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Strength Measurement of the Sertoli-Spermatid Junctional Complex

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ABSTRACT: The Sertoli cell ectoplasmic specialization (ES) is a specialized domain of the calcium-dependent Sertoli cell–spermatid junctional complex. Not only is it associated with the mechanical adhesion of the cells, but it also plays a role in the morphogenesis and differentiation of the developing germ cells. Abnormal or absent Sertoli ESs have been associated with step-8 spermatid sloughing and subsequent oligospermia. With a micropipette pressure transducing system (MPTS) to measure the force needed to detach germ cells from Sertoli cells, this study examined, for the first time, the strength of the junction between Sertoli cells and spermatids and between Sertoli cells and spermatocytes. The mean force needed to detach spermatocytes from Sertoli cells was 5.25×10^{-7} pN, pre–step-8 spermatids from Sertoli cells was 4.73×10^{-7} pN, step-8 spermatids from Sertoli cells was 8.82×10^{-7} pN, and spermatids plus EDTA was 2.16×10^{-7} pN. These data confirm the hypothesis that step-8 spermatids are more firmly attached to Sertoli cells than are spermatocytes and pre–step-8 spermatids and that calcium chelation reduces binding strength between Sertoli cells and spermatids. The MPTS is a useful tool in studying the various molecular models of the Sertoli–germ cell junctional strength and the role of reproductive hormones and enzymes in coupling and uncoupling of germ cells from Sertoli cells.

Key words: Ectoplasmic specialization, testis, micropipette, adherens junction.

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The complicated process of spermatogenesis occurs throughout the reproductive life of the male. It is a remarkable process in which spermatogonia undergo mitosis to become spermatocytes, which then undergo meiosis to become round spermatids, which then enter the process of spermiogenesis to differentiate into elongated spermatids (sperm) (Leblond et al, 1963). At any given point in time several generations of germ cells are present in the seminiferous epithelium but in different stages of maturation (Courot et al, 1970). During spermatogenesis, germ cells form different types of junctions with Sertoli cells, including the specialized ectoplasmic specialization between Sertoli cells and spermatids (Russell, 1977b).

Several types of intercellular junctions, including occluding junctions, adherens junctions (AJ), and gap communicating junctions, are believed to play crucial roles in spermatogenesis. The actin based cell-cell AJs between the Sertoli cell and the germ cell in the mammalian testis are important not only in mechanical adhesion of the cells, but in the morphogenesis and differentiation of the germ cells (Russell, 1993). Turnover of these calcium-dependent junctions occurs during the process of germ cell migration from the basal to the adluminal epithelial compartment (Lui et al, 2003b).

The Sertoli ectoplasmic specialization (ES), a cytoskeletal structure of the Sertoli cell, is associated with Sertoli-spermatid binding at the AJ (Russell, 1977a, 1980). Abnormal or absent Sertoli ESs have been associated with a reduction of mature sperm in semen (Russell et al, 1988; Boekelheide et al, 1989; O'Donnell et al, 1996, 2000) and conditions associated with oligospermia (Cameron and Griffin, 1998). ESs are found basally in the Sertoli cell near Sertoli-Sertoli tight junctions and apically between Sertoli cells and spermatids. They consist of hexagonally packed bundles of actin filaments situated between the plasma membrane and a cistern of endoplasmic reticulum (Russell, 1993). The ES is an important cell-cell adhesion mechanism in the seminiferous epithelium to ensure the retention of spermatids as they mature into spermatozoa. ESs are first seen in the rat at Stage VIII of rat spermatogenesis, when the step-8 spermatid appears. It is thought the ES forms in the Sertoli cell to strongly anchor the step-8 spermatid to the seminiferous epithelium; however, this has yet to be actually measured. The ES is present at the AJ until appropriate release of the step-19 spermatid and inappropriate release of earlier stage spermatids (ie, spermatid sloughing) is related to abnormal ES structure and oligospermia (O'Donnell et al, 1996, 2000).

