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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)

ECTOPLASMIC SPECIALIZATION (ES) is a testis-specific cell-cell actin-based adherens junction (AJ) type (for reviews, see Refs. 1–4). It is found between Sertoli cells at the basal compartment of the seminiferous epithelium, which is known as the basal ES. ES is also found between Sertoli cells and round, elongating, and elongate spermatids at the adluminal compartment, and is known as the apical ES (for reviews, see Refs. 1–3). Basal ES, together with tight junction and the basal tubulobulbar complex, creates the blood-testis barrier that divides the seminiferous epithelium into the basal and adluminal compartments (5). Preleptotene and leptotene spermatocytes that reside in the basal compartment must traverse the blood-testis barrier to enter the adluminal compartment for further development (for reviews, see Refs. 3, 4). Apical ES, on the other hand, facilitates spermatid movement across the epithelium, whereas it anchors spermatids onto Sertoli cells until their release to the tubule lumen at spermiation (for reviews, see Refs. 1–3). Therefore, ES is crucial for germ cell development, cell movement, and their orientation during spermatogenesis. Ironically, whereas the ES in the testis has been studied morphologically for more than three decades, its biology and regulation remain largely unexplored. This is largely due to the lack of suitable *in vivo* models to study ES dynamics.

Recent studies from this laboratory (for reviews, see Refs. 3, 4) reported an *in vivo* model using 1-(2,4-dichlorobenzyl)-indazole-3-carbohydrazide (AF-2364), a derivative of indazole-3-carboxylic acid, to induce germ cell loss from the epithelium without affecting the serum levels of FSH, LH, and testosterone, as a novel approach to study ES dynamics in the rat testis. Collectively, these studies have demonstrated that ES dynamics are regulated by several distinctive signaling pathways (for reviews, see Refs. 3, 4). Interestingly, one of these pathways, namely the integrin β1/focal adhesion kinase (FAK)/phosphatidylinositol-3-kinase (PI 3-kinase)/p130 Crk-associated protein (p130Cas) pathway (6, 7), is also being used in other epithelia to regulate focal adhesion (FA) function, an anchoring junction type confined to the cell-extracellular matrix interface (for reviews, see Refs. 8, 9). However, it is still arguable that results obtained by using this AF-2364 model might be due to the acute or chronic toxicity of AF-2364 because this is not a naturally occurring substance, even though this compound was shown not to be toxic to the treated animals at doses that were effective to

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Abbreviations: AF-2364, 1-(2,4-Dichlorobenzyl)-indazole-3-carbohydrazide; AJ, adherens junction; E, estradiol; ES, ectoplasmic specialization; FA, focal adhesion; FAK, FA kinase; FITC, fluorescein isothiocyanate; IP, immunoprecipitation; N.R., normal recovery without implant; p130Cas, p130 Crk-associated protein; p-ERK, activated form of ERK; PI 3-kinase, phosphatidylinositol-3-kinase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; T, testosterone.

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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. 00111 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 antiactin (catalog no. sc-1616, lot E0503), monoclonal mouse anti-Src (catalog no. sc-8056, lot J2103), mouse antivimentin (catalog no. sc-6260, lot B252), and mouse anti-α-tubulin (catalog no. sc-8035, lot G182) were 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-Tyr^{416} (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-phospho-Tyr (catalog no. 61–5800, lot 40286685) was purchased from Zymed Laboratories (San Francisco, CA). 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. Histostain-SP kit for immunohistochemistry, goat antimouse IgG-Cy-3, 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 770, 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 the testis and induces germ cell loss, in particular spermatids (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 cm^2) 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 used.

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 using 7.5 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 aprotinin (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 by 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 aprotinin (1 μg/ml)] four times by gentle resuspension and mild centrifugation (5 min, 1000 × *g*), and proteins in the immunocomplex were extracted from the 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.

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Fluorescent microscopy

Fluorescent microscopy was performed to colocalize β1-integrin with phospho-Src-Tyr^{416} 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 with 4′,6′-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA), and fluorescent microscopy was performed using a BX40 microscope (Olympus Corp., Melville, NY) equipped with Olympus UPlanF1 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-glycerolphosphate, 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 phospho-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 [25 mM Tris (pH 7.5)] at 22 C containing 5 mM glycerolphosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$), 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 (Ser^{383}) 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 first fixed in Bouin's fixative. Sections were treated with 3% H_2O_2 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, following 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 reddish brown 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. AQ: C

