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Crosstalk between desmoglein-2/desmocollin-2/Src kinase and coxsackie and adenovirus receptor/ZO-1 protein complexes regulates blood-testis barrier dynamics

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Abstract

Morphological studies in the testis reported the presence of ‘desmosome-like’ junctions between Sertoli cells at the blood-testis barrier, whose function is also constituted by tight junctions and basal ectoplasmic specializations. Unfortunately, little is known about the role of desmosomes in blood-testis barrier dynamics. This study aims to fill this gap with the functional investigation of two desmosomal cadherins, desmoglein-2 and desmocollin-2, by their specific knockdown in Sertoli cells cultured in vitro. Reminiscent of the blood-testis barrier in vivo, desmosome-like structures were visible by electron microscopy when Sertoli cells were cultured at high density, thereby forming a polarized epithelium with functional cell junctions. At this point, we opted to focus our efforts on desmoglein-2 and desmocollin-2 based on results which illustrated desmosomal mRNAs to be expressed by Sertoli and germ cells, as well as on results which illustrated desmoglein-2 to co-immunoprecipitate with plakoglobin, c-Src and desmocollin-2. Simultaneous knockdown of desmoglein-2 and desmocollin-2 not only led to a reduction and mislocalization of zonula occludens-1, but also perturbed the localization of c-Src and coxsackie and adenovirus receptor at the cell-cell interface, resulting in disruption of tight junction permeability barrier. We hereby propose a novel regulatory protein complex composed of desmoglein-2, desmocollin-2, c-Src, coxsackie and adenovirus receptor and ZO-1 at the blood-testis barrier.

Keywords

testis; desmosome; blood-testis barrier; Sertoli cell

Introduction

An important phenomenon in cell biology is the adhesion of cells to form an organized epithelium, and this is mediated by two types of junctions, namely adherens junctions and desmosomes. Desmosomes are generally found in tissues that are subjected to mechanical stress, but their presence in other organs has also been reported (Holthofer et al., 2007, Green and Simpson, 2007). In the mammalian testis, morphological studies in the late 1970s reported the presence of ‘desmosome-like’ structures between Sertoli cells at the blood-testis barrier (BTB), and between Sertoli cells and all non-elongating/elongated germ cells [*i.e.*, those up

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to, but not including, step 8 spermatids in the rat] (Russell, 1977a, Russell et al., 1983, Russell and Peterson, 1985). However, these structures were not typical of desmosomes found in other organs because they also appeared to have ultrastructural features of gap junctions (GJs), a type of communicating junction (Russell, 1993). Moreover, desmosome-like junctions in the testis were shown to lack a clearly defined dense mid-line which is common to conventional desmosomes (Russell, 1977a), revealing that these structures are unique and hybrid-like in character. Since their initial identification, however, there has not been a single study in the literature to address the biology of desmosome-like junctions in the testis.

Throughout spermatogenesis, leptotene spermatocytes traverse the BTB beginning at late stage VIII in order to gain entry into the adluminal compartment for further development (de Kretser and Kerr, 1988, Kerr et al., 2006). This cellular event requires extensive restructuring of the BTB, which in addition to desmosome-like junctions, is also constituted by co-existing tight junctions (TJs) and basal ectoplasmic specializations [basal ES] (Mruk and Cheng, 2004). At the same time, however, the integrity of the BTB must also be maintained since a breach in BTB function can result in exposure of unique antigens present on the surface of haploid germ cells to the host's immune system, leading to an arrest of spermatogenesis and infertility. As such, a tightly-regulated mechanism must underlie restructuring of the BTB in order to allow migration of leptotene spermatocytes without affecting the homeostasis of the adluminal compartment and spermatogenesis. To maintain homeostasis, it is conceivable that TJs, basal ES and desmosome-like junctions crosstalk within the junctional complex so that germ cell movement can be coordinated with barrier restructuring. Needless to say, a significant compromise in any one of these junctions can destabilize the BTB. Until now, we have acquired a relatively good understanding of some of the TJ and basal ES proteins that are critical for BTB function such as claudin-11 (Gow et al., 1999) and nectin-2 (Ozaki-Kuroda et al., 2002) whose knockdown resulted in sterility, but we do not yet know whether desmosomes play any role in BTB dynamics. In light of the unique arrangement of cell junctions at the BTB, we investigate herein whether desmosomes, similar to TJs and basal ES, contribute to BTB integrity.

Materials and Methods

Animals

The use of Sprague Dawley rats at 20 and 90 days old was approved by The Rockefeller University Laboratory Animal Use and Care Committee (Protocol numbers 06018 and 09016). Sertoli cells were isolated from 20-day-old animals, the age at which Sertoli cells are fully differentiated. This is also the age at which the testis does not contain a massive amount of germ cells, thereby facilitating Sertoli cell isolation. In all other instances, experiments were performed using adult (90 day old) rats, and this included the isolation of germ cells and seminiferous tubules, as well as the use of testes for immunohistochemistry and immunofluorescent microscopy.

RNA Extraction and RT-PCR

Different tissues, Sertoli and germ cells were homogenized with TRIzol reagent (Invitrogen), and RNA was extracted as instructed by the manufacturer. To degrade contaminating genomic DNA, 3 µg RNA was incubated with amplification-grade deoxyribonuclease I (Invitrogen). Subsequently, M-MLV reverse transcriptase (Promega) was used for reverse transcription of mRNA, and the cDNA of interest was amplified by PCR using *GoTaq* DNA polymerase (Promega). Primers used for PCR are listed in Suppl. Table 1. The authenticity of each PCR product amplified in the testis was verified by DNA sequencing. RT-PCR experiments were repeated at least five times using tissues from five different adult rats or five different batches of isolated Sertoli and germ cells.

Antibodies

Anti-desmoglein-2 antibody was generated as described (Yan and Cheng, 2006) with minor modifications (Suppl. Table 2). Commercially-obtained antibodies are listed in Suppl. Table 3.

