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Adhering Junction Dynamics in the Testis Are Regulated by an Interplay of $\beta 1$ -Integrin and Focal Adhesion Complex-Associated Proteins

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During spermatogenesis, the movement of developing germ cells across the seminiferous epithelium associates with extensive restructuring of cell-cell actin-based adherens junctions (AJs), such as ectoplasmic specialization (ES, a testis-specific AJ junction), between Sertoli and germ cells. Although this event of germ cell movement is essential to the completion of spermatogenesis, the mechanism(s) that regulates AJ restructuring is largely unknown. Using Sertoli-germ cells cocultured *in vitro* to study the regulation of AJ assembly, it was shown that this event associated with a transient induction of $\beta 1$ -integrin, vinculin, p-FAK-Tyr³⁹⁷, and phosphatidylinositol 3-kinase (PI3K) but not the nonphosphorylated form of focal adhesion kinase (FAK), paxillin, and p130 Cas. Furthermore, p-FAK-Tyr³⁹⁷ was shown to coimmunoprecipitate with $\beta 1$ -integrin, vinculin, and c-Src both *in vitro* and *in vivo* using Sertoli-germ cell cocultures and seminiferous tubules, respectively. These results seemingly suggest that the testis is using constituent proteins of the focal adhesion complex (FAC) found in other epithelia between cell and extracellular matrix to regulate AJ dynamics. To further confirm that p-FAK, a putative FAC protein in other epithelia, is indeed present at the site of ES, immunohistochemistry and immunofluorescent microscopy were used. The p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶ were found to localize almost exclusively at

the site of apical ES with weak staining at the basal ES in the seminiferous epithelium in a stage-specific manner, being highest at stages VI-VIII. In contrast, FAK was largely restricted to the basal compartment but with weak staining at the apical compartment. When rats were treated with 1-(2,4-dichlorobenzyl)-indazole-3-carbohydrazide (AF-2364) to perturb Sertoli-germ cell AJs, an induction of $\beta 1$ -integrin, perturb Sertoli-germ cell AJs, an induction of $\beta 1$ -integrin, vinculin, p-FAK-Tyr³⁹⁷, PI3K, and p130 Cas but not the non-phosphorylated form of FAK and paxillin was also detected in the testis, coinciding with the time spermatids began to deplete from the epithelium, indicating their involvement in AJ disassembly. Thereafter, the levels of vinculin, p-FAK-Tyr³⁹⁷, PI3K, and p130 Cas in the testis plunged, coinciding with the declining events of AJ disruption when virtually all spermatids were depleted from the epithelium. Taken collectively, these results suggest a bifunctional role of p-FAK, being involved in the events of Sertoli-germ cell AJ assembly and disassembly. In summary, the events of AJ dynamics in the testis, in particular at the site of ES, are regulated, at least in part, by proteins that are found in the FAC in other epithelia, such as $\beta 1$ -integrin, vinculin, and FAK utilizing the integrin/pFAK/PI3K/p130 Cas signaling pathway. (*Endocrinology* 144: 2141-2163, 2003)

DURING SPERMATOGENESIS, PRELEPTOTENE and leptotene spermatocytes must translocate across the blood-testis barrier (BTB), which is formed by the inter-Sertoli tight junctions (TJs) near the basal lamina, entering into the adluminal from the basal compartment of the seminiferous epithelium for further development (for reviews, see Refs. 1 and 2). This timely movement of developing germ cells is essential for spermatogenesis; it also associates with extensive restructuring of the actin-based cell-cell, intermediate filament-based cell-cell, and actin-based cell-matrix ad-

hering (or anchoring) junctions, also known as adherens junctions (AJs), desmosome junctions, and focal contacts in other epithelia, respectively (for reviews, see Refs. 1-3). Ectoplasmic specializations (ESs) are specialized actin-based cell-cell AJs unique to the testis. They are found in Sertoli cells at the sites in which developing and mature spermatids attach (apical ES) and between Sertoli cells at the basal compartment (basal ES; Refs. 1, 4, and 5). The turnover of basal and apical ES are essential for the movement of spermatocytes across the seminiferous epithelium (6), and the movement of spermatids and the release of sperm into the tubular lumen at spermiation (7), respectively.

Several proteins have been identified at the site of ES in the testis, which include $\alpha 6 \beta 1$ integrin (8, 9), vinculin (10), α -actinin (11), fimbrin (10), espin (12), myosin VIIa (13), c-Src (14), Csk (14), integrin-linked kinase (ILK) (15), gelsolin (16), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂; Ref. 16), phosphoinositide-specific phospholipase C (16), Fyn, a member of the Src family protein tyrosine kinase (17), Keap1 (18), and testin (19, 20). Yet little is known about these molecules regarding their function and regulation in the testis. In this report, we have investigated the roles of $\beta 1$ -integrin

Abbreviations: AF-2364, 1-(2,4-Dichlorobenzyl)-indazole-3-carbohydrazide; AJ, adherens junction; BTB, blood-testis barrier; Csk, protein tyrosine kinase that phosphorylates src family kinases; ES, ectoplasmic specialization; F12, Ham's F12 nutrient mixture; FAC, focal adhesion complex; FAK, focal adhesion kinase; FITC, fluorescein isothiocyanate; GCCM, germ cell-conditioned medium; GJ, gap junction; ILK, integrin-linked kinase; IP, immunoprecipitation; M_r, relative molecular mass; NP-40, Nonidet P-40; PI3K, phosphatidylinositol-3-kinase; p130 Cas, protein encoded by Crkas gene also called Crk-associated protein; PMSE, phenylmethylsulfonyl fluoride; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; SH2, Src homology 2 domain; Src, a protein tyrosine kinase of the transforming gene of Rous sarcoma virus (besides Src, this family includes Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk, and Yrk proteins); TJ, tight junction.

and vinculin in AJ dynamics using Sertoli-germ cell cocultures *in vitro*. These proteins were selected because they are known to colocalize to the actin filament bundles at the site of ES in a stage-dependent manner (9, 15, 21) and are putative constituent proteins of ES. Recent studies have shown that 1-(2,4-dichlorobenzyl)-indazole-3-carbohydrazide (AF-2364) is a potential male oral contraceptive that can induce germ cell loss from the seminiferous epithelium possibly by disrupting the Sertoli-germ cell AJs, such as ES, in the rat testis, without affecting the hypothalamus-pituitary-testicular axis (22, 23) and AJ structures in other epithelia (for review, see Ref. 1). Also, this compound is neither nephrotoxic nor hepatotoxic (1, 22, 23) unlike its analog, lonidamine [1-(2,4-dichlorobenzyl)-indazole-3-carboxylic acid], which is known to disrupt the stress fibers and microfilament network in Sertoli cells when administered *in vivo* by gavage but is toxic (for reviews, see Refs. 1 and 24). As such, this AF-2364-induced germ cell loss is being used as a model to study the cascade of events leading the disassembly of Sertoli and germ cell AJs both *in vivo* and *in vitro*.

