAM1 is a 64-kDa protein, the proteolytically cleaved secretory form that is released after the AR is 53 kDa [9].

Our lab reported a significant increase of Spam1 in caudal mouse sperm compared to caput ones [10]. This finding led to the discovery that *Spam1* is also expressed in all three regions of the mouse epididymis: epididymal Spam1 and its transcript were found in both wild-type and sperm-free mutant (germ cell-deficient) mice as well as in cultured epididymal epithelial cells, completely eliminating the possibility that the protein is merely being transported into the epididymis [11,12]. Expression was shown to be in the principal cells of the epididymal epithelium and to occur preferentially in the distal regions of the tract. Additionally, Zhang and Martin-DeLeon (2001) found evidence that mouse epididymal Spam1 is secreted *in vivo* and *in vitro* and that the gene is differentially regulated in testis and epididymis. Consistent with epididymal expression, putative androgen responsive elements (AREs) were discovered in the murine *Spam1* 5'-flanking region [12].

Since *SPAM1* is widely conserved among mammals [13] it is likely that its pattern of expression may also be conserved. Therefore, human SPAM1 might also be expressed in the epididymis, where it could play an important role in sperm maturation. For this reason, it was thought worthwhile to investigate the presence of SPAM1 in the human epididymis and vas deferens for which a possible role in sperm maturation has been proposed [14]. Because of the difficulty in obtaining fresh human tissue we included in our study tissues of *cynomolgus* macaque which is a primate model for studying human sperm maturation [15].

Materials and Methods

Procurement of human and macaque tissues

Human specimens were collected from five (5) individuals of various ages. The first was a surgical specimen from the human proximal corpus epididymis. It was obtained from a 45-year old man (Subject #1) of known fertility who underwent surgical excision of the epididymis because of a large spermatocele (a cystic swelling) at the level of the corpus epididymis. After surgical removal, a portion (about 1 cm in length) of the intact, non-dilated corpus epididymis was placed in PBS for the experiment. It was then washed in PBS, embedded in OCT medium, and stored at -80°C. Eight and 12-μm thick sections were made from the frozen specimen using a Leica Cryostat 3050 S.

Post-mortem reproductive tissues were obtained from four males. One was a 42-year-old who had died from a myocardial infarction and was not known to have any reproductive problems (Subject #2). The tissues (testis, epididymis, and vas deferens) were obtained through the National Disease Research Interchange (NDRI; Philadelphia, PA) and were either snap frozen in liquid nitrogen or embedded in OCT or paraffin for sectioning. Tissues from Subject #3 and 4, who were 27 and 68 years old, were obtained in the form of slides made from paraffin-embedded sections of testis, epididymis, vas deferens, and seminal vesicles through Novagen (Madison, WI). The tissues were analyzed by a pathologist and found to be morphologically normal and there were no known reproductive problems or medical pathologies that could affect reproductive function. Similarly morphologically normal tissues were used for epididymal sections from a 56 year-old male (Subject #5), and the slides were obtained from the histology core at the University of North Carolina Medical School. None of the subjects were known not to be on hormone therapy prior to death. Macaque (*cynomolgus*) reproductive issues were obtained from Covance (Alice, TX) from two 2 males who were 5 years old and of known fertility. Testis, epididymis, and vas deferens from each animal were snap frozen in liquid nitrogen, embedded in OCT or paraffin after standard formaldehyde fixation for sectioning.

Laser Microdissection (LM)/RT-PCR

To analyze the mRNA expression of *SPAM1* in the epididymal epithelium, frozen sections from Subjects #1 and #2 and paraffin-embedded sections from Subject #3 were used. For macaques, frozen sections of the corpus were obtained from Animal #1 and paraffin-embedded sections of the cauda were from the Animal #2. All paraffin-embedded sections were fixed with 4% paraformaldehyde. The PALM Microbeam system (PALM Microlaser Technologies AG, Bernreid, Germany) was used for laser cutting and separation of selected tissue regions.