Production of Desmoglein-2 Recombinant Protein and Preparation of Antiserum

In short, a cDNA fragment of rat desmoglein-2 was amplified by PCR with specific primers containing cloning sites (Suppl. Table 2) using AccuPrime™ *Taq* DNA polymerase (Invitrogen) and was cloned in-frame into the pET-41 Ek/LIC Vector (Novagen). Recombinant desmoglein-2 protein containing an N-terminal His₆ tag was expressed in *E. coli* BL21 (DE3) cells after induction with IPTG and was purified with a nickel-chelated column (Pierce). Two New Zealand female rabbits were immunized with recombinant desmoglein-2 to generate antiserum. Anti-desmoglein-2 IgG was prepared by (NH₄)₂SO₄ precipitation and purified by DEAE-chromatography (Cheng et al., 1988).

Seminiferous Tubule Isolation

Testes from adult rats were decapsulated and incubated in collagenase (0.5 mg/ml in F12/DMEM) at 37 °C for 30 min. Seminiferous tubules were then washed five times by sedimentation under gravity to remove Leydig cells and homogenized in lysis buffer (50 mM Tris, pH 7.4 at 22 °C containing 150 mM NaCl, 2 mM EGTA, 10% glycerol [v/v], 1% NP-40 [v/v] containing protease and phosphatase inhibitors [Sigma-Aldrich]). Seminiferous tubule lysates were used for co-immunoprecipitation.

Lysate Preparation, Immunoblotting and Co-Immunoprecipitation

Sertoli cell and seminiferous tubule lysates were prepared with lysis buffer. Protein concentrations were estimated by the *DC* Protein Assay (Bio-Rad). Approximately 25 µg protein was subjected to immunoblotting, whereas anywhere from 100 µg-3 mg protein was used for co-immunoprecipitation as described (Yan and Cheng, 2005). All co-immunoprecipitation experiments using different antibodies (Suppl. Table 3) were repeated at least three times with three different batches of Sertoli cell and seminiferous tubule lysates.

Immunofluorescent Microscopy

Sertoli cells were cultured on Matrigel-coated glass coverslips and hypotonically treated as described below. Two days after transfection of siRNA duplexes, cells were fixed with 4% paraformaldehyde [w/v], permeabilized with 0.1% Triton X-100 [v/v] and blocked with 1% BSA [w/v]. Thereafter, cells were incubated with different antibodies (1:50) at room temperature overnight (Suppl. Table 3), followed by a 30-minute incubation with Alexa Fluor-labeled secondary antibodies (1:100, Invitrogen). For desmoglein-2 immunostaining in vitro, Sertoli cells cultured for 4 days were pre-extracted for 5 minutes in ice cold 0.3% Triton X-100 [v/v] in CSK buffer [50 mM NaCl, pH 6.8 at 22 °C containing 300 mM sucrose, 10 mM PIPES and 3 mM MgCl₂]. Cells were subsequently fixed with 4% paraformaldehyde [w/v]. This was followed by using the above staining protocol with anti-desmoglein-2 IgG. These immunofluorescent microscopy experiments were repeated three times using Sertoli cells from three different cultures. For desmoglein-2 immunostaining in vivo, Bouin's fixed paraffin sections from adult rat testes were deparaffinized, rehydrated and heated at 98 °C for 10 minutes in antigen retrieval buffer [10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9.0]. Again, this was followed by the above staining protocol using anti-desmoglein-2 (H-145) antibody (Suppl. Table 3). Desmoglein-2 immunostaining was also performed with frozen sections that were fixed with 4% paraformaldehyde [w/v]. Both anti-desmoglein-2 and anti-desmoglein-2 (H-145) IgG were used at a final concentration of 0.02 mg/ml. These immunofluorescent microscopy experiments were repeated three times using different testes. Cells and testis

sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), and images were acquired with MicroSuite FIVE software (Version 1.224, Olympus Soft Imaging Solutions Corp) and an Olympus DP70 12.5 MPa digital camera attached to an Olympus BX61 motorized microscope (Olympus America Inc.). Images were uniformly adjusted for brightness and contrast with Photoshop in Adobe Creative Suite Design Premium software package (Version 3.0).

Electron Microscopy

Electron microscopy was performed at the Bio-Imaging Resource Center, The Rockefeller University. In brief, Sertoli cells were cultured on Matrigel -coated 60-mm culture dishes at high density. On day 4 in vitro, cells were transfected with either non-targeting or desmoglein-2-specific siRNA duplexes. Normal and transfected Sertoli cells were terminated on day 6 and fixed with 0.1 M cacodylate buffer, pH 7.5 at 22 °C containing 2.5% glutaraldehyde [v/v] and 2.5% paraformaldehyde [w/v]. Thereafter, cells were post-fixed with 1% OsO₄ [v/v] in 0.1 M cacodylate buffer and stained with 2% uranyl acetate [w/v]. Following dehydration with ethanol, Sertoli cells were detached from culture dishes with propylene oxide treatment and embedded in EPON (Electron Microscopy Sciences). Silver sections were obtained with a Reichert Ultracut II ultramicrotome and examined with a JEOL 100CXII electron microscope (JEOL USA Inc.).