Studies by immunohistochemistry using a specific phospho-Tyr antibody have shown intensive staining at the site of ES (14, 15), indicating that at least some molecules at this site can be tyrosine phosphorylated. There are also reports illustrating focal adhesion kinase (FAK) can become tyrosine phosphorylated in cells upon integrin clustering or during integrin-mediated cell adhesion (for reviews, see Refs. 25–28). Activation of FAK occurs during its interaction with the cytoplasmic tail of the clustered $\beta 1$ -integrin (for review, see Ref. 26). On activation, autophosphorylation of FAK at Tyr³⁹⁷ takes place; this in turn creates a binding site for the Src homology 2 (SH2) domain of Src or Fyn (29, 30) and other effector molecules, such as phosphatidylinositol-3-kinase (PI3K; Ref. 31). The coupling of Src family protein tyrosine kinases to this FAK/Src complex can further modify FAK by inducing phosphorylation of FAK at Tyr⁵⁷⁶ and Tyr⁵⁷⁷ in the kinase domain activation loop, which enhances its catalytic activity (32), or else at Tyr⁹²⁵, which creates a binding site for the adaptor Grb2 SH2 domain (33). Besides, Src kinases can phosphorylate other focal adhesion-associated substrates, such as paxillin (34) and p130 Cas (35, 36). Treatment of cells with cytochalasin D, also known to disrupt actin filaments in ES (37, 38), that disrupts the actin cytoskeleton can also inhibit the phosphorylation of FAK (39, 40). It is therefore logical to speculate that $\beta 1$ -integrin at the site of ES activates FAK, leading to changes in Sertoli-germ cell AJ dynamics.

To test this hypothesis, we have investigated: 1) the protein levels of FAK, p-FAK-Tyr³⁹⁷, PI3K p85 α paxillin, and p130 Cas during Sertoli-germ cell AJ assembly and disassembly both *in vitro* and *in vivo*; 2) the localization of FAK, p-FAK-Tyr³⁹⁷, and p-FAK-Tyr⁵⁷⁶ in the seminiferous epithelium from normal and AF-2364-treated rats; 3) colocalization of p-FAK-Tyr³⁹⁷ with vinculin, a putative ES-associated protein (10, 15, 21), FAK, or ZO-1, a putative TJ-associated protein (41) in the seminiferous epithelium by immunofluorescent microscopy; and 4) the constituents of the molecular complex that is present at the site of ES by immunoprecipitation using an anti-p-FAK-Tyr³⁹⁷ antibody. These results suggest that the dynamics of ES, a testis-specific, cell-cell actin-based AJ structure, are regulated by some of the same component

proteins, such as FAK and $\beta 1$ -integrin, that are found in focal adhesion complex (FAC) in other epithelia at the site of cell-matrix focal contacts.

Materials and Methods

Animals

Male Sprague Dawley rats ranging between 1 and 120 d of age were obtained from Charles River Laboratories, Inc. (Kingston, MA). Rats were killed by CO₂ asphyxiation, and testes were removed immediately for the isolation of testicular cells or for RNA extraction. The use of animals for studies described herein was approved by the Rockefeller University Animal Care and Use Committee with protocol no. 97117 and 00111.

Preparation of testicular cells and spent media

Sertoli cell cultures. Sertoli cells were isolated from 20-d-old rats as previously described (19, 42). At low cell density (5×10^4 cells/cm²), inter-Sertoli TJs could not form because of the lack of close proximity between cells rendering them incapable of assuming the columnar shape, which is common in Sertoli cells when they form TJs *in vitro* (42–44). Nonetheless, both AJs and communicating gap junctions (GJs) were present (19, 45). Isolated cells were plated at 5×10^4 cells/cm² in 100-mm Petri dishes in 9 ml serum-free Ham's F12 nutrient mixture and DMEM (F12/DMEM, 1:1, vol/vol; $\sim 4.5 \times 10^6$ cells/9 ml/100-mm dish) supplemented with 15 mM HEPES, 1.2 g/liter sodium bicarbonate, 10 μ g/ml bovine insulin, 5 μ g/ml human transferrin, 2.5 ng/ml epidermal growth factor, 20 mg/liter gentamicin, and 10 μ g/ml bacitracin. At high cell density (0.5 or 1×10^6 cells/cm²), all three types of junctions (TJs, AJs, and GJs) were formed. In some experiments, cells were plated on Matrigel-coated (Collaborative Biochemical Products, Bedford, MA; diluted 1:7 with F12/DMEM, vol/vol) 12-well dishes (Corning, Inc., Corning, NY) at a density of 1×10^6 cells/cm² as previously described (19, 45, 46). These cultures were incubated in a humidified atmosphere of 95% air and 5% CO₂ (vol/vol) at 35 C. Unless specified otherwise, time 0 represents Sertoli cell cultures that were terminated approximately 3 h after plating. After 48 h of incubation, cultures were hypotonically treated with 20 mM Tris (pH 7.4) for 2.5 min to lyse the residual germ cells (47), to be followed by two successive washes with F12/DMEM to remove germ cell debris. Media were replaced every 24 h. For adult Sertoli cell cultures, Sertoli cells were isolated from rats at 45 and 90 d of age with a purity of approximately 95% as previously described (48). The purity of these Sertoli cell cultures were analyzed microscopically (48, 49) and by RT-PCR using primer pairs specific to markers of Leydig cells (3β -hydroxysteroid dehydrogenase), germ cells (c-kit receptor), and peritubular myoid cells (fibronectin; Ref. 50).