Statistical analysis

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). F1

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 F2

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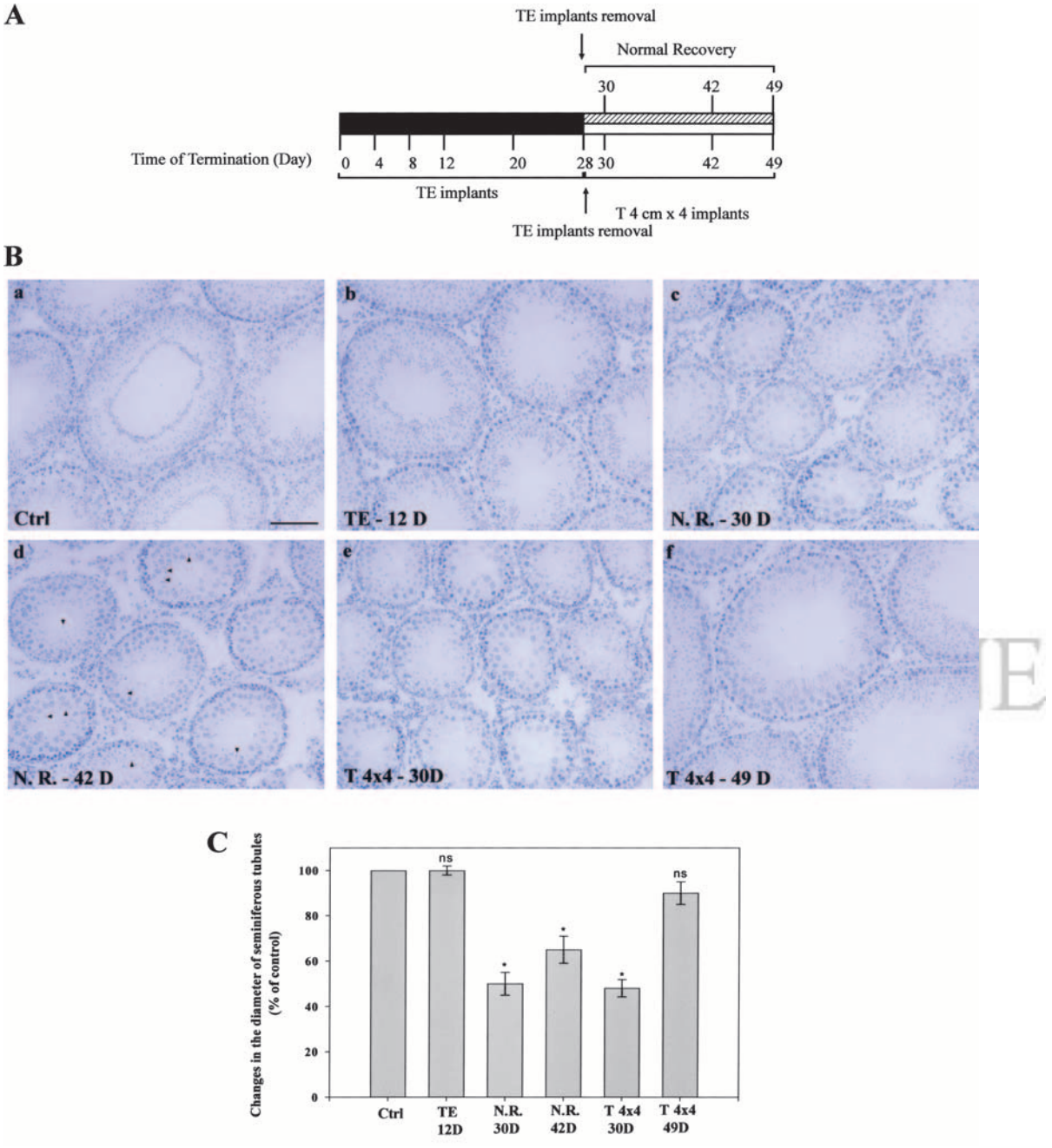

FIG. 1. A–C, Induction of germ cell loss from the seminiferous epithelium of the rat testis by suppressing endogenous testicular T level using TE implants. A, A schematic drawing that illustrates the treatment regimen. One experimental group of rats was treated with T (3 cm) and E (0.4 cm) implants for 28 d to induce germ cell loss. The other two groups of animals either underwent N.R. or received a T (4×4 cm)-induced recovery after TE implants were removed on the 28th day. B, a-f, Morphological analysis showing the status of spermatogenesis and the loss of germ cell from the epithelium after TE treatment using frozen sections stained with hematoxylin at selected time points. *Arrowheads* (B-d) show the elongating spermatids that reappeared in the epithelium after TE implant removal. C, The percent change in the average diameter of seminiferous tubules after the treatment was also scored and plotted. About 200 cross-sections of tubules from three rat testes at each time point were analyzed. Statistics were performed by *t* test, comparing the treatment groups with controls (Ctrl). ns, Not significantly different from control. *, Significantly different by *t* test, $P < 0.05$. *Bar*, B-a, 80 μm, which applies to b-f.

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).

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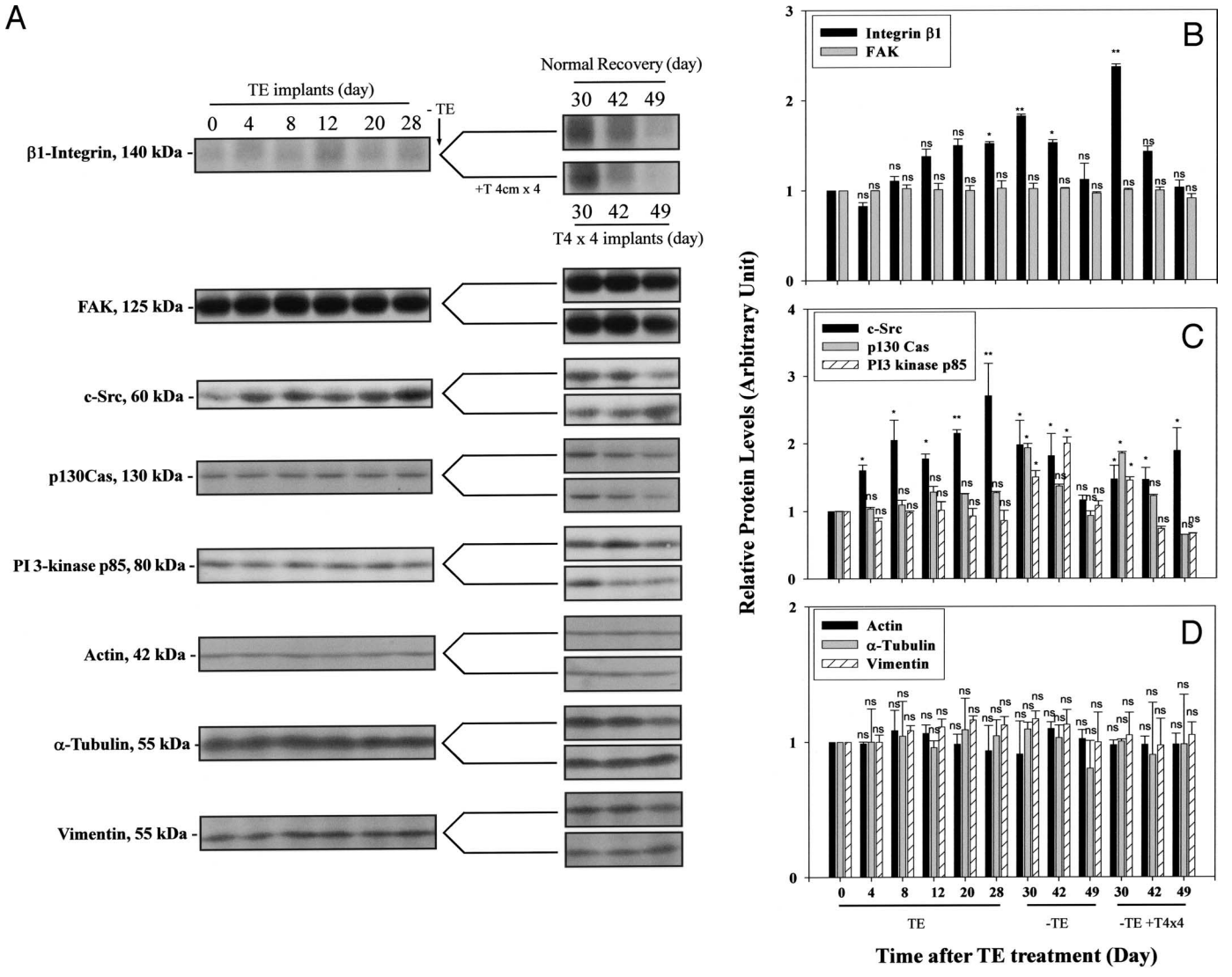