Sertoli Cell Cultures, and Specific Knockdown of Desmoglein-2, Desmocollin-2 or Desmoglein-2/Desmocollin-2 by RNAi

Sertoli cells were isolated from 20-day-old rat testes and cultured in F12/DMEM supplemented with growth factors as described (Mruk et al., 2003). On day 2, contaminating germ cells were removed (Galdieri et al., 1981) to yield Sertoli cells with a purity of ~98%. For harvesting cells for protein lysates and RNA, endocytosis assays and electron microscopy, cells were plated on Matrigel - (BD Biosciences) coated culture plates at high density (0.5×10^6 cells/cm²). For transepithelial electrical resistance (TER) and cell viability assays, Sertoli cells were plated (1.0×10^6 cells/cm²) on Matrigel -coated Millicell-HA culture plate inserts (Millipore) and 96-well culture plates, respectively. TER across the Sertoli cell epithelium was quantified as described (Cheng and Mruk, 2006). For immunofluorescent microscopy, cells were seeded on Matrigel -coated coverslips at a lower density ($0.03\text{--}0.05 \times 10^6$ cells/cm²) but at one which still permitted assembly of functional TJs, basal ES and desmosomes. Cells were cultured for 3–4 days to permit assembly of a functional barrier which mimicked the BTB in vivo (Chung and Cheng, 2001, Yan et al., 2008). Specific knockdown of desmoglein-2, desmocollin-2 or desmoglein-2/desmocollin-2 was performed by transfecting Sertoli cells on day 3 or 4 with corresponding siRNA duplexes (Suppl. Table 4, Thermo Scientific Dharmacon) using RiboJuice™ (Novagen). For experiments using 0.5×10^6 cells/cm² as the cell density, a non-targeting siRNA duplex (100 or 150 nM) was used as the control (Suppl. Table 4). A final concentration of 100 nM siRNA was used to silence either desmoglein-2 or desmocollin-2, whereas 150 nM (*i.e.*, 75 nM each) was used to silence both genes simultaneously. For TER and XTT cell viability assays, all treatment groups were transfected with a final concentration of 200 nM siRNA. For immunofluorescent microscopy, Sertoli cells were co-transfected with 60–75 nM siRNA and 1 nM siGLO red transfection indicator (Thermo Scientific Dharmacon) to track transfected cells and to estimate silencing efficiency by counting cells exhibiting red fluorescence. In selected experiments, nuclease-free water (vehicle Ctrl) was included as a control, in addition to the non-targeting siRNA duplex. Sertoli cells were rinsed 24 hours after transfection and incubated for an additional 24 hours in F12/DMEM before cells were terminated with lysis buffer, TRIzol reagent or immunohistological fixative. The final concentrations of all siRNA duplexes used in this study were determined in several pilot experiments, which demonstrated the efficacy of RNAi knockdown to be dependent on Sertoli cell plating density.

Endocytosis Assay

Two days after transfection with desmoglein-2/desmocollin-2-specific siRNA duplexes, cell surface proteins were biotinylated with 0.8 mM sulfo-NHS-SS-biotin (Pierce) in DPBS, pH 7.4 at 22 °C containing calcium chloride and magnesium chloride (GIBCO) for 30 min at 4 °C (Yan et al., 2008). The reaction was quenched with 50 mM Tris in DPBS for 15 min at 4 °C, and cells were then incubated in growth factor supplemented-F12/DMEM at 35 °C for various durations to allow protein endocytosis. Remaining cell surface biotin was stripped with 50 mM MESNA in 100 mM Tris-HCl, pH 8.6 at 22 °C containing 100 mM NaCl and 2.5 mM CaCl₂ for 30 min at 4 °C. Thereafter, the reaction was quenched with iodoacetamide (5 mg/ml) in DPBS for 15 min at 4 °C. Cell lysates were harvested in lysis buffer, and biotinylated proteins were isolated by incubating 100 µg protein with UltraLink Immobilized NeutrAvidin™ Plus beads (Pierce) overnight at 4 °C. Protein endocytosis was semi-quantified by immunoblotting using an anti-coxsackie and adenovirus receptor (CAR) antibody (Suppl. Table 3). This assay was repeated three times using Sertoli cells from three independent cultures.

Sertoli Cell Viability Assay

Sertoli cells were cultured in 96-well plates and transfected under identical conditions as described for TER measurements. Thirty-two hours thereafter, a cell viability assay was performed as instructed by the manufacturer using sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) which was supplied in the Cell Proliferation Kit II (Roche Applied Science). The relative level of metabolically-active cells in each well was reflected by the conversion of XTT into an orange formazan dye which was quantified colorimetrically. Absorbance was measured at 450 nm with a reference wavelength at 655 nm using a microplate reader (Model 680, Bio-Rad). Measurements were taken between 4–24 hours after the addition of XTT reagents (*i.e.* 36–52 hours after transfection). This assay was repeated three times using Sertoli cells from three different cultures.

General Methods

TER across the Sertoli cell epithelium was quantified as described (Chung et al., 2001, Cheng and Mruk, 2006). TER experiments were repeated three times using Sertoli cells from three independent cultures. Statistical analysis was performed with GB-STAT software (Version 7.0, Dynamic Microsystems). One-way ANOVA followed by Dunnett's post-test was used for multiple comparisons, and Student's *t*-test was used for paired comparisons.

Results

Functional desmosomes are present between Sertoli cells in vitro

RT-PCR was used to investigate the expression of desmosomal genes in different tissues and cell types, including Sertoli and germ cells (Fig. 1) since a study of this nature was lacking in the testis. Indeed, Sertoli and germ cells were found to differentially express genes from each of the three desmosomal families, indicating that the testis is equipped with the necessary building blocks to form desmosomes. Specifically, desmogleins-1, -2 and -4; desmocollins-1, -2 and -3; plakoglobin (*i.e.*, γ -catenin); plakophilins-1, -2 and -4; and desmoplakin were expressed by the testis (Fig. 1). At this point in our study, we chose to focus our efforts on desmoglein-2 due to its relatively high abundance in Sertoli cells (Fig. 1). Hence, a monospecific antibody was raised in rabbits against rat desmoglein-2 recombinant protein (17.8 kDa, Fig. 2) and used for immunoblotting. Thereafter, Sertoli cells cultured at high density were used to study the biology of desmosomes because previous reports have shown this system to mimic the BTB in vivo in that functional TJs (Fig. 3A) and basal ES are