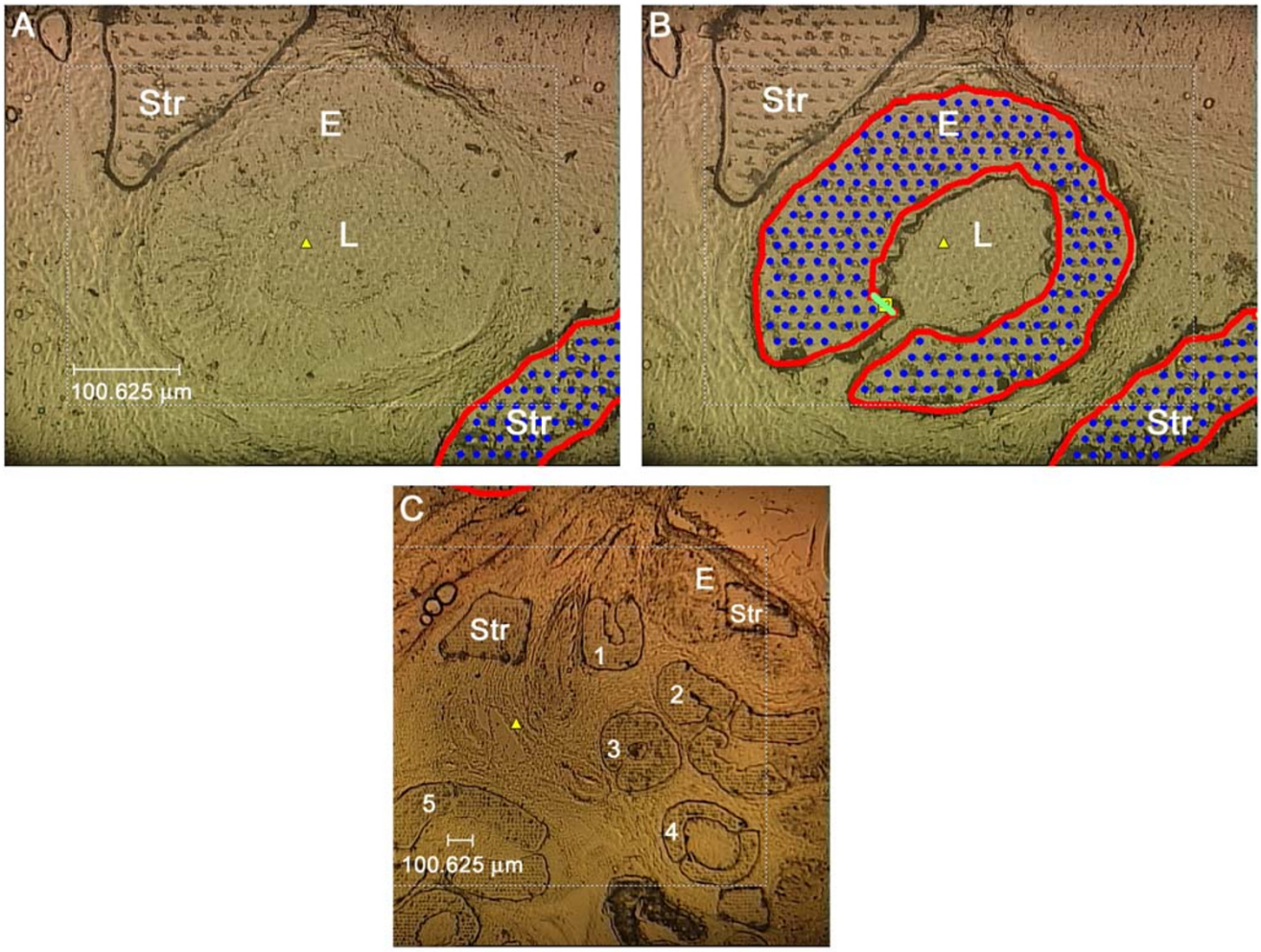

Figure 1
LM-Mediated Isolation of Epithelial cells from 8 µm thick sections of the human corpus epididymis. A) shows a cross-section of a tubule prior to microdissection flanked by two regions of stromal cells (Str) that were microdissected for use as a negative control. The lumen of the tubule is marked with an (L) while the epithelium is marked with an (E). B) shows the tubule in A) after microdissection of the epithelium outlined in red and blue-dotted, and C) displays a low power view of a microdissected section on a slide showing microdissected tubules (1–5), microdissected stromal cells (Str) and an undissected tubule (E).

Immediately prior to microdissection, water was removed from frozen sections by soaking the slides in 100% ethanol for 5 min followed by air-drying for 5 min. Paraffin-embedded sections were treated to remove the paraffin with xylene, prior to the ethanol treatment. Once the tissues were air-dried, the target cells, (epididymal epithelial cells) were carefully laser-microdissected according to the manufacturer's instructions with the cells being collected in mineral oil. The microdissection was performed with care to exclude sperm present in the lumen (Fig. 1). In addition to microdissection of the epithelial cells, stromal cells were also microdissected from the sections to be used as a negative control (Fig. 1). LM conditions were as follows: laser focus 67–69, laser energy 72–76 cut speed 8. These values varied slightly depending on the thickness (8–12 µm) of the sections. The total areas of epididymal cells that were microdissected ranged from approximately 350,000 to 450,000 μm^2. The samples in mineral oil were kept at -80°C until RNA extraction was performed.

RNA Extraction and Reverse Transcription-Polymerase Chain reaction (RT-PCR)

RNA was extracted from LM samples using the Ambion Cells-to-DNA II Kit (Ambion Inc., Austin, Texas)

according to the manufacturer's protocol. Briefly, cells were lysed in Cell Lysis II Buffer after incubation at 75°C for 10 min. DNase I was added and the lysate incubated at 37°C for 15 min. After inactivation of the DNase at 75°C for 5 min 5 μl of the lysate was used for first strand synthesis under the conditions recommended by the manufacturer. Control experiments were performed simultaneously without the addition of reverse transcriptase (RT). Two microliters of each reverse transcription product was subjected to PCR amplification using a pair of primers (forward: nt 1524–1543; reverse 1827–1843) designed from the last exon and the 3' UTR of the human *SPAM1* cDNA sequence (GenBank Accession No. 003117), to yield a product of 320 bp. The identical primer set was used for both humans and macaques. As an added negative control, primers (forward: nt 268–285; reverse 360–377, yielding a 110 bp product) for the transcript of the human Protamine1 gene, *PRM1* (GenBank Accession No. NM 002761), which is spermatid-expressed and so should be sperm-specific, were used to attempt to amplify the cDNA from the epithelial cells. Amplification of the 110 bp fragment of this cDNA would indicate contamination of the epididymal cells with sperm. Human genomic DNA was used as a positive control.

The PCR reactions for *SPAM1* were performed under the following conditions: 94°C for 2 min: 39 cycles at 94°C for 1 min, 59°C for 2 min, 72°C for 2 min, 72°C for 10 min, and 4°C hold. For *PRM1* they were 94°C for 2 min, 35 cycles at 94°C for 1 min, 57°C for 2 min, 72°C for 2 min, 72°C for 10 min, and 4°C hold. The PCR products were resolved on 1% agarose gel and stained with ethidium bromide, and the experiments were repeated.

Sequencing of the RT-PCR product

The gel-purified PCR products were cloned into either a pSTBlue-1 vector (Novagen, Madison, WI) or pCR 4-TOPO TA vector (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Several clones were isolated and sequenced in our core facility.

In situ transcript hybridization

In situ transcript hybridization was performed on tissue sections as described by Deng et al. [11]. Antisense and sense RNA probes generated from PCR of a 320-bp fragment (nt 1524–1843) from the last exon including the 3' UTR of human *SPAM1* were labeled with digoxigenin-11-UTP (DIG) using an in vitro transcription system [Riboprobe® System, (Promega, Cat. No. P1440)] in accordance with the manufacturer's protocol. Tissue sections were fixed for 30 min in 4% paraformaldehyde in DEPC-treated PBS, washed twice for 15 min in PBS containing 0.1% active DEPC, and equilibrated for 15 min in DEPC-treated 5x SSC. Sections were prehybridized for 2 h at 54°C in 500 μl hybridization buffer (50% formamide, 5x SSC, 40 μg/ml salmon sperm DNA) before hybridization with heat denatured RNA probes which had been diluted to 400 ng/ml in hybridization buffer.