A number of health-related conditions are associated

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with reduced fertility potential and oligospermia in men, including varicocele, hyperprolactinemia, diabetes, and idiopathic oligospermia (Cameron and Griffin, 1998). These conditions all are associated with reduced sperm in the semen, that is, oligospermia, and ultrastructural pathology unique to the junctional apparatus of the seminiferous epithelium (Cameron and Griffin, 1998). Cap stage spermatids in the human (step-8 spermatids in the rat) are presumed to be tightly anchored to the seminiferous epithelium at a Sertoli cell AJ, which includes the unique Sertoli ectoplasmic specialization (Russell, 1977a, 1980). In both in vitro and in vivo observations of experimental animal models, disruption of this junction results in spermatid sloughing and subsequent oligospermia (Russell et al, 1988; Boekelheide et al, 1989; O'Donnell et al, 1996, 2000).

This project was designed to measure the strength of junctions between germ cells and Sertoli cells and to determine whether the presence of the unique ES between Sertoli cells and step-8 spermatids actually results in an increase in the binding strength between these 2 cell types. To do this, we have modified a micropipette pressure transducing system for the purpose of testing junctional strengths between cells in a Sertoli–germ cell coculture model optimized for cell-cell binding (Cameron and Muffly, 1991). It is hypothesized that the junctions between step-8 spermatids and Sertoli cells are stronger than those between pre–step-8 spermatids and Sertoli cells and between spermatocytes and Sertoli cells.

Materials and Methods

Sertoli and germ cells were isolated from Sprague-Dawley rats, as previously described (Cameron et al, 1987). Sixteen- to seventeen-day-old rats were used for Sertoli cell isolation, and adult rats were used for germ cell isolation.

Sertoli Cell Isolation, Culture, and Pretreatment

Briefly, testes were excised from prepubertal male rats, and the parenchyma was digested using routine sequential enzymatic treatments with trypsin (0.25%, Sigma Chemical Co, St Louis, Mo) and collagenase (0.20%, Becton Dickinson, Sparks, Md). Isolated cells were plated to confluence on 13-mm round plastic coverslips coated with undiluted Matrigel in 24-well cell culture dishes. Cultures were incubated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (Ham) (DMEM:F12) (supplemented with 0.01 mol/L retinol and 1000 μL/100 mL insulin-transferrin-selenium [ITS]) at 39°C in a humidified incubator with 5% CO_2-95% air for 48 hours to expedite the removal of contaminating germ cells. After the 48-hour preincubation, the cultures were exposed to a 20 mM Tris-HCl buffer for 2.5 minutes to hypotonically lyse remaining germ cells, then incubated in supplemented DMEM:F12 at 33°C in a humidified incubator with 5% CO_2-95% air for 24 hours. After the 24-hour incubation, the media were replaced with supplemented DMEM:F12 containing 0.06 μg/mL follicle-stimulating hormone (FSH) (NIDDK-oFSH-20, AFP7028D, 175xNIH-FSH-S1) and 100 nM testosterone (Sigma). These pretreated Sertoli cell cultures were used in all coculture experiments.

Spermatocyte and Round Spermatid Isolation and Unit Gravity Velocity Sedimentation

Spermatocytes and pre–step-9 spermatids (round spermatids) were isolated from an adult male rat testis. Briefly, the decapsulated adult testis was digested with 0.10% collagenase (Gibco, Carlsbad, Calif; 37°C, 80 oscillations/min, 30 minutes) to separate seminiferous tubules from the testicular interstitial tissue. The washed seminiferous tubules then were digested with 0.25% trypsin (Sigma; 37°C, 90 oscillations/min, 15 minutes) to separate the peritubular cells from the seminiferous epithelium and to expedite the release of germ cells from the seminiferous epithelium. A 0.20% trypsin inhibitor solution (Sigma) was added to terminate the trypsin reaction. The resulting cell suspension (mixed germ cells and Sertoli cells) was resuspended in 25 mL McCoy media plus 0.5% bovine serum albumin (BSA).

