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Disruption of Sertoli-Germ Cell Adhesion Function in the Seminiferous Epithelium of the Rat Testis Can be Limited to Adherens Junctions Without Affecting the Blood–Testis Barrier Integrity: An In Vivo Study Using an Androgen Suppression Model

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During spermatogenesis, both adherens junctions (AJ) (such as ectoplasmic specialization (ES), a testis-specific AJ type at the Sertoli cell-spermatid interface (apical ES) or Sertoli–Sertoli cell interface (basal ES) in the apical compartment and BTB, respectively) and tight junctions (TJ) undergo extensive restructuring to permit germ cells to move across the blood–testis barrier (BTB) as well as the seminiferous epithelium from the basal compartment to the luminal edge to permit fully developed spermatids (spermatozoa) to be sloughed at spermiation. However, the integrity of the BTB cannot be compromised throughout spermatogenesis so that postmeiotic germ cell-specific antigens can be sequestered from the systemic circulation at all times. We thus hypothesize that AJ disruption, unlike other epithelia, can occur without compromising the BTB-barrier, even though these junctions, namely TJ and basal ES, co-exist side-by-side in the BTB. Using an intratesticularly androgen suppression-induced germ cell loss model, we have shown that the disruption of AJs indeed was limited to the Sertoli-germ cell interface without perturbing the BTB. The testis apparently is using a unique physiological mechanism to induce the production of both TJ- and AJ-integral membrane proteins and their associated adaptors to maintain BTB integrity yet permitting a transient loss of cell adhesion function by dissociating N-cadherin from β-catenin at the apical and basal ES. The enhanced production of TJ proteins, such as occludin and ZO-1, at the BTB site can supersede the transient loss of cadherin-catenin function at the basal ES. This thus allows germ cell depletion from the epithelium without compromising BTB integrity. It is plausible that the testis is using this novel mechanism to facilitate the movement of preleptotene and leptotene spermatocytes across the BTB at late stage VIII through early stage IX of the epithelial cycle in the rat while maintaining the BTB immunological barrier function. J. Cell. Physiol. 9999: 1–17, 2005. © 2005 Wiley-Liss, Inc.

Cell junctions are ubiquitous structures in multicellular organisms serving an array of biological functions. These include adhering cells to form specialized epithelia, sensing the environment, and providing the means for cell–cell communications (for[Q2] reviews, see Alberts et al., 2002; Braga, 2002; Perez-Moreno et al., 2003; Mruk and Cheng, 2004a). In virtually all the epithelia found in mammals including the skin, collecting tubules in the kidney, the respiratory tract, and the intestine, tight junctions (TJ) are located at the apex of a cell epithelium, furthest away from the basal lamina, to be followed by cell–cell actin-based adherens junctions (AJs) that form the adhesion belt, underneath of which lies the cell–cell intermediate filament-based desmosomes, these structures are referred to as the junctional complex (for reviews, see Alberts et al., 2002; Braga, 2002; Perez-Moreno et al., 2003; Lee and Cheng, 2004b; Mruk and Cheng, 2004a,b). Because of this intimate relationship between these junctions, a disruption of AJs can impair the TJ-barrier function and vice versa in many epithelia, such as those found in keratinocytes, which is a generally accepted cell physiological phenomenon (Troxell et al., 1999; Man et al., 2000; Gassler et al., 2001; Venkiteswaran et al., 2002; Guo et al., 2003). Interestingly, in the seminiferous epithelium of mammalian testes, such as rats, TJ, AJ, and desmosomes are not distinctly separated from one another to form the junctional complex, instead TJ co-exists side-by-side with two testis-specific AJ types called basal ectoplasmic specialization (ES) and basal tubulobulbar complex (TBC), forming the blood–testis barrier (BTB), which lies adjacent to the basement membrane, a modified form of extracellular matrix; whereas other AJs, such as apical ES, are found between round/elongating/elongate spermatids and Sertoli cells in the seminiferous epithelium (for reviews, see Russell and Peterson, 1985; Dym, 1994; Vogl et al., 2000; Toyama et al., 2003; Mruk and Cheng, 2004b; Siu and Cheng, 2004a,b). Unlike other TJ-barriers such as the blood-brain, the blood-epididymal, and the blood–retinal barriers, BTB must physically open to facilitate the migration of developing preleptotene and leptotene spermatocytes, which are in micrometers in diameter, at late stage VIII through early stage IX of the epithelial cycle (Russell, 1977) while maintaining the BTB integrity so that postmeiotic germ cell antigens can be sequestered from the systemic circulation. Furthermore, extensive AJ restructuring between Sertoli and germ cells occur during spermatogenesis to facilitate the timely migration of developing germ cells across the seminiferous epithelium in the rat

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testis without compromising the BTB integrity. As such, the testis must have a mechanism in place that regulates the TJ-barrier integrity at the BTB while permitting AJ restructuring. We hypothesize that Sertoli-germ cell AJ disruption in the seminiferous epithelium, unlike other epithelia, does not lead to a disruption of the TJ-barrier function. To test this hypothesis and to address this intriguing cellular physiology phenomenon pertinent to spermatogenesis regarding the functional interrelationship between TJ and AJ in the seminiferous epithelium, we have used an androgen suppression-induced Sertoli-germ cell AJ restructuring model.

Testosterone is one of the most important regulators of spermatogenesis and it is also one of the prime targets for hormonal male contraception (for reviews, see McLachlan et al., 2002b; Kamischke and Nieschlag, 2004). In previous studies, an increase in endogenous testosterone in the systemic circulation using testosterone and estrogen implants in adult rats can lead to a suppression of intratesticular testosterone, inducing depletion of spermatids (from step 9 and beyond) from the seminiferous epithelium (McLachlan et al., 1994; O'Donnell et al., 1996). Although the detailed mechanism of this spermatid sloughing is still unclear, it was shown to involve Sertoli-germ cell AJ disruption, in particular, at the apical ES (O'Donnell et al., 2000; McLachlan et al., 2002a,b). As such, this model was used to assess if a disruption of AJs that led to spermatid loss would compromise the BTB integrity. Recent studies have identified three putative ES-associated protein complexes in the rat testis crucial to ES dynamics, including the cadherin/catenin, the nectin/afadin, and the integrin/laminin protein complexes (for a review, see Lee and Cheng, 2004b). We therefore sought to investigate the loss of spermatids from the epithelium during intratesticular androgen suppression, is mediated via changes in protein–protein interaction in the ES protein complex. These are the subjects of this report.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing between 250 and 280 g were purchased from Charles River Laboratories (Kingston, MA). The use of animals for studies reported herein was approved by the Rockefeller University Animal Care and Use Committee, with Protocol Numbers 00111 and 03017.

Preparation of steroid implants

Androgen and estrogen implants were prepared by filling testosterone (Cat. #T-1500) or estradiol-17β (Cat. #E-8875) (Sigma, St. Louis, MO) to ethylene and vinyl acetate (EVA) tubing (Elvax 770, 9% VA; 2.15 mm ID × 2.4 mm OD, DuPont[Q3]). Earlier studies have estimated that implants using EVA had a steroid (e.g., MENT, 7α-methyl-19-nortestosterone) release rate of 90 μg/cm/day in vivo (in humans) when placed under the skin (Noe et al., 1999; von Eckardstein et al., 2003) versus ~30 μg/cm/day for Silastic implants (Kincl et al., 1968; Robaire et al., 1979). Both ends of the EVA tubing were sealed by heat. T implants were either 3 or 4 cm in length; E implants were 0.4 cm in length. Empty implants, 4 cm, without any steroid, were also prepared that served as vehicle controls.

Experimental design

Rats were randomly assigned to three groups with three rats for each time point in each group as follows. Group I: rats received one 3-cm T and one 0.4-cm E implants on day 0; Group II: TE implants were administered in rats on day 0 and removed on day 28, and rats were allowed to recover spontaneously (i.e., natural recovery); Group III: TE implants were administered on day 0 and removed on day 28, and four 4-cm T implants were inserted to the same site to permit rapid recovery. All implants were inserted subdermally to the dorsal side of adult rats. About three rats per time point in Group I were terminated on day 4, 8, 12, 20, and 28; in Group II, rats were terminated on day 29, 35, 42, 49, and in Group III, on day 29, 30, 42, 49. Controls included rats without any implants but terminated on day 0, 12, 28, 42, and rats received TE implants, but replaced with 4 × 4 cm empty implants on day 28, and terminated on day 29 and 35 thereafter.

Animal surgery

Animal surgery was performed with rats under anesthesia using Ketamine HCl (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) at 75 mg/kg b.w. administered via i.m. Hair at the surgical area were removed, and cleansed by scrubbing 70% alcohol and Betadine (2× each). A small insertion site of ~2.5 cm was opened, and implants were carefully inserted subcutaneously. Prior to their use, implants were briefly cleansed by immersing into 70% alcohol for decontamination. For TE treatment, one 3-cm T implant and one 0.4-cm E implant were embedded; and for recovery under high T condition, 4 × 4 cm T implants were embedded under the same site after TE implants were removed on day 28. For rats that underwent spontaneous natural recovery (SR), no implants were used after TE implants removal. Surgical site was stitched and was removed on day 7 after operation.

Preparation of samples

Rats were terminated at specific time points by CO_2 asphyxiation and body weights were recorded. Testes with epididymides attached were removed and photographed. Testes were weighed and snap frozen in liquid nitrogen, and stored at −80°C until use.

Lysates preparation and immunoblottings

Lysates were prepared essentially as earlier described using a lysis buffer (10 mM Tris, 0.15 M NaCl, 2 mM PMSF, 2 mM EDTA, 2 mM *N*-ethylmaleimide, 1% NP-40 [vol/vol], 1 mM sodium orthovanadate, 0.1 μM sodium okadate, and 10% glycerol [vol/vol]) (Lee et al., 2003; Lui et al., 2003c; Siu et al., 2003b) and protein estimation was carried out using the Coomassie blue dye-binding assay (Bradford[Q4], 1976) with BSA as a standard. For subsequent co-immunoprecipitation (Co-IP) experiments, lysates were prepared in the same lysis buffer. Antibodies and the sources of antibodies used in this study were summarized in Table 1. Immunoblottings were carried out using ~150 μg protein from each sample for SDS–PAGE as previously described (Lee et al., 2003; Lui et al., 2003c; Siu et al., 2003b; Wong et al., 2004) using 7.5%–12.5% T SDS polyacrylamide gels (Laemmli, 1970). All samples within an experimental group were processed simultaneously to eliminate interexperimental variations.

Immunohistochemistry

Immunohistochemistry was performed essentially as previously described (Siu et al., 2003b). Frozen testes were embedded in OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and sectioned at 7 μm in thickness using a microtome in a cryostat (Hacker, Fairfield, NJ) at −20°C, mounted onto poly-L-lysine coated slides and stained for JAM-1 with a Histostain-SP™ kit (Zymed Laboratories, South San Francisco, CA). Sections were counterstained by hematoxylin as described (Siu et al., 2003b; Siu and Cheng, 2004c). Sections were examined under an Olympus BX-40 microscope (Olympus Corp., Melville, NY) and photographed using an Olympus DP70 12.5 MPa Digital Camera. All images were acquired using the QImaging QCapture Suite (Version 2.6) Software Package from Quantitative Imaging Corp. (Burnaby, BC, Canada) and analyzed with Adobe PhotoShop (Version 7.0). Controls included the use of normal rabbit serum, rabbit IgG, or PBS to substitute the primary antibody.

