Journal of Andrology, Vol. 28, No. 5, September/October 2007
Copyright © American Society of Andrology

Microvillar Size and Espin Expression in Principal Cells of the Adult Rat Epididymis Are Regulated by Androgens

NADIA PRIMIANI,* MARY GREGORY,† JULIE DUFRESNE,† CHARLES E. SMITH,‡ YE LAUREN LIU,* JAMES R. BARTLES,§ DANIEL G. CYR,*† AND LOUIS HERMO*

*From the *Department of Anatomy and Cell Biology, McGill University, Montreal, Canada; †INRS-Institut Armand Frappier, Université du Québec, Pointe-Claire, Canada; ‡Département de Stomatologie, Université de Montréal, Montreal, Canada; and §Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois.*

ABSTRACT: Principal cells of the epididymis are the most prominent cell type and are noted for an apical cell surface studded with microvilli. The latter contain channel proteins that condition the microenvironment of epididymal lumen and promote sperm maturation; however, the regulation of the structure and integrity of microvilli is not well known. Espins are a family of proteins implicated in microvillar growth. The objectives of this study were to assess the regulation of espin in epididymal principal cells both in vitro and in vivo. Treatment of immortalized rat caput epididymal (RCE) cells with increasing doses of a homogenized testicular extract revealed a dose-dependent increase in the size of microvilli. Reverse transcriptase–polymerase chain reaction (RT-PCR) of adult rat epididymal RNA using espin-specific primers indicated the presence of a band at about 290 base pairs (bp) in all regions. Western blot analysis using affinity-purified espin antibody confirmed the presence of an approximately 110-kDa band in the epididymis, corresponding to espin isoform 1. In adult rats, immunocytochemistry revealed espin expression over principal cells. In orchidectomized rats, espin expression was significantly reduced, whereas ligation of the efferent ducts resulted in a decrease of espin expression but not to the extent of orchidectomy. The fact that espin expression was restored to control levels in orchidectomized rats supplemented with high levels of testosterone indicated that its expression was dependent on androgens and not on other lumicrine factors derived from the testis. Taken together, these data indicate that espin is expressed in the epididymis and is regulated by androgens.

Key words: RT-PCR, immunocytochemistry, RCE cell line, orchidectomy, testicular extract.

J Androl 2007;28:659–669

As spermatozoa transit through the male reproductive tract, they undergo various modifications to their plasma membrane, and the most important of these occur in the epididymis (Robaire and Hermo, 1988; Cornwall et al, 2002; Robaire et al, 2006). The mammalian epididymis is a highly coiled duct that links the efferent ducts to the vas deferens and serves as the site where spermatozoa become motile and fertile (Orgebin-Crist, 1967, 1969). The epididymis is made up of several distinct segments (Reid and Cleland, 1957; Hamilton, 1975; Hermo et al, 1994) and is composed of distinct epithelial cell types, with each one differing both structurally and functionally in the different segments giving rise to a complex pattern of gene expression and regulation along the length of the epididymis (Cornwall et al, 2002; Dacheux and Dacheux, 2002; Hermo and Robaire, 2002; Robaire et al, 2006).

Principal cells, the most abundant epithelial cell type, have been implicated in diverse functions including the absorption and secretion of water, ions, solutes, proteins, and lipids (Hermo and Robaire, 2002; Turner, 2002; Breton, 2003). These activities allow for the formation of a specific luminal microenvironment that changes dramatically along the length of the epididymis, and it is in this highly specialized milieu that sperm undergo their maturational modifications (Hermo and Robaire, 2002; Turner, 2002; Breton, 2003). This microenvironment is maintained by the presence of a blood-epididymal barrier that is formed apically between adjacent epithelial cells and serves to maintain the necessary ionic composition and pH of the lumen (Cyr et al, 2002). In addition, tall microvilli extend from the apical plasma membranes of principal cells and form a uniform brush border with occasional branching. These microvilli, termed stereocilia, provide the cell surface with a large surface area potentially available to interact with molecules present in the lumen. Of particular note is the fact that microvilli

Supported by grants from the CIHR (L.H.) and NSERC/CIHR Collaborative Health Grant (D.G.C., L.H.), NSERC (D.G.C.), and the National Institutes of Health (C.E.S., DE013237; J.R.B., DC004314).

Correspondence to: Dr Louis Hermo, Department of Anatomy and Cell Biology, McGill University, 3640 University St, Montreal, Quebec, H3A 2B2 (e-mail: louis.hermo@mcgill.ca).

Received for publication February 1, 2007; accepted for publication March 22, 2007.

DOI: 10.2164/jandrol.107.002634

contain a variety of membrane pumps, channels, and transporter systems and in this way maintain the proper volume as well as appropriate pH of both the principal cell and epididymal lumen (Wong et al, 2002; Turner, 2002; Hermo et al, 2002, 2005; Breton, 2003). While past studies have demonstrated that microvilli on principal cells are reduced in size after orchidectomy (Wahlqvist et al, 1996), the specific testicular factor(s) involved in their maintenance and the protein(s) involved has yet to be identified.

Microvilli are built around a common cytoskeletal element—the parallel actin bundle (Bartles, 2000). This bundle consists of tightly packed collections of actin filaments crosslinked by actin-bundling proteins, which serve to maintain many of the characteristics of microvilli. Three major classes of actin-bundling proteins have been detected in microvilli of vertebrate cells: villin, fimbrins/plastins, and espins (Bartles, 2000). Espins are actin-bundling proteins that come in multiple isoforms from a single gene and that differ markedly in size and their complement of ligand-binding sites (Sekerková et al, 2004, 2006a). The different espin isoforms or combinations of isoforms are expressed in different cell types and in complex spatiotemporal patterns during development (Sekerková et al, 2006a). In epithelial cells, they serve to elongate parallel actin bundles and thereby determine the steady-state length regulation and integrity of microvilli and stereocilia (Sekerková et al, 2006a).

The prototype espin was first identified as an approximately 110-kDa protein localized in the junctional plaque of the Sertoli cell ectoplasmic specialization of the testis, from which it derives its name: espin = ectoplasmic specialization + in (Bartles et al, 1996; Chen et al, 1999). To date there are 4 major espin isoform size classes ranging from about 110 to about 25 kDa and designated as espin 1–4 in order of decreasing size; splice variants are further specified alphabetically (Sekerková et al, 2004). Present in multiple actin-rich structures, espins are especially prominent in stereocilia of cochlear and vestibular hair cells and the microvilli of other sensory cells (Zheng et al, 2000; Sekerková et al, 2004, 2005). The stereocilia of principal cells of the human epididymis have been shown to contain an internal core of actin filament bundles crosslinked by fimbrin and associated with the membrane linker ezrin. Unlike brush border microvilli in the intestine and kidney, the microvilli of epididymal epithelial cells lack the bundling protein villin, but they also contain a different class of actin crosslinking protein, alpha actinin, in the stem portion of their long microvilli (Hofer and Drenckhahn, 1996).