With sterile technique, the gradient chambers on a STA-PUT velocity sedimentation cell separator were filled with the appropriate McCoy media plus BSA medium (2% and 4% BSA), and a linear gradient (2%–4%) was built under the cell suspension, at the loading rate initially at 10 mL/min. After 20 minutes, the rate was increased to 40 mL/min. Eighty minutes before the end of the collection time (4 hours), media with germ cell fractions were collected using a Fractomat automatic fraction collector (10 mL/vial at 160 drops/min). Spermatocytes and round spermatids (pre–step-9) were identified by phase contrast microscopy and pooled, washed, and resuspended in McCoy media. The number of cells in the spermatocyte and spermatid fractions were counted by hemocytometric analysis and assayed for viability by trypan blue exclusion.

Sertoli–Germ Cell Coculture

Approximately 400 000 isolated germ cells (spermatocytes and round spermatids) were added directly to the pretreated Sertoli cell–enriched monocultures. The Sertoli–germ cell cocultures were incubated with 0.06 μg/mL FSH plus 100 nM testosterone in a humidified chamber at 33°C with 5% CO_2-95% air for 36 hours.

Measurement of Junctional Strength Using a Micropipette Pressure Transducing System

The Sertoli–germ cell cocultures were imaged on an inverted interference contrast microscope (Axiovert 100, Zeiss, Germany) with a 20× objective. The microscope was fitted with the micropipette pressure transducing system (MPTS), which consisted of a 3-dimensional water robot micromanipulator (Narishige Scientific Instruments Lab, Tokyo, Japan), a micropipette holder, a glass micropipette, a water reservoir system to control the micropipette pressure, and a video system to record experiments (Figure 1).

The Micropipette

Micropipettes were created from 1-mm outer diameter, 0.5-mm inner diameter glass capillary tubes (A-M Systems Inc, Carlsbad,

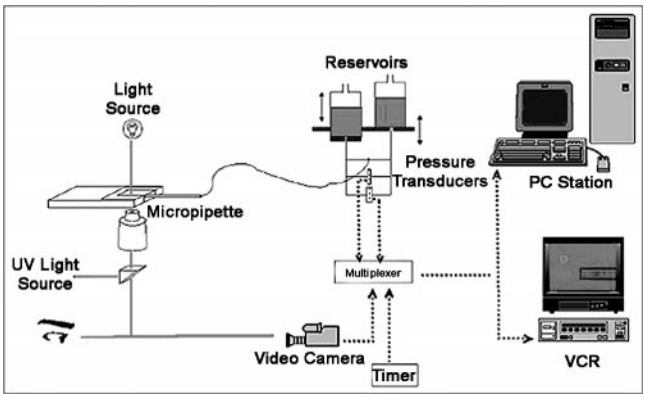

Figure 1. A schematic drawing of the micropipette pressure transducing system (MPTS).

Wash). The capillary tube was mounted onto a pipette puller (model PB-7, Narishige Scientific Instruments), heated, and pulled into a pipette with a tip of a few microns. To ensure a flat tip, this pipette was then mounted onto a microforge (model MF 83, Narishige Scientific Instruments). The microforge consists of a horizontal microscope, a micromanipulator, and a glass bead on a platinum wire. Upon heating of the wire, the glass bead melted and the tip of the micropipette was inserted into the melted glass. The bead/micropipette was allowed to cool. The micropipette was then pulled up, and the tip was broken by quick fracture, leaving a flat tip. The tip was filled with a saline solution to avoid plugging. To prevent rupture of the cells on the glass surface, the micropipette was coated with plasma proteins. The diameters of the pipettes used were 13.32 μm for spermatids (diameter 10 μm) and 16.65 μm for spermatocytes (diameter 15 μm).