Germ cell cultures. Total germ cells were isolated from 90-d-old rats by a mechanical procedure as previously described (51). Sequential filtrations were performed to remove cellular debris, spermatozoa, and elongated spermatids. When the final preparation was analyzed by DNA flow cytometry as previously described (51) and direct microscopic examination (51), it consisted largely of spermatogonia (17%), spermatocytes (18%), and round (prestep 8; 57%) and elongating (step 8) spermatids (8%). These germ cells had a purity of greater than 95% with negligible somatic cell contamination when examined microscopically and assessed by other criteria, such as RT-PCR to amplify testin; a known Sertoli cell product (51–53); c-kit receptor; a spermatogonium product (54); 3β -hydroxysteroid dehydrogenase; a Leydig cell product (55); and fibronectin, a peritubular myoid cell product (56). The cell number of freshly isolated germ cells was determined by a hemocytometer. The desired cell density was obtained by reconstituting cells in F12/DMEM, supplemented with 6 mM sodium lactate, 2 mM sodium pyruvate, 20 mg/liter gentamicin, and 10 μ g/ml bacitracin and were used within 1 h for coculture experiments. In selected experiments (see Fig. 7B, lower panel), elongated spermatids were not removed by omitting the glass wool filtration steps.

To assess changes in target gene expression in germ cells during maturation, germ cells were also isolated from rat testes at 10, 15, 20, 45, 60, 90, and 200 d of age as described above. Freshly isolated germ cells

were terminated by RNA STAT-60 (Tel-Test Inc., Friendswood, TX) for RNA extraction.

Preparation of germ cell-conditioned medium (GCCM). For the preparation of GCCM, freshly isolated germ cells were cultured at 0.3×10^6 cells cm^{-2} in a 100-mm dish (22.5×10^6 cells \times 9 ml/100-mm dish) and incubated in a humidified atmosphere of 95% air and 5% CO_2 (vol/vol) at 35°C for 16 h. Spent media were collected, centrifuged at $800 \times g$ for 1 h to remove residual germ cells, followed by $3000 \times g$ for 1 h to remove cellular debris and designed GCCM. GCCM were concentrated by a Minitan tangential ultrafiltration unit (Millipore Corp., Bedford, MA) equipped with eight Minitan plates [relative molecular mass (M_r) cut-off at 10 kDa], and filtered through 0.2- μm filter units. The protein content was estimated by Coomassie blue dye-binding assay using BSA as a standard (57). To study the effects of GCCM protein on Sertoli cell target gene expression, GCCM were incubated with Sertoli cells cultured alone for 5 d at 5×10^4 cells cm^{-2} in a 100-mm dish ($\sim 4.5 \times 10^7$ cells \times 9 ml/medium per dish) at a concentration of 1, 50, 500, and 1500 μg protein/ml for 2 h before termination for RNA extraction.

Preparation of nonviable germ cells. To analyze the effects of nonviable germ cells on Sertoli cell $\beta 1$ -integrin and vinculin expression, germ cells isolated from adult rat testes were stored at 4°C for approximately 24 h in F12 DMEM with 200 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM EDTA. These nonviable cells, confirmed by erythrosine red dye exclusion test (58), were washed twice in F12 DMEM by centrifugation at $800 \times g$, 10 min each, and layered onto the Sertoli cell (4.5×10^4 cells/9 ml F12 DMEM per 100-mm dish) monolayer that had been cultured alone for 5 d using a germ:Sertoli cell ratio of 1:1 and terminated at specified time points.

Sertoli-germ cell cocultures. To assess changes in target gene expression during the assembly of Sertoli-germ cell AJs, freshly isolated Sertoli cells as described above were first plated at high cell density (0.5×10^6 cells cm^{-2}) on Matrigel-coated 12-well dishes. These cells were cultured alone for 5 d to allow the establishment of a cell epithelium with TJ, GJ, AJ, and desmosome-like junctions (45) and the endogenous target gene expression to subside before their use for coculture experiments because cell-substratum structures were also formed in these cultures. Freshly isolated germ cells as described above were added onto this cell epithelium on d 6 and cocultured using a Sertoli:germ cell ratio of either 1:1, 1:3, or 1:5 to initiate Sertoli-germ AJ. [Although the assembly of desmosome-like junctions also take place in these cultures (59, 60), we limited our discussion on only AJ assembly throughout the text because we had not included any desmosome-like junction protein markers in our investigation because none of the desmosome junction proteins found in other epithelia, such as desmocollins, desmogleins, desmoplakin, and plakophilin, have been positively identified in the testis (for reviews, see Refs. 1 and 3-5).]

Earlier studies by electron microscopy have shown that anchoring junction structures, such as desmosome-like junctions, are found between Sertoli and germ cells (up to step 7 round spermatids) within 24-48 h when these cells were cocultured in serum-free media *in vitro* (59, 60). The presence of functional AJ structures in these cocultures was subsequently characterized in our laboratory by light and fluorescent microscopy and other biochemical analysis (45, 50, 61). The isolated germ cells used in the studies reported herein contained up to step 8 spermatids as earlier described (51, 58), and ultrastructures similar to ES found *in vivo* had been detected between Sertoli cells and step 8 spermatids *in vitro* (60). This is not entirely surprising because ES can be formed between Sertoli cells and steps 7-8 round spermatids *in vitro* (62, 63). Furthermore, current investigations in our laboratory by immunofluorescent microscopy have also identified espin, a putative ES-specific marker (12, 62), in these cocultures (Mruk, D. D., and C. Y. Cheng, unpublished observations). In addition, recently completed electron microscopy study using Sertoli-germ cell cocultures from our laboratory performed at the Rockefeller University BioImaging Resource Center has conclusively identified functional ES structure at the ultrastructural level consistent with earlier published reports (Refs. 59 and 60 and Siu, M. K. Y., and C. Y. Cheng, unpublished observations). Taken collectively, these results clearly illustrate functional AJ structures, such as actin-based ES, are present in the Sertoli-germ cell cocultures used in our studies as reported herein.

Cocultures were terminated at specific time points by RNA STAT-

60™ (Tel-Test) for RNA extraction or lysed by 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 PMSF, 1% 2-mercaptoethanol (vol/vol), 1 mM sodium orthovanadate (a protein tyrosine phosphatase inhibitor), and 0.1 M sodium okadaate (a protein Ser/Thr phosphatase inhibitor)] under reducing conditions for immunoblotting. For immunoprecipitation experiments, lysates of cocultures were obtained by using an immunoprecipitation (IP) buffer containing Nontidet P-40 (NP-40) instead of SDS and mercaptoethanol [0.125 M Tris, pH 6.8, at 22°C containing 1% NP-40 (vol/vol), 2 mM EDTA, 2 mM N-ethylmaleimide, 2 mM PMSF, 1 mM sodium orthovanadate, and 0.1 M sodium okadaate] to permit antigen-antibody interactions. In selected experiments (see Fig. 7B, later panel), total germ cells with elongated spermatids were also used for the Sertoli-germ cell coculture experiments in which the glass wool filtration steps were omitted. These cocultures were then processed for immunoprecipitation on d 2 using an anti-FAK-Tyr³⁹⁷ as described below.