In this study, we sought to explore how testicular factors regulate the growth and molecular composition of epididymal cell microvilli. These analyses were performed in vivo and using an immortalized rat caput epididymal (RCE) cell line, which has recently been developed by Dufresne et al (2005). We identify a specific espin isoform in the microvilli of epididymal principal cells and discover that its expression and microvillar dimensions are regulated by testicular factors.

Materials and Methods

Animals

All experimentation on animals was carried out according to guidelines defined by the McGill University Animal Care Committee and the Northwestern University Animal Care and Use Committee. Adult Sprague-Dawley rats (300–400 g) were purchased from Charles River Canada, Ltd (St Constant, Canada) and maintained on a 12-hour light-dark regime. All rats were fed Purina rat chow and given water ad libitum.

Experimental Protocols

In Vitro Regulation—Immortalized RCE cells were grown on 6-well plates coated with collagen IV in DMEM/HAM F12 medium (Sigma-Aldrich, Mississauga, Canada). Cells were incubated at 32°C with 5% CO_2. The cultured cells adhere to the collagen and polarize with microvilli extending from 1 surface. The cells were removed from the wells by trypsin digestion (0.05%). Procedures for culturing these cells are described by Dufresne et al (2005). Preparation of testicular extract was as follows: adult rat testes were homogenized in culture medium (2 vol/wt). Homogenates were centrifuged at maximum speed for 10 minutes at 4°C. The supernatant was collected and stored at −86°C. The supernatant was filtered using a 0.22-μm filter prior to use. Separate wells containing the RCE cells were cultured in the presence of 0% (control), 0.05%, 0.5%, and 5% testicular extract (TE) for 48 hours. At the end of the incubation period, the cells from 4 separate wells for each experimental group were centrifuged at 1000 × *g* at room temperature and the pellets fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 hours at 4°C. Cells were washed overnight in 0.1 M sodium cacodylate buffer, dehydrated in acetone, and embedded in Epon. Ultrathin sections were cut with a diamond knife and mounted on copper grids. Sections were stained with lead citrate and uranyl acetate prior to examination by electron microscopy (JEOL-USA Inc, Peabody, Mass).

Electron micrographs of microvillar areas at 1 pole of the RCE cells exposed to different concentrations of testicular extract were taken at a raw magnification of 4300 × from which prints were made at a final enlargement of 10 500 × . The total boundary area occupied by microvilli projecting from the apical side of the cells was measured using a MOP-3 image analyzer (Carl Zeiss Canada Ltd, Toronto, Canada). The area occupied by microvilli projecting into this space was then outlined and summed, and the dead space between microvilli was computed as the difference between the total boundary area and the summed microvillar areas in the microscopic field. Microvillar areas from 70–85 different cells were measured in this fashion for each

treatment group. Mean profile areas for the boundary, microvillar, and dead space in each treatment group and accompanying standard deviations and 95% confidence intervals were computed using Statistica software (version 7.1; Statsoft Inc, Tulsa, Okla). Initial analyses indicated that the data were not distributed normally, and $\log_{10}$ transformations were done prior to carrying out 1-way analysis of variance for multiple groups with unequal N, Student's *t* tests for independent samples (controls vs any one of the treatment groups), and power tests. Significance was established at $P < .05$.

In Vivo Regulation—Adult rats were anesthetized with an intraperitoneal injection of ketamine-xylazine (50:10 mg/kg). Four rats were sham operated as controls. Four other rats were bilaterally orchidectomized via an abdominal incision and killed 14 days later. Eight other orchidectomized rats were implanted with either an empty 2.5-cm capsule (4 rats) or 18.6-cm capsules (3 measuring 6.2 cm each) (4 rats) containing testosterone. Testosterone-filled polydimethylsiloxane capsules were prepared according to the method outlined by Stratton et al (1973) and have well-characterized steroid release rates (Brawer et al, 1983). These capsules mimic epididymal (18.6 cm) testosterone levels, which are 10 times greater than blood levels. Additional carrier rats were implanted with the testosterone implants prior to the start of the experiment to ensure that the implants functioned properly. These implants were removed from the carrier rats 3 days later, cleaned, and inserted subcutaneously on the backs of experimental animals at the time of orchidectomy. All of these rats were killed 14 days after surgery. At the completion of each experiment, the epididymides were fixed by retrograde perfusion with Bouin fixative and subsequently dissected and processed as described below for immunocytochemical analyses.

Efferent duct ligations were performed as follows. Adult rats (350–400 g) were anesthetized with an intraperitoneal injection of ketamine-xylazine (50:10 mg/kg). An abdominal incision was made through the peritoneum, and the testis and epididymis were gently retrieved. The efferent ducts were exposed, and the fat surrounding the ducts was displaced with tweezers. Using a black silk suture, the efferent ducts of each epididymis were ligated close to the rete testis leaving the vascularization intact. The testis and epididymis were then gently replaced in the scrotum and the incision sutured. Four sham operated rats were used as controls. Rats were sampled 14 days following surgery. At the end of the experiment, the epididymides were fixed by perfusion with Bouin fixative.

Identification of Espin mRNA in Adult Rat Epididymis—Four adult male rats were anesthetized with an intraperitoneal injection of ketamine-xylazine (50:10 mg/kg) and killed. Epididymides were removed and divided into 4 segments (initial segment [IS], caput [CT], corpus [CS], cauda [CA]); testes were used as positive controls. RNA was isolated from each segment using a commercial kit (Absolutely RNA RT-PCR Miniprep kit; Stratagene, Cedar Creek, Tex) according to the manufacturer's instructions. Espin-specific primers were designed according to the rat espin sequence. Reverse transcription was carried out using the reverse primer specific for the espin sequence (5′-TTC AAG ATG ACC TGT CGC TGC-3′). Specific forward and reverse espin primers (reverse: 5′-CTC GTC GTA TCC TAG TGT CCG-3′; forward: 5′-CCC TCA TCC CCA CAC TTG ATG AGC-3′) were used for polymerase chain reaction (PCR). Espin was linearized at 94°C and amplified using 35 cycles of 94°C (30 seconds), 60°C (1 minute), and 68°C (1 minute). Resulting amplicons were separated on 1.5% agarose gels in Tris-Borate Ethylenediammine Tetraacetic Acid (90 mM Tris-Borate, 2 mM Ethylenediammine Tetraacetic Acid [EDTA]; TBE) buffer and stained with ethidium bromide (0.05 µg/mL). The intensity of bands was determined using a Bio-Rad Fluor Image analyzer (Bio-Rad Laboratories, Mississauga, Canada).

Immunocytochemical Localizations of Espin—Testes and epididymides from 6 adult male rats were fixed by perfusion through the abdominal aorta in a retrograde manner with either Bouin (n = 3) or St Marie fixative (n = 3). After 10 minutes of fixation, tissues were removed and placed for 24 hours in fresh fixative at room temperature and then placed in 70% alcohol for several days prior to dehydration and embedding in paraffin. Tissue sections of 5-µm thickness were cut and mounted on glass slides. Affinity-purified rabbit polyclonal anti-espin antibodies were utilized at a concentration of 10 µg/mL. These antibodies were purified and characterized as previously described (Bartles et al, 1996).

