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Regulation of Ectoplasmic Specialization Dynamics in the Seminiferous Epithelium by Focal Adhesion-Associated Proteins in Testosterone-Suppressed Rat Testes


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Apical ectoplasmic specialization (ES) is a unique cell-cell actin-based adherens junction type restricted to the Sertoli-round/elongating/elongate spermatid interface in the seminiferous epithelium. An endogenous testosterone (T) suppression model was used to study the regulation of apical ES dynamics in the testis. By providing sustained releases of T and estradiol using subdermal implants in rats, this treatment reduced endogenous testicular T level. This in turn led to sloughing of spermatids (step 8 and beyond) from the seminiferous epithelium, which can be reversed by removing the implants, or replacing them with a higher dose of T implants. This model thus allows us to study the restructuring events at the apical ES. It was shown that apical ES restructuring involved proteins that were usually restricted to the cell-matrix focal adhesion site in other epithelia. For instance, the protein levels of β1-integrin, Tyr-phosphorylated focal adhesion kinase (p-FAK), and c-Src were induced during the T suppression-induced germ cell loss and recovery, implicating that these proteins are putative regulators of ES dynamics. Indeed, the formation of p-FAK/c-Src protein complex, but not their association with β1-integrin, was stimulated during T suppression-induced germ cell loss. ERK, a MAPK known to regulate focal adhesion turnover, was also activated during the androgen suppression-induced spermatid loss and the early phase of the recovery when germ cells began to repopulate the epithelium. Collectively, these data suggest that the apical ES is a cell-cell adherens junction type with the characteristics of cell-matrix focal contacts. In addition to its role in conferring cell adhesion formation, the p-FAK/c-Src protein complex apparently also regulates apical ES disruption via the ERK signaling pathway. (Endocrinology 146: 0000–0000, 2005)
induce transient infertility based on serum microchemistry analysis to assess liver and kidney function (for reviews, see Refs. 3, 4). As such, they are simply the artifacts of the drug effects on the seminiferous epithelium. Therefore, to verify the physiological significance of these earlier findings, studies using another model seem necessary, perhaps even essential. Herein we reported the use of a well-established in vivo model that induces germ cell loss from the epithelium via an endogenous testosterone (T) suppression using T and estradiol (E) implants (for a review, see Ref. 10). This model provides an excellent platform for studying apical ES dynamics because a loss of intratesticular T led to a progressive disruption of apical ES adhesion function, which can be used to investigate changes in selected target proteins at this site. This model was also used to evaluate the hypothesis that apical ES uses a cell-matrix FA-like structure, namely the β1-integrin/p-FAK/c-Src protein complex, as one of its functional adhesion complexes. We also investigated the possible involvement of MAPK pathways, such as the ERK, in the regulation of apical ES dynamics. These are the subjects of this report.

Materials and Methods

Animals

Male Sprague Dawley rats were obtained from Charles River Laboratories (Kingston, MA). The use of animals reported herein was approved by the Rockefeller University Animal Care and Use Committee with protocol no. 0011 and 03017.

Antibodies and reagents

Monoclonal mouse antibody against β1-integrin (catalog no. 610468, lot 7) was purchased from BD Transduction Laboratories (San Diego, CA). Polyclonal rabbit anti-FAK (catalog no. sc-558, lot D1204), goat anti-FAK (catalog no. sc-8056, lot J2103), mouse anti-FAK (catalog no. 06–500, lot 19950), rabbit anti-p130Cas (catalog no. sc-1616, lot H9251), monoclonal mouse anti-Src (catalog no. sc-6260, lot B252), and mouse anti-α-tubulin (catalog no. sc-8035, lot G182) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-p130Cas (catalog no. 06–500, lot 19950), rabbit anti-pI-3-kinase p85 (catalog no. 06–497, lot 25086), and monoclonal mouse anti-phospho-Src-Tyr416 (catalog no. 05–677, lot 26419) were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit anti-ERK (catalog no. 9102, lot 3) and rabbit anti-phospho-ERK (catalog no. 9101, lot 15) were obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-β1-integrin (catalog no. AB1952, lot 22111029) used in immunofluorescent microscopy was purchased from Chemicon (Temecula, CA). Protein A/G-PLUS agarose and horseradish peroxidase-conjugated secondary antibodies against IgG of different species were obtained from Santa Cruz Biotechnology. Detection of target proteins in immunoblots was performed using enhanced chemiluminescence kits purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The ERK intrinsic activity assay kit was purchased from Cell Signaling Technology. His-tostain-SP kit for immunohistochemistry, goat antimouse IgG-Cy3, and goat antirabbit IgG-fluorescein isothiocyanate (FITC) were obtained from Zymed Laboratories.