Seminiferous tubule cultures and lysate preparation

Seminiferous tubules were isolated from testes of adult rats (~ 300 g body weight) by enzymatic treatment as earlier described from this laboratory with negligible Leydig cell contamination (64). Tubules virtually freed of interstitial cell contamination were then trimmed into approximately 2-mm fragments and incubated for 36-48 h at 35°C in F12 DMEM with insulin ($2 \mu\text{g}/\text{ml}$), transferrin ($2 \mu\text{g}/\text{ml}$), gentamicin ($100 \mu\text{g}/\text{ml}$), and penicillin ($10^3 \text{ IU}/\text{ml}$) in a final volume of tubules from one testis/25 ml F12 DMEM. Thereafter, lysates were obtained from seminiferous tubules by treating tubules with the IP buffer [0.125 M Tris, pH 6.8, at 22°C containing 1% NP-40 (vol/vol), 2 mM EDTA, 2 mM N-ethylmaleimide, 2 mM PMSF, 1 mM sodium orthovanadate, and 0.1 M sodium okadaate] using an IP buffer tissue ratio of 1:1, vortexed for 30 sec, sonicated (two times, 5 sec each interspaced by 2 sec) with samples incubated in melting mice at 4°C, centrifuged at $15,000 \times g$ for 20 min to remove pellets. The clear supernatant was used as seminiferous tubule lysates.

Treatment of rats with AF-2364 to perturb Sertoli-germ cell AJs leading to germ cell loss from the seminiferous epithelium

Treatment of rats with AF-2364 by gavage. AF-2364 was synthesized as previously described from this laboratory with a purity of greater than 99.8% when assessed by elemental analysis, nuclear magnetic resonance, HPLC, and mass spectrometry (23). This compound was shown to perturb Sertoli-germ cell adhesion function in the testis inducing premature loss of germ cells from the seminiferous epithelium in rats, causing reversible infertility in treated animals (22, 23). Yet AF-2364 is neither nephrotoxic nor hepatotoxic (22, 23) and recent mutagenicity and acute toxicity studies in mice and rats were completed, conducted by licensed toxicologists and have shown that it is safe for further development (for review, see Ref. 1). The apparent site by which AF-2364 exerts its action is one of the multiprotein complexes composed of at least integrin-testin-cadherin in the testis-specific ES (for review, see Ref. 1). Adult rats weighing between 25 and 300 g were fed with one dose of AF-2364 at 50 mg/kg body weight, which is known to perturb Sertoli-germ cell AJs inducing germ cell loss in particular round and elongated spermatids from the seminiferous epithelium. The time when rats were administered with AF-2364 was designated time 0 (control). Thereafter, rats were housed separately for 8 d. Testes were removed at a specified time point with a group of three rats used for each time point. For immunoblotting, testes were homogenized using a TissueLyser (Tekmar, Cincinnati, OH) for protein extraction either by SDS lysis buffer (immunoblotting) or by IP buffer. For immunohistochemistry, testes were immediately frozen in liquid nitrogen and stored at -80°C until sectioning in a cryostat.

Treatment of Sertoli-germ cell cocultures with AF-2364. Sertoli cells (0.5×10^6 cells cm^{-2}) isolated from 2-d-old rats were cultured for 5 d to form an epithelium with TJs and AJs. On d 6, germ cells isolated from 90-d-old rats were added onto this cell epithelium using a Sertoli:germ cell ratio of 1:1 and were cocultured for an additional 2 d. On d 8, Sertoli-germ cell cocultures were incubated with either vehicle (ethanol) or different

doses of AF-2364 (1, 50, and 500 ng/ml) and terminated at specified time points.

Treatment of Sertoli cells with AF-2364 and lonidamine [1-(2,4-dichlorobenzyl)-indazole-3-carboxylic acid]. Sertoli cells (0.5×10^6 cells/cm²) isolated from 20-d-old rats as described above were cultured for 8 d alone and then incubated with 1, 50, and 500 ng/ml AF-2364 for 1 h and terminated thereafter. For lonidamine treatment, Sertoli cells were cultured for 2 d and then incubated with 1, 50, and 500 ng/ml lonidamine for 24 h and terminated thereafter.

Treatment of germ cells with AF-2364. Germ cells (22.5×10^6 cells/9 ml per 100-mm dish) isolated from 90-d-old rats were incubated immediately with 1, 50, and 500 ng/ml AF-2364 for 1 h and terminated thereafter.

Semiquantitative RT-PCR

Total RNA was isolated from tissues or cells by RNA STAT-60 (Tel-Test), and RNA concentration was quantified by spectrophotometry at 260 nm using an RNA/DNA calculator (model GeneQuant II, Pharmacia Biotech, Uppsala, Sweden). Semiquantitative RT-PCR was performed essentially as previously described (45, 46, 65, 66). To enhance the detection sensitivity and to yield semiquantitative data for analysis and comparison after densitometric scanning of the resultant autoradiograms, a trace amount of [γ -³²P]-labeled primers were also included in the RT-PCR tubes. Briefly, the sense primers of a target gene and S16 were labeled at the 5'-end with [γ -³²P]-dATP (specific activity, 6000 Ci/mmol, Amersham) using T₄ polynucleotide kinase (Promega Corp., Madison, WI). Approximately, 1×10^6 cpm were used per PCR and the ratio of the [γ -³²P]-labeled sense primer of a target gene to [γ -³²P]-labeled S16 was the same as the unlabeled primers.