FIG. 2. A–D, Changes in protein levels of ES-associated proteins including β1-integrin, FAK, c-Src, p130Cas, and PI 3-kinase in the testis during T suppression and recovery. Immunoblot analysis was performed using testis lysates (~100 μg protein per lane) from different treatment groups at specified 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 α-tubulin 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, $P < 0.01$.

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 Tyr^{397} and Try^{576} 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 and an anti-FAK antibody to pull out FAK (Fig. 3B, *lower panel*). The same blot was probed with an anti-phospho-Tyr antibody to quantify the p-FAK-Tyr content (see Fig. 3, B, *top panel*, and C). At 42 d after TE implant administration in the normal recovery group and 30 d in the T 4 × 4 cm recovery group, the levels of p-FAK-Tyr in the testis lysates were increased when compared with controls (Fig. 3B, *top panel*); whereas the level of total FAK in the immunocomplex remained unaltered, which is consistent with the immunoblotting result (Figs. 3, B, C *vs.* A, and 2). These results indicated that more FAKs were phosphorylated at the tyrosine residues during androgen suppression-induced germ cell loss. Because tyrosine-phosphorylated FAK is capable of recruiting and activating Src to form a protein complex that can further activate their downstream signaling pathways (17, 23), we also sought to investigate any changes in association between FAK and c-Src during the TE treatment by IP (Fig. 3, D and E). In normal testis, FAK and c-Src could indeed form a complex (Fig. 3D), which is consistent with an

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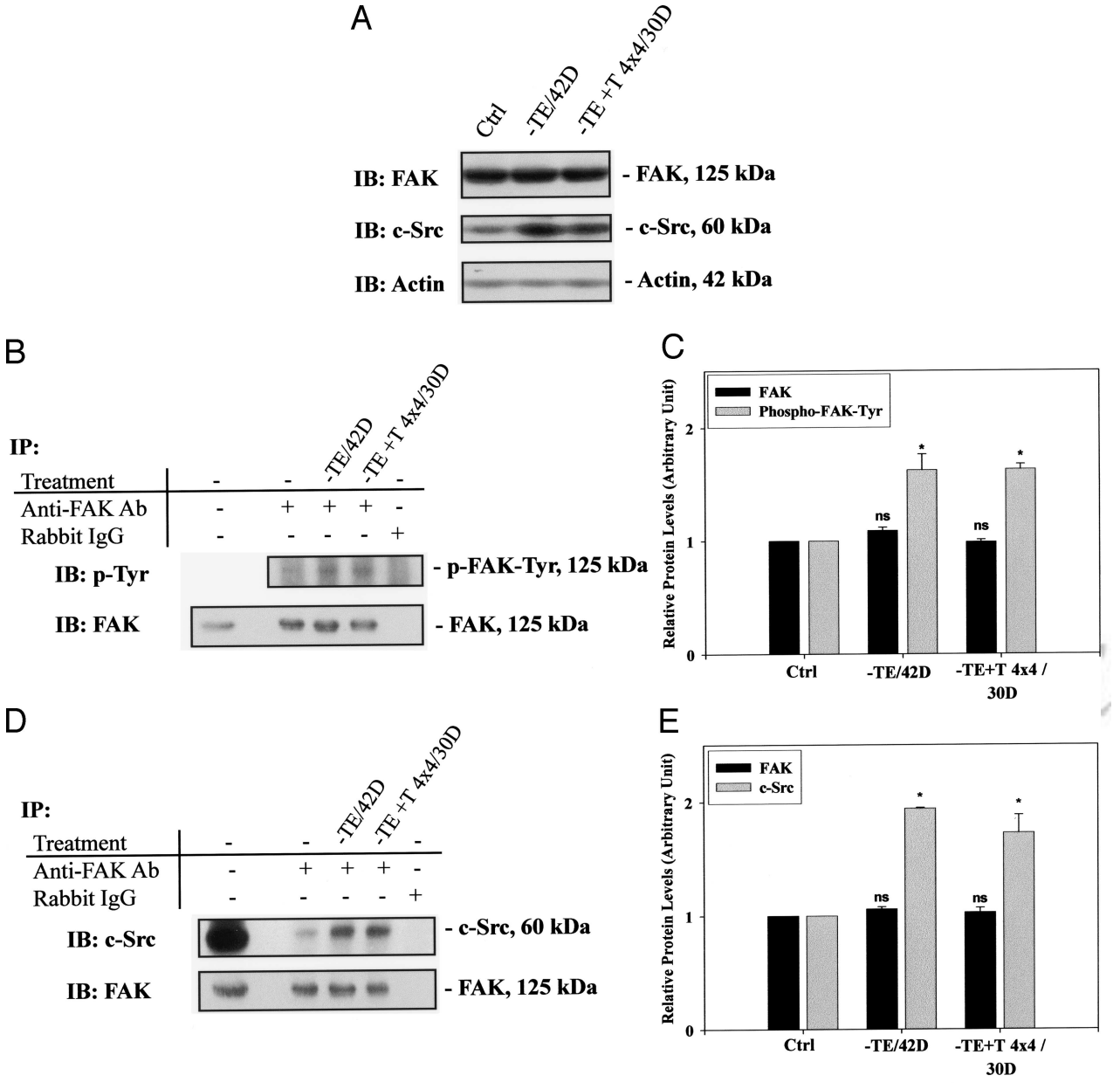