After hybridization, slides were washed for 30 min in 2x SSC at room temperature, 10 min in 2x SSC at 65°C, 10 min in 1x SSC at 65°C and 10 min in 0.1x SSC at 65°C. Slides were equilibrated for 5 min in TBS pH 7.5 (100 mM Tris, 150 mM NaCl), and then incubated for 2 h with alkaline phosphate-conjugated anti-digoxigenin antibody (Roche, Cat No. 1093274) diluted 1:5000 in TBS containing 1% BSA. Slides were then washed twice for 15 min in TBS, and equilibrated for 5 min in TBS pH 9.5 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$). They were then stained in NBT/BCIP solution (Pierce, Cat No. 34042) until a dark purple color was visible (up to 6.5 h). The reaction was stopped simultaneously for both test and control by rinsing with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 15 min. Non-specific background was removed by washing in 95% ethanol as needed (20 – 40 s) and slides were rinsed in water for 15 min. They were then counter-stained in 1% neutral red, dehydrated in an ethanol series, cleared in xylene, and mounted in permount. Imaging was done using a Zeiss Axioskop and a Kodak MDS digital camera using proprietary interface software and Adobe Photoshop.

Preparation of protein extracts

Protein extracts from tissue and sperm were prepared based on the methods of Cherr et al. [16] and Deng et al. [11]. Epididymis and vas deferens tissues were minced in PBS, shaken, and allowed to gravity settle before the supernatant was carefully removed. This was repeated at least six times or until the washing was sperm-free, by microscopic examination. Supernatant was retained and sperm pelleted from it by centrifugation at 1,000×g for 10 min. Protein extracts were prepared by lysing sperm or homogenized tissues with a solubilization buffer (62.5 mM Tris-HCl, 10% glycerol, 1% SDS, pH 6.8) containing protease inhibitor (1 mM PMSF) at 4°C. The suspension was vortexed and then centrifuged at 10,000×g for 10 min, and the supernatant containing proteins was retained.

SDS-PAGE and Western blot

Western blot was performed to detect SPAM1 in protein extracts. An equal mass of each protein sample was subjected to reducing conditions (99°C for 5 min in 100 mM dithiothreitol (DTT)), separated on a 15% SDS-PAGE, and transferred to nitrocellulose membrane according to standard protocols. The protein was detected using a 1:1000 dilution of rabbit polyclonal antibody generated against recombinant macaque SPAM1 (a generous gift from Dr. James Overstreet, UC Davis) and the Western-Breeze Chemiluminescent Western Blot

Immunodetection Kit (Invitrogen, Cat No. WB7106) according to the manufacturer's protocol. A Dot blot was also performed to compare the intensities of the protein staining in the different regions of the epididymis and the testis, when equal amounts of protein were loaded. Protein amounts were measured using a BCA assay kit (Pierce, #23227) and spectrophotometry.

Hyaluronic Acid Substrate Gel Electrophoresis

Hyaluronidase activities in tissues were measured using HASGE performed as described by Deng et al. [11]. Briefly, hyaluronic acid from bovine vitreous humor was added to 15% SDS-polyacrylamide gel at a final concentration of 0.15 mg/ml. Gel was run at 15 mAmps constant current until the leading dye band (bromophenol blue) migrated near the bottom. After electrophoresis, gels were incubated at room temperature for 2 h in PBS containing 3% Triton X-100 on a rocking platform to remove SDS. They were then incubated in 100 mM sodium acetate (pH 7.0) at 37°C for 24–48 h. To visualize the digestion of the hyaluronic acid, gels were stained with 0.5% Alcian blue in 3% acetic acid for at least 2 h, destained in 7% acetic acid, and then counterstained with Coomassie brilliant blue G-250. Undigested hyaluronic acid is stained with Alcian blue and shows a dark blue background against an unstained area with digested hyaluronic acid. The gels were scanned with a laser densitometer. Images showing the results of the digestion of the hyaluronic acid by hyaluronidase activity present were captured by scanning the gels.

Immunohistochemistry

To confirm the results obtained from the Western analysis and to verify the SPAM1-expressing cell type, immunohistochemistry was performed. Paraffin-embedded slides were dewaxed with three washes in xylene followed by dehydration in ethanol. After dewaxing, some of the human tissue sections were stained with 0.5% toluidine blue for 5 min to quench autofluorescence. Tissue sections were fixed with 4% paraformaldehyde in 1x PBS (or alternatively methanol) for 30 min and blocked for 60 min in 2% BSA in 1x PBS. In some sections antigen unmasking or retrieval was performed by boiling slides in a microwave for 10 min in 0.01 M citrate buffer, pH 6 [17]. Sections were incubated for 1 h at room temperature in rabbit polyclonal antibody against recombinant macaque SPAM1 (diluted 1:500 in the blocking solution), and then washed three times for 5 min each in 1x PBS. Preimmune rabbit serum was used in place of SPAM1 antibody as a control. Sections were then incubated for 30 min at 4°C in fluorescein isothiocynate (FITC)-conjugated anti-rabbit antibody (Sigma) diluted 1:500 in blocking solution, and then washed three times for 5 min each in 1x PBS. They were then mounted in p-phenylenediamine antifade (1 mg/ml) with or without propidium iodide (1.5 μg/ml) or DAPI (1.5 μg/ml) and viewed on a Zeiss axiophot fluorescence microscope with images taken with a CCD cooled camera, or with a Zeiss LSM 510 NLO multiphoton confocal microscope using the 488 nm and 647 nm lines of the Ar/Kr laser for FITC.

In silico analysis of transcription factor binding sites

The 5' flanking sequence of human *SPAM1* gene was analyzed using the TESS program [18]. This web-based program searches for putative transcription factor binding sites listed in the TRANSFAC database [19]. Statistically significant transcription factor binding site matches were returned. Those factors that are known to function in the testis or epididymis were recorded.