Tissue sections were deparaffinized in Histoclear (Fisher Scientific, Ottawa, Canada) and rehydrated by immersion in a series of graded ethanols. Immunocytochemistry was done using the DAKO Catalyzed Signal Amplification System (DAKO, Carpenteria, Calif) according to the manufacturer's instructions. Control sections were incubated with preimmune serum.

Immunofluorescence—Specimens fixed in St Marie fixative and embedded in paraffin were utilized for all immunofluorescent localizations done with adult in vivo epididymal tissues. Sections were rehydrated and blocked with phosphate buffered saline (PBS) + 5% bovine serum albumin before incubation with anti-espin antibodies (diluted 1:200) for 90 minutes at 37°C. At the end of the incubation period, the sections were washed in PBS and subsequently incubated with a secondary antibody labeled with a Rhodamine Red-X tag (diluted 1:200; Jackson Immunoresearch, West Grove, Pa) for 30 minutes. Sections were washed in PBS and mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, Calif). Similar incubation procedures were carried out for the RCE cells fixed in ice-cold methanol. Control slides were incubated with preimmune serum.

Western Blot Analyses—Western blots were done according to the methods described by Sekerková et al. (2006b). Briefly, epididymides were homogenized in 20 vol (milliliters per gram of wet tissue) of 0.25 M sucrose, 3 mM imidazole-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride at 4°C using a 5-mL Potter–Elvehjem homogenizer (8 strokes, 3000 rpm) and immediately extracted at a concentration of about 40 mg of wet tissue per milliliter by heating at 100°C in sodium dodecyl sulfate (SDS) gel sample buffer for 3 minutes, with intermittent agitation on a vortex mixer. The samples were centrifuged at 16 000 × *g* in a microcentrifuge, and the resulting supernatants were resolved in SDS gels and analyzed on Western blots using the ECL system (GE Healthcare, Baie d'Urfé, Canada) with or without prior stripping according to the procedure recommended by the manufacturer.

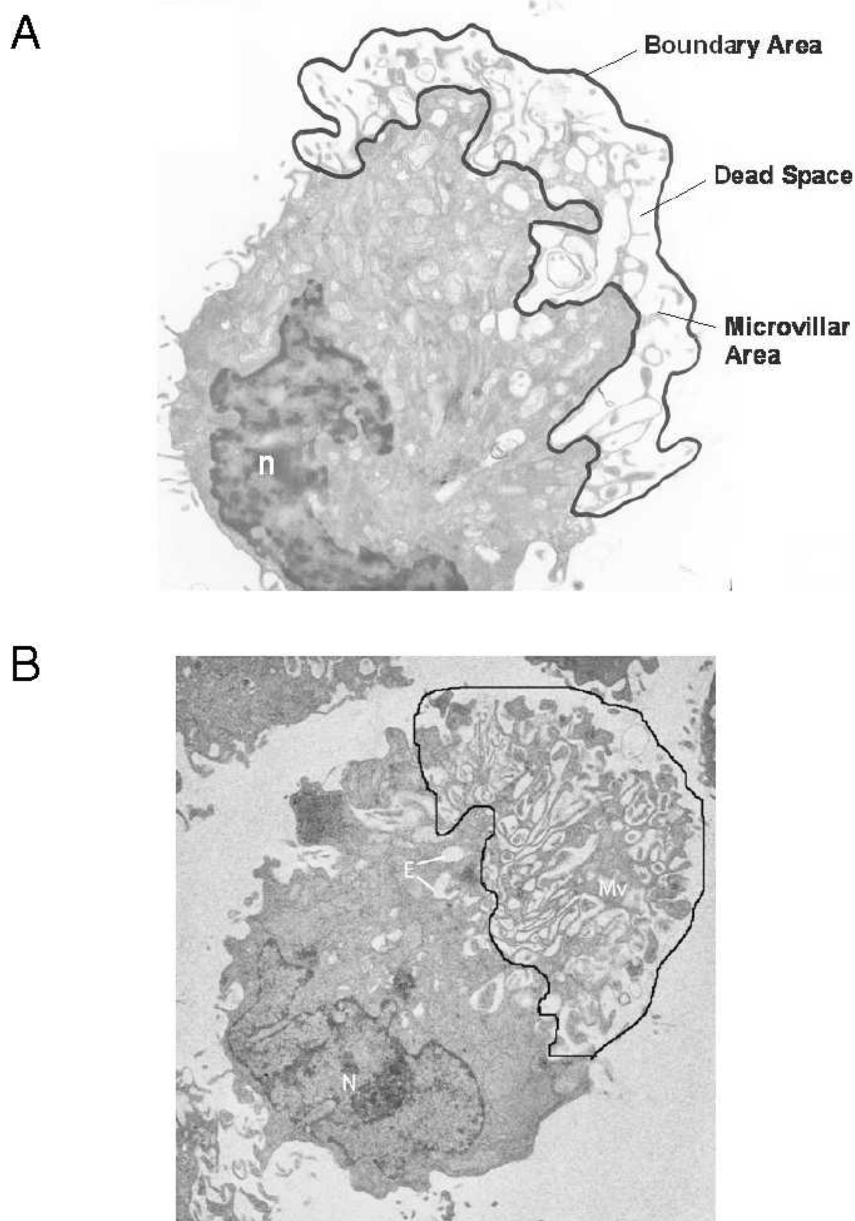

Figure 1. **(a)** Electron micrograph of an untreated rat caput epididymal (RCE) principal cell with superimposed lines demarcating the boundary area, microvillar area, and dead space area that were measured to calculate the size of microvilli in cells exposed to increasing concentrations of testicular extract. n indicates nucleus. Original magnification = 6500 ×. **(b)** Electron micrograph of an RCE principal cell treated with 5% testicular extract. Note the extensive microvillar development along 1 pole of the cell. mv indicates microvilli; E, endosomes. Original magnification = 6500 ×.

Results

Effects of Testicular Extract on Microvillar Growth in the RCE Cell Line

In the electron microscope, control RCE cells and those incubated with 0.05% testicular extract had prominent nuclei, a well-developed Golgi apparatus, abundant cisternae of endoplasmic reticulum, and occasional endosomes and lysosomes in addition to cytoskeletal elements (Figure 1a), all features characteristic of principal cells in vivo (Hamilton, 1975; Robaire and Hermo, 1988). A few scattered microvilli were evident along their apical surface (Figure 1a). In contrast, whereas RCE cells exposed to 0.05%, 0.5%, and 5% concentrations of testicular extract showed secretory and endocytic organelles comparable in size and

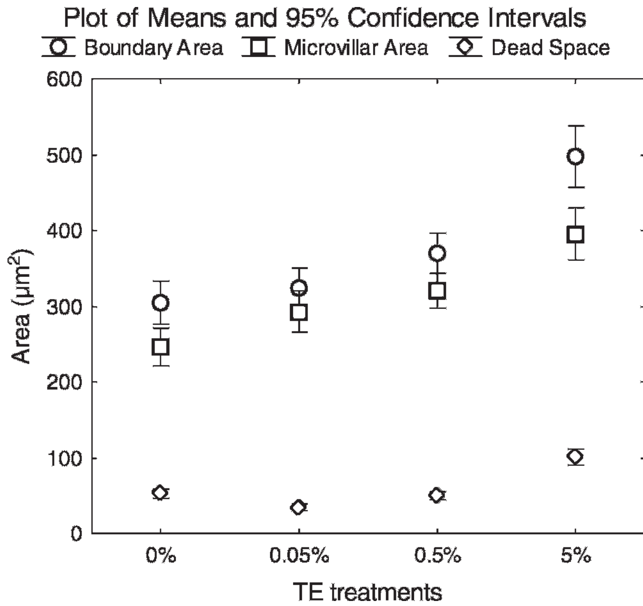

Figure 2. Quantification of boundary area, microvillar area, and dead space area in principal cells cultured with 0%, 0.05%, 0.5%, or 5% testicular extract. Results show significant increases in all 3 parameters.