FIG. 3. A–C, Changes in Tyr phosphorylation of FAK and its interaction with c-Src during T suppression-induced germ cell loss from the epithelium. A, Testis lysates at specified time points of different experimental groups were prepared in modified radioimmunoprecipitation assay buffer (see *Materials and Methods*). Samples were subjected to immunoblot (IB) analysis with anti-FAK and anti-Src antibodies before their use for IP, and equal protein loading was determined using an anti-β-actin antibody. Ctrl, Control. B, The extent of FAK Tyr phosphorylation (the activated form of FAK) during TE implant treatment was investigated by IP in which samples (~400 μg protein per sample) were immunoprecipitated using an anti-FAK antibody (Ab). Thereafter, immunocomplexes were resolved by SDS-PAGE, and the blots were probed with an anti-phospho-Tyr antibody (*top panel*) and an anti-FAK antibody (*bottom panel*). Whereas FAK level remained unchanged, an increase in phosphorylation of FAK was detected. C, Densitometrically scanned results using immunoblots, such as those shown in B. D, To determine whether there were any changes in FAK and c-Src association during T suppression-induced germ cell depletion, IP was performed using an anti-FAK antibody, and the immunocomplexes were analyzed by immunoblotting with an anti-Src antibody. The same blot was also stripped and reprobed with an anti-FAK antibody to validate the IP result. This experiment illustrates an increase in c-Src association with FAK during germ cell loss. E, Histogram showing the densitometrically scanned results using immunoblots, such as those shown in C. Each *bar* in C and E represents the mean ± SD of results from three separate IP experiments using different sets of samples. The protein level of a target protein in the control group was arbitrarily set at 1, against which Student's *t* test was performed. ns, Not significantly different; *, Significantly different, $P < 0.05$.

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 × 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.

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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(Tyr^{416}) 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 × 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