Induction of germ cell loss from the seminiferous epithelium by androgen suppression in adult rats in vivo using T and E implants

T and E implants were prepared in ethyl vinyl acetate (ELVAX 70) DuPont tubing as described (11). T was packed in implants of 3 or 4 cm in length, whereas 17β-estradiol was packed in 0.4-cm implants. A single 3-cm T and 0.4-cm E implant were placed under the skin along the dorsal surface of each 90-d-old male Sprague Dawley rat to provide a sustained release of steroids (n = 3 rats for each specified time point). This treatment with TE implants leads to a suppression of endogenous T level in vivo that induces germ cell loss from the seminiferous epithelium (step 8 and beyond), from the epithelium (12). The surgical procedure was carried out as follows. Rats were placed under anesthesia by ketamine-HCl (70 mg/kg body weight, im). Hair at the surgical site (−2 × 1 cm2) on the dorsal surface was shaved and cleaned twice with 70% ethanol and Betadine. Sterile implants were inserted under the skin through a small incision (−1.5 cm in length), and the site was stitched immediately thereafter using nonabsorbable surgical sutures (Ethicon Inc., Somerville, NJ). Sutures were removed after the wound healed on d 7. TE implants were removed from all rats on d 28. In one experimental group, rats received 4 × 4-cm T implants to facilitate the resumption of spermatogenesis (T 4 × 4 cm) (n = 3 rats for each time point). In another group, rats (n = 3 rats for each time point) were allowed to recover normally without any implants (N.R.). Rats were terminated and testes removed at specified time points, frozen in liquid nitrogen, and stored at −80°C until use.

Immunoblottings

Immunoblot analyses were performed using testes lysates prepared in sodium dodecyl sulfate (SDS) lysis buffer [0.125 M Tris (pH 6.8), at 22°C containing 1% SDS (wt/vol), 2 mM EDTA, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1.6% 2-mercaptoethanol (vol/vol), 1 mM sodium orthovanadate, and 0.1 μM sodium okadate] as described earlier (6). Equal amount of proteins from lysates (~100 μg protein per lane) was resolved by SDS-PAGE following 5% or 10% T SDS-polyacrylamide gels under reducing conditions. Proteins were electroblotted onto nitrocellulose membranes and probed with the following antibodies of target protein in the selected dilutions as determined in preliminary experiments: β1-integrin (1:1000), FAK (1:200), Src (1:200), p130Cas (1:1000), PI 3-kinase p85 (1:1000), ERK (1:1000), phospho-ERK (1:1000), actin (1:200), vimentin (1:500), α-tubulin (1:500), and phospho-Tyr (1:1000). Blots were then probed with the corresponding secondary antibodies, and target proteins were visualized by an enhanced chemiluminescence system as described (6). Each blot was stripped using an SDS stripping buffer as described (6) and reprobed with another primary antibody for up to four to five different target proteins.

Immunoprecipitation (IP)

Testis lysates used in IP was prepared in modified radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4) at 22°C containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (vol/vol), 0.25% Na-deoxycholate (vol/vol), 0.05% SDS (vol/vol), 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, leupeptin (1 μg/ml), and aprotonin (1 μg/ml)]. About 400 μg proteins from lysates of normal rat testes, testes from rats terminated at 42 d after TE treatment from the N.R. group, and rats terminated at 30 d after TE treatment from the T 4 × 4 cm recovery group were used for IP reactions. Testis lysates were first precleared by 2 μg of rabbit or mouse IgG for approximately 2 h, following by 10 μl protein A/G-PLUS agarose for 2 h to eliminate nonspecific interactions with IgG or agarose. IP was then performed by adding either 2 μg of antibodies (anti-FAK or anti-Src) or 2 μg of IgG (rabbit or mouse) as negative control to lysates and incubated at 4°C overnight with rotation (~30 rpm). Thereafter immunocomplexes were precipitated by adding 20 μl protein A/G-PLUS agarose to the sample and incubated for 6 h. The immunocomplexes were washed with 300 μl wash buffer [50 mM Tris-HCl (pH 7.4) at 22°C containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (vol/vol), 1 mM PMSF, 1 mM sodium orthovanadate, leupeptin (1 μg/ml), and aprotonin (1 μg/ml)] four times by gentle resuspension and mild centrifugation (5 min, 1000 × g), and proteins in the immunocomplexes were extracted from agarose at 100°C in SDS-sample buffer [0.125 M Tris (pH 6.8) at 22°C containing 1% SDS (wt/vol), 1.6% 2-mercaptoethanol (vol/vol), and 20% glycerol (vol/vol)]. Proteins were resolved by SDS-PAGE, and immunoblotting was performed as described in the previous section. Lysates from normal rat testes served as positive controls to assess the specificity of primary antibodies.
Fluorescent microscopy