Differences and significance tests of means for testicular extract treatments

Parameter*	Change, %	*P* †	Power‡
Boundary area			
0.05%	6	.5589§	0.2430
0.5%	21	.0025	0.9325
5%	63	.0000	1.0000
Microvillar area			
0.05%	19	.0145	0.7930
0.5%	30	.0001	0.9889
5%	61	.0000	0.9999
Dead space			
0.05%	−34	.0000	0.9891
0.5%	−6	.9990§	0.0520
5%	93	.0000	1.0000

* Number of observations for treatments: 0%, n = 80; 0.05%, n = 77; 0.5%, n = 72; and 5%, n = 85.

† For 1-way analysis of variance of $\log_{10}$ transformed data for unequal N; *P* values below .05 are considered significantly different (NS indicates not significant).

‡ The power associated with rejecting the null hypothesis that the 2 means are equal.

§ Not significantly different.

distribution to that of controls, there was a noticeable enrichment of microvilli on 1 pole of the cell (Figure 1b). Such microvilli presented an extensive, irregular branching pattern that at times formed an anastomotic network. This was unlike the uniform brush border–like appearance of microvilli documented for in vivo principal cells (Hamilton, 1975; Robaire and Hermo, 1988).

Quantitative measurements were made on profile areas of the boundary area, defined as the total area encompassing the microvilli located on 1 pole of the cell. In addition, measurements were made on the dead space area and microvillar area located within the boundary area (Figure 1a). Morphologic and quantitative measurements indicated that there were significant changes in profile areas of each of these parameters relative to controls at all concentrations of testicular extract tested (Figures 1b and 2; Table). This included a gradual and relatively linear increase in total boundary area in cells treated with 0%–0.5% extract followed by a very sharp increase in boundary area in cells treated with 5% extract (Figure 2; Table). The microvillar area showed a trend to increase at all testicular extract concentrations tested, whereas the dead space area between microvilli initially showed a large decrease in area in the 0.05% extract group followed by increases thereafter and in particular with the 5% extract (Figure 2; Table). The large increase in total boundary area seen with 5% extract was due to large increases in both microvillar area and dead space area relative to values measured in control cells (Figure 2; Table).

Immunofluorescent staining of the RCE cells with an anti-espin antibody revealed a polarized reaction over the apical region of these cells (Figure 3a), indicating that these cells express espin and that its localization resides over their microvilli. Immunofluorescence was also performed on the adult epididymis, where a reaction was observed over the microvilli of principal cells (Figure 3b). No reaction was noted when tissue sections were treated with preimmune serum (not shown).

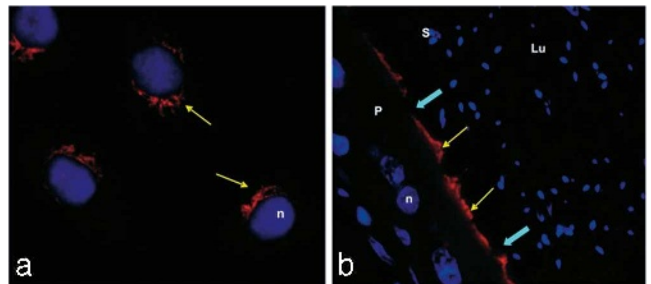

Figure 3. Immunofluorescent staining with anti-espin antibody in cultured rat caput epididymal (RCE) cells **(a)** and in the cauda epididymidis of a control adult rat **(b)**. In the photomicrograph on the left, an intense reaction appears over 1 pole of the RCE cells (arrows). In the photomicrograph on the right, an intense reaction is seen over the microvilli (thin arrows) of epithelial principal cells (P) extending into the epididymal lumen (Lu) but not over microvilli of clear cells (thick arrows). S indicates spermatozoa in the lumen; n, nucleus of principal cell. **(a)** Original magnification = 640 ×; **(b)** original magnification = 350 ×.

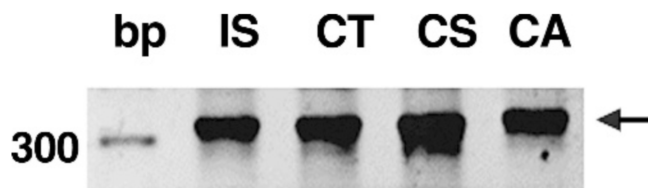

Figure 4. Reverse transcriptase–polymerase chain reaction of rat espin in the epididymis of adult rats. In the epididymis, RNA was isolated from 4 epididymal segments (initial segment [IS], caput [CT], corpus [CS], and cauda [CA]). There is an intense 290–base pair (bp) band in all segments (arrow).

Identification of Espin in the Adult Rat Epididymis In Vivo

Reverse transcriptase (RT)–PCR done on adult epididymal tissue using espin-specific primers revealed the presence of a distinct band of about 290 base pairs (bp) in all epididymal regions (Figure 4). Western blot analysis confirmed that espin was expressed at the protein level in the adult rat epididymis (Figure 5).

Regulation of Epididymal Espin on In Vivo Adult Rat Epididymis

Immunostaining with an anti-espin antibody demonstrated the presence of an intense reaction over the microvilli of principal cells of the epididymal epithelium of control adult rats (Figure 6a through c). The reaction was homogeneous over the entire length of each microvillus and was similar for these cells in each epididymal segment (Figure 6a through c). There was no reaction over epithelial clear cells anywhere in the epididymis (Figure 6c). In the testis, as a positive control, a prominent reaction was present at the ectoplasmic specializations found between Sertoli cells and the heads of elongating spermatids and between neighboring Sertoli cells (not shown). No reaction was present over the epithelium or intertubular spaces of the testis or epididymis when sections were incubated with preimmune serum (Figure 6d).

Espin expression at 14 and 21 days after orchidectomy revealed the complete absence of a reaction over the microvilli of principal cells in all epididymal segments (Figure 7a). In orchidectomized rats that received implants of testosterone (at high levels comparable to that occurring in the epididymal lumen), espin immunostaining was restored to an intensity comparable to that seen in control rats (Figure 7b and c). Ligation of the efferent ducts resulted in a decrease of espin staining but not to the extent of that seen in orchidectomized rats (Figure 7d).