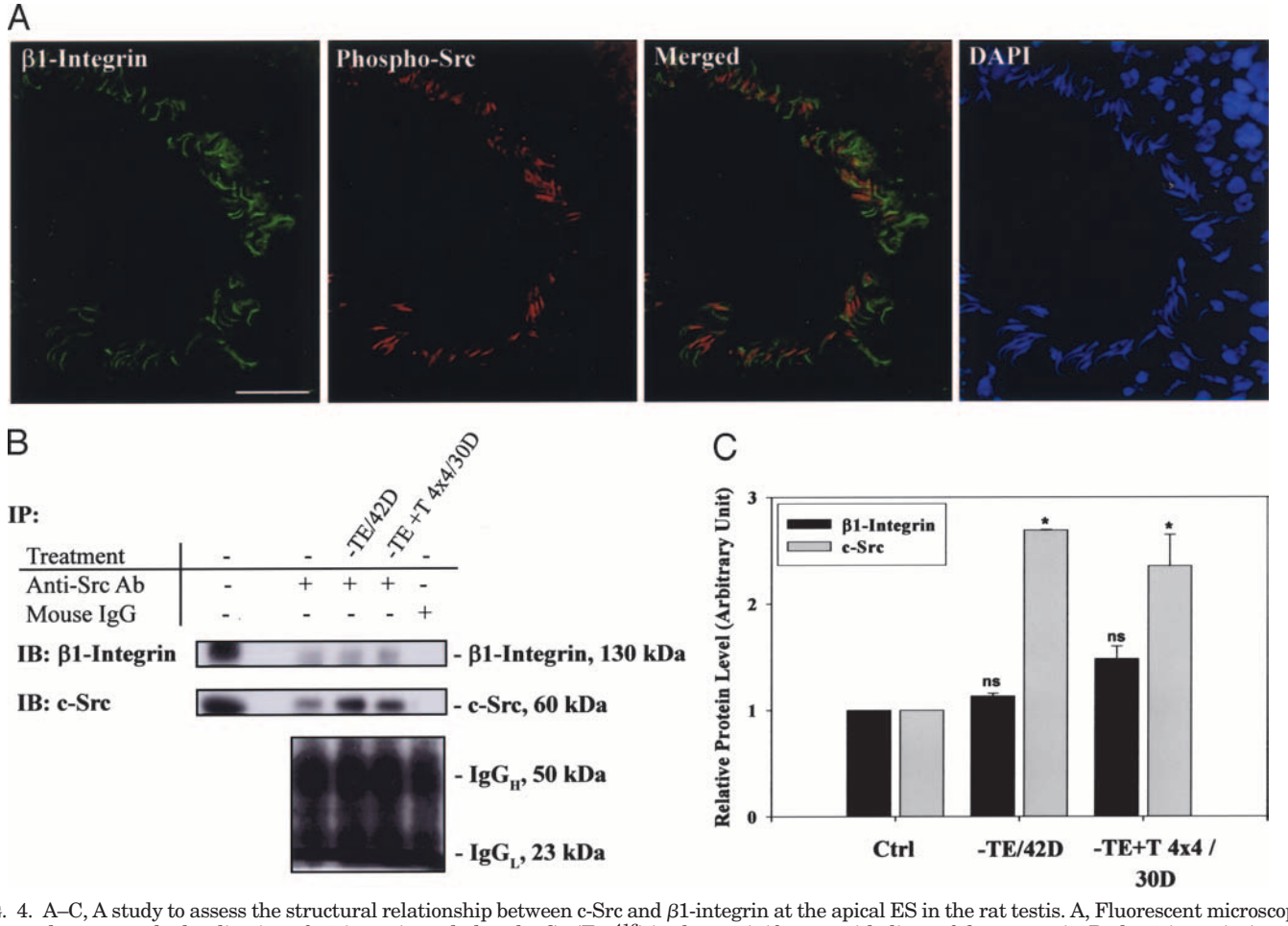

FIG. 4. A–C, A study to assess the structural relationship between c-Src and β1-integrin at the apical ES in the rat testis. A, Fluorescent microscopy was used to assess the localization of β1-integrin and phospho-Src(Tyr^{416}) in the seminiferous epithelium of the rat testis. Both β1-integrin (*green*, FITC) and p-Src(Tyr^{416}) (*red*, Cy-3) were found near the heads of elongate spermatids and colocalized to the same site (*orange*, merged image), which was consistent with their localization at the apical ES. The 4′,6′-diamino-2-phenylindole staining shows the nucleus of Sertoli and germ cells in the epithelium. B, An IP experiment using an anti-Src antibody as described in *Materials and Methods*. β1-Integrin was shown to associate with c-Src using testis lysates from control (Ctrl) and treatment groups. In control testis (Ctrl), β1-integrin was found to associate with c-Src. Yet an induction of c-Src during androgen suppression-induced germ cell loss (see *middle panel*; note: this result is consistent with immunoblot data shown in Fig. 2 using testis lysates without IP) was not accompanied by an increase in β1-integrin association (*middle vs. top panel*). The *bottom panel* shows the heavy and light chains of IgG, which served as a loading control. C, Results of the IP experiment, such as those shown in B, were densitometrically scanned and plotted. Each **bar** in C is a mean ± SD of three independent experiments. The amount of β1-integrin and c-Src present in the immunoprecipitated protein complex in control rat testes was arbitrarily set at 1, against which *t* test was performed. *, Significantly different by *t* test, $P < 0.05$. *Bar in left panel* (A), 40 μm, which applies to the remaining panels.

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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 immunoblottings 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 testis 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-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, PI 3-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

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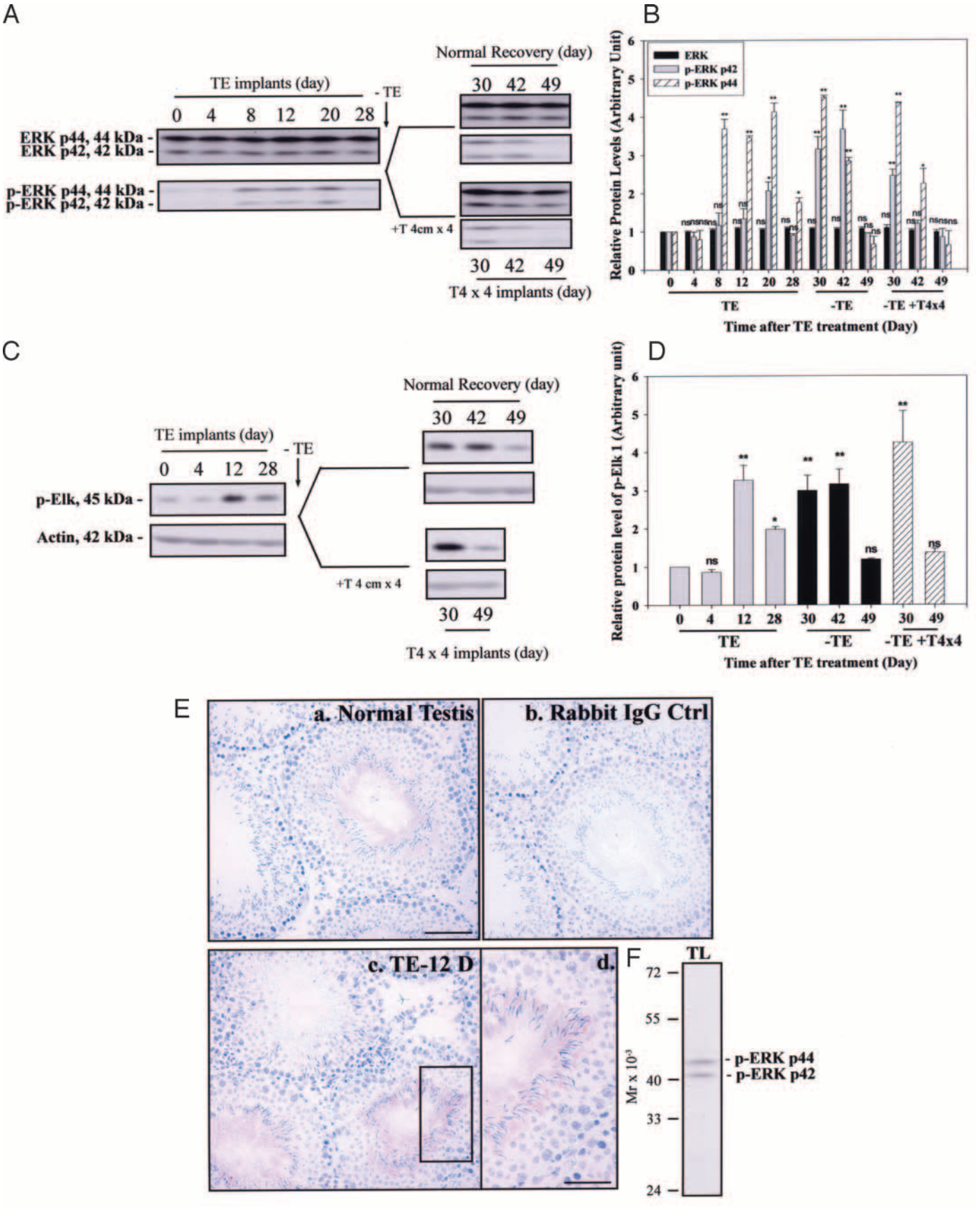

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functional domains of these proteins that are involved in either junction disruption or re-construction.