To ensure the linearity of a target gene and S16 during their amplification, 10- μ l aliquots of PCR products at 18, 20, 22, 24, and 26 cycles were withdrawn and resolved onto 5% T polyacrylamide gels using 0.5 \times TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) as a running buffer in preliminary experiments. Also different concentrations of primer pairs, reverse transcription products and annealing temperatures were used in preliminary experiments for each target gene to ensure its production and S16 in each PCR experiment were in linear phase. Because of the disparity between the endogenous levels of a target gene and S16, the linear phase of the housekeeping gene, such as S16, was close to its saturation phase. For the target gene, its linearity was at its exponential phase in the PCR. It is because of this disparity issue we had made every effort to include results of immunoblot analysis to verify data of RT-PCR. Furthermore, the additional protein analysis ensures that the detected changes in mRNA levels indeed translate into functional proteins, which are the effectors to induce any phenotypic and/or cellular changes. The PCR products were visualized by ethidium bromide staining and autoradiography was performed using X-OMAT AR film (Eastman Kodak Co., Rochester, NY) after gels were dried. The authenticity of the PCR products for β 1 integrin, vinculin, and S16 (Table 1) was confirmed by direct nucleotide sequencing as previously described (46, 61).

Immunoblotting

Total Sertoli-germ cell lysates were obtained from cocultures plated in 12-well dishes at specified time points. Briefly, spent media were removed from each well, and 1 ml SDS lysis buffer was added to the remaining cells. The solubilized cell suspension was transferred to a microfuge tube, vortexed, incubated at room temperature for 5–10 min,

and centrifuged at $15,000 \times g$ to remove cellular debris. The clear supernatant was used as whole-cell lysate. Protein content was estimated by Coomassie blue dye-binding assay using BSA as a standard (57). Equal amounts of proteins ($\sim 100 \mu$ g/lane) were resolved onto 7.5% or 10% T SDS-polyacrylamide gels by SDS-PAGE under reducing conditions (67) and electroblotted onto nitrocellulose papers. Membranes were blocked with 6% nonfat dry milk (Nestle, Solon, OH) in PBS-Tris (10 mM sodium phosphate, 0.15 M NaCl, 10 mM Tris, pH 7.4 at 22 C) containing 0.1% Tween 20 (vol/vol). For immunoblotting, the following primary antibodies were used: rabbit anti- β 1-integrin (catalog no. sc-8978, lot no. B221), rabbit anti-FAK (catalog no. sc-558, lot no. J051), and mouse anti-P13K p85 α (catalog no. sc-1637, lot no. J101) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse antivinulin (catalog no. V9131, lot no. 70K4877) was from Sigma (St. Louis, MO). Rabbit anti-p-FAK-Tyr³⁹⁷ (catalog no. 07-012, lot no. 21019) and rabbit anti-p-FAK-Tyr⁵⁷⁶ (catalog no. 07-157, lot no. 20262) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse antipaxillin (catalog no. P13520, lot no. 16) and mouse anti-p130 Cas (catalog no. P27820, lot no. 7) were from Transduction Laboratories, Inc. (Lexington, KY). These antibodies were known to cross-react with the corresponding target proteins in rats as indicated by the manufacturers.

After the primary antibody incubation, membranes were incubated with either one of the following secondary antibodies depending on the source of the primary antibody. These include goat antirabbit IgG-horseradish peroxidase (catalog no. sc-2004, lot no. G091) and goat antimouse IgG-horseradish peroxidase (catalog no. sc-2005, lot no. H231), which were purchased from Santa Cruz Biotechnology. The blots were then developed with an enhanced chemiluminescence system using a kit from Amersham Pharmacia Biotech (Piscataway, NJ). We have listed both the catalog and lot numbers for each specific antibody used in this report because preliminary experiments have shown that several antibodies from other vendors failed to yield satisfactory results.

IP

About 500 μ g protein of whole-cell lysates of Sertoli-germ cell cocultures (in ~ 250 – 500μ l sample size) terminated on d 2 after addition of germ cells onto the Sertoli cell epithelium and seminiferous tubules after 36–48 h in culture, and lysates of adult rat testes were pretreated by incubating with 5 μ l normal rabbit serum for 3 h at room temperature to be followed by 20 μ l protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 1 h to eliminate proteins in the lysates that would otherwise nonspecifically bind to rabbit serum proteins and subsequently bound to protein A/G PLUS-agarose. The lysates were then centrifuged at $1000 \times g$ for 5 min to pellet the agarose beads and supernatant was collected. Two microgram of either anti-FAK or anti-p-FAK-Tyr³⁹⁷ antibody were added to the supernatant and incubated overnight. Thereafter, 20 μ l protein A/G PLUS-agarose was added to the lysates and incubated for 4 h. The samples were centrifuged at $1000 \times g$ for 5 min to collect pellet. Supernatant was discarded and the immune complexes were washed four times with IP buffer. After the final wash, the immunocomplexes were resuspended in 50 μ l SDS-PAGE sample buffer [0.125 M Tris, pH 6.8 at 22 C containing 1% SDS (wt/vol), 20% glycerol, 1.6% 2-mercaptoethanol (vol/vol)] and heated for 10 min at 100 C to extract the proteins. Beads were pelleted by centrifugation and supernatant was collected and resolved by SDS-PAGE using a 7.5% T polyacrylamide gel under reducing conditions and were immunoblotted with mouse anti-p-Tyr (p-Tyr-100; catalog no. 9411) (Cell Signaling Technology, Inc., Beverly, MA) or anti-FAK antibody (for FAK immunoprecipitation experiment); and anti- β 1-integrin, anti-vinculin, anti-c-

TABLE 1. Primers used for semiquantitative RT-PCR to assess the steady-state mRNA levels of β 1-integrin, vinculin, and S16

Target gene	Primer sequence	Orientation	Position	Length (bp)	Annealing temp. used (C)	Ref.
β 1-Integrin	5'-GAGGAGGATTACTTCAGAC-3'	Sense	519–537	303	58	81
	5'-AGCAGTCGTGTTACATTTC-3'	Antisense	804–821			
Vinculin	5'-CTGGTGGACGAGGCTAT-3'	Sense	2134–2150	290	59	82
	5'-ATGTTTCCAGCCACAGC-3'	Antisense	2407–2423			
S16	5'-TCCGCTGCAGTCCGTTCAAGTCTT-3'	Sense	15–38	385	58–59	83
	5'-GCCAAACTTCTGGATTTCGACGC-3'	Antisense	376–399			

Src (catalog no. sc-8056, lot no. C051; Santa Cruz Biotechnology), or p-FAK-Tyr³⁹⁷ antibody (for p-FAK-Tyr³⁹⁷ immunoprecipitation experiment). Whole-cell lysates of Sertoli-germ cell cocultures isolated on d 2, and testicular lysates and/or seminiferous tubular lysates without incubation with antibodies or with incubation with normal rabbit serum were used as negative controls.