Results

SPAM1 mRNA is Present in the Epididymal Epithelium of Humans and Macaques

In all LM epididymal samples from humans (Subjects 1 and 3 corpus, Subject 2 caput/corpus) and macaques (#1, corpus and 2, cauda) the expected 320 bp *SPAM1* cDNA fragment was amplified in the RT-PCR reactions. The control RT (-) reactions for the epithelium as well as the cDNA from the stromal cells of Subject #2 gave no 320 bp product, unlike the test samples and the positive control which was from human genomic DNA (Fig. 2A). The 320 bp band was verified to be a human *SPAM1* fragment by sequencing. Absence of a *SPAM1* cDNA band from the stromal cells demonstrates that epididymal *SPAM1* mRNA is localized to the tubule epithelium.

The primers for *PRM1* amplified the expected 110 bp product only in the genomic DNA. The absence of this sperm-specific band in the cDNA from the epithelial cell lysate (Lane 11) verifies that the epithelial cells were free from sperm contamination and that the cDNA amplified from the cells is endogenous to the epididymis. In Fig. 2B the results for macaques, which differ from humans at only one nucleotide in the expected 320 bp fragment, show a positive band only in RT (+) reactions, indicating the absence of DNA contamination from the samples. Importantly, sequencing verified the products to be *SPAM1*. These results clearly show that *SPAM1* mRNA is synthesized in the human caput/corpus and the macaque corpus and cauda epididymides.

In situ transcript hybridization was performed on human caput/corpus epididymis (Subject #2; Fig. 3) to confirm the presence of *SPAM1* transcript detected by LM/RT-PCR. The analysis indicates that the transcript is present in the epithelial cells where the purple label can be seen (Fig. 3A). The mRNA was located predominantly in the perinuclear region of the principal cells. Unlike test sections hybridized with the antisense probe, the control sections probed with the sense riboprobe showed only the neutral