Fluorescent microscopy was performed to colocalize β1-integrin with phospho-Src-Tyr416 in the seminiferous epithelium of adult rat testes. In brief, cryosections (~8 μm) of testes were mounted on poly-l-lysine-coated slides, fixed in Bouin’s fixative, and nonspecific binding sites blocked with 10% normal goat serum. Sections were then incubated with a rabbit anti-β1 integrin antibody (1:100 in 10% normal goat serum) and a mouse anti-p-Src antibody (1:75) overnight. This was followed by an incubation with goat antirabbit IgG-FITC and goat antimouse IgG-Cy3 for 1 h. Sections were then washed and mounted in Vectashield Hardset fluorescent optics and an Olympus DP70 12.5MPa digital camera. All images were acquired using the QCapture software package (Suite version 2.60, Quantitative Imaging Corp., Burnaby, BC, Canada) and processed in Photoshop (version 7.0, Adobe, San Jose, CA). Either IgGs or 1% goat serum was used to replace primary antibodies to serve as negative controls.

ERK intrinsic kinase assay

The intrinsic ERK activity in lysates of testes obtained from control rats, rats from the TE treatment group, and the two recovery groups was quantified using a nonradioactive kinase assay. Testis lysates used in the assay were prepared in a cell lysis buffer [20 mm Tris (pH 7.5), at 22 C containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton (vol/vol), 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM PMSF]. Activated form of ERK (p-ERK) in approximately 200 μg of lysate proteins from each sample was pulled down selectively by a phosho-ERK-specific antibody immobilized to agarose at 4 C overnight. Thereafter beads were washed twice with the cell lysis buffer and twice with a kinase buffer [29 mM Tris (pH 7.5) at 22 C containing 60 mM NaCl, 10 mM sodium orthovanadate, 0.1 mM sodium orthovanadate, and 10 mM MgCl2], and kinase assay was immediately carried out in the presence of 200 μM cold ATP and 2 μg Elk-1 fusion protein (a downstream transcription factor of ERK) at 30 C for 30 min. Under this condition, the exogenous Elk-1 fusion proteins were phosphorylated by the activated ERKs. Phosphorylation of Elk-1 was subsequently quantified by immunoblotting using an anti-phospho-Elk-1 (Ser383) antibody. The assay was performed three times using different sets of samples. To ensure equal protein loading in each reaction, immunoblotting using an antiactin antibody was performed for all sets of lysates used in the study.

Immunohistochemistry

Immunohistochemical localization of phospho-ERK in the rat seminiferous epithelium was performed in cross-sections of testes from normal rats and rats that received TE treatment for 12 d. Cryosections (~8 μm) of testes were fixed in Bouin’s fixative. Sections were treated with 3% H2O2 in methanol (vol/vol) to block the endogenous peroxidase activity, following by an incubation with 10% normal goat serum to block nonspecific binding. Sections were subsequently incubated with an anti-phospho-ERK (1:100) antibody in a moist chamber at room temperature overnight. Thereafter sections were incubated with a biotinylated goat antirabbit antibody for 30 min, followed by streptavidin-peroxidase for 10 min. Aminoethylcarbazole mixture was used as the substrate to allow color development for approximately 10 min. Immunoreactive p-ERK appeared as red-brown (1:4-6) precipitates on the sections. Slides were then counterstained with hematoxylin and mounted in GVA mount. Controls included sections incubated with normal rabbit IgG instead of the primary antibody, PBS in substitution of the primary antibody, and normal goat serum replacing the secondary antibody.

Statistical analyses

Statistical analyses were performed by ANOVA with Tukey’s honestly significant difference tests or Student’s t tests using the GB-STAT statistical analysis software package (version 7.0; Dynamic Microsystems, Silver Spring, MD).

Results

Endogenous T suppression by TE implants induces spermatid loss from the seminiferous epithelium

In this study, the endogenous T level in the rat testis was manipulated by using TE implants, which was known to induce the release of round, elongating, and elongate spermatids from the seminiferous epithelium because of a loss of apical ES function (for a review, see Ref. 10). During the early course of the TE treatment (e.g. at 12 d), seminiferous tubules still appeared normal (Fig. 1, B-b vs. B-a). However, after 28 d of T suppression, the number of step 8 (and beyond) spermatids found in the epithelium drastically plummeted, and this trend sustained to the early time points of the two recovery groups (Fig. 1B-c and B-e). Elongating spermatids began to reappear in the epithelium 14 d after TE implants were removed (i.e. d 42) (Fig. 1B-d, see black arrowheads), and the spermatogenesis returned to an almost normal level by 3 wk after TE implants were removed (i.e. d 49) (Fig. 1, B-f vs. B-a). The diameter of the seminiferous tubules was also largely reduced under during T suppression because of the loss of spermatids. At 2 d after TE implants were removed, the diameters of the tubules in both recovery groups were only approximately 50% of that of the normal (Fig. 1C), which bounced back to the normal level by 49 d when spermatogenesis resumed to the control level (Fig. 1, C and B-f).