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 autophosphorylate at tyrosine residues, most abundantly at Tyr^{397}, on activation such as via its interaction with integrins (for reviews, see Refs. 21, 22, 36). The autophosphorylation 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, T 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 Tyr^{416} 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 Tyr^{925} 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

FIG. 5. A–F, Changes in the protein level and intrinsic activity of ERK in the testis during androgen suppression-induced germ cell loss. A, Changes in protein levels of ERK and phospho-ERK (activated form) during TE treatment and the subsequent recovery were assessed by immunoblottings. B, The densitometrically scanned results using immunoblots, such as those shown in A, were plotted. C, The activation of ERK during TE treatment was estimated by using a nonradioactive kinase assay as described in *Materials and Methods*. The β-actin content in each sample assayed was also quantified by immunoblotting to confirm equal protein loading and uniform protein transfer from gels to nitrocellulose membranes. D, Results from immunoblots, such as those shown in C, were densitometrically scanned and plotted. Each *bar* in B and D is the mean ± SD of three experiments using different sets of samples from different rats. The protein level of target proteins at time 0 was arbitrarily set at 1, against which one-way ANOVA was performed. E, Immunohistochemistry study localizing phospho-ERK using frozen cross-sections of normal rat testes and testes from rats that underwent T suppression for 12 d, at the time when ERK was activated (see A–D). Phospho-ERK appeared as reddish precipitates in the seminiferous epithelium (see a, c, and d). Rabbit IgG in the same dilution was used to substitute the primary antibody in the control, which yielded no detectable signal (b), illustrating the staining for p-ERK shown herein was specific. Micrographs shown herein are the representative results from three experiments using different samples. F, To further ensure the specificity of the p-ERK antibody used for immunohistochemistry shown in E, an immunoblot using testis lysate for SDS-PAGE was probed with the same antibody, and the result is shown herein. Only two immunoreactive bands corresponding to the electrophoretic mobilities of the p-ERK p44 and p42 isoforms were identified. ns, Not significantly different by ANOVA; *, Significantly different, $P < 0.05$; **, Significantly different, $P < 0.01$. *Bar* (C-a), 80 μm, which applies to C-b and C-c; *bar* (C-d), 40 μm.

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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 spermiation 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 protein 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/pFAK/c-Src 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, 52), 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.

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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.

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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) spermiation, respectively, if it can be specifically targeted to the

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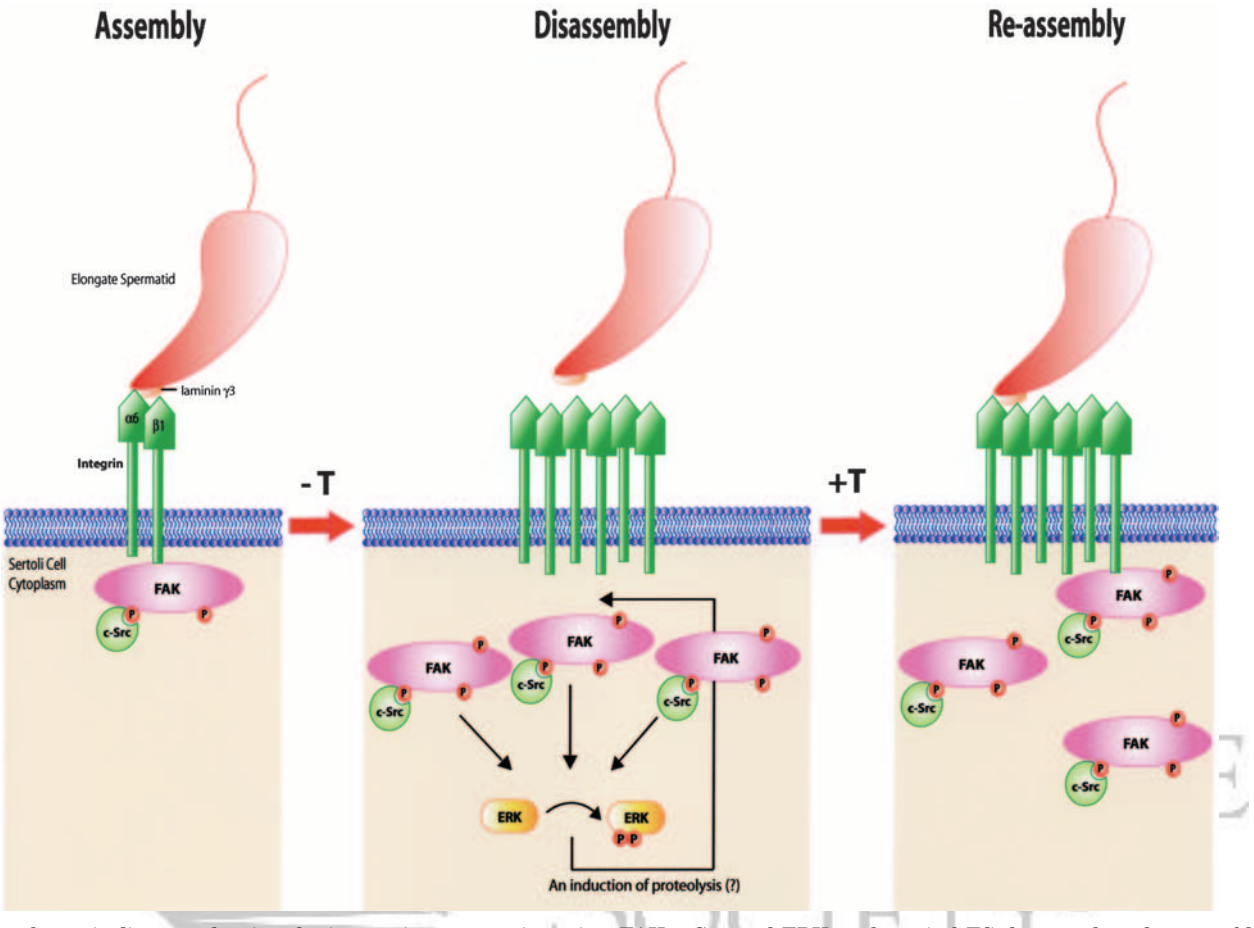