The Water Reservoir System

The pressure at the tip of the micropipette was controlled by a system consisting of 2 water reservoirs and a pressure transducer (model DP15-30, Validyne, Northridge, Calif) connected between the 2 reservoirs. One reservoir was a reference reservoir, and the other one was an adjustable reservoir. The reference reservoir was adjusted so that no pressure would be applied at the micropipette. This was achieved by connecting the reference reservoir directly to the micropipette and positioning it at the same level as the micropipette. As a result, there were no movements from particles or cells in front of the micropipette. The adjustable reservoir was then positioned to create the desired pressure, as read by the pressure transducer. A valve switch was used to connect the micropipette either to the reference reservoir or the adjustable reservoir. The pressure transducer output signal was decoded via a carrier demodulator (model CD 280-2, Validyne). The pressure range of the transducer was 80 000 dyn/cm^2 with an accuracy of 400 dyn/cm^2.

Cell Measurements

Cover slips containing Sertoli–germ cell cocultures were carefully removed from the wells and placed in an engineered cover slip holder for use with the MPTS-fitted inverted microscope. The detachment of germ cells from Sertoli cells was measured and analyzed. In some experiments, 2 mM or 4 mM EDTA was added to the cultures immediately before the measurements, as controls. To detach germ cells from Sertoli cells, the micropipette was brought to the surface of the individual spermatocyte or the individual spermatid at 200× magnification making sure not to touch the underlying Sertoli cells. If the Sertoli cells were touched by the micropipette, the measurement was abandoned. The pressure required to detach the germ cell from the underlying Sertoli cell monolayer was then recorded. Each detachment event (a maximum of 4) consisted of a 5-second suction pressure interval. If the germ cell did not dissociate, it was abandoned, and the last pressure reading was recorded. The recorded pressure (in cm-H_2O) was used to calculate force via the equation $F = \Delta P \times \pi R^2 p$, where F (pN) is the force on a static cell, ΔP is the suction pressure (N/μm^2), and $\pi R^2 p$ is the cross-sectional area of the pipette (μm^2). To convert the pressure reading received in cm-H_2O to N/μm^2, for use in the above equation, the conversion factors 1 cm-H_2O = 98.06 Pa and 1 Pa = N/m^2 were used, since the international unit of force is Newtons (1 N = 1 kg m/s^2), and the international unit of pressure is Pascal (Pa).

Statistics

To determine statistical significance of the mean force (set at the 0.05 level), a 1-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference test.

Results

The mean force required to detach spermatocytes, pre–step-8 spermatids, and step-8 spermatids from Sertoli cells in the optimized Sertoli–germ cell in vitro binding model was determined following multiple measurements acquired from the modified MPTS. The mean force necessary to detach spermatocytes from Sertoli cells was 5.25×10^{-7} pN (SE = 3.43×10^{-8}, n = 38), pre–step-8 spermatids from Sertoli cells (ie, Sertoli-spermatid junctions with no ES) was 4.73×10^{-7} pN (SE = 2.17×10^{-8}, n = 38), step-8 spermatids from Sertoli cells (ie, Sertoli-spermatid junctions with ES) was 8.82×10^{-7} pN (SE = 3.37×10^{-8}, n = 33), and spermatids plus EDTA was 2.16×10^{-7} pN (SD = 3.12×10^{-8}, n = 6). These results are presented in Figure 2. Measurements were not made between step-8 spermatids and spermatocytes and the underlying Sertoli cells following EDTA treatment because, in most cases, these cells detached from the Sertoli cells before measurements could be made.

A 1-way ANOVA determined a significant difference between the mean force of spermatocytes and the mean force of step-8 spermatids, between the mean force of pre–step-8 spermatids and the mean force of step-8 spermatids, and between the mean force of spermatids versus spermatids plus EDTA, where $P < .05$. There was no significant difference between the mean force of spermatocytes and the mean force of pre–step-8 spermatids.