Induction of β1-integrin, an ES-associated integral membrane protein, adaptor proteins, kinases, and downstream signaling molecules in the testis during T suppression-induced spermatid loss

To study the changes in the cell adhesion structure at the apical ES during T suppression, immunoblot analyses were performed to assess the protein levels of different ES-related proteins. β1-Integrin, a transmembrane receptor and a known component of the apical ES structure residing in Sertoli cells (7, 13, 14), was induced during the androgen suppression-induced germ cell loss. Its expression was highest 2 d after TE implant removal (d 30) in the T 4 × 4 cm recovery group, about 2.5-fold vs. the control (Fig. 2, A and B). On the other hand, the level of FAK, a nonreceptor protein tyrosine kinase that is known to bind to β-integrins (15, 16), remains almost constant during the course of treatment (Fig. 2, A and B). However, c-Src, a member of the Src family of protein tyrosine kinase and a binding partner of FAK (17, 18), was also induced by T suppression as early as 4 d after the TE treatment and increased to approximately 2.5-fold after 28 d (Fig. 2, A and C). The level of c-Src returned gradually to normal during the normal recovery phase but remained relatively high in the T 4 × 4 cm recovery treatment group (Fig. 2, A and C). An induction in the protein levels of p130Cas, an adaptor protein that binds to the C-terminal domain of FAK (19), and PI 3-kinase p85, the regulatory subunit of this signaling molecule that interacts with FAK (20), was also detected in the recovery phase (Fig. 2, A and C). The
expression of p130Cas was highest 2 d after TE implant removal in both groups, whereas PI3 kinase p85 expression was induced by up to approximately 2-fold vs. normal 42 d during normal recovery phase (Fig. 2, A and C). To ensure that the changes in protein levels described here were not the result of uneven protein loading and non-uniform protein transfer from gels to nitrocellulose membranes, the amount of cytoskeleton proteins including actin, vimentin, and α-tubulin were quantified between samples in each experimental set. These cytoskeletal proteins were not significantly affected in the testis during the course of treatment (Fig. 2, A and D).
Germ cell loss induced by T suppression in the testis induces FAK phosphorylation and increases FAK-c-Src association

In the focal adhesion complex, clustering of integrins recruits FAK to the FA site and triggers its autophosphorylation on tyrosine residues (for reviews, see Refs. 21, 22). Interestingly, it was shown that activated FAK via phosphorylation at Tyr397 and Tyr576 could indeed bind to β1-integrin in Sertoli cells (6). To investigate whether FAK was also phosphorylated during the germ cell loss induced by T suppression in the testis, IP was performed using testis lysates (~100 μg protein per lane) from different treatment groups at specified time points. Target proteins were detected by their corresponding antibodies as indicated in Materials and Methods. Each blot used in immunoblotting was stripped and reprobed with anti-β-actin, vimentin, and c-Src antibodies to assess equal protein loading (A). Results from immunoblots, such as those shown in A, were densitometrically scanned and compared as shown in B–D. Each bar is a mean ± SD of three determinations from three rats. The protein level of a target protein at time 0 was arbitrarily set at 1, against which one-way ANOVA was performed. ns, Not significantly different by ANOVA; *, Significantly different, \( P < 0.05 \); **, Significantly different, \( P < 0.01 \).
earlier report (6). Yet a significant increase in the association of c-Src with FAK was detected at 42 d in the normal recovery group and at 30 d in the T 4 cm recovery group (Fig. 3, D and E). Collectively, these observations illustrate that when the Sertoli-germ cell adhesion function at the apical ES was perturbed, it was accompanied by an increase in FAK phosphorylation and protein-protein association of the FAK/c-Src complex.
c-Src and β1-integrin are components of the protein complex at the apical ES in the seminiferous epithelium of the rat testis

To further investigate whether the FAK/c-Src complex is a regulatory protein unit that activates the downstream signal transduction necessary for junction restructuring at the apical ES, we sought to examine whether c-Src is present at the ES and can interact with β1-integrin. Immunofluorescent microscopy was performed to confirm the colocalization of β1-integrin and phospho-Src(Tyr416) at the apical ES in normal testes (Fig. 4A). Additionally, using an anti-Src antibody for IP, it was shown that β1-integrin was indeed associated with c-Src in normal rat testes and TE-treated testes (Fig. 4B). However, their interactions in testes of rats 14 d after TE implant removal (d 42) in the normal recovery group and 2 d after TE implant removal (d 30) in the T 4 cm treatment group (at the time of germ cell loss) were not induced (Fig. 4, B and C). This was in contrast to the interaction between p-FAK and c-Src, which was induced (see Fig. 3, C and D). The immunoblot of this IP experiment shown in Fig. 4A (upper panel) was also probed with an anti-Src antibody to ensure that the amount of c-Src pulled down in the IP step was consistent with that of the immunoblotting result shown earlier (see Figs. 2, A and C, and 3A vs. Fig. 4, B and C). These findings clearly support the notion that FAK, c-Src, and β1-integrin are putative binding partners that can form a dynamic functional protein complex to regulate germ cell adhesion function at the apical ES.