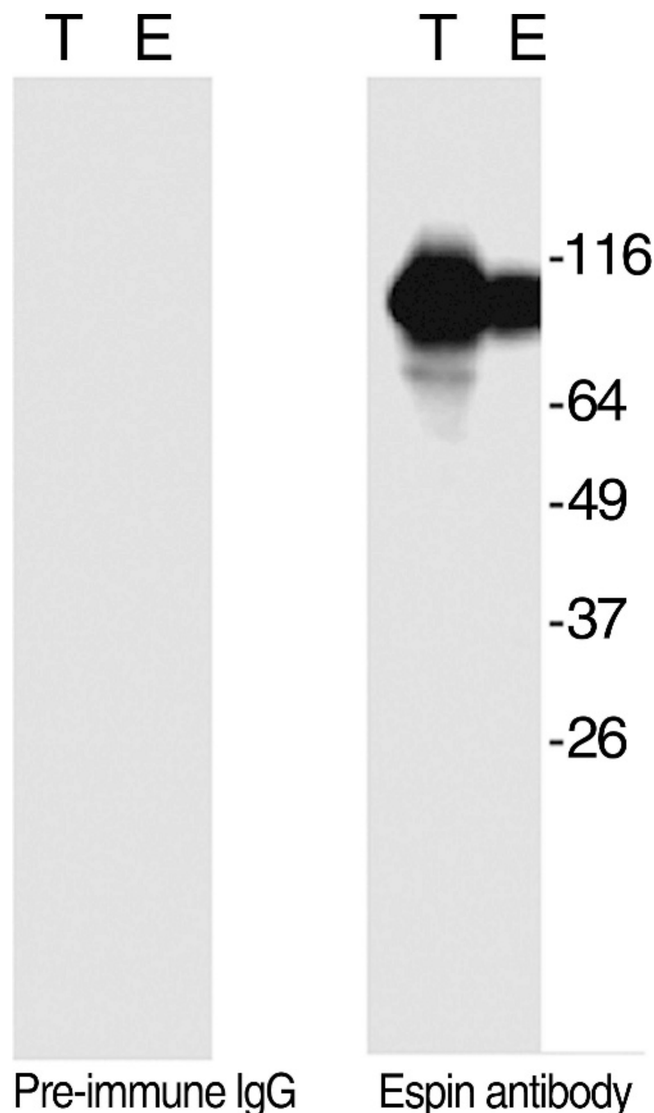

Figure 5. Western blot of espin in the testis (T) and epididymis (E). No bands were detected in the immunoblots incubated with preimmune serum (left panel). In the right panel, espin is detected in protein extracts of the testis and epididymis at about 100 kDa.

Discussion

In the present study, espin was distributed over microvilli of principal cells in all epididymal segments of the adult rat epididymis in vivo. The presence of espin in the adult rat epididymis was also confirmed by RT-PCR and Western blot analysis. Espin was also expressed over microvilli located at 1 pole of the RCE cell line. Using electron microscopy, we demonstrated using the RCE cells that the size of microvilli on principal cells is regulated by an extract made from the testis. This analysis revealed a significant increase in microvillar surface area, with extensive branching in an

anastomotic manner, in the presence of increasing doses of testicular extract. While we did not pursue the identification of the factor within the testicular extract that causes this increase on our RCE cells, our finding that espin is a component of microvilli and that it is regulated by high doses of testosterone would suggest that espin expression is important in maintaining the integrity and size of these microvilli. Indeed, previous studies have shown that espin causes a dramatic concentration-dependent lengthening of microvilli and their parallel actin bundles in cultured kidney cells (Loomis et al, 2003). In sensory hair cells of the cochlea and vestibular apparatus it was also demonstrated that espin was important for stereociliary growth and maintenance of length (Rzadzinska et al, 2005). It would be of interest in future experiments to determine if espin expression correlates with microvillar length in the RCE cell line. Conversely, while the size of microvilli of the RCE cells correlates with increasing doses of testicular extract, it was noted that the microvilli do not form a typical uniform brush border as seen in principal cells in vivo. This could be attributed to the fact that the RCE cells, while establishing contacts with one another, do not aggregate to form a tube with an enveloping lumen; nor are other epithelial cell types, intertubular cells, or spermatozoa present in the culture. One or several of these parameters may prove to be important in maintaining the proper architecture of the microvillar border of principal cells, as seen in the in vivo epididymis.

Espin expression, however, was absent from the epithelial clear cells. The microvilli of these cells do not form a uniform brush border but rather display very long, irregularly shaped, branching structures. Clear cells are highly endocytic in nature (Hamilton, 1975; Robaire and Hermo, 1988; Hermo et al, 1994), and such microvilli may provide for more effective interactions of receptors on their surface with substances (proteins) needed to be removed from the lumen. The absence of espin from clear cells may provide their microvilli with more flexibility to perform such needed functions.

Parallel bundles of actin are important for cell shape and physiology, including their presence in microvilli, and are assembled through sequential action of multiple actin bundling proteins such as espin (Bartles, 2000). Parallel actin bundles are commonly associated with relatively long-lived specializations of the cell surface, such as microvilli, where they function in part as a scaffold to support evaginations or invaginations of the plasma membrane. Its proposed role with actin and presence in microvilli of principal cells would suggest that espin plays an important role in their microvilli. It has been suggested that espin plays a role in the outgrowth of actin filaments, forming the inner core of microvilli, and in this way is important for steady-state length regulation and integrity of microvilli (Loomis et al, 2003; Rzadzinska et al, 2005). In the epididymis, the presence of microvilli on the apical surface of principal cells allows for the proper transport of ions, solutes, and water across the epididymal epithelium, as these structures express various pumps, channels, and transporters and in this way maintain the luminal microenvironment in a manner that is essential for the maturation of spermatozoa (Hermo et al, 2000; Hermo and Robaire, 2002; Breton, 2003).

In the testis, earlier studies demonstrated that low-dose treatments with testosterone and estradiol led to the premature detachment of step 8 spermatids from Sertoli cells. It was concluded that adhesion molecules between these 2 distinct cell types were dependent on these hormones (O'Donnell et al, 2000). More recently, it was demonstrated that FSH regulates the formation of ectoplasmic specializations, whereas testosterone alone did not affect these junctions (Sluka et al, 2006). In the epididymis, it has been documented that following orchidectomy there is a decrease in epithelial cell height, a striking loss of apical microvilli from their surface, and lysosomal accumulation and vacuolation (Wahlqvist et al, 1996; Delongeas et al, 1987; Orgebin-Crist and Davies, 1974; Moore and Bedford, 1979). This led to the suggestion that factors emanating from the testis are important in the proper maintenance of epididymal structure and functions. It is now well documented that such factors could reach the epididymis via the circulation and epididymal lumen (Robaire and Hermo, 1988; Cornwall et al, 2002; Robaire et al, 2006). In the latter case, factors of testicular origin entering the epididymis via the lumen are referred to as lumicrine factors, and several have been identified as regulating specific protein expression in the epididymis (Hinton et al, 2000; Robaire et al, 2006).