assembled by day 4 in vitro (Chung and Cheng, 2001, Yan et al., 2008). These findings are now expanded by electron microscopy to show that desmosome-like junctions are also present between Sertoli cells in vitro, coexisting with TJs and basal ES (Fig. 3B). During assembly of TJs, basal ES and desmosomes in vitro, the steady-state levels of desmoglein-2, plakoglobin and vimentin were shown to be up-regulated (Fig. 3C–D). Desmoglein-2 was also visualized at the Sertoli cell interface in vitro, partially co-localizing with TJ and basal ES proteins zonula occludens-1 (ZO-1) and N-cadherin, respectively (Fig. 3E). In the adult rat testis, desmoglein-2 was observed as a discontinuous belt at the BTB, partially co-localizing with N-cadherin (Fig. 3F). While the staining pattern was different from N-cadherin, these results illustrate that desmoglein-2 was present at the BTB. Specificity of anti-desmoglein-2 antibodies used for immunostaining was demonstrated by immunoblots using Sertoli cell and seminiferous tubule lysates (Fig. 2C, D). Interestingly, the partial co-localization of desmoglein-2 with N-cadherin is consistent with a recent report (Young et al., 2009). Finally, the presence of functional desmosomes in the testis was corroborated by co-immunoprecipitation when desmoglein-2 was shown to interact with plakoglobin in vitro and in vivo when Sertoli cell and seminiferous tubule lysates were used, respectively (Fig. 3G), similar to published reports (Kowalczyk et al., 1994, Witcher et al., 1996). Desmoglein-2 also associated with c-Src, a protein kinase known to regulate junction dynamics in other epithelia and endothelia (Dejana et al., 2008, Perez-Moreno and Fuchs, 2006). Moreover, results from co-immunoprecipitation experiments illustrated that desmoglein-2 interacts with desmocollin-2 in vitro and in vivo (Fig. 3H).

Desmoglein-2 knockdown in Sertoli cells in vitro results in a decrease in the steady-state level and cellular localization of ZO-1, leading to BTB restructuring

The function of desmoglein-2 in BTB dynamics was investigated by its transient knockdown in Sertoli cells in vitro by RNAi. When compared to the non-targeting control, a ~60% reduction in the desmoglein-2 protein level was achieved 2 days after transfection (Fig. 4A, B). To investigate whether a compromise in desmosome function (as evidenced by desmoglein-2 silencing) could affect barrier integrity, the levels of different BTB-constituent proteins were examined by immunoblotting (Fig. 4A, C). While the levels of most proteins remained unchanged following RNAi, ZO-1 was consistently shown to decrease by ~20%. Moreover, a mislocalization of ZO-1 was detected by immunofluorescent microscopy. In desmoglein-2-silenced Sertoli cells, ZO-1 staining at the cell-cell interface became discontinuous and showed signs of internalization (Fig. 4D, a versus d). On the contrary, following desmoglein-2 knockdown, occludin (Fig. 4D, b versus e) and N-cadherin (Fig. 4D, c versus f) were unaffected in both protein level (Fig. 4A) and cellular localization (Fig. 4D). When the integrity of the Sertoli cell barrier was assessed by electron microscopy after desmoglein-2 silencing, areas of junction restructuring were noted (Fig. 5), likely resulting from the down-regulation and mislocalization of ZO-1, an important TJ adaptor. However, sites adjacent to junction restructuring remained intact (Fig. 5). Areas of junction restructuring were not observed in control Sertoli cells (*i.e.*, those cultured with [Fig. 5A] and without [Fig. 3B] non-targeting siRNA duplexes), which were processed identically to Sertoli cells treated with desmoglein-2 siRNA duplexes.

Simultaneous knockdown of desmoglein-2 and desmocollin-2 in Sertoli cells in vitro results in a further reduction in the steady-state level and mislocalization of ZO-1

Since functional desmosomes are constituted by both desmogleins and desmocollins, we opted to simultaneously knockdown desmoglein-2 and desmocollin-2 (*i.e.*, desmoglein-2/desmocollin-2) in anticipation of more profound phenotypic changes. Desmocollin-2 was selected for simultaneous silencing based on RT-PCR results which showed this gene to be expressed abundantly by Sertoli cells (Fig. 1), as well as based on co-immunoprecipitation results which demonstrated desmoglein-2 to interact with desmocollin-2 (Fig. 3H). Two days

after transfection, a ~60% decrease in the desmoglein-2 protein level (Fig. 6A, B) and a ~60% decrease in the desmocollin-2 mRNA level were attained (Fig. 6A, C) when compared to the non-targeting control. Consistent with desmoglein-2 silencing (Fig. 4), a ~30% reduction in ZO-1 was noted by immunoblotting after desmoglein-2/desmocollin-2 silencing (Fig. 6A, D), as opposed to the ~20% reduction that was observed after knockdown of desmoglein-2 alone (Fig. 4A, C). Mislocalization and internalization of ZO-1 following desmoglein-2/desmocollin-2 knockdown (Fig. 6E, a versus e) were also observed by immunofluorescence microscopy. No changes in the levels (Fig. 6A) or cellular localizations of other BTB-constituent proteins such as occludin (Fig. 6E, b versus f), N-cadherin (Fig. 6E, c versus g) and plakoglobin (Fig. 6E, d versus h) were observed following double knockdown. We had also attempted to immunostain claudin-11 in Sertoli cells following desmoglein-2 and desmoglein-2/desmocollin-2 knockdown but were unsuccessful because of the lack of a working antibody.