Immunofluorescent microscopy

Fluorescent microscopy was performed essentially as previously described (Lee et al., 2003, 2004; Siu et al., 2003b; Siu and Cheng, 2004c). Fluorescein isothiocyanate (FITC)- and

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TABLE 1. Summary of primary antibodies used for different immunological experiments

Vendor	Antibody against target protein	Animal source	Catalog #	Lot #	Usage	Working dilution
Santa Cruz Biotechnologies (Santa Cruz, CA)	Actin	Goat[a]	sc-1616	D052	IB	1:1,000
	Claudin-11	Goat	sc-13641	A162	IB	1:200
	N-cadherin	Rabbit	sc-7939	J1502	IB	1:200
					IP	1:40
	E-cadherin	Rabbit	sc-7870	C212	IB	1:200
	α-catenin	Rabbit	sc-7894	G3003	IB	1:200
	β-catenin	Rabbit	sc-7199	L0203	IB	1:200
					IP	1:40
	Nectin-3	Goat	sc-14806	K261	IB	1:100
	Laminin-γ3	Goat	sc-16601	G032	IB	1:200
Zymed Laboratories, Inc. (South San Francisco, CA)	Occludin	Rabbit	71-1500	30979485	IB	1:200
					IF	1:100
	JAM-1	Rabbit	36-1700	30979650	IB	1:250
					IHC	1:100
					IF	1:250
	ZO-1	Rabbit	61-7300	30175033	IB	1:250
	ZO-1-FITC	Mouse, monoclonal	33-9111	30879018	IF	1:100
	N-cadherin	Mouse, monoclonal	33-3900	30778768	IF	1:100
	β-catenin	Rabbit	71-2700	30477187	IF	1:100
	Phospho-Tyr	Mouse, monoclonal	13-6600	40186105	IB	1:500
Sigma-Aldrich (St. Louis, MO)	l-afadin	Rabbit	A0349	012K4875	IB	1:1,000
BD Transduction Laboratories (San Diego, CA)	Integrin-β1	Mouse, monoclonal	610468	8	IB	1:500

IB, immunoblotting; IHC, immunohistochemistry; IF, immunofluorescent microscopy.
[a]All primary antibodies used in this study were polyclonal antibodies except otherwise specified.

Cy3-labeled goat anti-rabbit IgG were used to co-localize N-cadherin and β-catenin in the seminiferous epithelium of rat testes. A FITC-conjugated ZO-1 antibody and a Cy3-labeled goat anti-rabbit IgG were used to co-localize JAM-1 (or occludin) with ZO-1 to assess the integrity of the BTB during androgen suppression-induced germ cell loss from the epithelium as described (Wong et al., 2004). Frozen sections were obtained in a cryostat and prepared as described above. All samples from different rats within an experimental group, such as during androgen suppression-induced germ cell loss from the epithelium, were processed simultaneously with 3–4 cross-sections per slide so that all samples could be placed on 2–3 slides. This step is essential to eliminate interexperimental variations in particular differences in antibody incubation time, staining conditions and subsequent color development. Sections were treated with 10% normal goat serum (Zymed, Cat. #01-6201, diluted in PBS) and incubated with the corresponding primary antibody pair at room temperature overnight. Sections were then washed in PBS to be followed by incubation with fluorescein-labeled secondary antibody pair. Sections were subsequently mounted in Vectashield with DAPI [4′, 6-diamidino-2-phenylindole, dihydrochloride] (Vector Laboratories, Burlingame, CA), and viewed under an Olympus BX-40 microscope equipped with fluorescent optics.

Image analysis

To estimate the diffusion of proteins (e.g., N-cadherin and β-catenin) from the site of its localization in the testis during androgen suppression-induced germ cell loss from the epithelium, assessing changes in protein–protein association besides Co-IP, fluorescent images of N-cadherin and β-catenin from fluorescence microscopy were acquired and photographed at the same magnification and parameters. At least 50 tubules from testes of two different rats were randomly selected and examined as follows. First, fluorescent images were photographed and printed using an Epson 890 Inject printer at 1,440 × 720 dpi resolution on Epson Photo papers. Second, the diffusion of a target protein staining, such as N-cadherin, from its arbitrary site of origin is defined as the distance (in mm) from the BTB site near the basement membrane to the edge of visible staining away from the BTB in a treatment group, or control testes. Thus, the relative diffusion (RD) of a target protein during treatment = distance of the target protein diffused away from BTB at specified time point/the distance in control testis (mean) at the same time point. The magnification (or the absolute distance) used in our measurement did not interfere RD results because they were being canceled out in the numerator and denominator.

Co-IP and an assessment of Tyr-phosphorylation content of β-catenin

About 500–1,200 μg protein lysates from each sample were used for Co-IP studies to assess changes in protein–protein interactions during androgen depletion-induced germ cell loss. All samples within an experimental group were processed simultaneously to eliminate interexperimental variation. Co-IP was performed as described (Lee et al., 2003, 2004). In brief, lysates were pretreated with 1% (vol/vol) IgG for at least 1 h with agitation using a rotator (GlasCol, Terre Maute, IN) at 24 rpm. Thereafter 10 μl Protein A/G-PLUS agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added and incubated for another hour. After a brief centrifugation (1,000*g*, 5 min) to remove non-specifically interacting proteins in the pellet, supernatant was collected and incubated with the corresponding primary antibody of a target protein overnight with agitation at 4°C. Thereafter, 20 μl Protein A/G-PLUS agarose was added, and incubated for 2 h to precipitate immunocomplexes. Immunocomplexes were washed four times with Co-IP buffer by gentle resuspension and centrifugation. The resultant complexes were denatured in SDS-sample buffer and resolved by SDS–PAGE. Target proteins were visualized by the corresponding antibodies. Scaled-up Co-IP (∼1,200 μg protein per sample) was carried out to assess Tyr-phosphorylation content of β-catenin. In short, about 1/3 of the immunoprecipitated proteins in the scaled-up Co-IP using an anti-β-catenin antibody as the precipitating antibody were resolved by SDS–PAGE, electroblotted onto nitrocellulose and probed with an anti-phospho-Tyr antibody. Immunostained protein band on the blot that had the same electrophoretic mobility as of β-catenin (∼92 kDa) was identified. Because β-catenin protein levels were induced during androgen suppression-induced germ cell loss, measures were taken to correct for the increasing β-catenin protein level as follows. First, the same amount of SDS sample buffer was added to each sample to extract immunocomplexes from the scale-up Co-IP experiment. Second, only 1/3 of this IP product was resolved by SDS–PAGE and the β-catenin protein levels were estimated by densitometric scanning of the protein blot. Third, based on this semi-quantitative scanning data, the same amount of β-catenin that was immunoprecipitated between samples within

the treatment group was resolved on another SDS–polyacrylamide gel. Fourth, after phospho-Tyr immunostaining, this same blot was stripped and reprobed with a β-catenin antibody confirming the level of β-catenin was indeed uniform in all samples within the treatment group. As such, any changes in phospho-Tyr level in β-catenin is the result of putative changes in its phosphorylation status.

Statistical analysis

Different parameters (e.g., body and testis weights, steady-state levels of different target proteins interpolated from densitometrically scanned results after normalized against β-actin) from samples within an experimental group were compared between samples at different times versus controls and/or between pairs of samples at other time points by ANOVA using Honest Significant Test (HST) with the JMP IN software package (Version 4, SAS, Inc., Cary, NC).

RESULTS

Changes in testes weight and size, and body weight during androgen suppression-induced germ cell loss from the seminiferous epithelium

The treatment regimen that was used to induce AJ disruption, causing germ cell loss from the seminiferous epithelium by suppressing the endogenous T level via TE implants and the subsequent recoveries, is shown in Figure 1A. A progressive and significant loss in testes weight was detected following TE implants insertion at the time of germ cell loss (Fig. 1B, part a). By the time TE implants were removed (see solid arrow), testes weight had decreased to about half of normal testes and this trend continued for several additional days. During recovery, either spontaneously or under high T condition, testes weight recovered rapidly and was similar to normal rat testes within 3 weeks (Fig. 1B, part a). Such changes of testes weight were consistent with changes in testes size (Fig. 1C). For instance, testis length along the longitudinal axis decreased from ~20 mm (control) to ~15 mm (29D) after ~4 weeks of T suppression, and returned to the size virtually indistinguishable from normal testes in both recovery groups (Fig. 1C). The body weights of these rats also gained steadily from ~300 to ~420 g in control groups when housed with access to food and water ad libitum (Fig. 1B, part b). The rate of body weight gaining was significantly smaller than controls after TE implants were placed under the skin; however, body weights were rapidly recovered in both recovery groups and the differences found between treatment groups and control rats were negligible at the end of the experimental period (Fig. 1B, part b).

Did the androgen suppression induced-germ cell loss from the seminiferous epithelium disrupt the BTB integrity?

Selected TJ-integral membrane proteins and adaptors were upregulated in androgen suppressed rat testes. Three TJ-integral membrane proteins: occludin, claudin, and JAM; and an adaptor known to exists at BTB: ZO-1, were examined. An increase in the protein levels of occludin, JAM-1 and ZO-1, but not claudin-11, was observed beginning on day 20 after TE implants (Fig. 2A,B), reaching a peak of almost three- to fivefold versus control rats on days 28–30 at the time spermatids (step 8 and beyond) were depleting from the epithelium (see below). Thereafter, the protein levels of occludin, JAM-1, and ZO-1 declined gradually when implants were removed on day 28 to permit recovery, and returned to their normal level by day 49 when the epithelium was fully recovered (Fig. 2A,B).