In the present study, while efferent duct ligation lowered the expression of espin, it was not entirely abolished, suggesting that espin expression could in part be regulated by circulating levels of androgens or a lumicrine factor(s). However, the fact that orchidectomy resulted in the complete absence of a reaction for espin over microvilli of principal cells and that microvillar espin levels were restored to control levels by administration of high doses of testosterone suggested that espin is regulated by androgens. Because testosterone levels in the lumen of the epididymis are approximately 10 times higher than in the blood (Turner, 2002), the levels in ligated rats appear to be insufficient in maintaining normal expression levels of espin. Hence, regulation of espin and microvillar size by androgens indicates their importance in regulating processes implicated in creating a luminal environment

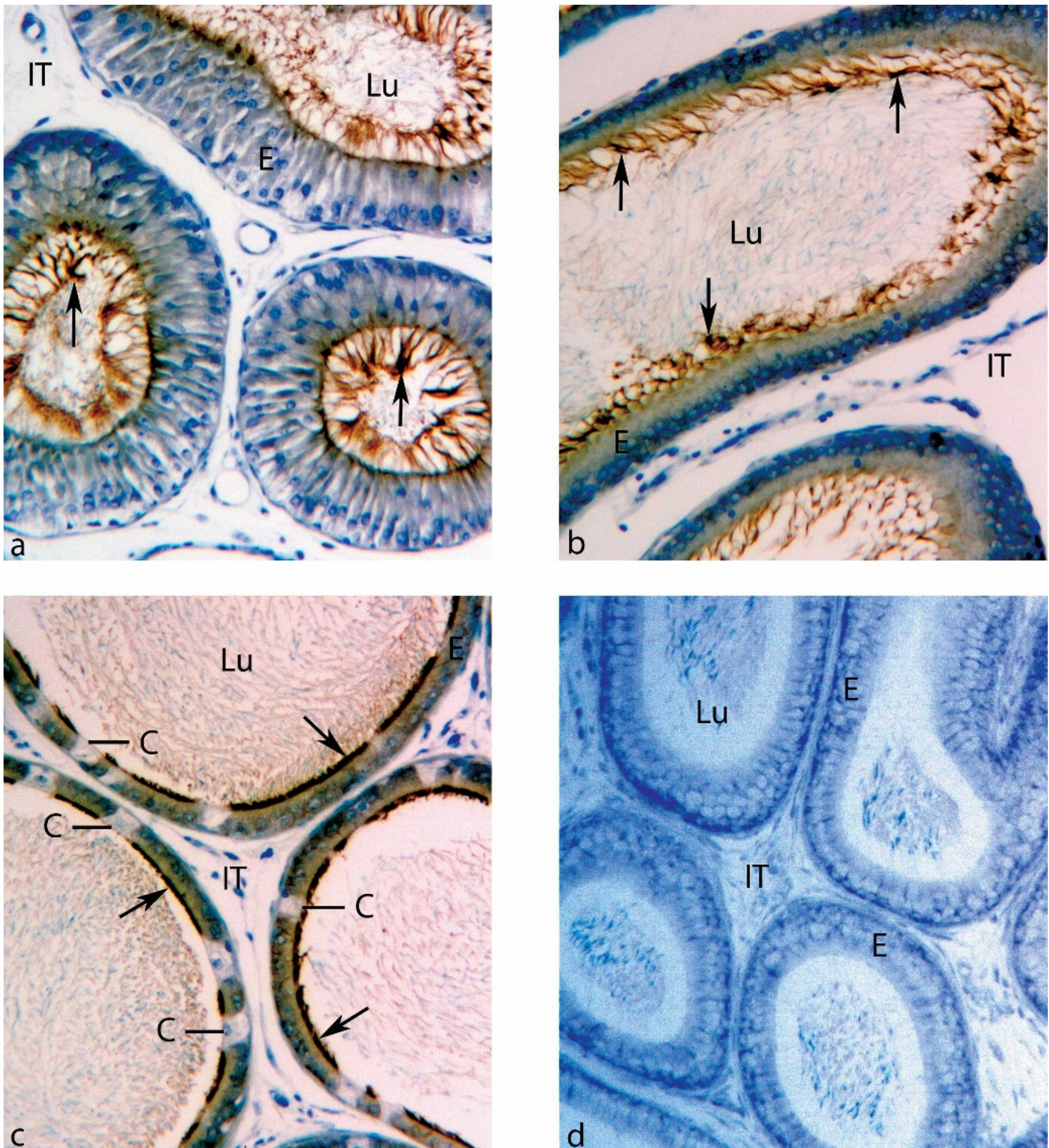

Figure 6. Immunostaining of the initial segment **(a)**, caput **(b)**, and cauda **(c)** epididymidis of an untreated adult rat with anti espin antibody reveals an intense reaction (arrows) over the microvilli of epithelial (E) principal cells. Clear cells (C) are unreactive. There is no reaction over sperm in the lumen (Lu). Control sections employing preimmune serum **(d)** revealed no reaction over the entire epithelium (E), lumen (Lu), or intertubular space (IT). In all panels, original magnification = 100 ×.