Simultaneous knockdown of desmoglein-2/desmocollin-2 results in a loss of c-Src and CAR from the Sertoli cell surface, an increase in CAR endocytosis and a disruption of BTB function

Following single (*i.e.*, desmoglein-2 or desmocollin-2) and/or double (*i.e.*, desmoglein-2/desmocollin-2) knockdown, both c-Src and CAR mislocalized from the Sertoli cell surface (Fig. 7A, B), but no changes in their steady-state protein levels were detected following silencing (Fig. 4, 6). Moreover, loss of CAR from the Sertoli cell surface was more profound in double silencing as opposed to single silencing experiments (Fig. 7B, b, c versus d). To validate immunofluorescent microscopy results, changes in the kinetics of CAR endocytosis were assessed by an endocytosis assay (Fig. 7C, D). While the kinetics of CAR endocytosis reached a plateau by ~30 min in the control, a statistically significant increase in the rate of CAR internalization was observed up to 60 min when Sertoli cells transfected with desmoglein-2/desmocollin-2 duplexes were compared to those transfected with control duplexes. Since both CAR and c-Src have been implicated in TJ dynamics, the functional significance of these effects was assessed by TER. Indeed, simultaneous knockdown of desmoglein-2/desmocollin-2 perturbed Sertoli cell barrier function (Fig. 7E), resulting from collective changes that we observed with CAR, ZO-1 and c-Src as reported herein. However, no significant changes in TER were observed when either desmoglein-2 or desmocollin-2 was silenced. To validate that the decrease in TER in all treatment groups on day 4 and beyond was not the result of Sertoli cell death (Fig. 7E), an XTT cell viability assay was performed. Sertoli cell viability was not affected significantly 48 hours after transfection with control, desmoglein-2, desmocollin-2 or desmoglein-2/desmocollin-2 duplexes (Fig. 8A). Repeated measurements were taken 36–52 hours after transfection, and similar results were obtained in each reading. The moderate decrease in TER, which was observed in all treatment groups (including the control) following transfection, was very likely caused by the use of RiboJuice. Finally, to further validate the key finding in this study, TER and selected cell staining experiments were repeated using nuclease-free water as a control, in addition to the non-targeting siRNA duplex (Fig. 8B, C).

Discussion

The desmosome functions as a signaling platform at the BTB

Since the initial report three decades ago, which identified the desmosome as a constituent junction of the BTB coexisting with TJs, basal ES and GJs (Vogl et al., 2008, Russell, 1977a), this is the first study to illustrate that the rat testis is equipped with the structural components of desmosomes. Generally speaking, the role of desmosomes in different epithelia is to reinforce the function of adherens junctions, revealing that these junction types co-exist within the junctional complex together with TJs, and this is the subject of investigation in this

study. Thus, crosstalk between these individual structures is a key element in the maintenance of tissue integrity. In contrast to other blood-tissue barriers, the BTB is a dynamic structure that must 'disassemble' or restructure at stages VIII-IX of the seminiferous epithelial cycle to accommodate leptotene spermatocyte transit (Russell, 1977b), while at the same time maintaining an immunological barrier to sequester post-meiotic germ cell development from the systemic circulation. As such, it is envisioned that a unique mechanism is in place to facilitate restructuring of the BTB during the seminiferous epithelial cycle in the testis. While several TJ (*i.e.*, occludin/ZO-1, JAM-A/ZO-1 and claudin-11/ZO-1) and basal ES (*i.e.*, N-cadherin/ β -catenin) multi-protein complexes have been identified at the BTB (Mruk and Cheng, 2004, Mruk et al., 2008), how they are regulated during BTB restructuring is virtually unknown. Without this critical information, it is difficult to execute functional experiments to investigate how these proteins regulate junction dynamics in order to facilitate the transit of leptotene spermatocytes across the BTB. In this study, desmoglein-2 was shown to interact with the non-receptor protein tyrosine kinase c-Src, a known regulator of TJs and a mediator of integrin signaling in vitro and in vivo (Boutros et al., 2008). This is significant because Src-mediated integrin signaling is known to affect cell shape and motility, as well as TJ barrier integrity in other epithelia and endothelia (Boutros et al., 2008). As such, c-Src is likely to be an important regulator of BTB dynamics. It is conceivable that desmosomes may also serve as one of the major docking sites for membrane-associated c-Src since this protein was shown to be enriched in unique membrane microdomains (Galbiati et al., 2001). This notion is further supported by mislocalization of c-Src after desmoglein-2/desmocollin-2 silencing in Sertoli cells. Moreover, activated c-Src can regulate TJs and GJs by disrupting occludin/ZO-1 and connexin-43/ZO-1 interactions (Kale et al., 2003, Gilleron et al., 2008), respectively, illustrating the significance of desmosomes as a integrated component of the BTB by coexisting with TJs (*i.e.*, occludin) and GJs (*i.e.*, connexin-43). Other desmosomal component proteins, besides desmoglein-2, may also be substrates of c-Src. For instance, plakoglobin was shown to be tyrosine phosphorylated, which transduced signals to the nucleus (Green and Simpson, 2007). As such, these findings are consistent with the emerging concept that the desmosome can serve as a signaling hub at sites of cell-cell contact (Runswick et al., 2001, Green and Simpson, 2007). It is likely that c-Src mediates its effects via ZO-1 which can indirectly affect other TJ, basal ES and GJ proteins at the BTB. Indeed, a recent report has illustrated that c-Src forms a complex with Cx43/ZO-1 in the 43GPA9 Sertoli cell line and that these proteins can be endocytosed in response to a non-genomic carcinogen (Gilleron et al., 2008). Thus, the findings reported herein have opened up new avenues for future investigation on desmosome dynamics in the testis.

What possible role do desmosomes play in the transit of leptotene spermatocytes across the BTB during spermatogenesis?