BTB integrity was not compromised during androgen suppression-induced germ cell loss from the epithelium. The above immunoblot data have illustrated that occludin and ZO-1 levels in the testis

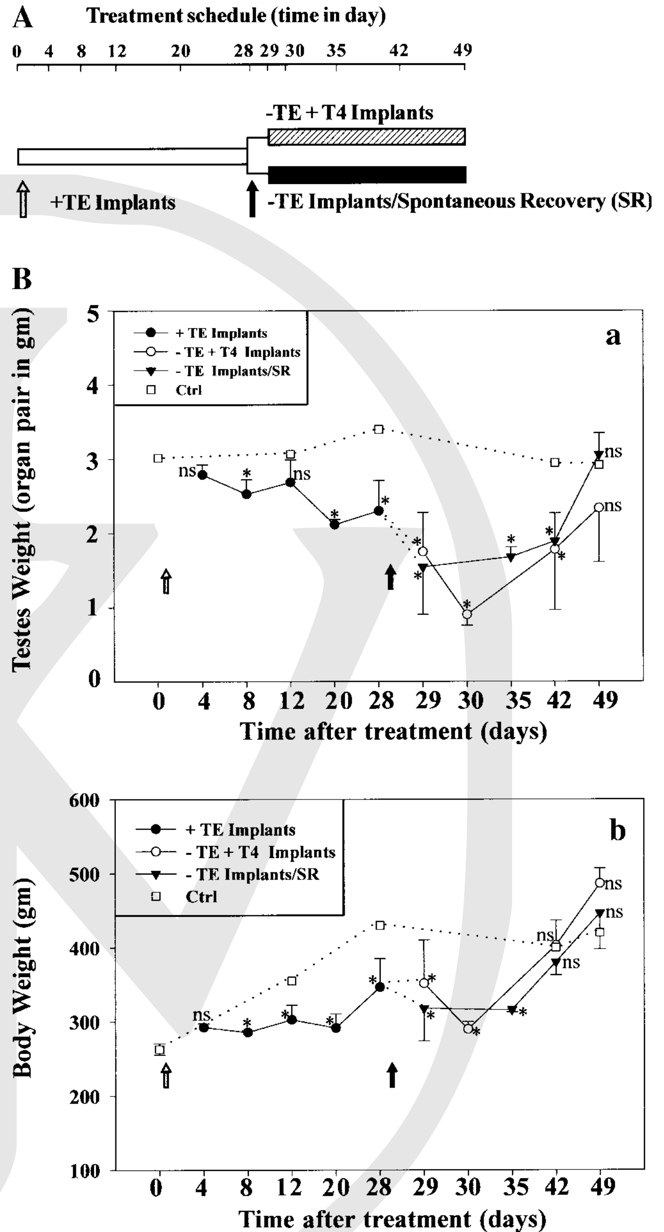

Fig. 1. **A–C**: A study of using TE implants to suppress intratesticular androgen levels to deplete germ cells from seminiferous epithelium and its effects on testes and body weight, and testis size in adult rats. A: The treatment regimen that was used in studies in this report. B: This figure shows changes in testes weight (**a**) representing organ pair (g) from each animal (n = 3 for each time point). Changes in body weight were shown in part (**b**). On day 0 (the date when surgery was performed), rats received TE implants. On day 28, TE implants were removed in one group of rats and replaced with 4 × 4 cm T implants (T4) (−TE + T4 implants). In another group of rats, TE implants were removed and rats underwent naturally spontaneous recovery (SR) without any implants (−TE implants/SR). Open arrow indicates when TE implants were administered; closed arrow indicates when TE implants were removed to allow either androgen-induced (T4 implants) or spontaneous recovery. Each data point is the mean ± SD SD of three rats. ns, not significantly different from control as determined by ANOVA; *, significantly different, $P < 0.05$; (C, **a–g**) shown[Q5] herein are representative photographs of testes with epididymides displaying changes in their sizes during treatment and recovery. The labeling used here is applicable to all other figures in this report. T, testosterone; E, estrodiol-17β.

COLOR

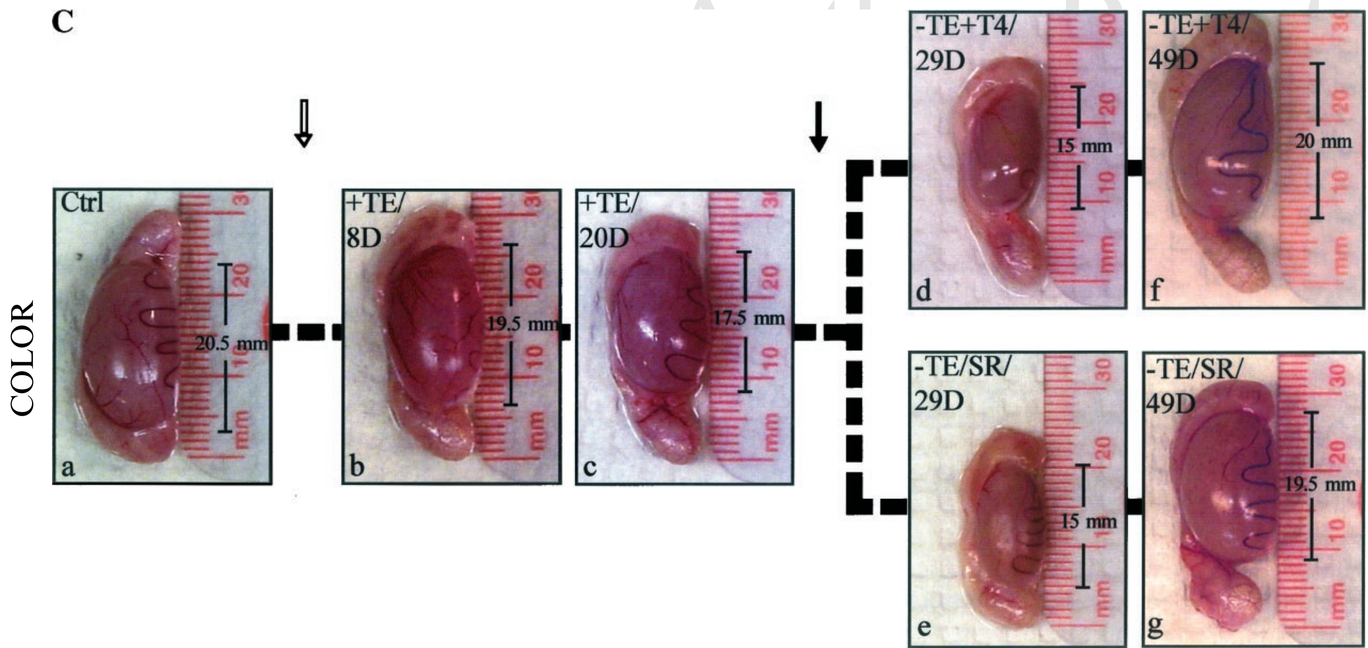

Fig. 1. (*Continued*)

were induced by the TE-implants mediated androgen suppression (Fig. 2A–L), yet this did not give any indication if the site of BTB had been disrupted. A recent study using fluorescence microscopy to co-localize occludin and ZO-1 to the epithelium has proven to be a novel tool to assess the BTB integrity (Wong et al., 2004), it was therefore used in this study. Both occludin and ZO-1 were localized to the same site in the basal compartment consistent with their localization at the BTB site (Fig. 3A–D), forming an almost continuous superimposable immunoreactive ring in the epithelium of a normal rat testis (Fig. 3A–C). This pattern of localization persisted in testes on day 20 (Fig. 3E–G) and 29 (Fig. 3I–K) at the time of extensive spermatids loss from the epithelium (see Fig. 4) except that there was an increase in fluorescence of occludin or ZO-1 versus control testes (Fig. 2E–G,I–K vs. A–C), illustrating the BTB integrity had not been compromised, consistent with results of immunoblots that illustrate an increase in protein levels (Fig. 2).

Study using JAM-1 to further validate that BTB remained intact during androgen suppression-induced germ cell loss from the seminiferous epithelium. To further validate the above observations, the study was extended to another TJ-integral membrane protein at the BTB namely JAM-1, since an increase in JAM-1 protein was detected during androgen suppression-induced germ cell loss (Fig. 2). Immunohistochemistry was preformed in normal rat testes in which JAM-1 appears as reddish-brown precipitates at the basal compartment of the epithelium, consistent with its localization at the BTB (Fig. 4A). JAM-1 was found to be a stage-specific protein, being highest at stages IX–XIV, but lowest at stages IV–VI. At stages IV–VI, the staining of JAM-1 at the BTB site diminished greatly (see inset I in Fig. 4A). Some JAM-1 staining was also found in the interstitium surrounding the endothelial cells of the microvessel, illustrating it is also used as a TJ-integral membrane protein in vascular TJ-barrier (Fig. 4A). Positive (Fig. 4A, part e) and negative (Fig. 4A, part d) controls were also included to assure the staining shown in Figure 4 was specific for JAM-1.

We next performed an additional immunohistochemistry experiment to examine the pattern of JAM-1 in the testis during androgen suppression-induced germ cell loss from the epithelium (Fig. 4B). Consistent with the immunoblotting data shown in Figure 2, JAM-1 staining at the basal compartment was indeed greatly induced. The insets shown in different parts in Figure 4B were at lower magnification, corresponding to the selected time points of the androgen suppression regimen (See Fig. 1A). This result illustrates that amidst the progressive shrinkage of the seminiferous tubules (Fig. 4B, parts c, d vs. part a), the reddish-brown immunoreactive JAM-1 signals also gradually intensified, yet this protein continued to maintain an almost uninterruptive ring consistent with the data of occludin shown in Figure 3. This immunohistochemistry also implicated that the BTB integrity had been maintained during androgen suppression-induced germ cell loss from the epithelium (Fig. 4B).

Since JAM-1 was shown to interact with ZO-1 in other epithelia (Bazzoni et al., 2000), we sought to use fluorescence microscopy to co-localize JAM-1 and ZO-1 at the site of BTB in control testes as well as in testes by 29D after TE implants at the time of germ cell loss (see Fig. 4B). As shown in Figure 4C, parts a–d, the merged images of JAM-1 and ZO-1 was not entirely yellowish for all tubules because JAM-1 was not expressed uniformly at all stages of the epithelial cycle (Fig. 4C vs. A). Nonetheless, JAM-1 that was co-localized with ZO-1 always maintained a non-interruptive ring at the BTB site in the seminiferous epithelium during androgen suppression-induced germ cell loss (Fig. 4C–g).

What is the mechanism(s) by which androgen suppression induces germ cell loss from the seminiferous epithelium?

Changes in ES-associated protein levels during androgen suppression-induced germ cell loss from the seminiferous epithelium. Three ES-structural protein complexes have been identified in the apical ES of rat testes (for reviews, see Cheng and Mruk, 2002; Toyama et al., 2003; Lee and Cheng, 2004a,b; Mruk and