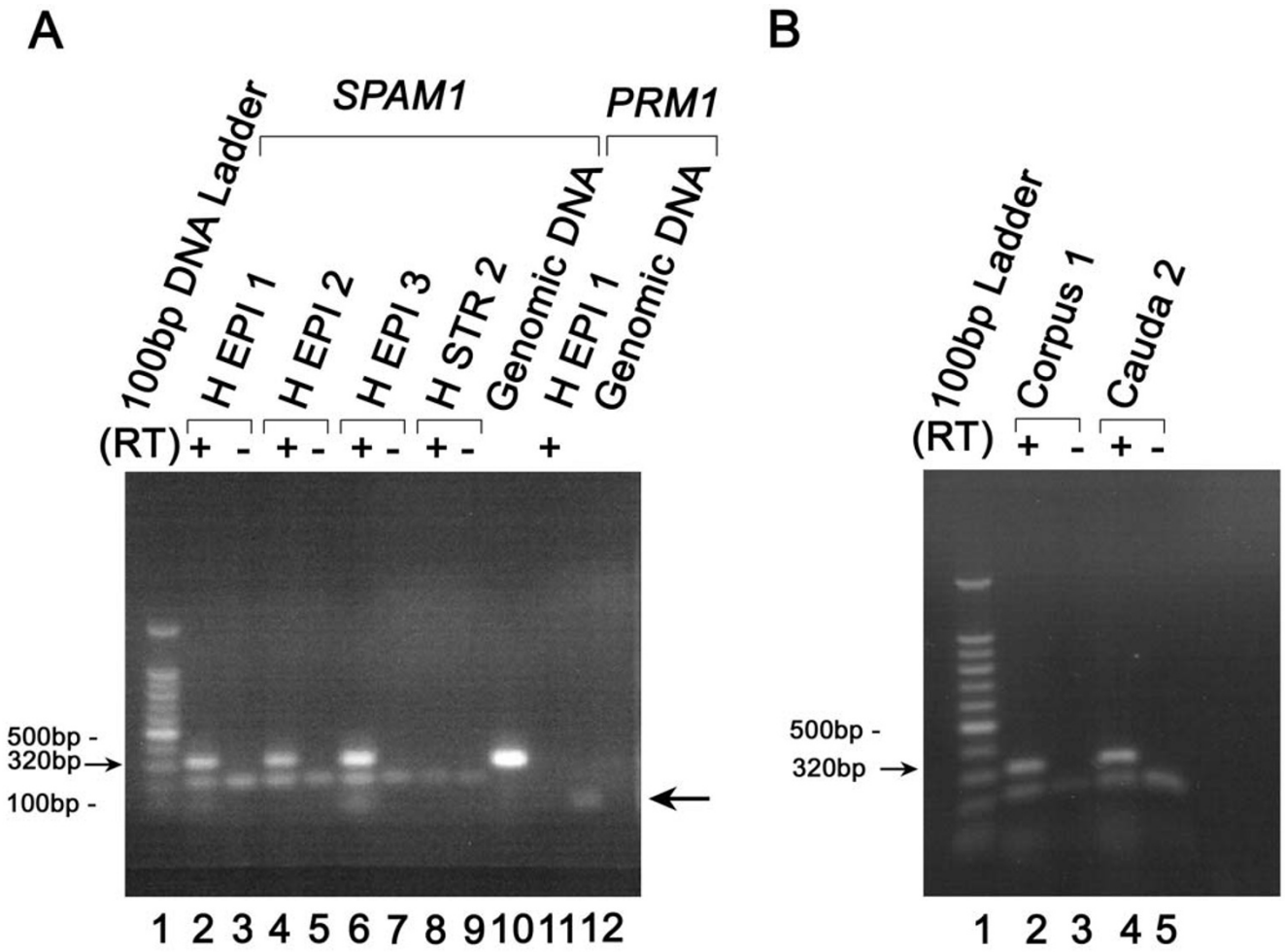

Figure 2
The presence of steady-state *SPAM1* mRNA as detected by LM/RT-PCR of the lysate recovered from human epididymal epithelial cells (H EPI, test) and stromal cells (H STR, control). RNA was subjected to first strand synthesis with (+) or without (-) reverse transcriptase (RT), followed by PCR amplification. The expected 320 bp band for the *SPAM1* cDNA fragment is seen in A) for Subjects 1–3 in Lanes 2, 4, 6, for RT(+) reactions. This band was verified to be *SPAM1* by sequencing. No 320 bp band is detected in Lanes 3, 5, 7, in the absence of RT showing that the *SPAM1* bands are not from DNA contamination. In Lanes 8 and 9 no *SPAM1* bands are detected for the stromal cells, but one is seen in Lane 10 for genomic DNA, the positive control. In Lane 11, no sperm-specific band for *PRM1* cDNA could be amplified from the cDNA of epithelial cells of Subject #1, verifying the homogeneity of the cells. In Lane 12 the 110 bp band (arrowed on the right) is the *PRM1* DNA fragment amplified from genomic DNA, as a positive control. A band of the same size should be generated from the cDNA in the presence of mRNA from the lysate. Note that the primer cloud for *SPAM1* is not present for *PRM1* in Lanes 11 and 12. In B) the 320 bp band in Lanes 2 and 4 are from the corpus of Animal #1 and the cauda of Animal #2, respectively. They were also verified to be *SPAM1* by sequencing. The marker, 100 bp ladder, is shown in Lane 1 of both A) and B). The same results were obtained for replicate experiments.

red staining (Fig. 3B). The purple label seen in the connective tissue in control and test sections may result from endogenous alkaline phosphatase activity.

SPAM1 Protein is Present in the Primate Epididymis and Vas Deferens

SPAM1 protein was detected by Western blot analysis in extracts from testis, epididymis, vas deferens and sperm of Subject #2 and of macaque (Fig. 4A). The intact protein (~64 kDa) was found in each sample studied. Partial

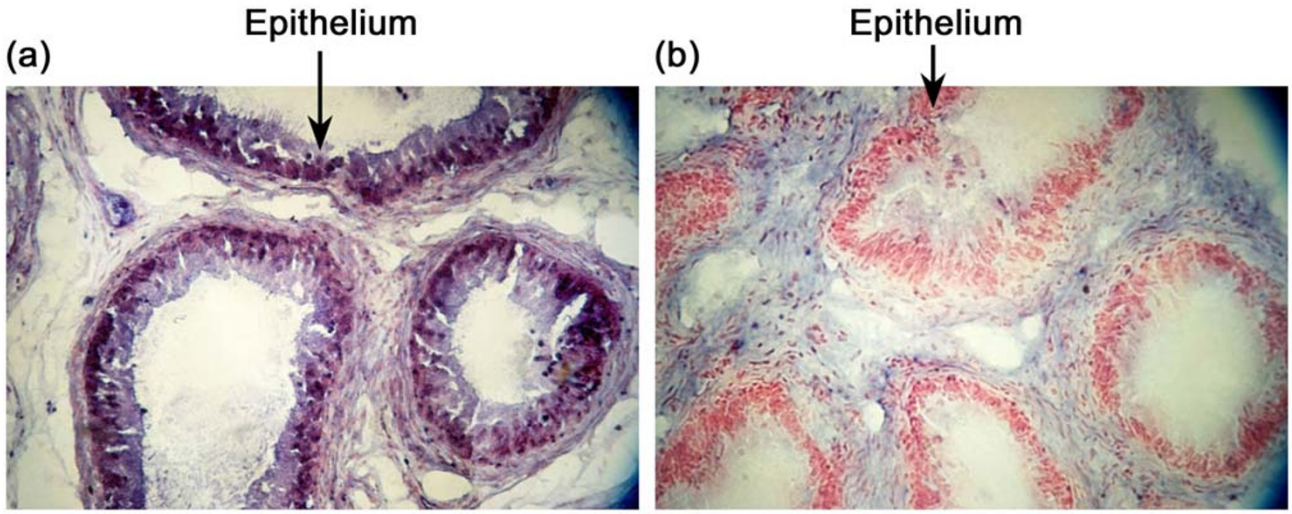

Figure 3
In situ hybridization localization of *SPAM1* transcripts in human corpus epididymal epithelium. Histological sections were hybridized with either an anti-sense (A) or sense (B) *SPAM1* RNA probe. *SPAM1* transcripts localized by immunolabeling appear as a purple stain in the epithelium of (A). This stain is absent from the negative control (B) which shows only the neutral red counterstaining and which was incubated for the same period of time as A) in the developing solution.

degradation products (~60 kDa) appear in epididymal sections. Proteolytically cleaved SPAM1 (~53 kDa) was seen in human sperm samples. Also, a low molecular weight band (<50 kDa) was seen in vas deferens in both species. In macaque where all three regions of the epididymis were studied, the levels of the protein in the epididymis were approximately equal to that seen in the testis, and the distal regions tended to have stronger bands than the caput. The Dot blot which showed that the corpus has the highest level of expression (Fig. 4B) confirmed this tendency. Due to the limited availability of fresh human tissue, only the caput epididymis was studied. The vas deferens of both species seemed to have lower amounts of SPAM1 than the epididymis.

Epididymal SPAM1 Demonstrates Hyaluronidase Activity

Using HASGE, neutral hyaluronidase activity (pH 7.0) was demonstrated in protein extracts from the epididymis, with testis and sperm serving as positive controls. Hyaluronidase activity was demonstrated by a non-staining area corresponding to the size of the intact protein at ~64 kDa (Fig. 5). In samples from the human caput, human sperm and macaque corpus there was also a narrow band with a higher MW (>114 kDa, as indicated by the smaller arrow) which may represent dimerization of the molecule. This experiment was repeated multiple times, and the results seen in Figure 5 are a concatenation of bands from several HASGE gels.