FIG. 6. A schematic diagram showing the interactions among integrins, FAK, c-Src, and ERK at the apical ES that regulate the assembly and disassembly of the Sertoli-germ cell adhesion function. Under normal physiological conditions, when the apical ES is intact, adhesion between Sertoli cells and spermatids is mediated, at least in part, by the integrin $\alpha6\beta1$/laminin-$\gamma3$ protein complex, which interacts with the activated FAK/c-Src complex in the Sertoli cell to form a cell-matrix FA-like structure at the apical ES (*left panel*). However, when apical ES is disrupted, such as induced by T suppression in the testis via TE implants, the FAK/c-Src complex dissociates from integrins, even though the protein levels of $\beta1$-integrin, p-FAK, and c-Src are induced as reported herein (*middle panel*). It is also noted that not only the Tyr phosphorylation of FAK is induced so as its association with c-Src. This protein complex is likely activating the ERK signaling pathway, which in turn facilitates adhesion structure disassembly (*e.g.* by increasing proteolysis, favoring protease activity at the apical ES site). This postulate is based on recent studies (7) that have illustrated the presence of proteases, such as matrix metalloproteinases, at the apical ES that is associated with the $\beta1$-integrin/laminin-$\gamma3$ protein complex. The increases in protein levels of integrins, p-FAK, and c-Src in the testis are plausibly an attempt used by the testis to counteract the loss of spermatids from the epithelium. However, this induction in proteins fails to rescue germ cell loss because of a loss of protein-protein association between $\beta1$-integrin and p-FAK/c-Src, which destabilizes the $\alpha6\beta1$-integrin/laminin-$\gamma3$ complex and leads to ES disruption. When the intratesticular T level is being restored (*e.g.* N.R. or by using T-only implants), the protein levels of $\beta1$-integrin, p-FAK, and c-Src were also induced (*right panel*). At the same time, the protein-protein interaction between $\beta1$-integrin and p-FAK/c-Src is being reestablished. This, in turn, stabilizes the $\alpha6\beta$-integrin/laminin-$\gamma3$ protein complex and restores the ES function (*right panel vs. middle panel*).

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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

1. **Russell LD** 1980 Sertoli-germ cell interrelations: a review. Gamete Res 3:179–202
2. **Vogl AW, Pfeiffer DC, Mulholland DJ, Kimel G, Guttman J** 2000 Unique and multifunctional adhesion junctions in the testis: ectoplasmic specializations. Arch Histol Cytol 63:1–15
3. **Cheng CY, Mruk DD** 2002 Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. Physiol Rev 82:825–874
4. **Mruk DD, Cheng CY** 2004 Sertoli-Sertoli and Sertoli-germ cell interations and

balt3/zee-end/zee-end/zee00305/zee0732-05a | saylora | S=4 | 12/29/04 | 11:54 | 4/Color Figure(s): 1,4-6 | Art: 1195119 | Input-ss