**ERK activity was elevated during the T suppression-induced germ cell loss from the epithelium and the recovery phase**

To investigate the possible downstream signaling pathway(s) involved in this T suppression-induced germ cell loss
in the testis, one of the MAPK pathways that was known to be activated on integrin clustering/activation and FAK phosphorylation, namely ERK (24, 25), was also examined using immunoblotting (Fig. 5, A and B). Whereas the protein level of total ERK did not change during the entire experimental period, phosphorylated ERK was induced 8 d after TE implants and stayed at a high level (up to ~4-fold) until the end of normal and T 4 × 4 cm recovery on d 49 (Fig. 5, A and B). We also sought to examine whether ERK was activated during the T suppression treatment and recovery using intrinsic activity assays specific for ERK. The results of these assays revealed that the activity of ERK was significantly induced by TE implants, beginning 12 d after TE treatment (Fig. 5, C and D). The level of ERK activity remained high up to 42 d after TE treatment in the normal recovery group and returned to the basal level at 49 d (Fig. 5, C and D). The intrinsic activity of ERK also increased by approximately 4-fold 2 d after TE implant removal in the T 4 × 4 cm treatment group vs. the control. To determine whether activated ERK was indeed present at the apical ES, immunohistochemistry was performed to localize phospho-ERK in the seminiferous epithelium. In normal rat testis, phospho-ERK was confined largely to stage VII-VIII tubule around the heads of the elongating spermatids (Fig. 5E-a), consistent with its localization at apical ES reported in an earlier study (26). Furthermore, 12 d after TE implant treatment, the intensity of phospho-ERK immunostaining was drastically increased, similar to findings obtained by immunoblotting and intrinsic kinase assays (Fig. 5, E-c and E-d). This finding also illustrates that activated ERKs were present predominantly at the apical ES. Normal rabbit IgG control (Fig. 5E-b) and other controls (data not shown) yielded no detectable staining, indicating that the staining shown herein was specific for phospho-ERK. Figure 5F is an immunoblot using tests lysate stained with the anti-p-ERK antibody, illustrating the immunostaining result shown herein is specific for ERK.

**Discussion**

*T* suppression-induced germ cell loss from the seminiferous epithelium involves the induction of several FA-associated proteins that are found at the apical ES

It has been known for decades that subdermal sustained-release of T and E via steroid implants can reduce the weight and sperm count of rat testes (27, 28). Recent studies have shown that a suppression of endogenous T using TE implants can induce failure on the transformation of step 7 to step 8 spermatids in the seminiferous epithelium as well as perturbing the cell adhesion function of spermatids (from step 8 and beyond) (12, 29), leading to spermatid loss from the epithelium. However, after the removal of implants that permitted either normal recovery or recovery under a higher dose of T via T-only implants, testicular weight and testis sperm count rebounded (11). Conversion of step 7 to 8 spermatids also resumed, and round spermatids were found to reattach to the epithelium (29, 30). In this study, this androgen suppression model was used to study the physiology of cell junction restructuring in the seminiferous epithelium pertinent to spermatogenesis. We investigated changes of the β1-integrin/p-FAK/c-Src protein complex and its downstream ERK signaling function during androgen suppression-induced damage to the adhesion function between Sertoli cells and elongating spermatids.

Previous studies have shown that the apical ES structure, referring to the actin filament bundles sandwiched between the Sertoli cell membrane and the cisternae of ectoplasmic reticulum restricted to the Sertoli cell side at the Sertoli-cell spermatid interface, remained morphologically intact in Sertoli cells even after spermatids had detached from the epithelium during androgen suppression-induced germ cell loss from the epithelium (30). It was thus suggested that the TE-induced germ cell loss was not due to the absence of this structure but is rather caused by the defects in cell adhesion molecules present in Sertoli cells and spermatids (30). On the other hand, there is increasing evidence suggesting that the actin-based AJ that confers cell adhesion function at the apical ES is constituted, at least in part, by components of the FA complex at focal contacts that are usually restricted to the cell-matrix interface in other epithelia (for reviews, see Refs. 31, 32).

These findings imply that the apical ES is using the efficient mechanisms of FA that facilitate cell movement to regulate Sertoli–germ cell junction dynamics during spermatogenesis. As such, in this study, we focused our investigation on the FA-associated proteins that are present at the apical ES in the rat seminiferous epithelium. Among the FA components found at the apical ES, integrin is by far the best-studied protein (13, 14). In the testis, α6β1-integrin, residing on Sertoli cells, is the binding partner of laminin γ3, which is restricted to germ cells in particular elongating spermatids at the apical ES (7). During testicular T suppression, β1-integrin was significantly induced when elongate spermatids were depleted from the epithelium during the early recovery phase. Other molecules that were known to be recruited to integrins via FAK to exert signaling functions that can elicit changes in cell adhesion function during FA restructuring include c-Src, PI3-kinase, and p130Cas (for reviews, see Refs. 21, 22). Interestingly, these proteins were indeed induced during the course of treatment with their peaks of expression overlapped with that of β1-integrin.