Immunohistochemistry

Immunohistochemistry was performed to localize FAK and p-FAK in the seminiferous epithelium of normal and AF-2364-treated rat testes essentially as previously described (68–70) using Histostain SP kits (catalog no. 95-6143) from Zymed Laboratories, Inc. Corp. (Burlingame, CA). Briefly, animals were killed by CO₂ asphyxiation. Testes were removed immediately, embedded in O.C.T. compound (Miles Scientific, Elkhart, IN), and frozen in liquid nitrogen. All tissue blocks were stored at –80°C until used. Frozen sections (8 μm thick) were cut at –20°C with a disposable blade in a cryostat (Hacker, Fairfield, NJ) and mounted on poly-L-lysine (Sigma, M_r > 150 kDa)-coated glass slides. Sections were air dried at room temperature, fixed in modified Bouin's fixative for 5–10 min, and washed thoroughly with PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 at 22°C). Streptavidin-biotin peroxidase immunostaining was performed as follows. Briefly, fixed sections were treated with 3% hydrogen peroxide in methanol for 5 min to block endogenous peroxidase activity. To minimize the nonspecific antibody binding, sections were incubated with a serum blocking solution (Zymed Laboratories, Inc. Corp.) or 10% nonimmune goat serum. Sections were then incubated with primary antibodies in a moist chamber at 35°C overnight. Primary antibodies were used with the following dilution: rabbit anti-FAK (1:50) to 1:100, rabbit anti-p-FAK-Tyr³⁹⁷ (1:100 to 1:250), and rabbit anti-p-FAK-Tyr⁵⁷⁷ (1:20 to 1:150). Sections were washed thoroughly with PBS and incubated with biotinylated goat antirabbit IgG for 30 min and then with the streptavidin-peroxidase conjugate for 10 min. Sections were treated with the aminoethylcarbazole mixture (substrate-chromogen mixture) for 5–10 min. Sections were counterstained in hematoxylin and mounted. Sections were examined and photographed in a BX-40 (Olympus Corp., Melville, NY) using planapochromat ×10, ×20, and ×40 objectives and an 82A blue filter.

All micrographs were digitally acquired using a digital imaging camera (Olympus Corp.) interfaced to a Macintosh G4 computer running under Mac OS 9.22 and analyzed by Adobe Photoshop (version 7.0). At least 50–100 sections were examined from each testis and at least three rats were used per time point in each experimental set. Controls consisted of: 1) sections incubated with PBS instead of the specific primary antibody; 2) sections incubated with normal rabbit serum at the same dilution as of the specific primary antibody but omitting the primary antibody incubation; 3) the secondary antibody replaced with normal rabbit serum; and 4) sections incubated with the primary antibody that had been preabsorbed with lysates of seminiferous tubules or control peptides provided by the manufacturer (such as Santa Cruz Biotechnology). Control and experimental slides were immunostained simultaneously in the same experiment session. For preabsorbed controls, approximately 500 μg protein of seminiferous tubule lysate (or 5–10 μg blocking peptide if available from the antibody vendor) in 10 μl immunoprecipitation buffer (see above) was added to 200 μl diluted rabbit anti-p-FAK-Tyr³⁹⁷, rabbit anti-p-FAK-Tyr⁵⁷⁷, or rabbit anti-FAK and incubated overnight at 4°C with agitation before it was used for immunostaining. In all experiments, control slides yielded nondetectable staining illustrating specificity of the staining results.

Immunofluorescent microscopy for colocalization of p-FAK, vinculin, ZO-1, and FAK to the ES in the seminiferous epithelium of rat testes

To confirm results of the immunoprecipitation experiments that p-FAK, the phosphorylated (activated) form of FAK, indeed associates with the apical ES at the adluminal compartment of the seminiferous epithelium, immunofluorescent microscopy was performed essentially as previously described for testin (20) and cadherin-catenin (50) from this laboratory. Double-fluorescent probes, namely fluorescein isothiocyanate (FITC) and Cy3, were used to colocalize p-FAK with vinculin [a putative ES-associated protein (10, 15, 21), which served as a positive control], ZO-1 [a TJ-associated protein in the testis (41), which served as

a negative control], or FAK in the same tissue sections. The colocalization of p-FAK with vinculin to the same site of apical ES in the seminiferous epithelium will strengthen results of the immunoprecipitation and immunohistochemistry experiments, indicating that the phosphorylated form of FAK is indeed associated with the ES structure. Briefly, testes removed from adult rats were embedded in O.C.T. compound (Miles Scientific) and frozen in liquid nitrogen. Frozen sections of testes (8 μm thick) were cut at –20°C in a cryostat and mounted on poly-L-lysine (M_r > 150 kDa)-coated glass slides. Sections on slides were placed at 4°C for 10 min and then air dried at room temperature and fixed in modified Bouin's solution (4% formaldehyde in picric acid). Sections were then treated with 3% H₂O₂ in methanol to block endogenous peroxidase activity, followed by 10% nonimmune goat serum to minimize nonspecific antibody binding as earlier described (2, 51, 68–70).

Sections were subsequently incubated with a rabbit anti-p-FAK-Tyr³⁹⁷ antibody (Upstate Biotechnology, Inc. catalog no. 17-12, lot no. 21019) followed by a goat-antirabbit IgG-FITC (Zymed Laboratories, Inc. Corp., catalog no. 62-6111, lot no. 1-66552). Thereafter, sections were washed in PBS (10 mM sodium phosphate, 15 M NaCl, pH 7.4 at 22°C) and incubated with a mouse anti-vinculin antibody (Sigma, catalog no. V9131, lot no. 70K4877) or a mouse anti-FAK antibody (Transduction Laboratories, Inc., catalog no. 61-088, lot no. 21), followed by a goat antimouse IgG-Cy3 (Zymed Laboratories, Inc. Corp., catalog no. 81-6515, lot no. 11067429). For colocalization study for p-FAK and ZO-1, the first pair of primary and secondary antibody that was rabbit anti-p-FAK-Tyr³⁹⁷ antibody and goat-antirabbit IgG-Cy3 (Zymed Laboratories, Inc. Corp., catalog no. 81-6515, lot no. 1-66552), respectively. And the second antibody was mouse anti-ZO-1-FITC conjugate (Zymed Laboratories, Inc. Corp., catalog no. 35-111, lot no. 1-665662). Sections were then mounted in Vectashield (Vector Laboratories, Burlingame, CA) and fluorescent microscopy was performed using a BX40 microscope (Olympus Corp.) equipped with UPlanFL fluorescent optics (Olympus Corp.). All images were digitally acquired in Adobe Photoshop (version 7.0) and analyzed with Image-Pro Plus (version 4.5) software (Media Cybernetics, Silver Spring, MD) in a Compaq 5877 workstation running under Windows XP. Controls included: 1) sections incubate with normal rabbit serum instead of the primary antibody; 2) secondary antibody alone without the use of primary antibody; and 3) primary antibody preabsorbed with seminiferous tubule lysates or blocking peptide as described above. For all controls, they failed to yield detectable fluorescent staining, illustrating specificity of the antibody used for immunofluorescent microscopy.