The most intriguing finding in this study was that knockdown of desmoglein-2 and desmocollin-2 in Sertoli cells, which had assembled a functional TJ-permeability barrier, resulted in changes in the localization of CAR, a TJ and basal ES component protein (Wang et al., 2007), as well as in c-Src which is known to interact with CAR (Wang et al., 2007). Based on studies using dual-labeled immunofluorescent analysis, both CAR and c-Src translocated away from the Sertoli-Sertoli cell interface and into the cytosol. This was supported in part by results from the protein endocytosis assay which employed biotinylation of cell surface proteins to track protein internalization, illustrating that knockdown of desmoglein-2/desmocollin-2 led to an increase in the endocytosis of CAR which partly contributed to barrier disassembly. However, endocytosis of CAR was not the only factor responsible for barrier disassembly; changes in the steady-state levels and/or localizations of ZO-1 and c-Src also played important roles. While mislocalization of ZO-1 was also observed after knockdown of desmoglein-2/desmocollin-2, but given that occludin was largely unaffected, it is possible that the decrease in the ZO-1 level at the cell-cell interface is only a

secondary effect of CAR mislocalization because ZO-1 is recruited to cell junctions by CAR (Cohen et al., 2001). While results from silencing experiments have illustrated that desmoglein-2 and desmocollin-2 are critical to barrier function, additional experiments are needed to address protein-protein interactions at the BTB. For instance, the decrease in TER observed after simultaneous knockdown of desmoglein-2 and desmocollin-2 does not indicate whether homophilic or heterophilic interactions were perturbed, and this conclusion is similar to a previous report which used blocking peptides against a desmoglein and a desmocollin (Runswick et al., 2001).

Based on these observations, we hypothesize that BTB restructuring at stages VIII–IX of the seminiferous epithelial cycle (Russell, 1977b; Yazama, 2008), which facilitates transit of leptotene spermatocytes, is mediated by crosstalk between desmosome- and CAR-based protein complexes. CAR, a TJ- and basal ES-associated integral membrane protein, was previously shown to be expressed by both Sertoli and germ cells at the site of the BTB (Wang and Cheng, 2007; Mirza et al., 2007). Similar to trans-epithelial/endothelial migration of viruses and leukocytes across a barrier, leptotene spermatocytes are likely to traverse the BTB by assembling stable CAR-CAR interactions with adjacent Sertoli cells, in essence to replace ‘disassembled’ CAR-CAR interactions between Sertoli cells (Wang and Cheng, 2007). Herein, it was shown that down-regulation of desmoglein-2 and desmocollin-2 by RNAi resulted in an increase in CAR internalization, as well as in changes in ZO-1 and c-Src, which collectively disrupted Sertoli cell tight junction barrier function. As such, it is envisioned that primary spermatocytes in transit across the BTB trigger a tightly controlled mechanism to down-regulate desmosomal proteins such as desmoglein-2 and desmocollin-2 in a localized manner. This would destabilize the BTB by breaking down existing CAR-CAR interactions between Sertoli cells, thereby inducing the formation of nascent CAR-CAR interactions between the Sertoli cell and the traversing leptotene spermatocyte. In this way, migrating germ cells would contribute directly to Sertoli cell function and to the maintenance of BTB function. This is an attractive hypothesis because the function of other junctional complexes at the BTB, namely those of occludin and N-cadherin, would not be affected and the immunological barrier would be maintained during germ cell movement. In the physiological context of the testis, the transient down-regulation of desmosomal cadherins may be achieved by an increase in cytokine levels at the BTB since cytokines such as TNF α are known to regulate the steady-state levels of integral membrane proteins at this cellular site (Mruk and Cheng, 2004). In this respect, we are currently investigating whether different cytokines can up-/down-regulate the steady-state levels of desmosomal proteins such as desmoglein-2 in Sertoli cells. Alternatively, these events may be mediated via proteases. Indeed, desmosomal cadherins were shown to be substrates of matrix metalloproteases [MMPs] (Weiske et al., 2001), which are produced by both Sertoli and germ cells (Mruk and Cheng, 2004). While the exact identity of the trigger(s) responsible for BTB restructuring beginning at late stage VIII remains to be identified, it is likely to be derived from preleptotene spermatocytes as it is these cells that must communicate with Sertoli cells to initiate BTB restructuring in a timely manner. As such, knowing the identity of this molecule(s) would prove critical to understanding BTB remodeling and spermatogenesis. In summary, this study provides the first biochemical evidence for the existence of functional desmosomes at the Sertoli cell barrier/BTB. More importantly, we have shown that desmosomes play a role in Sertoli cell barrier/BTB dynamics and may balance BTB integrity by acting on other protein complexes at this cellular site.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

basal ES	basal ectoplasmic specialization
BTB	blood-testis barrier
GJ	gap junction
TER	transepithelial electrical resistance
TJ	tight junction

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Fig. 1. Desmosomal genes are expressed in testis, Sertoli and germ cells. (A–C) Presence of desmosomal transcripts from three gene families was investigated by RT-PCR. Kidney, heart and skin served as positive controls. Sk, skin; K, kidney; H, heart; T, testis; SC, Sertoli cells; GC, germ cells. (A) Cadherin family. Dsg, desmoglein; Dsc, desmocollin. S-16 served as an internal control. (B) Armadillo family. Pkg, plakoglobin; Pkp, plakophilin. (C) Plakin family. Dpk, desmoplakin. (D) Purity of isolated germ and Sertoli cells was verified by the absence of testin and c-Kit receptor mRNA, respectively.

Fig. 2. Production of desmoglein-2 recombinant protein and specificity of anti-desmoglein-2 antibodies. (A) Nucleotide sequence of rat desmoglein-2 mRNA (GenBank accession no. XM_001054396) encoding intracellular domains. The nucleotide sequence that was used for recombinant protein expression, as well as its translation, is noted in blue. The sites corresponding to the protein/nucleotide sequence are also noted in blue in the schematic drawing of desmoglein-2 domains. EC, extracellular repeating elements; EA, extracellular anchoring domain; TM, transmembrane domain; IA, intracellular anchoring domain; ICS, intracellular cadherin-type segment; IPL, intracellular proline-rich linker; RUD, repeat unit domains; DTD, desmoglein-specific terminal domain. (B) Detection of expressed His-tagged

desmoglein-2 recombinant protein with an anti-His Tag antibody. Recombinant desmoglein-2 (17.8 kDa) was detected in the soluble fraction of bacterial cultures (Lane 3) but not before IPTG induction (lane 1) and not in culture medium (lane 2). (C) Immunoblot of Sertoli cell (SC) lysate probed with anti-desmoglein-2 IgG produced in-house. (D) Immunoblot of seminiferous tubule (ST) lysate probed with a commercially- obtained anti-desmoglein-2 antibody (H145).