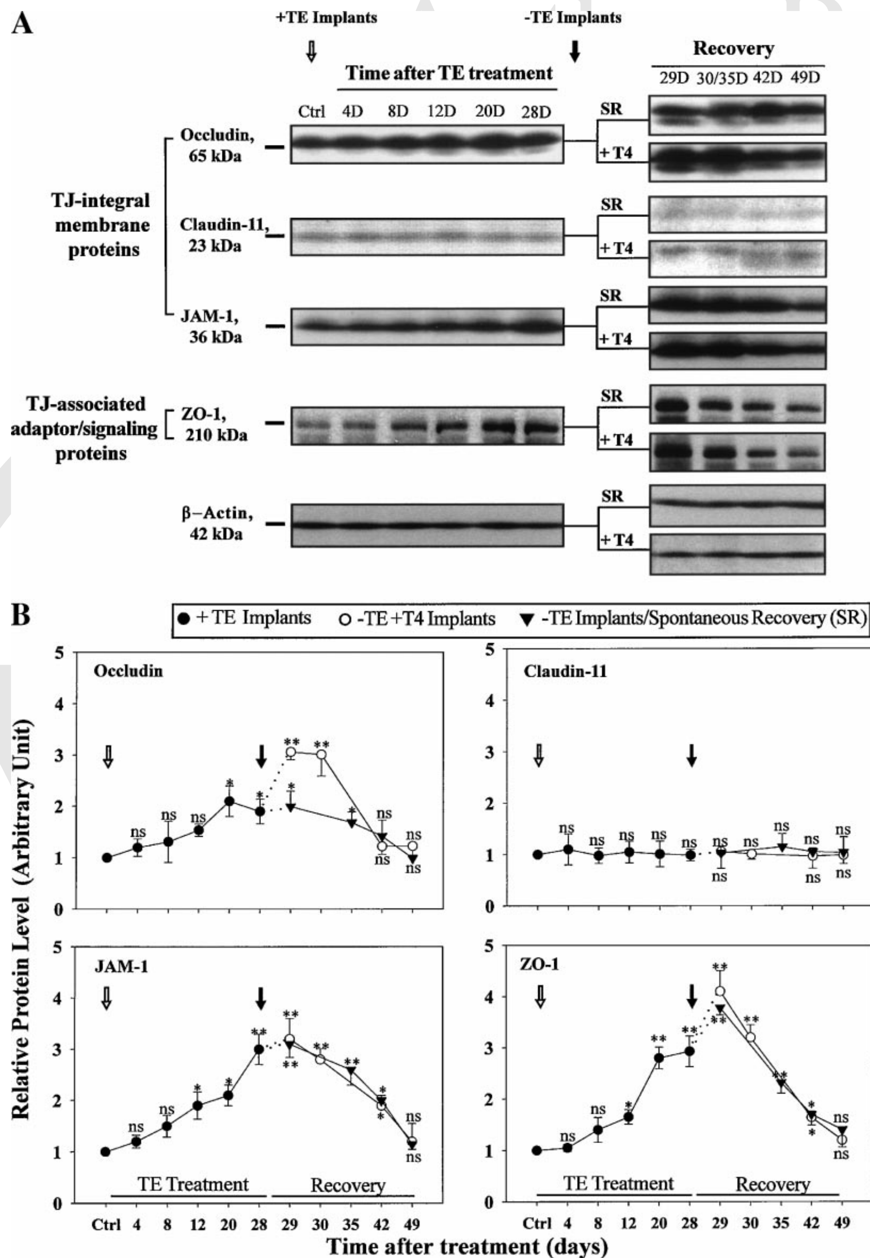

Fig. 2. **A, B**: A study by immunoblotting to assess changes in TJ-associated proteins in the rat testis during androgen suppression-induced germ cell loss and its recovery. A: Immunoblotting results in which lysates of testes containing ~150 μg protein from each sample within an experimental group were resolved by SDS–PAGE, and the blots were probed with different primary antibodies. Immunoblot data were arranged into two groups: androgen depletion induced by TE implants and the two recovery groups. In the recovery phase, the column labeled as 30/35D represents recovery under high T condition on day 30 or spontaneous recovery on day 35. The bottom part is the same blot as those shown above, but reprobed with an anti-actin antibody to assess equal protein loading. B: These are densitometrically-scanned data using immunoblots such as those shown in (A). All data were normalized against β-actin to account for uneven protein loading and the level of a target protein in control rats was arbitrarily set at 1. Each data point is the mean ± SD of three rats. ns, not significantly different from control as determined by ANOVA; *, significantly different, $P < 0.05$; **, significantly different, $P < 0.01$.

Cheng, 2004a,b; Siu and Cheng, 2004a,b): the cadherin/catenin complex, the nectin/afadin complex, and the integrin/laminin complex. Thus, these proteins were selected to examine changes in their levels during androgen suppression-induced germ cell loss to assess AJ disruption (Fig. 5). All cadherins and catenins were induced in the testis during androgen suppression-induced germ cell loss, which declined thereafter in both recovery groups and returned to control levels (Fig. 5A,B). N-Cadherin, α-catenin, and β-catenin, apparently sharing a common pattern of changes in protein levels, were induced by almost four- to fivefold of the control by days 28–30 after TE treatment (Fig. 5A). A noted exception is E-cadherin, which protein level was reduced after TE treatment, but returned to that of normal testes at the end of recovery (Fig. 5A,B). l-Afadin and integrin-β1 protein levels (Fig. 5A,B) displayed a pattern similar to N-cadherin, α-catenin, and β-catenin which were induced during germ cell loss but subsided thereafter during recovery. Like E-cadherin, nectin-3 protein level declined when spermatids were depleted from the seminiferous epithelium during TE treatment

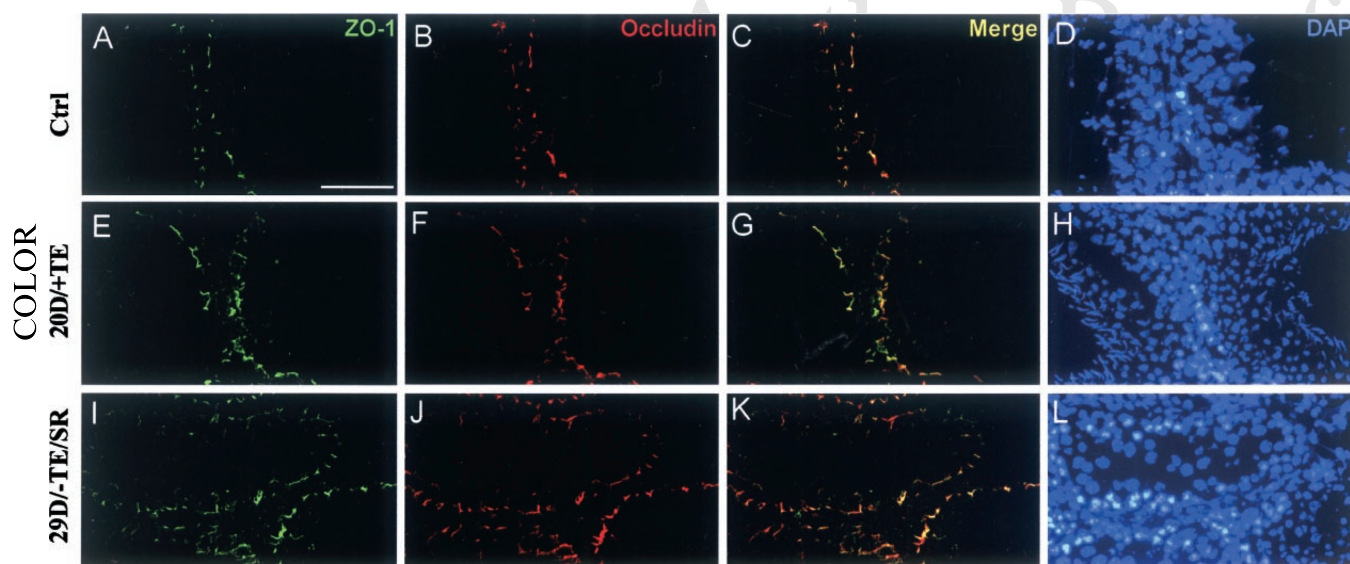

Fig. 3. **A–L**: An assessment of the BTB integrity in rat testes by immunofluorescent microscopy during androgen suppression-induced germ cell loss from the seminiferous epithelium. A–D, control testes; E–H, testes of rats with TE implants for 20 days (20D/+TE); I–L, testes of rats terminated on day 29 in spontaneous recovery group (29D/−TE/SR). A, E, I: ZO-1 staining (FITC-conjugated, green). B, F, J: Occludin staining (Cy3-conjugated secondary antibody, red). C, G, and K are the corresponding merged images of A and B, E and F, and I and J (yellowish-orange). D, H, L: DAPI staining. Bar = 80 μm in (A), which applies to (B–L).

and returned to the level of normal testes in the recovery phase (Fig. 5A,B). Laminin-γ3 showed no obvious changes in its protein level (Fig. 5A,B).

N-cadherin and β-catenin diffuse away from the BTB site, dissociating from each other during androgen suppression-induced germ cell loss from the epithelium. Most of the AJ-associated proteins examined displayed a pattern of induction during germ cell loss from the epithelium, such as cadherins, catenins, afadins, and integrin-β1 (see Fig. 5A,B). For those that were downregulated, such as E-cadherin and nectin-3, their losses may have weakened adhesion between Sertoli and germ cells, leading to germ cell loss from the epithelium. The observation that an induction of AJ-proteins can still lead to a loss of cell adhesion function is unexpected (see Figs. 4B and 5). We hypothesize that such an induction may be a physiological response of the epithelium in the testis to the declining endogenous testosterone level, trying to retain the depleting spermatids. To investigate how these germ cells lost their ability to adhere to the epithelium, we performed two additional experiments. First, we sought to examine the pattern of distribution of N-cadherin and β-catenin in the seminiferous epithelium using fluorescence microscopy during androgen suppression-induced germ cell loss (Fig. 6A). In control rat testes, N-cadherin and β-catenin co-localized to the same site in the basal compartment consistent with their localization at the BTB (Fig. 6A, parts a–d). Interestingly, there was evidence that a thickening of the belt-like fluorescent immunostaining of both N-cadherin and β-catenin had occurred during germ cell loss induced by androgen suppression using TE implants (see white square bracket in Fig. 6A, parts a, e, i, and m); and this staining became diffused from the BTB site. Figure 6B summarized the result of an analysis quantifying the RD of N-cadherin and β-catenin from BTB in epithelium during androgen suppression-induced germ cell loss versus control rats, illustrating a significant increase in protein diffusion. Second, we speculated that such an increase in the diffusion of N-cadherin and β-catenin away from the BTB site might reflect a loss of protein–protein association between N-cadherin and β-catenin. We therefore sought to investigate this possibility by Co-IP experiments using an antibody against N-cadherin or β-catenin to pull down the corresponding protein in lysates and the blots were probed for β-catenin (Fig. 7A). The relative level of β-catenin in control testes versus samples from rats during androgen suppression-induced germ cell loss from the epithelium detected in this Co-IP experiment using an anti-β-catenin antibody had a trend similar to that using lysates alone for immunoblotting, showing an induction at the time of germ cell loss (Fig. 7A,B). Yet using an anti-cadherin antibody for Co-IP, it failed to pull down more β-catenin at the time of germ cell loss (Fig. 7A), indicating that at the late stage of the TE treatment, this protein pair had weakened protein–protein association. The relative protein levels in the Co-IP experiment were normalized against control and plotted as a bar chart in Figure 7B. To estimate these results semi-quantitatively, the amount of β-catenin that was pulled down by the corresponding N-cadherin or β-catenin antibody was compared and plotted as a scatter-line chart (Fig. 7B). It is noted that there was a 50% loss in protein–protein association between N-cadherin and β-catenin at the time of germ cell loss (Fig. 7B vs. Fig. 4B). This result hence suggests that the loss of germ cells from the seminiferous epithelium may be the result of a loss of association between a cell adhesion protein (e.g., N-cadherin) with its adaptor (e.g., β-catenin) that led to a weakened or disrupted junction.