Statistical analysis

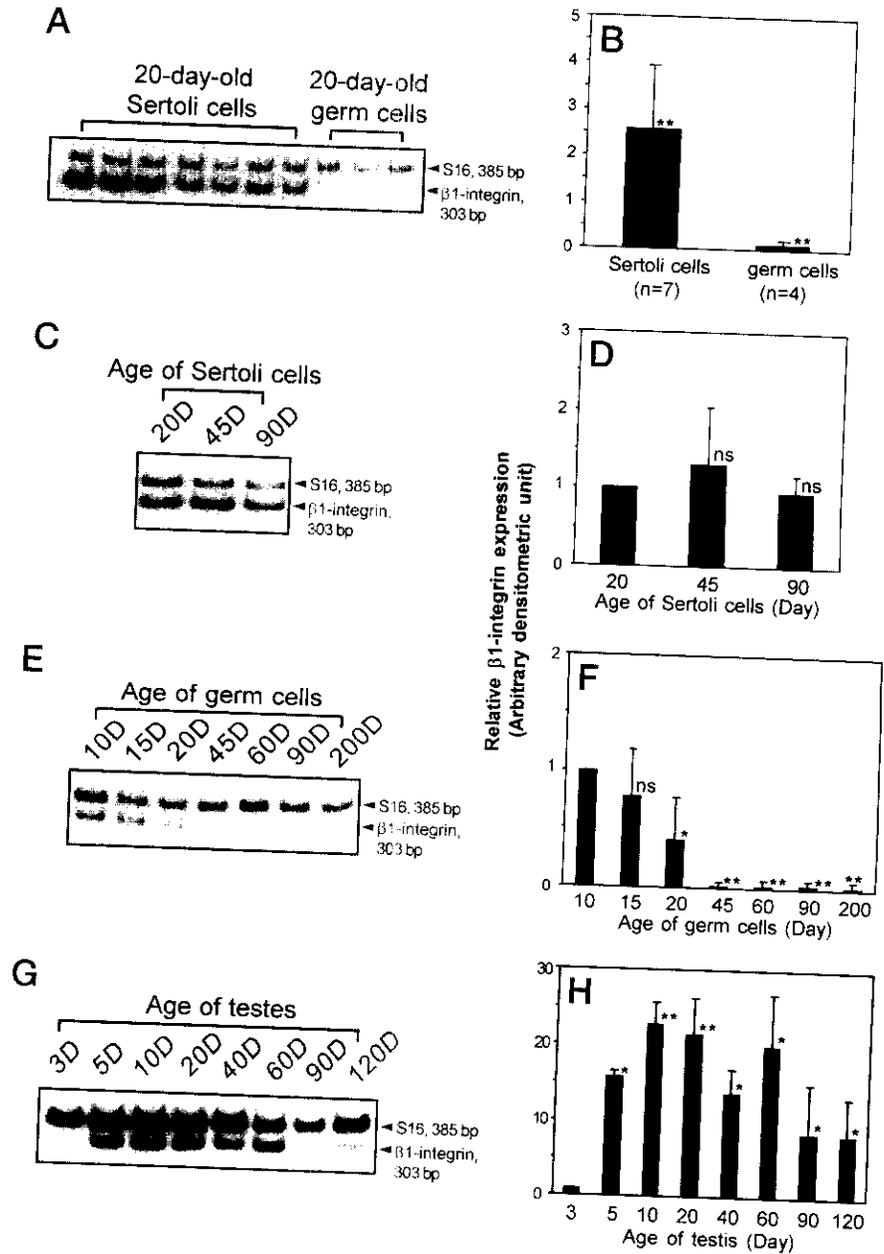
Multiple comparisons were performed using one-way ANOVA followed by Tukey's honestly significant difference test to compare selected pairs of experimental groups so that changes in the expression of a target gene at a selected time point between samples within an experimental group can be compared. In selected experiments, *t* test was also performed by comparing treatment groups with the corresponding controls. Statistical analysis was performed using the GR-STAT statistical analysis software package (version 7.0; Dynamic Microsystems, Inc., Silver Spring, MD).

Results

Relative expression of β1-integrin in Sertoli and germ cells and its developmental regulation in Sertoli and germ cells and the testis

It is noted that most of the β1-integrin in the seminiferous epithelium is contributed by Sertoli rather than germ cells in all ages examined except in prepubertal rats (Fig. 1, A–F). The steady-state mRNA level of β1-integrin remained relatively steady during Sertoli cell maturation (Fig. 1, C and D). Its expression by germ cells plummeted drastically and became almost undetectable after 20 d of age (Fig. 1, E and F). During testis maturation, the steady-state mRNA level of β1-integrin became clearly detectable at 5 d of ages coinciding with the initiation of spermatogonial proliferation at 3–6 d after birth