Fig. 3.

Functional desmosomes are present at the Sertoli cell barrier in vitro and in vivo. (A) Sertoli cells cultured at high density (1.0×10^6 cells/cm²) on Matrigel-coated bicameral inserts assemble a functional tight junction barrier as demonstrated by the increase in TER. (B, a) Electron micrograph showing desmosome-like junctions between Sertoli cells (0.5×10^6 cells/cm², day 6). (b) Magnified view of the boxed area in a. Arrowheads indicate desmosomes which are typified by patches of electron-dense material. SC, Sertoli cell; n, nucleus; Id, lipid droplet. (C) Immunoblots investigating steady-state levels of different proteins in Sertoli cells (0.5×10^6 cells/cm², day 0–7) during desmosome assembly. Actin served as a loading control. D, day. (D) Histogram summarizing results in C after normalizing each data point against its

corresponding control (0D) and actin. 0D was arbitrarily set at 1 for all proteins except for desmoglein-2 which was barely detectable at this time point and set at 0.1. Instead, 1D was arbitrarily set at 1 for desmoglein-2. Each bar represents mean $\pm$ SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (one-way ANOVA followed by Dunnett's test against 0D). (E) Sertoli cells ($0.03\text{--}0.05 \times 10^6$ cells/cm², day 4) were co-immunostained for desmoglein-2 (a–d, red) with either N-cadherin (c, green) or ZO-1 (d, green). Nuclei were visualized with DAPI (blue). Dsg-2, desmoglein-2. Bar in a (23 μ m) also applies to b–d. (F) Adult rat testis paraffin section co-immunostained for desmoglein-2 (a and c, red) and N-cadherin (b and c, green), showing partial colocalization at the BTB (arrowheads in c). Bar in a (23 μ m) also applies to b and c. (G, H) Lysates from Sertoli cells (SC, 0.5×10^6 cells/cm², day 6) and adult seminiferous tubules (ST) were used for co-immunoprecipitation with different antibodies (Suppl. Table 3) as listed above each lane. Immunoblotting was performed with anti-desmoglein-2 and anti-plakoglobin antibodies. Co-IP, co-immunoprecipitation; lysate (lane 1 in all immunoblots in G, H) represents 10 μ g Sertoli cell (SC); PKG, plakoglobin (positive control; plakoglobin is known to bind to desmoglein-2); CX-43, connexin-43; Rb, rabbit; Ms, mouse; Dsg-2, desmoglein-2; Dsc-2, desmocollin-2. Rb and Ms IgG represent negative controls.

Fig. 4.

Steady-state level and cellular localization of ZO-1 are affected by desmoglein-2 knockdown in Sertoli cells in vitro. Sertoli cells were transfected on day 4 with non-targeting (Ctrl) or desmoglein-2- (Dsg-2) specific siRNA duplexes, and cells were harvested two days thereafter. (A) Immunoblots examining steady-state levels of BTB-constituent proteins following desmoglein-2 silencing by RNAi. Lysates obtained from transfected Sertoli cells (0.5×10^6 cells/cm²) were used for immunoblotting. Actin served as a loading control. GJ, gap junction. (B, C) Histograms summarizing results in A after normalizing each data point against its corresponding control (arbitrarily set at 1) and actin. Proteins that did not change significantly are not represented by histograms. Each bar represents mean $\pm$ SD from 3–5 independent

experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$ (Student's t -test). (D) Transfected Sertoli cells ($0.03\text{--}0.05 \times 10^6$ cells/cm²) tracked with siGLO transfection indicator (red) were immunostained for ZO-1 (a, d), occludin (b, e) and N-cadherin (c, f; green) 2 days after transfection. Nuclei were visualized with DAPI (blue). Micrographs are presented as merged images. Arrowheads (d) show internalization of ZO-1 from the cell-cell interface following desmoglein-2 knockdown. Results are representative of at least three independent Sertoli cell cultures in which at least 50 images were collected using a 20X objective and analyzed in Photoshop. Bar in a (20 μm) also applies to b–f.

Fig. 5.

Desmoglein-2 knockdown in Sertoli cells affects BTB integrity. On day 4, Sertoli cells (0.5×10^6 cells/cm²) having functional TJs, basal ES and desmosomes (*i.e.*, BTB) were transfected with either non-targeting or desmoglein-2-specific siRNA duplexes. Two days after transfection, cells were processed for electron microscopy. (A) Sertoli cells transfected with control siRNA duplexes. Arrowheads indicate functional Sertoli cell junctions. (B) Sertoli cells transfected with desmoglein-2 siRNA duplexes. Areas of BTB restructuring were observed (asterisks), while adjacent areas remained intact (arrowheads). (C) Magnified view of the boxed area in B. Separation of the Sertoli cell plasma membrane was observed (white arrowheads). SC, Sertoli cell; n, nucleus.

Fig. 6.