An increase in Tyr-phosphorylation of β-catenin may contribute to the cadherin/catenin protein complex dissociation. One mechanism that can account for the cadherin/catenin protein complex dissociation is Tyr-phosphorylation of the adaptor, β-catenin (for a review, see Daniel and Reynolds, 1997). To estimate the Tyr-phosphorylation status of β-catenin, we first used an anti-β-catenin antibody to pull down the target protein, which was resolved on SDS–PAGE, electroblotted to nitrocellulose membrane and probed for a phosphor-Tyr antibody and detected an

increase in Tyr-phosphorylation at the time of Sertoli-germ cell adhesion impairment (day 20 and 30). However, the total Tyr-phosphorylation increase in β-catenin could be the result of an increase in β-catenin protein, which is known to occur during germ cell loss (see Figs. 5A, 6A, and 7A). To circumvent this issue, we had corrected uneven β-catenin protein level during androgen suppression-induced changes in the immunoprecipitated complexes as described in Materials and Methods (see part 3 in Fig. 8A vs. part 1). Interestingly,

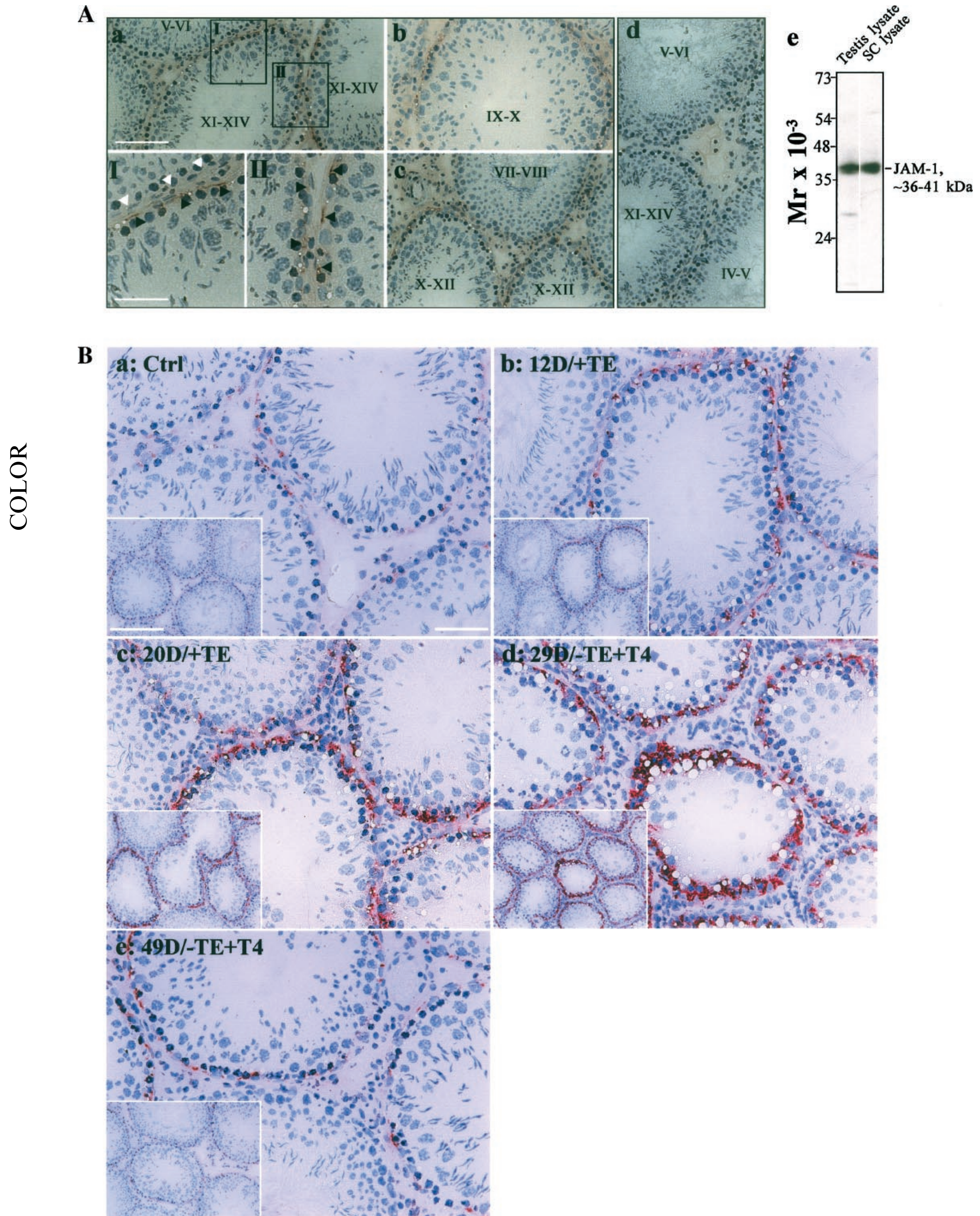

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Fig. 4.

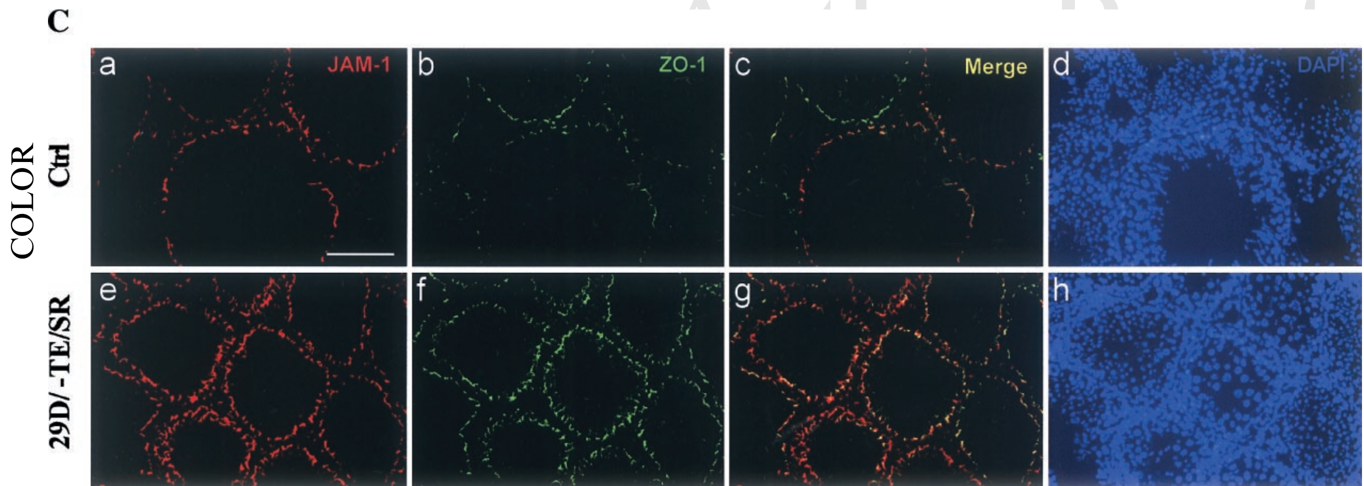

Fig. 4. (*Continued*)

an increase in Tyr-phosphorylation status in β-catenin was still detected (Fig. 8A,B). Figure 8 has thus illustrated an increase in Tyr-phosphorylation of β-catenin during germ cell loss from the epithelium.

DISCUSSION

Does a disruption of AJs in the seminiferous epithelium affect TJs, analogous to junctional complex disruption in other epithelia?

In this study, we have illustrated that a decline of intratesticular androgen level induced by TE implants can lead to a loss of adhesion function at the ES, leading to sloughing of spermatids (steps 8 and beyond) from the seminiferous epithelium, consistent with several earlier studies using this model (O'Donnell et al., 1996, 2000; Beardsley and O'Donnell, 2003). It was anticipated that TJs at the BTB would be compromised in this model since a disruption of AJs is known to perturb TJ-permeability barrier function in virtually all other epithelia examined to date including skin, kidney, and intestine (Troxell et al., 1999; Man et al., 2000; Gassler et al., 2001; Venkiteswaran et al., 2002; Guo et al., 2003). Indeed, both N-cadherin and β-catenin at the basal ES that co-exist with TJ in constituting the BTB were shown to be induced. However, a loss of their association was also detected by Co-IP experiment. Furthermore, both proteins at the basal ES apparently were diffusing away from the BTB site as detected by fluorescent microscopy consistent with biochemical study by Co-IP. Yet the BTB integrity was not compromised as illustrated by fluorescent microcopy. The technique of using fluorescent microscopy to monitor BTB integrity has been rigorously characterized in our laboratory in conjunction with electron microscopy and micropuncture technique assessing the leakage of [^{125}I]-BSA from the systemic circulation to the rete testis and seminiferous tubule fluids (Chung et al., 2001; Wong et al., 2004); and it was shown to be a reliable and sensitive approach to monitor the BTB integrity. It is apparent that the BTB integrity was maintained by a surge in the TJ-integral membrane proteins that constitute the TJ fibrils, such as occludin, JAM-1 and their common adaptor, ZO-1. This observation is physiologically significant, perhaps it is the same mechanism that is being used by the BTB to maintain the TJ integrity during extensive AJ restructuring pertinent to spermatogenesis. These data suggest that while the occludin/ZO-1, the JAM-1/ZO-1 and the cadherin/catenin protein complexes co-exist to constitute the BTB; at the time of AJ restructuring to facilitate germ cell movement during spermatogenesis, a disengagement of TJ (e.g., occludin/ZO-1) and AJ (e.g., cadherin/catenin) proteins can occur, thereby allowing the induced TJ proteins to supersede the function of AJ proteins at the BTB and vice versa. This thus permits the loss of AJ function without compromising the BTB integrity.

Herein, we have shown that the three ES structural protein complexes, namely the cadherin/catenin, the nectin/afadin, and the integrin/laminin were induced preceding germ cell detachment from the epithelium as detected histologically. In some cases, the protein levels were induced as early as 4–12 days after insertion of TE implants, such as N-cadherin and catenins, with no obvious germ cell loss detected as yet. It is obvious that when germ cell loss was most severe on days 28–29, the protein induction was also most drastic. Needless to say, it can be argued that since T suppression by TE implants

Fig. 4. **A–C**: A study to assess the integrity of the BTB in the rat testis using JAM-1 as a TJ-marker protein by immunohistochemistry and fluorescent microscopy during androgen suppression-induced germ cell loss. A: Immunohistochemical localization of JAM-1 in normal rat testes. **a–c**, JAM-1 staining appears as reddish-brown precipitates. **d**, control staining using normal rabbit IgG, illustrating the staining shown in (a–c) is specific to JAM-1. Stages of the epithelial cycle were labeled. A magnified view of the boxed areas shown in (a) was also included and shown in **I** and **II**, in which black arrowheads indicate the JAM-1 staining which was found in the basal compartment near the basement membrane at the site consistent with its localization at the BTB (I and II) in stage XI–XIV tubules, and white arrowheads indicate the lack of JAM-1 staining in the same location in a stage V–VI tubule (I). These micrographs are representative results of four different experiments. Bar = 100 μm in (a), which applies to b–d; bar = 50 μm in (I), which applies to II. The immunoblot shown in (**e**) illustrates the specificity of JAM-1 antibody using lysates of testes and Sertoli cells since only a prominent band with an electrophoretic mobility of JAM-1 at ∼36–41 kDa was detected. B: Immunohistochemical staining of JAM-1 in rat testes during androgen depletion-induced germ cell loss. **a**, normal testis; **b** and **c**, testes from rats with TE implants for 12 days (12D/+TE) and 20 days (20D/+TE); **d**, testis of rat in early recovery phase under high T condition after 1 day of TE implants removal with 4× T implants inserted in this rat (29D/−TE + T4). Bar = 80 μm in (a), which also applies to (b–d); and bar = 320 μm in the inset of (a), which applies to insets in (b–d). C: Immunofluorescent co-localization of JAM-1 and ZO-1 in normal and androgen-depleted testes. a–d, normal testis; **e–h**, testis of rat terminated on day 29 in the spontaneous recovery group (29D/−TE/SR) when the epithelium was virtually devoid of all spermatids (see B). **a** and **e**, JAM-1 staining revealed by a Cy3-conjugated goat anti-rabbit IgG (red); **b** and **f**, ZO-1 staining revealed by an FITC-conjugated mouse anti-ZO-1 antibody (green); **c** and **g**, merged images of (a and b), (e and f), respectively (yellowish-orange); e and h, DAPI staining. Bar = 120 μm in (a), also applicable to (b–g).