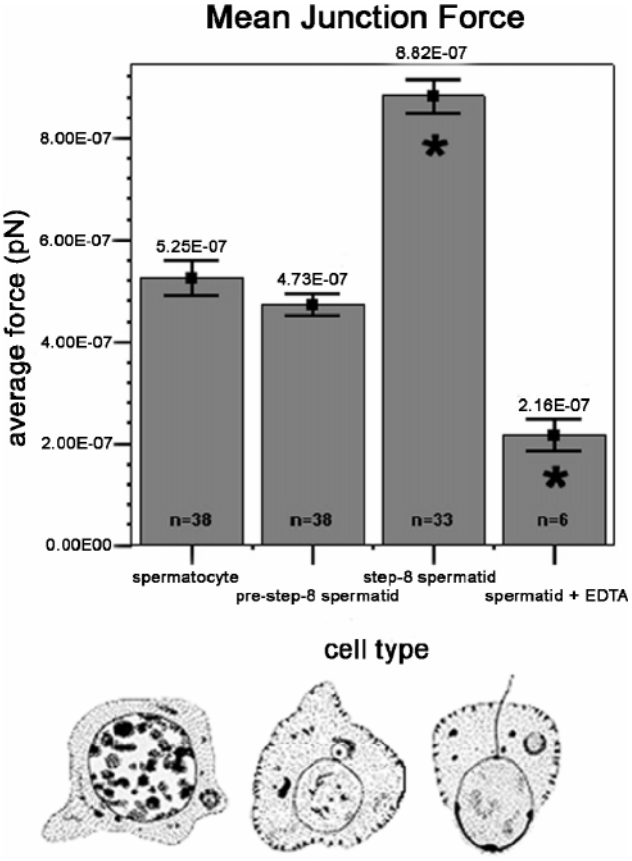

Figure 2. Bar graph displaying the mean force (pN) required to detach spermatocytes, pre–step-8 spermatids, and step-8 spermatids from Sertoli cells in vitro with FSH+T. Asterisk indicates a significant difference, as determined by 1-way ANOVA.

Discussion

It is theorized that spermatids associated with ESs adhere to Sertoli cells more strongly than all other germ cells and that this is essential for anchoring spermatids in the seminiferous epithelium during the final stages of spermiogenesis. Additionally, complete spermiogenesis is not observed in the absence of these unique Sertoli-spermatid junctions, which when disrupted lead to spermatid sloughing and oligospermia (Russell et al, 1988; Boekelheide et al, 1989; O'Donnell et al, 1996). However, the actual junctional strength between Sertoli cells and spermatids has never been measured to verify this unsubstantiated dogma central to the successful completion of spermatogenesis.

We have, for the first time, recorded actual force measurements necessary to detach germ cells from underlying Sertoli cells in vitro. The results suggest that the force measurements are related to the junctional strength between the various germ cell types and the Sertoli cells in a manner consistent with well-established theories related to germ cell–Sertoli cell attachments. On this basis, the data presented confirm the hypothesis that step-8 spermatids are more firmly attached to Sertoli cells than are pre–step-8 spermatids and spermatocytes. It is possible that the force recorded represents the membrane stability of just the germ cell type and not the actual strength of the junctional structure. More studies are needed to make this determination, but in either case, it was clearly more difficult to detach step-8 spermatids from Sertoli cells than the other germ cell types tested.

Of the cells tested, the ES is only present between Sertoli cells and step-8–step-19 spermatids and is conspicuously absent between Sertoli cells and spermatocytes and pre–step-8 spermatids (Russell, 1993). This suggests that the structural nature of the ES contributes to the actual junctional strength between these 2 cell types, ensuring that elongating spermatids (post–step-8 spermatids) are securely anchored to the seminiferous epithelium during the final stages of spermiogenesis. This also supports the hypothesis that when the ES does not form properly between the Sertoli cell and the periluminal step-8 spermatid, or is otherwise abnormal, the junction strength is significantly lessened, thereby leading to spermatid sloughing and oligospermia (Cameron and Griffin, 1998).