their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr Rev 25:747–806

5. **de Kretser DM, Kerr JB** 1988 The cytology of the testis. In: Knobil E, Neill JD, eds. The physiology of reproduction, 2nd ed. New York: Raven Press; 837–932
6. **Siu MKY, Mruk DD, Lee WM, Cheng CY** 2003 Adhering junction dynamics in the testis are regulated by an interplay of β1-integrin and focal adhesion complex-associated proteins. Endocrinology 144:2141–2163
7. **Siu MKY, Cheng CY** 2004 Interactions of proteases, protease inhibitors, and the β1 integrin/laminin γ3 protein complex in the regulation of ectoplasmic specialization dynamics in the rat testis. Biol Reprod 70:945–964
8. **Burridge K, Chrzanowska-Wodnicka M** 1996 Focal adhesions, contractility, and signaling. Ann Rev Cell Dev Biol 12:463–518
9. **Petit V, Thiery J-P** 2000 Focal adhesions: structure and dynamics. Biol Cell 92:477–494
10. **McLachlan RI, Wreford NG, O'Donnell L, de Kretser DM, Robertson DM** 1996 The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. J Endocrinol 148:1–9
11. **McLachlan RI, Wreford NG, Tsonis C, de Kretser DM, Robertson DM** 1994 Testosterone effects on spermatogenesis in the gonadotropin releasing hormone-immunized rat. Biol Reprod 50:271–280
12. **O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM** 1996 Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. Biol Reprod 55:895–901
13. **Mulholland DJ, Dedhar S, Vogl AW** 2001 Rat seminiferous epithelium contains a unique junction (ectoplasmic specialization) with signaling properties both of cell/cell and cell/matrix junctions. Biol Reprod 64:396–407
14. **Salanova M, Stefanini M, de Curtis I, Palombi F** 1995 Integrin receptor α6β1 is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. Biol Reprod 52:79–87
15. **Schaller MD, Otey CA, Hildebrand JD, Parsons JT** 1995 Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. J Cell Biol 130:1181–1187
16. **Tahiliani PD, Singh L, Auer KL, LaFlamme SE** 1997 The role of conserved amino acid motifs within the integrin β3 cytoplasmic domain in triggering focal adhesion kinase phosphorylation. J Biol Chem 272:7892–7898
17. **Schaller MD, Hildebrand JD, Parsons JT** 1999 Complex formation with focal adhesion kinase: a mechanism to regulate activity and subcellular localization of Src kinases. Mol Biol Cell 10:3489–3505
18. **Xing Z, Chen HC, Nowlen JK, Taylor SJ, Shalloway D, Guan JL** 1994 Direct interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. Mol Biol Cell 5:412–421
19. **Harte MT, Hildebrand JD, Burnham MR, Bouton AH, Parsons JT** 1996 p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. J Biol Chem 271:13649–13655
20. **Chen HC, Guan JL** 1994 Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA 91:10148–10152
21. **Parsons JT, Martin KH, Slack JK, Taylor JM, Weed SA** 2000 Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene 19:5606–5613
22. **Parsons JT** 2003 Focal adhesion kinase: the first ten years. J Cell Sci 116:1409–1416
23. **Cobb BS, Schaller MD, Leu TH, Parsons JT** 1994 Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. Mol Cell Biol 14:147–155
24. **Zhu X, Assoian RK** 1995 Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. Mol Biol Cell 6:273–282
25. **Chen HC, Chan PC, Tang MJ, Cheng CH, Chang TJ** 1998 Tyrosine phosphorylation of focal adhesion kinase stimulated by hepatocyte growth factor leads to mitogen-activated protein kinase activation. J Biol Chem 40:25777–25782
26. **Chapin RE, Wine RN, Harris MW, Borchers CH, Haseman JK** 2001 Structure and control of a cell-cell adhesion complex associated with spermiation in rat seminiferous epithelium. J Androl 22:1020–1052
27. **Ewing LL, Desjardins C, Irby DC, Robaire B** 1977 Synergistic interaction of testosterone and oestradiol inhibits spermatogenesis in rats. Nature 269:409–411
28. **Robaire B, Ewing LL, Irby DC, Desjardins C** 1979 Interactions of testosterone and estradiol-17β on the reproductive tract of the male rat. Biol Reprod 21:455–463
29. **O'Donnell L, McLachlan RI, Wreford NG, Robertson DM** 1994 Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle. Endocrinology 1994:2608–2614
30. **O'Donnell L, Stanton PG, Bartles JR, Robertson DM** 2000 Sertoli cell ectoplasmic specialization in the seminiferous epithelium of the testosterone-suppressed adult rat. Biol Reprod 63:99–108
31. **Siu MKY, Cheng CY** 2004 Dynamic cross-talk between cells and the extracellular matrix in the testis. Bioessays 26:978–992
32. **Lee NPY, Cheng CY** 2004 Ectoplasmic specialization, a testis-specific cell-cell actin-based adherens junction type: is this a potential target for male contraceptive development? Hum Reprod Update 10:349–369
33. **Richardson A, Parsons JT** 1996 A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125FAK. Nature 380538–380540
34. **Ruset PJ, Roy S, Shi E, Mernaugh RL, Hanks SK** 2000 Phosphospecific antibodies reveal focal adhesion kinase activation loop phosphorylation in a nascent and mature focal adhesions and requirement for the autophosphorylation site. Cell Growth Differ 11:41–48
35. **Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF** 2004 FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 6:154–161
36. **Guan JL** 1997 Focal adhesion kinase in integrin signaling. Matrix Biol 16:195–200
37. **Calalb MB, Polte TR, Hanks SK** 1995 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
38. **Giannone G, Ronde P, Gaire M, Beaudouin J, Haiech J, Ellenberg J, Takeda K** 2004 Calcium rises locally trigger focal adhesion disassembly and enhance residency of focal adhesion kinase at focal adhesions. J Biol Chem 279:28715–28723
39. **Schlaepfer DD, Mitra SK** 2004 Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev 14:92–101
40. **Lin EH, Hui AY, Meens JA, Tremblay EA, Schaefer E, Elliott BE** 2004 Disruption of Ca^{2+}-dependent cell-matrix adhesion enhances c-Src kinase activity, but causes dissociation of the c-Src/FAK complex and dephosphorylation of tyrosine-577 of FAK in carcinoma cells. Exp Cell Res 293:1–13
41. **Siu MKY, Cheng CY** 2004 Extracellular matrix: recent advances on its role in junction dynamics in the seminiferous epithelium during spermatogenesis. Biol Reprod 71:375–391
42. **Carragher NO, Frame MC** 2004 Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. Trends Cell Biol 14:241–249
43. **Carragher NO, Levkau B, Ross R, Raines EW** 1999 Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of $pp125^{FAK}$, paxillin, and talin. J Cell Biol 147:619–629
44. **Carragher NO, Westhoff MA, Fincham VJ, Schaller MD, Frame MC** 2003 A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. Curr Biol 13:1442–1450
45. **Schlaepfer DD, Hanks SK, Hunter T, van der Geer P** 1994 Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 372:786–791
46. **Schlaepfer DD, Hunter T** 1997 Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. J Biol Chem 272:13189–13195
47. **Eblen ST, Slack JK, Weber MJ, Catling AD** 2002 Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. Mol Cell Biol 22:6023–6033
48. **Potempa S, Ridley AJ** 1998 Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scarrer factor-induced adherens junction disassembly. Mol Biol Cell 9:2185–2200
49. **Glading A, Chang P, Lauffenburger DA, Wells A** 2000 Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. J Biol Chem 275:2390–2398
50. **Glading A, Lauffenburger DA, Wells A** 2002 Cutting to the chase: calpain proteases in cell motility. Trends Cell Biol 12:46–54
51. **Mruk D, Zhu LJ, Silvestrini B, Lee WM, Cheng CY** 1997 Interactions of proteases and protease inhibitors in Sertoli-germ cell cocultures preceding the formation of specialized Sertoli-germ cell junctions *in vitro*. J Androl 18:612–622
52. **Lee NPY, Cheng CY,** 2005 Protein kinases and adherens junction dynamics in the seminiferous epithelium of the rat testis. J Cell Physiol, in press
53. **Lui WY, Lee WM, Cheng CY** 2003 Sertoli-germ cell adherens junction dynamics in the testis are regulated by RhoB GTPase via the ROCK/LIMK signaling pathway. Biol Reprod 68:2189–2206
54. **Beardsley A, O'Donnell L** 2003 Characterization of normal spermiation and spermiation failure induced by hormone suppression in adult rats. Biol Reprod 68:1299–1307

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