causes testicular atrophy which is likely the result of loss of spermatids from the epithelium, as denoted by a reduction in testis weight to about 1/3 to 2/5 of that of control, proteins in Sertoli cells, spermatogonia, or spermatocytes might be "enriched" in the samples that were being analyzed instead of being physiologically "induced." If this is indeed the case, the level could plausibly be induced by 2.5- to 3-fold at the most. Yet in induction of ~five- to sixfold for these proteins were detected, illustrating that an increase in protein expression had indeed occurred. Furthermore, the induction of AJ-associated proteins was significantly higher than TJ proteins, arguing against the possibility that such changes are the result of a non-specific protein concentration effect as a result of declining testis weight. Nonetheless, such an increase in AJ proteins failed to retain germ cells in the epithelium since the association between these protein complexes, such as N-cadherin and β-catenin, was significantly weakened as shown herein. The N-cadherin-associated β-catenin level which was pulled down using an anti-N-cadherin antibody by Co-IP was similar to the control level when

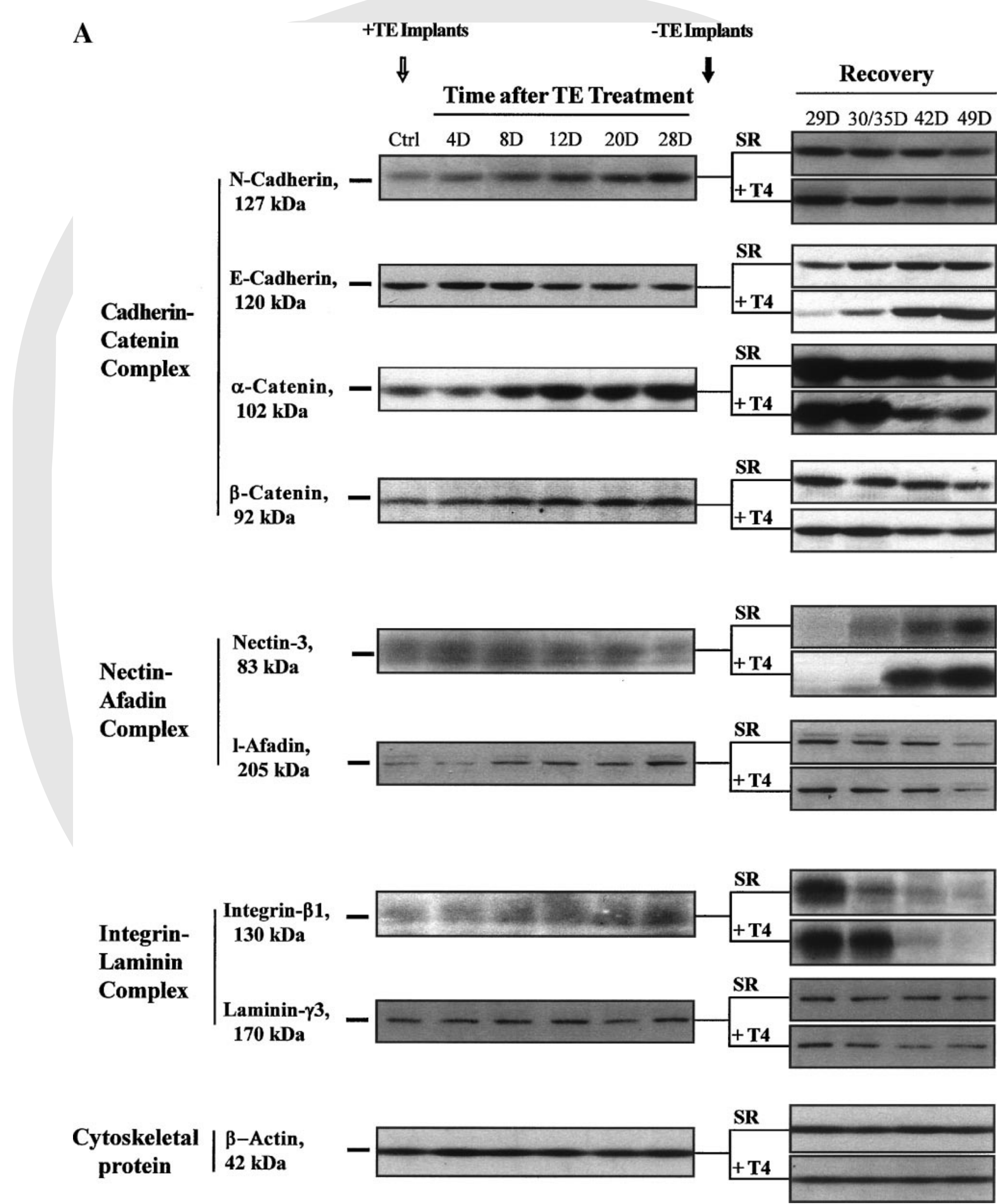

Fig. 5. **A, B**: A study by immunoblotting to assess changes in AJ-associated proteins in rat testes during androgen suppression-induced germ cell loss from the seminiferous epithelium. A: Lysates of testes from rats of different treatment groups containing ~150 μg protein per lane was resolved by SDS–PAGE, electroblotted onto nitrocellulose papers and immunostained using different primary target antibodies. B: These are densitometrically-scanned data using immunoblots such as those shown in (A) with n = 3 rats. The level of target protein in control rats (Ctrl) was arbitrarily set at 1, against which all data were compared. Each data point is the mean ± SD of three rats. ns, not significantly different from control as determined by ANOVA; *, significantly different, $P < 0.05$; **, significantly different, $P < 0.01$. Open arrowhead indicates where TE implants were placed in the dorsal region of the adult rats; solid arrowhead indicates where TE implants were removed, and rats were allowed to recover either spontaneously or with androgen implants.

Author Proof

Fig. 5. (*Continued*)

its level was induced by almost five- to sixfold at the peak of germ cell loss from the epithelium. This is physiologically important since the N-cadherin/β-catenin complex is more predominant at the basal ES (Lee et al., 2003, 2004), and if basal ES structure was largely retained when spermatids were depleted from the epithelium on day 30 (2 days into the recovery phase), the N-cadherin antibody should pull down significantly more β-catenin than it was detected, taking testicular atrophy into account. This thus implies that there was an increase in the 'free,' non-N-cadherin-bound β-catenin pool in the cytoplasm of the testicular cells [e.g., both Sertoli cells, spermatogonia, and spermatocytes were shown to express both cadherins and catenins (Wu et al., 1993; Lee et al., 2003)], and a loss of N-cadherin/β-catenin association. This argument is indeed supported by results of fluorescence microscopy, which detected a diffusing localization pattern of these two proteins in the epithelium. It is likely that their loss of protein–protein association leads to a fuzzy, less focused localization at the BTB.

What are the differences between the regulation of epithelial TJ/AJ and TJ/basal ES at the BTB?

Current studies in epithelial cell physiology have shown that the assembly of TJs between epithelial cells requires the presence of AJs, and changes in AJ integrity can modulate TJ formation and the epithelial permeability barrier function (Behrens et al., 1985; Troxell et al., 1999; Man et al., 2000; Guo et al., 2003). For instance, nectin-based adhesion is initially established between migrating epithelial cells, followed by cadherin-based adhesion to form mature AJs; thereafter JAMs, followed by claudin and occludin, are being recruited to the apical portion of AJ for assembling TJs (for reviews, see Takai and Nakanishi, 2003; Sakisaka and Takai, 2004), illustrating the intimate relationship between these junctions. When confluent epithelial cells are treated with agents that target specifically to AJ proteins (e.g., E-cadherin), both AJs and TJs can be compromised, as manifested by an increase in permeability function (Fujimori and Takeichi, 1993; Man et al., 2000).