SPAM1 is Immunolocalized in the Principal Cells of the Epididymal Epithelium

SPAM1 protein was localized within the human corpus (Subject #4, Fig. 6) caput and cauda epididymis (Subject #5, Fig. 6), using immunohistochemistry. The images were taken in the red confocal channel to visualize the morphology of the tissues while eliminating the effects of autofluorescence. The control slides (A, D, and F) contain no (or little) green staining, the SPAM1 signal, in the epithelium. For the corpus, epithelial staining is seen in the test samples (B and C). In C the slide is stained with propidium iodide to dye the nuclei red and therefore enhance the visualization of the yellow-green signal. Note that the yellow-green signal is of a lighter hue and is specific for the epithelium compared with the darker green autofluorescence seen in the stroma. Testis sections on the corpus slides were used as positive controls and showed the same color pattern as the epididymis for the presence and absence of the signal (data not shown).

The epithelium in the caput and cauda test sections, E and G, are differentially stained compared to the controls (D and F); although the presence of autofluorescence in G masks some of the green color. However, immuno-staining of the sperm in the lumen of the cauda sections (F and G) serves as an internal control, being similar in color to the epithelium for the presence and absence of the signal.

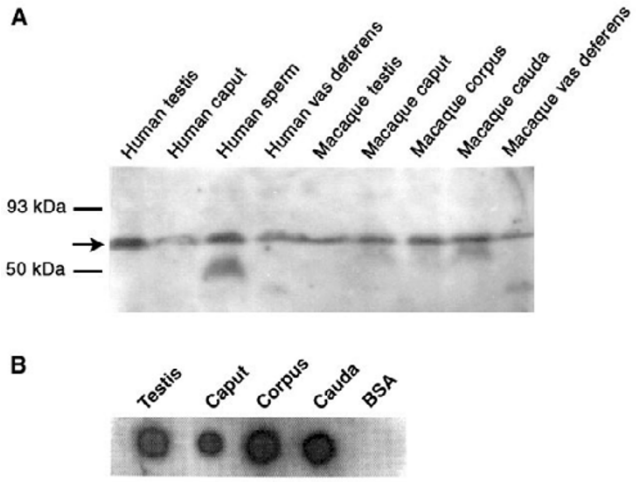

Figure 4
A). Western blot analysis of protein extracts from testis, epididymis, vas deferens and sperm of human (Subject #2) and macaque #1. Equal amounts (40 μg) of protein were loaded in each lane. The arrow points to the ~64 kDa SPAM1 band in all samples. This experiment was repeated three times. A 53 kDa band is also seen for human sperm, while the epididymis shows weaker bands (partially degraded products) at ~60 kDa. In both species the vas deferens show a weak <50 kDa band. B) Dot blot analysis of protein extracts from macaque testis, caput, corpus, and cauda. Bovine serum albumin (BSA) was used as a negative control. Equal volumes with approximately equal amounts of protein (40 μg) were blotted for each tissue.

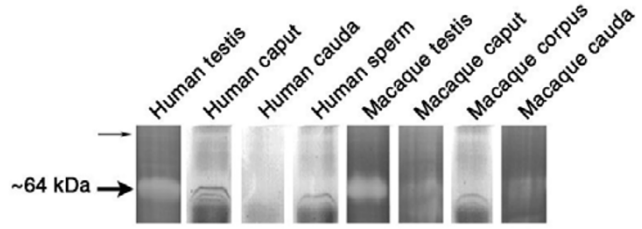

Figure 5
HASGE analysis at pH 7.0 of protein extracts from testis, epididymis, vas deferens and sperm of human and macaque. Equal amounts (60 μg) of protein were loaded in each lane. The large arrow shows a non-staining clear band at ~64 kDa, representing the digested substrate. A narrow band of a much higher MW (approximately 114 kDa) was seen in human caput, human sperm, and macaque corpus, as indicated by the smaller arrow.

For Subject #4 where the slides contained sections from the vas deferens and seminal vesicle (included by Novagen, the commercial supplier), the epithelia of these tissues were also immuno-stained, indicating the presence of SPAM1 (data not shown).

In the two animals studied the epididymal epithelia and the sperm in the lumen of test slides were consistently a brighter green (Fig. 7B) than those of control (Fig. 7A). In the latter the autofluorescence is high due to the nature of fixation. In the epididymides of both humans and macaques the protein was localized to the principal cells of the epithelium and in the heads of maturing sperm (positive control).

SPAM1 Protein is Predominantly Found at the Posterior Head of Immature Human and Macaque Epididymal Sperm

Immunohistochemistry analysis of the proximal corpus epididymal sections offered a unique opportunity to observe SPAM1 localization in immature human and macaque sperm (Fig. 7C). This analysis revealed that SPAM1 is predominantly found in the posterior head of immature sperm from the caput/corpus epididymis, unlike the case of ejaculated sperm where it is distributed across the whole or entire head.

Transcription Factor Binding Sites in the SPAM1 5' Flanking Region

An *in silico* analysis of the human *SPAM1* 5' flanking region was performed on the sequence of a 700 bp genomic fragment previously isolated and cloned in our lab from promoter walking, and on the human genome database sequence through NCBI. Several putative transcription factor binding sites were found which may be used for *SPAM1* expression in the epididymis, and these sites are likely distinct from those used in the testis (Table 1). SRY and CRE (cAMP-responsive element) sites were found in the more proximal promoter region: these are markers for germ cell expression in the testis. Particularly interesting is the presence of putative androgen responsive elements (AREs), to which androgen receptor transcription factors bind in response to androgens. These results underscore the finding of *SPAM1* expression in the epididymis.