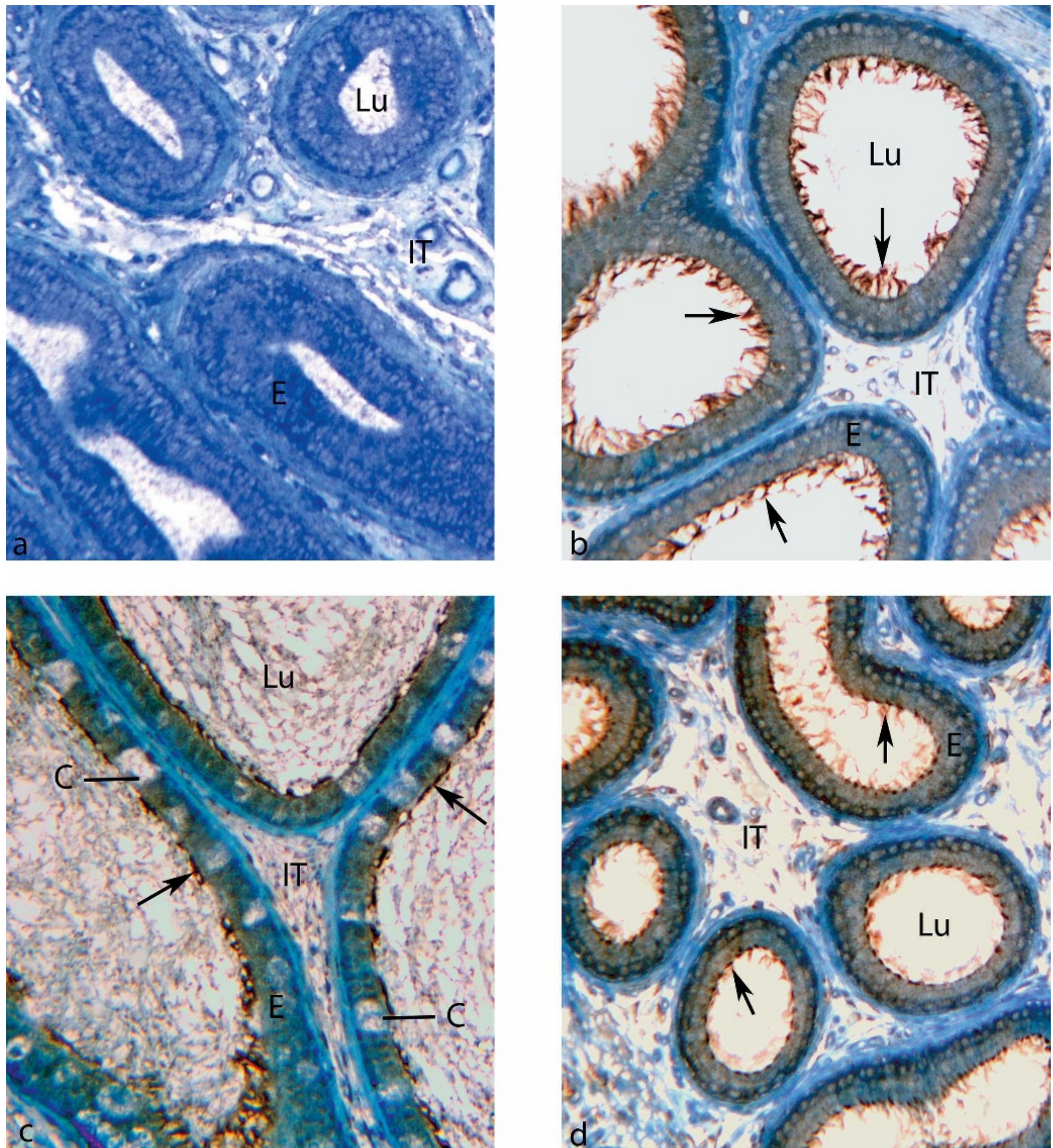

Figure 7. Immunostaining with antiespin antibody in the caput epididymidis of orchidectomized rats **(a)**, corpus **(b)**, and cauda **(c)** regions of orchidectomized rats supplemented with high doses of testosterone and efferent duct ligated rats **(d)**, all at the 14-day interval. In **panel a**, no reaction is apparent over the epithelium (E), whereas in **panels b** and **c**, the reaction is intense over microvilli of principal cells (arrows) comparable to control rats. No reaction is seen over clear cells (C). In **panel d**, a spotty reaction is present over epithelial (E) principal cells much weaker than in control rats. No reaction is ever detected over the intertubular spaces (IT) or luminal contents (Lu). In all panels, original magnification = 100 ×.

that is necessary for sperm maturation. Past studies have shown that many epididymal proteins are activated or down-regulated after orchidectomy (Orgebin-Crist et al, 1975; Holland et al, 1992; Robaire and Viger, 1995; Ezer and Robaire, 2002, 2003; Robaire et al, 2006). The present data suggest that androgens regulate espin expression in the adult epididymis.

In the male reproductive tract, many proteins have been shown to be regulated by estrogens in the efferent ducts and epididymis (Hess et al, 2002). To this end, we examined whether or not espin is regulated by estrogen. To access this parameter, we utilized wild-type and αERKO mice in conjunction with LM immunocytochemistry on a number of epididymal slides from such mouse models that we have used in previous studies (Ruz et al, 2006). Espin expression was intensely expressed on the microvilli of principal cells of wild-type mice and remained so in αERKO mice, suggesting that estrogen did not regulate espin expression in the epididymis (unpublished data).

Previous studies have shown that androgens regulate the expression of proteins implicated in forming adherens and tight junctions that are necessary for the formation of an intact blood-epididymal barrier (Cyr et al, 2002). Likewise, several pumps, transporters, and channels present on microvilli have also been shown to be androgen dependent (Badran and Hermo, 2002; Pastor-Soler et al, 2002; Ruz et al, 2004). Thus, one of the critical roles of androgens is to regulate epididymal functions that control the composition of epididymal lumen and hence sperm maturation, and this may in part be due to structurally and functionally active microvilli.

In summary, in the present study microvillar size in the RCE cell line is dependent on testicular factors, and espin is expressed on 1 pole of these cells. In the adult rat epididymis, espin is localized to the microvilli of principal cells, where it is regulated by high levels of testosterone. It is suggested that microvillar size and integrity are dependent on espin and its regulation by androgens.

Acknowledgments

Jeannie Mui and Greg Wernecke are thanked for their technical assistance.

References

Badran HH, Hermo L. Expression and regulation of aquaporins 1, 8, and 9 in the testis, efferent ducts, and epididymis of adult rats and during postnatal development. *J Androl*. 2002;23:358–373.

Bartles JR. Parallel actin bundles and their multiple actin-bundling proteins. *Curr Opin Cell Biol*. 2000;12:72–78.

Bartles JR, Wierda A, Zheng L. Identification and characterization of espin, an actin-binding protein localized to the F-actin-rich junctional plaques of Sertoli cell ectoplasmic specializations. *J Cell Sci*. 1996;109:1229–1239.

Brawer JR, Schipper H, Robaire B. Effects of long term androgen and estradiol exposure on the hypothalamus. *Endocrinology*. 1983;112:194–199.

Breton S. Luminal acidification in the epididymis and vas deferens. In: Hinton BT, & Turner TT, eds. *Third International Conference on the Epididymis*. Charlottesville, Va: Van Doren; 2003:60–72.

Chen B, Li A, Wang D, Wang M, Zheng L, Bartles JR. Espin contains an additional actin-binding site in its N-terminus and is a major actin-bundling protein of the Sertoli cell-spermatid ectoplasmic specialization junctional plaque. *Mol Biol Cell*. 1999;10:4327–4339.

Cornwall GA, Lareyre J-J, Matusik RJ, Hinton BT, Orgebin-Crist M-C. Gene expression and epididymal function. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002:169–199.

Cyr DG, Finnson K, Dufresne J, Gregory M. Cellular interactions and the blood-epididymal barrier. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002: 103–118.

Dacheux J-L, Dacheux F. Protein secretion in the epididymis. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002;151–168.

Delongeas J, Gelly J, Leheup B, Grignon G. Influence of testicular secretions on differentiation of the rat epididymis: ultrastructural studies after castration, efferent duct ligation and cryptorchidism. *Exp Cell Biol*. 1987;55:74–82.

Dufresne J, St-Pierre N, Viger RS, Hermo L, Cyr DG. Characterization of a novel rat epididymal cell line to study epididymal function. *J Endocrinol*. 2005;146:4710–4720.

Ezer N, Robaire B. Androgenic regulation of the structure and functions of the epididymis. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002: 297–316.

Ezer N, Robaire B. Gene expression is differentially regulated in the epididymis after orchidectomy. *Endocrinology*. 2003;144:975–988.

Hamilton DW. Structure and function of the epithelium lining the ductuli efferentes, ductus epididymidis and ductus deferens in the rat. In: Greep RO, & Astwood EB, eds. *Handbook of Physiology*. Sec 7. Vol 5. Washington, DC: American Physiological Society; 1975: 259–301..