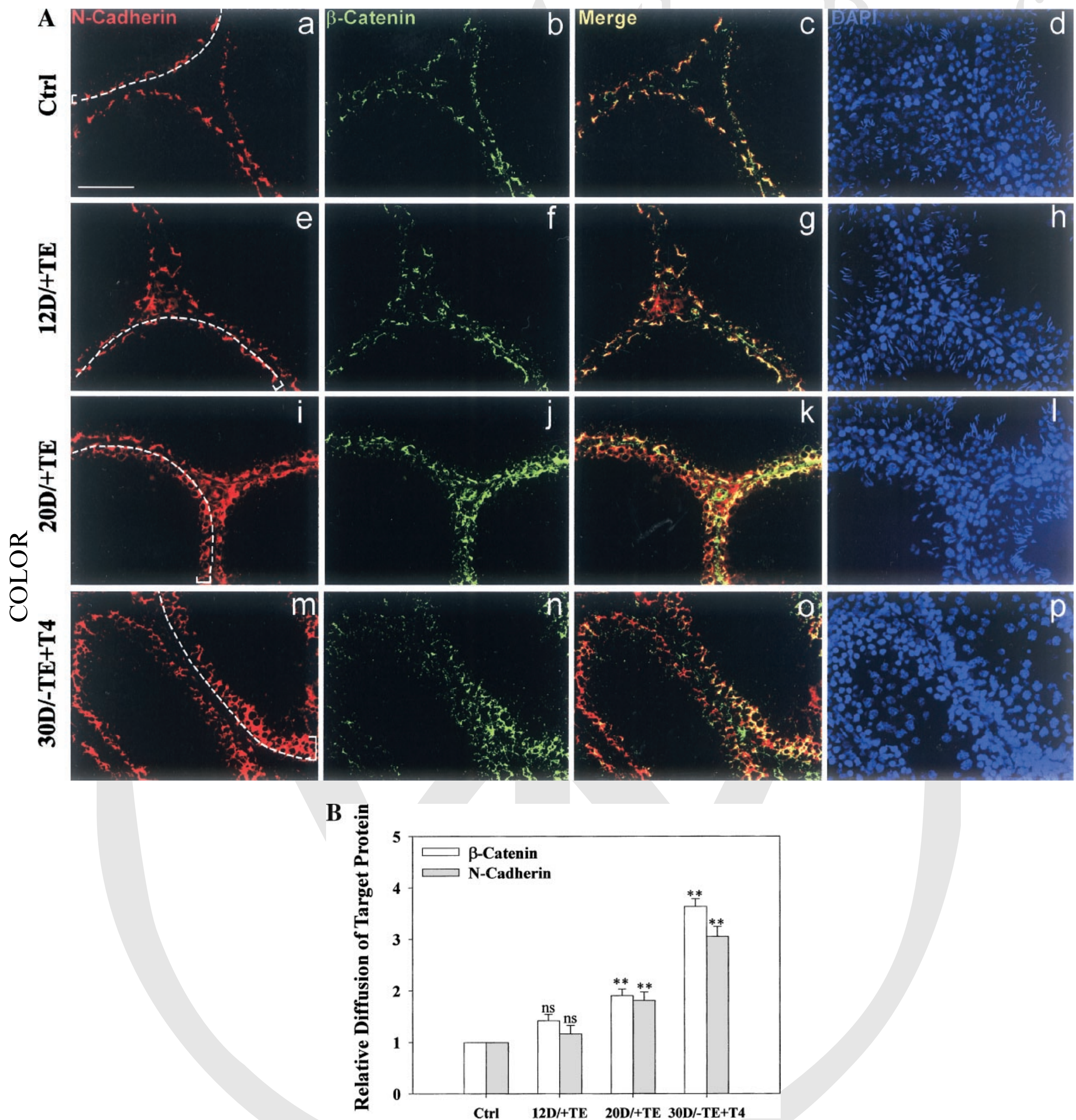

Fig. 6. **A, B**. A study to assess changes in the pattern of localization of N-cadherin and β-catenin in the seminiferous epithelium and their apparent loss of association by immunofluorescence microscopy during androgen suppression-induced germ cell loss from the testis. A: Immunofluorescent co-localization of N-cadherin and β-catenin to the epithelium in rat testes during androgen suppression-induced germ cell loss. **a–d**, control testes; **e–h**, testes of rats with TE implants for 12 days (12D/+TE); **i–l**, testes of rats after 20 days of TE treatment (20D/+TE); **m–p**, testes of rats terminated on day 30 with 28 days of TE treatment followed by 2 days of recovery under high T condition (30D/−TE + T4) when the seminiferous epithelium was virtually devoid of round, elongating, and elongate spermatids (see Fig. 3B). a, e, i, m: N-cadherin staining (Cy3, red); b, f, j, n: β-catenin staining (FITC, green); c, g, k, and o are the corresponding merged images of (a and b), (e and f), (i and j), (m and n) (yellowish-orange); d, h, l, p: DAPI staining. Bar = 100 μm in (a), which also applies to (b–p). White broken line indicates the location of the basal compartment corresponding to the BTB site nearing the basement membrane in (a, e, i, m); and the "bracket" indicates the RD of a target AJ-protein during androgen depletion-induced germ cell loss from the epithelium. B: This bar chart shows the RD of N-cadherin or β-catenin from its site of origin near the basement membrane. This is calculated by measuring the distance of the fluorescence that moved away from the BTB site indicated by the white broken line. As seen in (a and b), virtually all the immunoreactive N-cadherin and β-catenin resided closely to the white broke line in the basal compartment, which diffused away from the basal ES at BTB as shown in testes from time points at 12D/+TE, 20D/+TE, and 30D/−TE + T4. The distance of target protein that diffused away was compared to control testis (mean), which was arbitrarily set at 1. Each data point is the mean ± SD (n = 50 tubules scored from at least two testes of different rats). ns, not significantly different from control as determined by ANOVA; **, significantly different, $P < 0.01$.

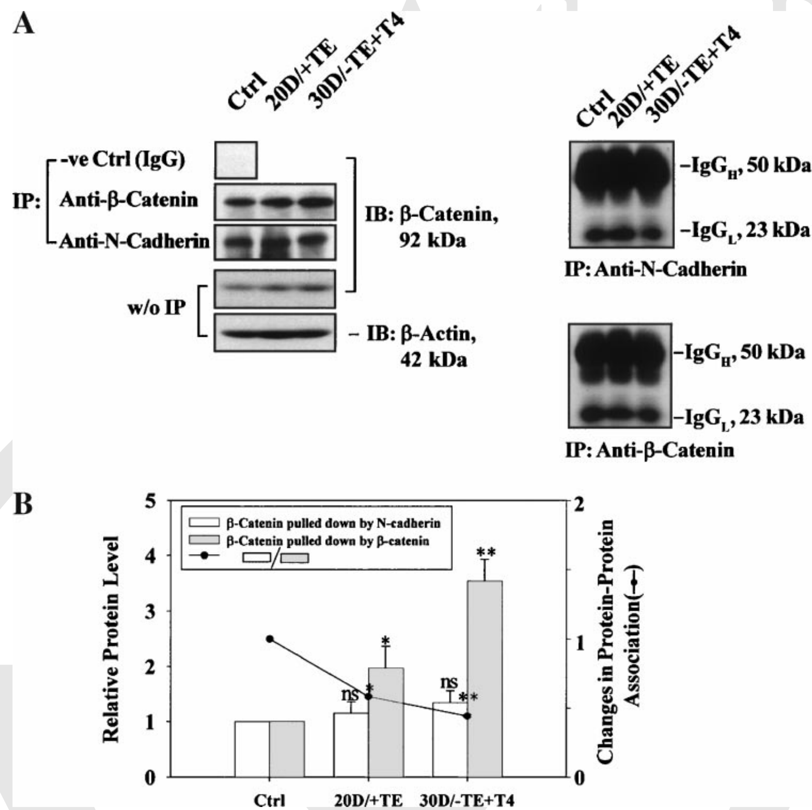

Fig. 7. **A, B**: A study by Co-IP to assess changes in protein–protein interactions of the N-cadherin-β-catenin protein complex in rat testes during androgen suppression-induced germ cell loss from the seminiferous epithelium. A: Equal amount of lysates (~500 μg protein) from control, 12D/+TE, and 30D/−TE + T4 testes were IP using an anti-N-cadherin or an anti-β-catenin antibodies. The immunoprecipitated complexes were analyzed by immunoblotting (IB) using a specific β-catenin antibody to estimate if there was a loss of protein–protein association in the cadherin/catenin complex. A negative control was also included in which the precipitating antibody was substituted by rabbit IgG. Following SDS–PAGE, proteins were detected by immunoblotting using an anti-β-catenin antibody. An aliquot of lysates used for IP were also probed for β-actin to assess if equal amount of protein was used. The right part shows the heavy and light chains of IgG following IP from the same immunoblots shown on the left part to illustrate equal protein loading and uniform protein transfer. A surge in β-catenin using lysates without (w/o) IP was detected. While total β-catenin pulled down by the anti-β-catenin antibody is consistent with the lysate data (see w/o IP), the anti-N-cadherin failed to pull out the same amount of β-catenin, illustrating a loss of protein–protein interaction. B: This bar chart was prepared using data such as those shown in (A) that were densitometrically scanned, and normalized to the control testis, which was arbitrarily set at 1. Each data point is the mean ± SD from three experiments. ns, not significantly different from control as determined by ANOVA; **, significantly different, $P < 0.01$.

This suggests that the proximity of AJ and TJ between epithelial cells has created a bi-directional cross-talk mechanism that a disruption of one junction type can transmit the disruptive signal to the other. If a similar mechanism(s) is in place in the seminiferous epithelium, the BTB integrity cannot be maintained at spermatogenesis when thousands of developing germ cells have to traverse the epithelium throughout the epithelial cycle, associating with extensive junction restructuring (for reviews, see Cheng and Mruk, 2002; Mruk and Cheng, 2004a).

Based on the data presented herein plus those found in the literature, it remains unclear regarding the intricate coordination of TJs and basal ES to facilitate germ cell movement. Yet the present study has clearly illustrated that, unlike other epithelia, the basal/apical ES cell adhesion function and the TJ barrier function can be "disengaged" so as to maintain the BTB integrity. For instance, during the androgen suppression-induced spermatids loss from the epithelium, signals that induce disruption of apical ES are being shielded from affecting TJ integrity, but not basal ES, at the BTB. In fact, these signals possibly reinforce the basal barrier function. This conclusion was reached based on the fact that both TJ and AJ proteins were induced at the time of spermatid loss. Yet N-cadherin and β-catenin at the basal ES were found to diffuse away from the BTB site, apparently disengaging from the adjacent TJ-protein complexes (e.g., the occludin/ZO-1 and the JAM-1/ZO-1 protein complexes) so that the TJ-barrier function was not compromised. This disengagement mechanism can thus permit the loss of spermatids from the epithelium because of a loss of the cadherin/catenin function that confers cell adhesion (via a loss of protein–protein interactions) while leaving the significantly induced proteins of the occludin–ZO-1 and the JAM-1–ZO-1 complexes at the BTB site to maintain the TJ-barrier function.

Recent studies have shown that TJ and AJ of gastrointestinal epithelial cells can be disrupted by progastrin hormone through activation of Src and PI 3-kinase, respectively. However, blocking either signal transducer failed to rescue the other junction type from disruption (Hollande et al., 2003). In Madin–Darby canine kidney cells, EGTA treatment-induced junction disruption involved a transition of ZO-1 association with TJ proteins occludin and claudin to AJ protein E-cadherin (Rothen-Rutishauser et al., 2002). These

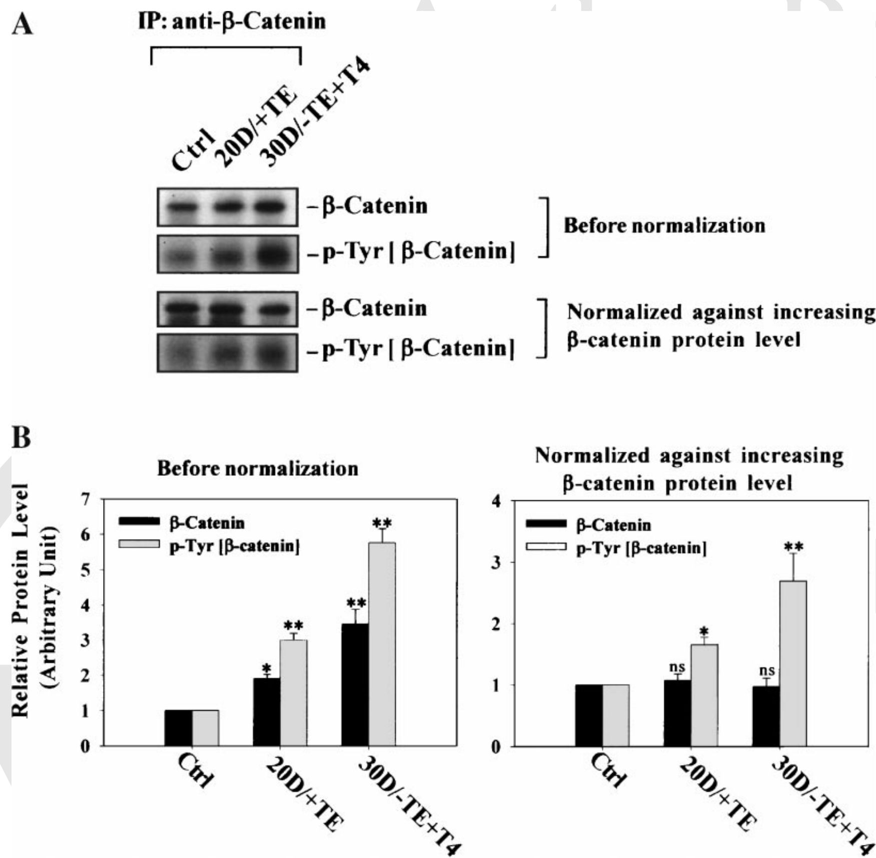

Fig. 8. **A, B**: A study to assess changes in the level of Tyr-phosphorylation of β-catenin in rat testes during androgen suppression-induced germ cell loss. A: To examine changes in Tyr-phosphorylation in β-catenin, sample lysates (~1,200 μg) from control (Ctrl), 12D/+TE, and 30D/−TE + T4 testes were IP using an anti-β-catenin antibody. Immunocomplexes after IP were divided into three equal aliquots as follows. The first aliquot (1/3) was examined by immunoblotting using an anti-β-catenin antibody (top part). This blot was stripped and reprobed with an anti-phospho-Tyr antibody illustrating an increase in Tyr-phosphorylation (p-Tyr) (2nd part). As such, both top and 2nd parts are results obtained before normalization against changes in β-catenin protein. But since there was an increase in β-catenin per se (see top part), the increase shown in the second part could be attributed to an increase in β-catenin protein. The blot in top part was therefore scanned and the protein level of β-catenin was normalized and the other 1/3 aliquot was used for a second immunoblotting experiment. The third part shows the IP products of β-catenin after it was normalized against the increase in β-catenin level. As such, the level of β-catenin between samples was uniform (3rd part). When this blot was stripped and reprobed with an anti-phospho-Tyr antibody, an increase in Tyr-phosphorylation indeed was detected (bottom part), showing a surge in β-catenin phosphorylation. B: This bar chart was prepared using immunoblotting data, such as those shown in (A), which were densitometrically scanned, and normalized to control testes, which was arbitrarily set at 1. Each data point is the mean ± SD from three experiments. ns, not significantly different from control as determined by ANOVA; **, significantly different, $P < 0.01$.