These results are not entirely unexpected. In contrast, they are analogous to a previous study using AF-2364 to perturb apical ES function to induce spermatid loss from the epithelium that they were up-regulated during the treatment (6). This suggests that FA components are indeed being used for ES restructuring, possibly via changes in protein-protein interactions between the ES-integral membrane proteins (e.g. β1-integrin) and its peripheral adaptors and signaling molecules (e.g. FAK, c-Src) as reported herein (see also below). Interestingly, some of the proteins that were induced at the time of germ cell loss [e.g. β1-integrin, c-Src, PI 3-kinase, p130Cas, and p-ERK (including its intrinsic activity)] remained to be stimulated during the recovery phase. In fact, this is not entirely unprecedented. For instance, FAK activation is required for both the assembly and disassembly of FA (33–35). Collectively, these latest findings have illustrated that many of these signaling molecules (e.g. FAK) and adaptors (e.g. p130Cas) are bifunctional molecules crucial to junction restructuring events including disassembly and reassembly. Future studies should therefore include deletion studies to pinpoint the
functional domains of these proteins that are involved in either junction disruption or re-construction.

**β1-integrin / FAK/Src is part of the adhesion protein complex present at the apical ES in the seminiferous epithelium of the rat testis**

Although FA-associated components are localized at the apical ES, little is known regarding how these molecules maintain cell adhesion function between Sertoli and germ cells. Herein we sought to investigate whether the FA components used similar mechanisms to regulate cell adhesion at the ES as in the cell-matrix focal contacts. FAK, one of the major adaptors and signaling proteins during FA formation, was known to autophostorylate at tyrosine residues, most abundantly at Tyr407, on activation such as via its interaction with integrins (for reviews, see Refs. 21, 22, 36). The auto-phosphorylation of FAK creates a high-affinity binding site for Src family kinases, which recruits c-Src to FAK to form a bipartite kinase complex by which Src can further phosphorylate FAK at other tyrosine residues (17, 23, 37). These events were also observed at the apical ES in the seminiferous epithelium of normal adult rats (6). Herein during the T suppression, more activated FAK/c-Src protein complexes are being formed in the seminiferous epithelium when ES adhesion function is disrupted (30 d, 4 × 4 cm) or is being reestablished (42 d, normal recovery). This suggests that the FAK/c-Src complex is important for not only maintaining cell adhesion but also regulating cell adhesion disruption (35, 38). In both cases, an increase in phosphorylation of FAK and its binding to Src are necessary, although different tyrosine phosphorylation sites and downstream signaling pathways may be involved (for a review, see Ref. 39).

To further verify whether the FAK/c-Src complex is indeed present at the site of apical ES, immunofluorescent microscopy and co-IP were performed to examine the association between c-Src and β1-integrin in the testis. It was shown that the phosphorylated form of c-Src indeed colocalized with β1-integrin in normal rat testes at the site consistent with their localization at the apical ES. Autophosphorylation of c-Src at Tyr416 is a typical feature of c-Src activation, which was shown to colocalize with FAK at the FA site from an earlier study in other epithelium (40). Co-IP experiments further validated this observation that c-Src and β1-integrin were indeed coimmunoprecipitated in normal testes and testes from the two recovery groups. These results further strengthen our hypothesis that β1-integrin/FAK/Src is part of the adhesion protein complex between Sertoli and germ cells at the ES, supporting the notion that ES has the characteristics of both cell-matrix and cell-cell junction types (for reviews, see Refs. 31, 41). However, in the IP experiment, the association between β1-integrin and c-Src was not induced in the recovery phases, even though their protein levels were both stimulated when compared with controls, which was different from the FAK/c-Src protein complex. Although more FAK was associated with c-Src when ES adhesion was disrupted by the TE treatment, the extent of association between this complex and β1-integrin did not change. This illustrates that the FAK/c-Src protein complex found in the testis does not rely only on integrin-mediated activation to confer cell adhesion function at the ES but it is also required (and activated) during ES disassembly. The activation of the protein complex in this latter event is independent of integrin binding. When a loss of adhesion function is being induced, the activated FAK/c-Src protein complex possibly dissociates from integrins and activates different signaling pathways that mediates junction restructuring (for a review, see Refs. 39, 42). For example, the loss of adhesion of smooth muscle cells induced by degraded collagen fragments was shown to be mediated by calpain, a protease that cleaves adhesion-associated proteins (43), and its recruitment to the FA site requires FAK and Src (44).