Discussion

Although human *SPAM1* mRNA expression is detected by Northern analysis only in the testis [20], RT-PCR assays reveal that the gene is also transcribed in somatic tissues where the message is rare. Rare *SPAM1* transcripts are however not ubiquitous as revealed by their absence from human ovary, spleen, and liver [21] and murine skeletal muscle [22]. Both rare and abundant *SPAM1* transcripts have been found in neoplastic breast tissue [23] and in a number of other cancers including pharyngeal [24], met-

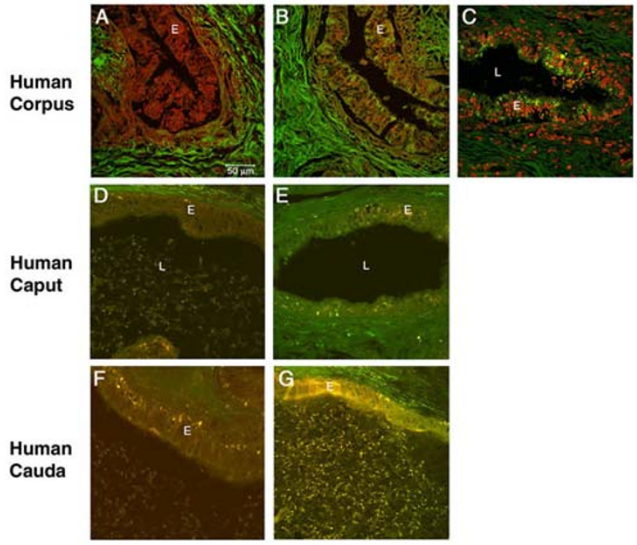

Figure 6
Immunohistochemical localization of SPAM1 in the human epididymis using multiphoton confocal microscopy. The magnification of tissue sections is the same as is shown in A. Images are taken in the red confocal channel to help eliminate the effect of autofluorescence which occurred in varying degrees depending on the laboratory in which the slides were fixed before sectioning. Preimmune serum was used for negative controls (A, D, and F). The epithelium (E) in A which is from the human proximal corpus (Subject #4) appears red, while SPAM1 is indicated by the yellow-green color in the epithelial lining of the test sections, B and C, also from the corpus of the same male. In C the nuclei are stained with propidium iodide to enhance the visualization of the FITC green color of the signal. Note that the green color in the elastic fibers in the stroma underlying the epithelium in test and control is due to strong autofluorescence and not the primary antibody. D and E, and F and G are from Subject #5, these slides as well as those from the macaques had very high levels of autofluorescence, however the sperm in the lumen (L) serve as a positive control, varying in staining intensity between F and G. Although no sperm are present in the caput test section, the epithelial lining for control (D) and test (E) are quite different in color.

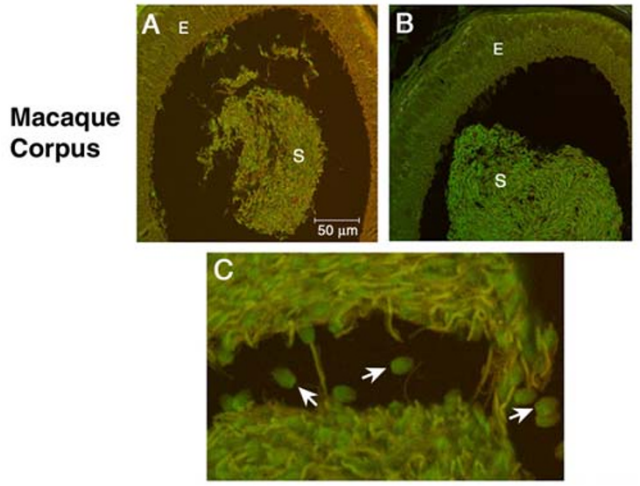

Figure 7
Immunohistochemical localization of SPAM1 in macaque epididymis and epididymal sperm using multiphoton confocal microscopy. In A and B (magnification seen in A) are shown the control and the test, respectively; after SPAM1 antibody staining in the corpus (Animal #2). As in Fig. 6, the images are taken in the red confocal channel but due to high autofluorescence the control is not as red as in Fig. 6. However sperm in the lumen serve as a positive control for the test whose epithelium and sperm are greener than the control. In C) are seen immature sperm from the corpus (magnification = ×1,000). SPAM1 is localized more heavily to the posterior region of the sperm head (as shown by the arrows) as was also seen for immature human sperm (data not shown).

astatic melanomas and gliomas [25]. In normal somatic cells rare transcripts have been found in breast tissue [23] and in fetal, placental, and prostate cDNA libraries [21].

Expression in the prostate is relevant to our findings in the present study where transcripts were detected in the human caput/corpus epididymis using LM/RT-PCR. This technique which has been effectively used to procure homogeneous populations of cells from tissue sections [26–28] revealed that *SPAM1* mRNA is confined to the epididymal epithelium. *In situ* hybridization demonstrated that the transcript is localized in the principal cells, confirming its epithelial location, similar to that reported for the mouse [11,12]. It must be stressed that because the probes used in the hybridization were generated from the unique 3' UTR of *SPAM1* cDNA the possibility of cross-hybridization to paralogous hyaluronidases is highly unlikely. This conclusion is supported by unique BLAT assignment of the probe sequence to the human genome [29].

SPAM1 protein was found to be present in both the human and macaque reproductive tracts. This indicates that in the epididymis and vas deferens *SPAM1* is both transcribed and translated. The results from Western analysis of protein extracts from the epididymis, which like the vas deferens were thoroughly washed to remove sperm, show conclusively that intact SPAM1 protein is synthesized in all three epididymal regions in macaques. It is noteworthy that the ~53 kDa band, representing proteolytically cleaved SPAM1 was seen only in sperm and not in the epididymis or the vas which had their own unique

Table 1: Putative transcription factor binding sites

Transcription Factor Binding Sequence	Binding Site Location (-bp)
ARE	661, 763
GATA family	92, 125, 313, 397
PEA3	696, 741, 900, 1063
SRY	18, 155, 661
CRE	207

In silico analysis of the human SPAM1 5' flanking region detected several putative transcription factor binding sites associated with epididymal expression. Androgen responsive elements (ARE), GATA-family sequences, and PEA3 sequences are markers for epididymal expression [43]. SRY and CRE are markers for testicular expression.

degradation products at ~60 kDa and <50 kDa, respectively. This suggests that the Western results may reveal an isoform that is endogenous to each tissue. It also indicates and that sperm is not the source of epididymal SPAM1. Also in macaque the intensity of the epididymal bands was equal to, or greater than, that of the testis. This also makes it highly unlikely that SPAM1 is present in the epididymis merely because it is transported there from the testis.