Hermo L, Adamali HI, Andonian S. Immunolocalization of CA II and H+V-ATPase in epithelial cells of the mouse and rat epididymis. *J Androl*. 2000;21:376–391.

Hermo L, Chong DL, Moffatt P, Sly WS, Waheed A, Smith CE. Region- and cell-specific differences in the distribution of carbonic anhydrases II, III, XII, and XIV in the adult rat epididymis. *J Histochem Cytochem*. 2005;53:699–713.

Hermo L, Oko R, Morales CR. Secretion and endocytosis in the male reproductive tract: a role in sperm maturation. *Int Rev Cytol*. 1994;154:106–189.

Hermo L, Robaire B. Epididymal cell types and their functions. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002;81–102.

Hess RA, Zhou Q, Nie R. The role of estrogens in the endocrine and paracrine regulation of the efferent ductules, epididymis and vas deferens. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002: 317–338.

Hinton BT, Lan Z-J, Lye RJ, Labus JC. Regulation of epididymal function by testicular factors: the lumicrine hypothesis. In: Goldberg E, ed. *The Testis: From Stem Cell to Sperm Function*. Serono Symposia, USA. New York, NY: Springer; 2000: 163–173..

Hofer D, Drenckhahn D. Cytoskeletal differences between stereocilia of the human sperm passageway and microvilli/stereocilia in other locations. *Anat Rec*. 1996;245:57–64.

Holland MK, Vreeburg JT, Orgebin-Crist M-C. Testicular regulation of epididymal protein secretion. *J Androl*. 1992;13:266–273.

Loomis PA, Zheng L, Sekerková G, Changyaleket B, Mugnaini E, Bartles JR. Espin cross-links cause the elongation of microvillus-type parallel actin bundles in vivo. *J Cell Biol*. 2003;163:1045–1055.

Moore HDM, Bedford JM. Short-term effects of androgen withdrawal on the structure of different epithelial cells in the rat epididymis. *Anat Rec*. 1979;193:293–311.

O'Donnell L, Stanton PG, Bartles JR, Robertson DM. Sertoli cell ectoplasmic specializations in the seminiferous epithelium of the testosterone-suppressed adult rat. *Biol Reprod*. 2000;63:99–108.

Orgebin-Crist M-C. Maturation of spermatozoa in the rabbit epididymis: fertilizing ability and embryonic mortality in does inseminated with epididymal spermatozoa. *Ann Biol Anim Biochem Biophys*. 1967;7:373–389.

Orgebin-Crist M-C. Studies on the function of the epididymis. *Biol Reprod*. 1969;1:155–175.

Orgebin-Crist MC, Danzo BJ, Davies J. Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. In: Greep RO, & Astwood EB, eds. *Handbook of Physiology*. Sec 7. Vol 5. Washington, DC: American Physiological Society; 1975: 319–338..

Orgebin-Crist M-C, Davies J. Functional and morphological effects of hypophysectomy and androgen replacement in the rabbit epididymis. *Cell Tissue Res*. 1974;148:183–201.

Pastor-Soler N, Isnard-Bagnis C, Herak-Kramberger C, Sabolic I, Van Hoek A, Brown D, Breton S. Expression of aquaporin 9 in the adult rat epididymal epithelium is modulated by androgens. *Biol Reprod*. 2002;66:716–1722.

Reid BL, Cleland KW. The structure and function of the epididymis. 1. The histology of the rat epididymis. *Aust J Zool*. 1957;5:223–246.

Robaire B, Hermo L. Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, & Neill JD, eds. *The Physiology of Reproduction*. New York, NY: Raven Press; 1988: 999–1080.

Robaire B, Hinton BT, Orgebin-Crist MC. The epididymis. In: Knobil E, & Neill JD, eds. *Physiology of Reproduction.* 3rd ed. New York, NY: Elsevier Publishers; 2006;1071–1147.

Robaire B, Viger RS. Regulation of epididymal epithelial functions. *Biol Reprod*.1995: 52:226–236.

Ruz R, Andonian S, Hermo L. Immunolocalization and regulation of cystic fibrosis transmembrane conductance regulator in the adult rat epididymis. *J Androl*. 2004;25:265–273.

Ruz R, Gregory M, Smith CE, Cyr DG, Lubahn DB, Hess RA, Hermo L. Expression of aquaporins in the efferent ductules, sperm counts, and sperm motility in estrogen receptor-alpha deficient mice fed lab chow versus casein. *Mol Reprod Dev*. 2006;73: 226–237.

Rzadzinska A, Schneider M, Noben-Trauth K, Bartles JR, Kachar B. Balanced levels of espin are critical for stereociliary growth and length maintenance. *Cell Motil Cytoskelet*. 2005;62:157–165.

Sekerková G, Freeman D, Mugnaini E, Bartles JR. Espin cytoskeletal proteins in the sensory cells of rodent taste buds. *J Neurocytol*. 2005;34:171–182.

Sekerková G, Zheng L, Loomis PA, Changyaleket B, Whitlon DS, Mugnaini E, Bartles JR. Espins are multifunctional actin cytoskeletal regulatory proteins in the microvilli of chemosensory and mechanosensory cells. *J Neurosci*. 2004;24:5445–5456.

Sekerková G, Zheng L, Loomis PA, Mugnaini E, Bartles JR. Espins and the actin cytoskeletal of hair cell stereocilia and sensory cell microvilli. *Cell Mol Life Sci*. 2006a;63:2329–2341.

Sekerková G, Zheng L, Mugnaini E, Bartles JR. Differential expression of espin isoforms during epithelial morphogenesis, stereociliogenesis and postnatal maturation in the developing inner ear. *Dev Biol*. 2006b;291:83–95.

Sluka P, O'Donnell L, Bartles JR, Stanton PG. FSH regulates the formation of adherens junctions and ectoplasmic specializations between rat Sertoli cells in vitro and in vivo. *J Endocrinol*. 2006;189:381–395.

Stratton ID, Ewing LL, Desjardins C. Efficacy of testosterone-filled polydimethylsiloxane implants in maintaining plasma testosterone in rabbits. *J Reprod Fertil*. 1973;35:235–244.

Turner TT. Necessity's potion: inorganic ions and small organic molecules in the epididymal lumen. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002: 131–150.

Wahlqvist R, Dahl E, Tveter KJ. Effects of castration upon the morphology of the accessory sex organs of the male rat – a scanning electron microscopy study. *Scanning Microsc*. 1996;10:1155–1162.

Wong PYD, Gong XD, Leung GPH, Cheuk BLY. Formation of the epididymal fluid microenvironment. In: Robaire B, & Hinton BT, eds. *The Epididymis, From Molecules to Clinical Practice*. New York, NY: Klumer Academic/Plenum Publishers; 2002: 119–130.

Zheng L, Sekerková G, Vranich K, Tilney LG, Mugnaini E, Bartles JR. The deaf jerker mouse has a mutation in the gene encoding the espin actin-bundling proteins of hair cell stereocilia and lacks espin. *Cell*. 2000;102:377–385.