An increase in intrinsic ERK activity is required for apical ES disruption during androgen suppression-induced germ cell loss from the seminiferous epithelium

The FAK/c-Src protein complex is known to activate several signaling pathways that lead to ERK activation. For instance, the binding of this complex to Grb2 adaptor protein at the FAK phosphorylated Tyr527 residue can trigger Ras-dependent ERK activation (45, 46). Another possible regulation for ERK activity is via the Rac/p21-activated kinase signaling pathway (47), in which the activation of p21-activated kinase requires FAK and Src (for a review, see Ref. 22). During T suppression-induced germ cell loss, activity of ERK was induced soon after an induction of c-Src protein had occurred during the TE treatment and remained significantly high until the end of the recovery phases. This was supported by immunohistochemistry studies using an anti-phospho-ERK antibody. Taken collectively, these results suggest that the increase in ERK activity is necessary to induce the loss of...
adhesion function at the apical ES. The fact that ERK activation is involved in junction disassembly is not entirely surprising. For instance, a blockade of ERK signaling pathway in Madin-Darby canine kidney cells can indeed block the hepatocyte growth factor/scatter factor-induced AJ disassembly (48). In the testis, the level of phospho-ERK is also the highest in the seminiferous epithelium when spermatiation is in progress, suggesting its role in promoting ES disassembly (26).

The result reported herein also implies that ERK activation is likely associated with the formation of the FAK/c-Src protein complex. Indeed, a recent report has shown that the FAK/c-Src signaling complex may regulate cell adhesion turnover at the FA site via this MAPK pathway (35). This can be achieved, at least in part, by the ERK-activated proteolysis at this site (49), which facilitates the cleavage of FA complex components (for a review, see Ref. 50), and this event occurs in a FAK-c-Src-dependent manner (44). Indeed, recent studies have shown that interactions of proteases and protease inhibitors are crucial in regulating apical ES dynamics in the testis and that proteases (e.g., matrix metalloproteinases) are indeed associated with the integrin/laminin complex at the apical ES (7, 51). Thus, it is plausible that FAK, c-Src, and ERK are involved in this event. However, it is of interest to note that an increase in staining of p-ERK and p-Src (see above) was not detected by immunohistochemistry at the seminiferous epithelium during the time that spermatids were being sloughed from the epithelium (data not shown).

These results, which apparently contradict the data obtained from immunoblotting and intrinsic kinase assays, can be caused by the ES-associated proteins being sloughed to the tubule lumen with germ cells after they completed their role in mediating apical ES restructuring but could not be stained on frozen sections of testes. However, to verify this hypothesis, studies such as by micropuncture techniques, which collect the luminal fluids to quantify the amount of these proteins, are warranted.

**Are the changes in protein levels and their activities mediated by androgen suppression indeed the result of germ cell loss?**

Whereas results from this study have validated earlier findings using the AF-2364 model regarding the significance of the β1 integrin/β1-integrin protein complex in regulating ES dynamics, a question remains: how can we be certain that the observed effects are attributable to germ cell loss rather than effects mediated by the implants per se independent of the cell loss? We offer several lines of evidence to address this interesting issue. First, studies using both models demonstrated the activation of β1 integrin, FAK, and c-Src during germ cell loss and the subsequent recovery in the seminiferous epithelium (6, 32), implicating their involvement in ES restructuring. Yet the most compelling evidence comes from studies using specific inhibitors. For instance, by blocking a specific signal transducer of the signaling pathway (e.g., ROCK), the disruptive effects of AF-2364 in depleting germ cells from the epithelium can indeed be significantly delayed (7, 53). This finding thus confirms unequivocally that Sertoli-germ cell ES dynamics are regulated by the integrin/RhoB/ROCK/LIMK/cofilin signaling pathway. Second, earlier studies using fluorescent and electron microscopy to survey the damage to the testis during androgen suppression have shown that apical ES is the only structure that was reversibly damaged (30, 54). This, in turn, leads to the loss of spermatids (step 8 and beyond) from the epithelium. As shown in this report, the proteins that were induced and activated were also localized precisely to the apical ES site. We have also demonstrated changes in protein-protein association between the β1-integrin and p-FAK/c-Src protein complexes at the ES. These findings thus support the notion that changes in these proteins and their activation are related to the event of germ cell loss rather than the result of nonspecific drug effect (e.g., the AF-2364 model) or androgen withdrawal.