results seemingly suggest that epithelial AJ and TJ disassembly, although interdependent with each other, is regulated via different signaling pathways. Furthermore, the shift of association between ZO-1, occludin/claudin, and E-cadherin also supports the disengagement hypothesis ascribed to BTB regulation. It is apparent that if adequately sealed within a microenvironment, the disruption of one junction type can be restricted without spreading over to others. An interesting question remains to be addressed: is this indeed the case in the testis? In the testis, several signaling pathways have been identified that are crucial to AJ dynamic regulation, by and large at the apical ES, which include the integrin/pFAK/PI 3-K/p130 Cas/ERK and the integrin/RhoB/ROCK/LIMK/cofilin pathways (Lui et al., 2003a; Siu et al., 2003b), whereas TJ dynamics are mediated via the TGF-β3/MEKK2/p38 MAPK and integrin/ILK/GSK-3/p130 Cas/JNK pathways (Lui et al., 2003b; Siu et al., 2003a). Some of these signaling pathways may act specifically to one junction type but some seem to be more versatile. For instance, the TGF-β3/p38 MAPK was found to regulate both AJ and TJ dynamics (Wong et al., 2004). Interestingly, TGF-β3 was recently shown to disrupt Sertoli-germ adhesion function at the AJ without affecting the TJ integrity at the BTB (Xia and Cheng, 2005). Thus, the testis is equipped with different transducers that are capable of directing multiple signaling pathways to regulate diversified functions pertinent to junction dynamics, but the details of how these signals are being utilized remain unexplored. Nonetheless, these findings illustrate the seminiferous epithelium is using a different strategy to regulate junction dynamics pertinent to spermatogenesis versus other epithelia.

Phosphorylation of adaptor proteins caused dissociation of cell adhesion complex

Although it has been known for almost a decade that phosphorylation of cadherins and β-catenin can alter the adhesive function of AJ (for reviews, see Daniel and Reynolds, 1997; Gumbiner, 2000), yet this possibility has not been explored in the testis until recently (Wine and Chapin, 1999; Chapin et al., 2001; Lui et al., 2003a). In this study, we have demonstrated unequivocally that an increase in Tyr-phosphorylation of β-catenin

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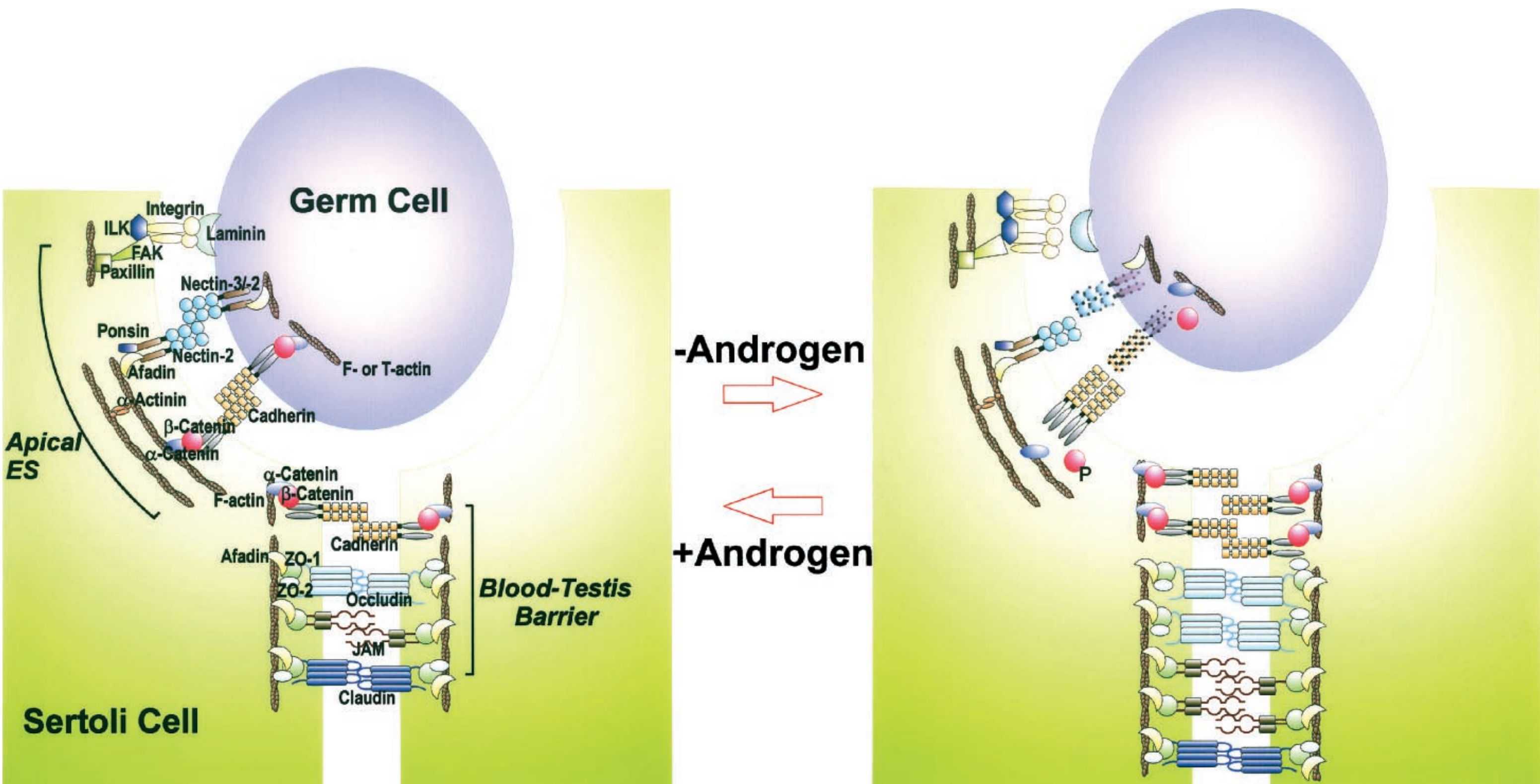

Fig. 9. A schematic drawing that illustrates the disruption of apical ES and the maintenance of BTB during androgen suppression in rat testes. This drawing is prepared based on results reported herein and recent reviews (Cheng and Mruk, 2002; Lee and Cheng, 2004a,b; Mruk and Cheng, 2004a,b; Siu and Cheng, 2004a,b). The left part illustrates the simplified molecular architecture of BTB and ES protein complexes present in the seminiferous epithelium of the rat testis. Claudins, JAMs, and occludins are the TJ-integral membrane proteins known to be present at the BTB site. Basal ES is also found at the BTB, but only cadherin/catenin complex is shown in this drawing since its presence at the basal ES has been conclusively demonstrated. At the apical ES, besides the cadherin/catenin complex, the nectin/afadin/ponsin and the integrin/laminin complexes are also present, which are the molecular constituent proteins that confer Sertoli-germ (mostly spermatids) cell adhesion function. Under physiological conditions with normal androgen level in the testis, which is about 100-fold higher than that of the systemic circulation, germ cells at different stages of development adhere to Sertoli cells in the seminiferous epithelium until spermiation. TE implants treatment that suppresses intratesticular T level result in germ cell loss, in particular spermatids from step 8 and beyond, without compromising BTB integrity, which is shown on the right part. Tyr-phosphorylation of β-catenin causes the disassociation of β-catenin from cadherin, thus weakening the adhesion function between Sertoli and germ cells, inducing the loss of spermatids from the epithelium. The interactions between nectin and afadin, as well as between integrin and laminin may also be weakened either via a downregulation of their protein production or a loss of protein–protein interactions. Tight junction (TJ) proteins (e.g., JAM and occludin) are upregulated, and remain restricted to the BTB site when the intratesticular androgen level is reduced to maintain the integrity of the TJ-barrier function at the BTB. Basal ES protein complexes (e.g., cadherin/catenin) are also upregulated, however, the protein–protein interactions between these proteins are disrupted, similar to apical ES, thereby leading to germ cell loss from the epithelium.

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occurred at the time of a weakened N-cadherin/β-catenin association, leading to spermatid detachment from the epithelium. Earlier studies have implicated the roles of protein phosphorylation in the regulation of spermiation (Wine and Chapin, 1999; Chapin et al., 2001). Using AF-2364 [1-(2,4-dichlorobenzyl)-indazole-3-carbohydrazide] to induce germ cell depletion from the epithelium, it has been shown that germ cell loss is also accompanied by an induction of N-cadherin and β-catenin, and other peripheral ES proteins such as Fer kinase, pFAK, p130Cas (Chen et al., 2003; Lee et al., 2003; Siu et al., 2003b). For instance, the cadherin/catenin complex was shown to associate with several protein kinases including c-Src, CK2, Csk and Fer, and adaptors (e.g, WASP, axin, zyxin) that link the protein complex to the underlying actin cytoskeleton (Chen et al., 2003; Lee and Cheng, 2005). Similar to earlier studies using the AF-2364 model, we speculate that these kinases are involved in the loss of spermatids from the epithelium when a surge in Tyr-phosphorylation of β-catenin occurs, thereby the cadherin and catenin proteins not only dissociate from each other but also from the actin cytoskeleton.

Summary and concluding remarks

In summary, a disruption of AJ (e.g., ES) in the androgen suppression model is limited to the apical and basal ES without affecting the BTB integrity, implicating the testis is able to direct the regulatory signals to a junction site specifically via a disengagement mechanism. Based on the results reported herein, we propose a schematic model shown in Figure 9 that regulates ES and TJ function in the rat seminiferous epithelium. It is of interest to note that both apical ES and basal ES at the Sertoli cell side are similar in structure except that basal ES co-exist with TJ at BTB. But how can a signal that targets the disassembly of apical ES and basal ES can limit its action at ES without perturbing the adjacent TJ-barrier? While this is likely via a disengagement mechanism as illustrated herein, it remains to be investigated how this signal is being transmitted between basal ES and TJ at the BTB, and between BTB and apical ES.

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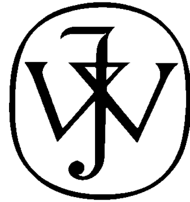

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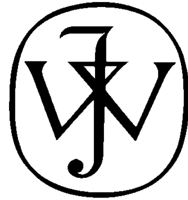

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