Steady-state level and cellular localization of ZO-1 are affected by simultaneous desmoglein-2/desmocollin-2 knockdown in Sertoli cells in vitro. Sertoli cells ($0.03\text{--}0.05 \times 10^6$ cells/cm²) were transfected on day 4 with non-targeting (Ctrl) or desmoglein-2/desmocollin-2 (Dsg2/Dsc2) specific siRNA duplexes, and cells were harvested two days thereafter. (A) Immunoblots investigating steady-state levels of BTB-constituent proteins following desmoglein-2/desmocollin-2 silencing by RNAi. Lysate or RNA from transfected Sertoli cells (0.5×10^6 cells/cm²) were used for immunoblotting or RT-PCR, respectively. Actin and S-16 served as loading controls for immunoblotting and RT-PCR, respectively. (B, C, D) Histograms summarizing results in A after normalizing each data point against its corresponding control and actin for immunoblots, and S-16 for RT-PCR. Proteins that did not change significantly are not represented by histograms. The control was arbitrarily set at 1. Each bar represents mean $\pm$ SD from 3–5 independent experiments performed in duplicate. **, $P < 0.01$ (Student's *t*-test). (E) Transfected Sertoli cells ($0.03\text{--}0.05 \times 10^6$ cells/cm²) tracked with siGLO transfection indicator (red) were immunostained for ZO-1 (a, e), occludin (b, f), N-cadherin

(c, g) and plakoglobin (d, h; green). Nuclei were visualized with DAPI (blue). Micrographs are presented as merged images. Arrowheads (e) show internalization of ZO-1 from the cell-cell interface following desmoglein-2/desmocollin-2 knockdown, similar to results shown in Fig. 4. Results are representative of at least three independent Sertoli cell cultures in which at least 50 images were collected using a 20X objective and analyzed in Photoshop. Bar in a (23 μ m) also applies to b–h.

Fig. 7.

Desmoglein-2/desmocollin-2 knockdown results in mis-localization of c-Src and CAR from the Sertoli cell surface, and increased CAR internalization. Sertoli cells ($0.03\text{--}0.05 \times 10^6$ cells/cm²) were transfected on day 4 (A–D) or day 3 (E) with one of the following: non-targeting (Ctrl), desmoglein-2- (Dsg-2), desmocollin-2- (Dsc-2) or a mixture of desmoglein-2/desmocollin-2- (Dsg-2/Dsc-2) specific siRNA duplexes, and cells were harvested two days thereafter (A–D). (A, B) Redistribution of c-Src and CAR from the cell-cell interface as shown by immunofluorescent staining. Transfected Sertoli cells ($0.03\text{--}0.05 \times 10^6$ cells/cm²) tracked with siGLO transfection indicator (red) were immunostained for c-Src (A) or CAR (B; green). Nuclei were visualized with DAPI (blue). Micrographs are presented as merged images. Arrowheads (A, b and B, b–d) show loss of c-Src and CAR from the cell-cell interface, respectively. Results are representative of at least three independent Sertoli cell cultures in which at least 50 images were collected using a 20X objective and analyzed in Photoshop. Bar in A, a (23μm) also applies to b. Bar in B, a (23 μm) also applies to b–d. (C) Increase in the rate of CAR endocytosis following desmoglein-2/desmocollin-2 silencing by RNAi. Transfected Sertoli cells (0.5×10^6 cells/cm²) were assessed for changes in the kinetics of CAR endocytosis by an endocytosis assay. Following isolation of biotinylated proteins, immunoblotting was used to quantify internalized CAR. Controls included (i) total biotinylated cell surface proteins without stripping (total, positive control) [note: lysates used for isolation of biotinylated proteins were diluted 10-fold, but no dilution was made in the actin immunoblot], (ii) lack of biotinylation (no biotin, negative control) and (iii) stripping of biotin without endocytosis (stripped, negative control). (D) A plot summarizing results in C after normalizing against actin. CAR internalization is expressed as a percentage of total cell surface

CAR. Each data point represents mean $\pm$ SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (Student's t -test). (E) Effects of desmoglein-2, desmocollin-2 and desmoglein-2/desmocollin-2 RNAi on TER. TER measurements across the Sertoli cell epithelium (1.0×10^6 cells/cm²) were taken daily at specified time-points, including both before and after transfection on day 3. Results are representative of three independent TER experiments with each experiment yielding similar results (mean $\pm$ SD, each experiment had triplicate data points). *, $P < 0.05$; **, $P < 0.01$ (Dunnett's test against Ctrl subsequent to one way ANOVA).

Fig. 8.

Relative cell viability following RNAi and validation of the use of non-targeting siRNA. Sertoli cells were transfected on day 3 (A–B) or day 4 (C) with one of the following: nuclease-free water (vehicle Ctrl), non-targeting (Ctrl) siRNA, desmoglein-2- (Dsg-2), desmocollin-2- (Dsc-2) or a mixture of desmoglein-2/desmocollin-2- (Dsg-2/Dsc-2) specific siRNA duplexes, and cells were harvested two days thereafter (A, C). (A) Sertoli cell viability after transfection with control (Ctrl), desmoglein-2 (Dsg-2), desmocollin-2 (Dsc-2) or desmoglein-2/desmocollin-2 (Dsg-2/Dsc-2) duplexes. Sertoli cells cultured in 96-well plates were transfected under the same conditions as described for TER experiments (200 nM siRNA for each group, *i.e.* Ctrl, desmoglein-2 RNAi and desmocollin-2 RNAi, and 100 nM siRNA each for desmoglein-2/desmocollin-2 RNAi). Relative cell viability, as reflected by absorbance at 450 nm, was measured 48 hours after transfection. Bar = mean $\pm$ SD ($n = 4$). (B) TER measurements across the Sertoli cell epithelium (1.0×10^6 cells/cm²) were taken daily at specified time-points, including both before and after transfection on day 3. Each data point represents mean $\pm$ SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$. (C) Transfected Sertoli cells ($0.03\text{--}0.05 \times 10^6$ cells/cm²) tracked with siGLO transfection indicator (red) were immunostained for ZO-1 (a, c, e) and

CAR (b, d, f, green). Arrowheads (e, f) indicate mislocalization of ZO-1 and CAR following desmoglein-2/desmocollin-2 knockdown.