Western blot analysis also suggests that SPAM1 protein is expressed at higher levels in the distal regions of the epididymis, with the highest level of expression in the corpus, a region associated with a lower volume of sperm. This is supported by the Dot blot, with equal protein loading, showing the macaque corpus to have the highest amount of SPAM1. This observation not only argues against the mere transportation of SPAM1 into the epididymis, but also suggests a region-dependent expression of the gene. This finding is consistent with that reported in the mouse [11,12] where there is differential expression of the protein in the epididymis with the highest expression in the corpus. Expression in the extratesticular pathway seems to be lowest in the vas deferens in both human and macaque. However, its presence prompted us to examine the vas in the mouse where it had not been previously studied: both the transcript and protein were found (Martin-DeLeon et al, unpublished results). Thus there seems to be a conservation of the pattern of expression of SPAM1 in the extratesticular pathways of mice and primates.

The finding from the Western blots was corroborated by those from immunohistochemistry. The latter indicates that SPAM1 is associated with the principal cells of the epithelium, consistent with the localization of the transcript and with findings in the mouse [11,12]. The finding of human SPAM1 in the seminal vesicle, which was examined only because it was included in the tissue series on the slides from Novagen, was unexpected but not surprising. CD52, a well-known human epididymal protein, is also known to be expressed in the seminal vesicle [30]. While the expression of human SPAM1 in the seminal vesicle remains to be confirmed, data from our lab have shown that the murine *Spam1* gene is both transcribed and translated in this tissue (Martin-DeLeon, unpublished results).

HASGE shows that epididymal SPAM1 has neutral hyaluronidase activity (Fig. 5), which is typical only of sperm hyaluronidase. This further supports the identity of the protein expressed in the epididymis. In general, the expression of the enzyme activity was less robust in the epididymis compared to the testis, and inconclusive for the vas deferens. The latter is not surprising because (1) Western analysis showed SPAM1 levels to be the lowest in the vas deferens and (2) it is reasonable to expect that HASGE is less sensitive than Western analysis. More importantly, it is possible that the hyaluronidase activity in cells from non-testicular origin may be lower than that of the testis due to differences in the pattern of glycosylation. Variations in glycosylation patterns have been shown to exist among tissues [31] and glycosylation levels have been reported to influence hyaluronidase activity of SPAM1 in the mouse and horse [10,32]. Note that in the mouse, tissue differences in glycosylation patterns have been reported for the testis and epididymis [33]. It is possible that in the epididymis and vas deferens instead of hyaluronidase activity, a more important role of SPAM1 (which is a glycosidase) may be to modify surface proteins of sperm during their maturation. Taken together, the data from tissues of 5 men and 2 macaques show that SPAM1 is both transcribed and translated in the epididymal epithelium of primates.

The finding of conserved expression patterns of epididymal SPAM1 between humans and mice is supported by the findings in the promoter of the human gene. Table 1 reveals that in human *SPAM1* there is a CRE transcription factor binding site, a site that was shown to be functional in the murine *Spam1* promoter region [34] as well as putative AREs which are also present in the mouse [12]. Of

special interest are the relative locations of the AREs which are associated with genes expressed in the epididymis [35] and the prostate at -661 and -763; and a CRE (cyclic-AMP responsive element) which is consistent with haploid expression in the testis at -207 [34]. The relative location of these elements are consistent with the findings of Hsia et al. [36] who observed that within the promoter regions of genes expressed in both the testis and the epididymis, the testis-expression elements are more proximal to the transcription start site than those mediating epididymal expression.

It should be mentioned that CRE and SRY are likely to be responsible for increasing the basal transcriptional level of *SPAM1*, leading to the robust expression of the mRNA that is detectable with Northern analysis only in the testis. Note that the relevant transcriptional binding factor and/or co-activator for CRE (CREM and ACT) and for SRY in the adult (SRY) are testis-specific [37,38]. Future studies are necessary to determine if the transcriptional binding sites revealed by this study are functional in humans.

In the immunohistochemical studies the sperm concentrations in some of the lumen of the tubules in sections of the caput/corpus epididymis were low enough to allow the observation of SPAM1 localization on immature sperm. Observations of sperm from Subject #1, the 45-year-old male of known fertility with an obstruction in his corpus epididymis, as well as macaques, revealed that SPAM1 is concentrated in the posterior and deficient in the anterior head of immature primate sperm. This localization is quite different from that reported for ejaculated human sperm where SPAM1 is found uniformly over the entire head surface [39]. Our finding suggests that SPAM1 undergoes redistribution on the sperm head during epididymal maturation in primates as it does in guinea pigs and mice [40,10], albeit with a reverse order going from a regionalized to uniform pattern.

The finding that SPAM1 with enzymatic activity is expressed in the epididymides of three species (mice, humans and macaques) in two classes of mammals indicates that epididymal SPAM1 is functionally important. It is likely that SPAM1 may play a role in sperm maturation, since its expression is highest in the corpus where a number of maturational changes occur [41,42]. Thus, this study increases the biomedical significance of findings regarding Spam1 in the mouse model. For example, it is reasonable to believe that like the mouse, human SPAM1 is secreted from the epididymal epithelium with an intact lipid anchor [12,33] and may impact sperm maturation. It should also be noted that a study of the glycosylation and 2D-gel patterns of Spam1 from the epididymis and testis of mice indicates that epididymal Spam1 may be a unique isoform rather than a redundant protein [33]. Such a unique isoform may play a specific role in sperm maturation in humans where SPAM1 is the only reproductive hyaluronidase in the cluster on 7q31. Currently studies are underway to determine the function of epididymal Spam1 in the mouse.

Authors' Contributions

EAE participated in the design, generated the riboprobes, performed the cryosections, the in situ hybrdizations, and immunohistochemistry, and drafted the manuscript. HZ carried out the RT-PCR, Westerns, immunohistochemistry, and the HASGE. PAM-D conceived of the study, participated in its design, performed the laser microdissections, and directed and coordinated the investigation.

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