**Summary and concluding remarks**

In summary, by using this androgen suppression model to study Sertoli-germ cell ES dynamics, the data reported herein have confirmed earlier results based on the AF-2364 model that delineated the regulation of ES dynamics with several unexpected findings. First, β1-integrin and the p-FAK/c-Src protein complex are crucial to ES dynamics. It was shown that the adhesion function between Sertoli cells and spermatids in the seminiferous epithelium is mediated, at least in part, by the integrin α6β1/laminin-γ3 protein complex. This, in turn, interacts with the activated FAK/c-Src complex at the ES site to form a FA-like structure. Second, during the T suppression-induced spermatid loss from the epithelium, pFAK/c-Src-induced ERK activation causes the dissociation of the protein complex from α6β1-integrin, destabilizing the adhesion function at the apical ES site (see Fig. 6). The induction in protein levels of β1-integrin, FAK, and c-Src may also be a physiological response to counteract the androgen depletion-induced ES disruption. During recovery, more integrins, FAK, and c-Src are being synthesized, and the association between integrins and the p-FAK/c-Src complex is also reestablished, stabilizing the α6β1-integrin/laminin-γ3 protein complex at the apical ES (Fig. 6). It is obvious that the model put forth herein will be revised with emerging information in the years to come. Nonetheless, it serves as a working model for investigators in the field. Needless to say, a thorough understanding of how ES is being regulated in the seminiferous epithelium remains to be elucidated. For examples, much work is needed to determine how the FAK/c-Src complex transmits signals between integrins and the actin cytoskeleton structure. Does this involve an adaptor, such as p130Cas? And how does the ERK signaling pathway mediate its signals with proteases and protease inhibitors at the apical ES to elicit the loss of cell adhesion function? Furthermore, it remains to be determined the mechanism by which a suppression on intratesticular T level induced by TE implants can induce activation of the α6β1-integrin/laminin-γ3 protein complex.

Furthermore, these results have revealed some potential targets that can be tackled to regulate cell adhesion function in the testis, leading to the development of novel male contraceptives. For example, a specific activator or inhibitor of ERK can be used to induce premature spermatid loss or to prevent (or delay) spermatiation, respectively, if it can be specifically targeted to the
testis, such as with the use of a deglycosylated FSH mutant protein that can bind to the FSH receptors on Sertoli cells without the hormonal activity. In either case, infertility will result. Using such an approach, it is likely that androgen implants (or TE implants) are needed to perturb the androgen microenvironment in the testis. As such, this should not interfere with the hypothalamic-pituitary-testicular axis because the site of action is at the Sertoli-germ cell interface.

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References

4. Mruk DD, Cheng CY 2004 Sertoli-Sertoli and Sertoli-germ cell interactions and
their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr Rev... Regulation of Apical ES in T-Suppressed Rat Testes Endocrinology, March 2005, 146(3):0000–0000

AQ: F

Aqu: G

AQ: H

Robaire B, Ewing LL, Irby DC, Desjardins C

27.

Chapin RE, Wine RN, Harris MW, Borchers CH, Haseman JK

10.


14.

Mulholland DJ, Dedhar S, Vogl AW

O'Donnell L, McLachlan RI, Wreford NG, Robertson DM 1995 Integrin receptor 0β1 is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. Biol Reprod 52:79–86


16.

Siu MKY, Cheng CY

1994 Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol Cell Biol 15:954–963

14.


14.

Salanova M, Stefanini M, de Curtis I, Palombi F 1995 Integrin receptor 0β1 is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. Biol Reprod 52:79–86

16.

Chen HC, Guan JL

5.

Siu MKY, Cheng CY


45.

Lui WY, Lee WM, Cheng CY

1999 Complex formation with focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol Cell Biol 15:954–963

53.

Lee NPY, Cheng CY

1996 A mechanism for regulation of the adhesion-disassembly and requirement for the autophosphorylated associated protein tyrosine kinase pp125FAK. Nature 380538–540

36.

Beardsley A, O'Donnell L

1998 Activation of both MAP kinase and phosphatidylinositol-3 kinase in nascent and mature focal adhesions and requirement for the autophosphorylated associated protein tyrosine kinase pp125FAK. Mol Cell 14:147–155


1994 Stable association of pp60src and pp59lyn with the focal contact-associated protein tyrosine kinase pp125FAK. Mol Cell 14:147–155

Cobb BS, Schaller MD, Leu TH, Parsons JT 1994 Direct interaction of p56lck with the focal contact-associated protein tyrosine kinase pp125FAK. Mol Cell 14:147–155


47.


1997 The role of conserved amino acid motifs within the integrin β3 cytoplasmic domain in triggering focal adhesion kinase phosphorylation. J Biol Chem 272:792–798

1999 Complex formation with focal adhesion kinase: a mechanism to regulate activity and subcellular localization of Src kinases. Mol Biol Cell 10:2348–2355


1997 Activation of both MAP kinase and phosphatidylinositol-3 kinase. Proc Natl Acad Sci USA 91:10148–10152


31.

Lee NPY, Cheng CY

1997 The role of conserved amino acid motifs within the integrin β3 cytoplasmic domain in triggering focal adhesion kinase phosphorylation. J Biol Chem 272:792–798


44.


2002 Cutting to the chase: calpain enzymes target the focal adhesion complex. Trends Cell Biol 12:46–54


49.


50.


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