Several molecular models of the structure and regulation of the ectoplasmic specialization at the Sertoli cell–spermatid junction have been proposed. One such model includes the controversial and most studied cadherin-catenin complex. In this model, it is proposed that the presence and regulation of the multiprotein cadherin-catenin complex at the ES controls the coupling and uncoupling of spermatids to Sertoli cells (Newton et al, 1993; Wine and Chapin, 1999; Lee et al, 2003). Disruption of this protein complex via phosphorylation of $p120^{ctn}$ (Daniel and Reynolds, 1997), tyrosine phosphorylation of β- and/or γ-catenin (Daniel and Reynolds, 1997), and/or the addition of an anti–N-cadherin antibody (Newton et al, 1993; Perryman et al, 1996) results in the loss of germ cells from the seminiferous epithelium. This step-8 sloughing, as described by O'Donnell et al (1996), is also related to testosterone reduction and possibly, therefore, N-cadherin expression (Newton et al, 1993; McLachlan et al, 1994; O'Donnell et al, 1994). In vitro, testosterone and dihydrotestosterone with a fixed concentration of FSH causes a dose-related increase in N-cadherin levels (Perryman et al, 1996). Increasing doses of FSH in the presence of a fixed concentration of testosterone also creates a dose-related increase in N-cadherin protein levels (Perryman et al, 1996). In the models studied, the ES is still present, indicating that testosterone has an effect on the cell adhesion molecules at this junction and not the ES structure itself (McLachlan et al, 1994; O'Donnell et al, 1994, 2000). The effects of reproductive hormones on cell adhesion and coupling and uncoupling dynamics be-

tween Sertoli cells and germ cells, as defined above, can be tested using the MPTS.

Other proposed molecular models of the Sertoli-spermatid ES consist of the nectin-afadin-ponsin complex and the integrin complex. Nectins are found in both Sertoli cells (nectin-2) and spermatids (nectin-3), with the strongest expression at stages IV–IX and decreasing at stage VIII (Bouchard et al, 2000; Ozaki-Kuroda et al, 2002). 1-Afadin, found in the testis, connects to the actin cytoskeleton (Mandai et al, 1997), and studies using afadin$^{-/-}$ mice have shown that afadin is essential in proper structural organization of tight junctions and cadherin-based AJs (Ikeda et al, 1999). Ponsin, of which mRNA is found in the testis (Mruk and Cheng, 2004), binds to afadin and allows it to colocalize with nectin to the cadherin-based AJ (Mandai et al, 1997). However, no biochemical or functional studies on the nectin-afadin-ponsin complex have been conducted.

The most studied integrin receptor in the testis is $\alpha 6\beta 1$, which is found in the Sertoli cell membrane (for review, Vogl et al, 2000). The binding partner of $\alpha 6\beta 1$ is not yet known, but recent studies have indicated that the laminin $\gamma 3$ chain is a putative binding partner (Koch et al, 1999; Mulholland et al, 2001; Siu and Cheng, 2004). The expression of $\beta 1$-integrin has been shown to be affected by hormones. Testosterone, in the presence of FSH, increases $\beta 1$-integrin levels in a dose-dependent manner (Pearce, 2003), as do increasing doses of FSH in the presence of testosterone. Integrins are important in cell adhesion not only structurally, but also in that they transmit signals to trigger events that activate signal transducers, such as Rho GTPase (Lui et al, 2003a), focal adhesion kinase (Mulholland et al, 2001; Siu et al, 2003), Src (Wine and Chapin, 1999), C-terminal Src kinase (Wine and Chapin, 1999), and integrin-linked kinase (Mulholland et al, 2001), to affect Sertoli–germ cell AJ dynamics (Lui et al, 2003b). Again, the effects of reproductive hormones on the integrin-based model of the Sertoli–germ cell junction and its role in coupling and uncoupling of germ cells from Sertoli cells can be tested with the MPTS.

Results from this study show that the junctional strength between Sertoli cells and germ cells can be measured in vitro, support long-held speculations regarding Sertoli-spermatid junctional interactions, and provide a means to actually test proposed mechanisms of junction dynamics between cells of the seminiferous epithelium.

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