FIG. 1. A-H. Differential expression of β 1-integrin in Sertoli and germ cells, and changes on its steady-state mRNA level in these cells and testes during maturation. Total RNA was extracted from Sertoli cells, germ cells, or testes. Sertoli cells isolated from rats at specified ages were cultured *in vitro* for 3 d before termination so that germ cells could be removed by a hypotonic treatment on d 2 to eliminate RNA contributed by germ cells (see *Materials and Methods*). For germ cell preparations, cells were terminated soon after their isolation because more than 90% of germ cells became nonviable within 16 h of their isolation (51, 58). These germ cells, however, had negligible somatic cell contamination using various criteria to confirm their purity as described (50, 51). Semiquantitative RT-PCR was performed to assess changes in the steady-state mRNA level of β 1-integrin using primer pair-specific to this target gene and coamplified with S16. A, An autoradiogram showing the relative steady-state mRNA level of β 1-integrin in Sertoli and germ cells isolated from 20-d-old rat testes. C, E, and G, Autoradiograms showing the changes in steady-state β 1-integrin mRNA level in Sertoli and germ cells and testes during development, respectively. B, D, F, and H, Corresponding densitometrically scanned results using autoradiograms, such as those shown in A, C, E, and G, and normalized against S-16. Each bar represents a mean \pm SD of two to three experiments using different batches of cells or testes from three rats. Statistical analysis was performed by *t* test by comparing the steady-state mRNA level of β 1-integrin in either cells or testes at other ages *vs.* d 20 (D), 10 (F), or 3 (H), which was arbitrarily set at 1, except for results shown in (B) in which Sertoli cells were compared with germ cells and vice versa. *, Significantly different, $P < 0.05$; **, significantly different, $P < 0.01$; ns, Not significantly different.



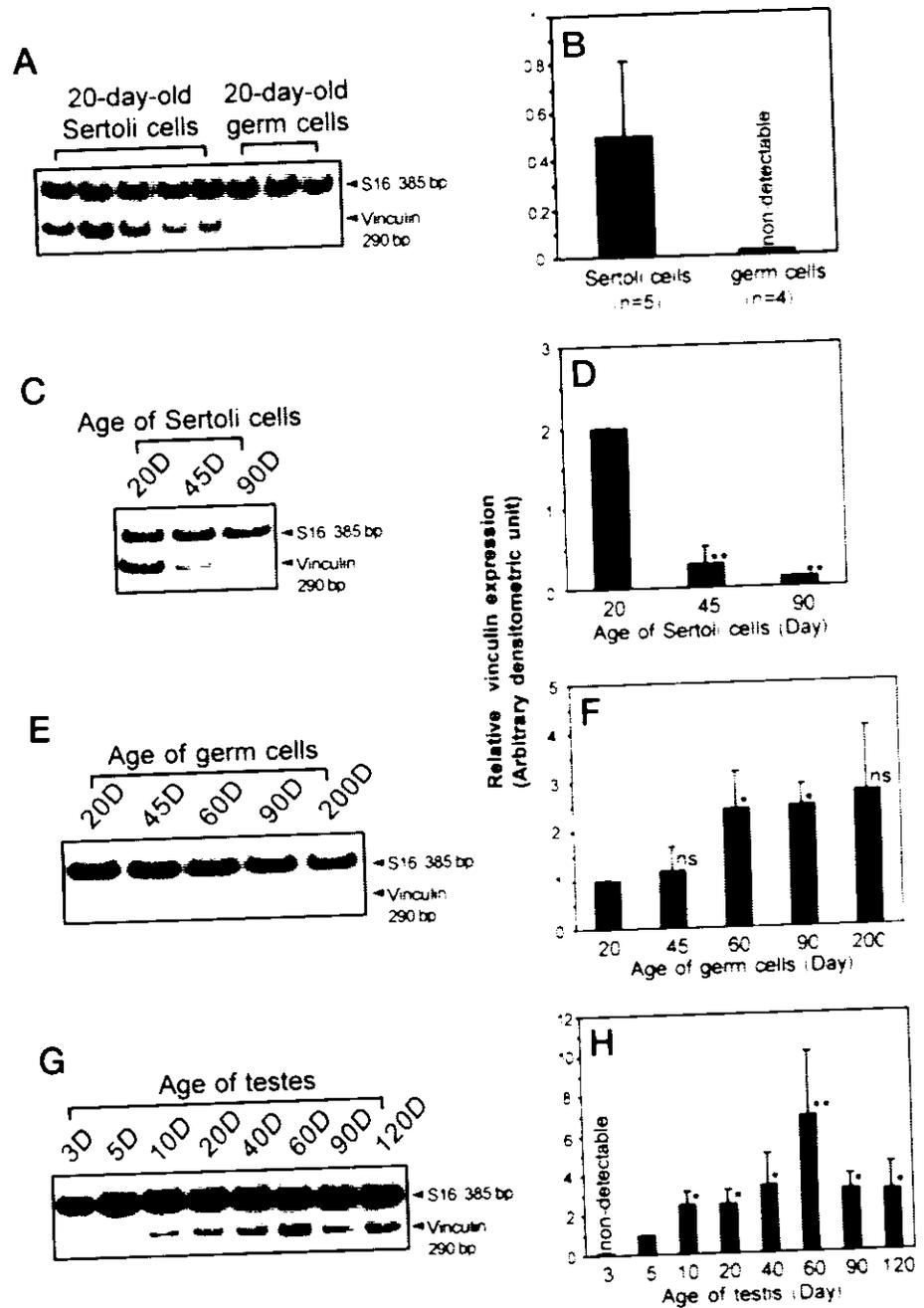
and peaked at 10–20 d of age coinciding with the assembly of the BTB, and by 90–120 d of age, it was plunged to a level approximately one third of those rats at 5–60 d of age (Fig. 1, G and H). This lowering in β 1-integrin level in adult rat testes could be the result of an increase in the ratio of germ cells *vs.* Sertoli cells in the seminiferous epithelium.

Relative expression of vinculin in Sertoli and germ cells and its developmental regulation in Sertoli and germ cells and the testis

RT-PCR was performed to study whether Sertoli and germ cells express vinculin. In 20-d-old rats, like β 1-integrin, Sertoli cells contributed most of the vinculin expression in the seminiferous epithelium (Fig. 2, A and B). The steady-state mRNA level of vinculin plummeted during Sertoli

cell maturation (Fig. 2, C and D) in contrast to the pattern of β 1-integrin, which remained relatively stable (Fig. 2, C and D *vs.* Fig. 1, C and D). Unlike β 1-integrin, its steady-state mRNA level in germ cells, although relatively low, compared with Sertoli cells, increased steadily and peaked at 90 d of age during germ cell maturation (Fig. 2, E and F). During testis maturation, the steady-state mRNA level of vinculin became detectable at 5 d of ages and peaked at 60 d of age (Fig. 2, G and H). Because germ cells contributed more RNA than Sertoli cells in the testis samples being analyzed at 90 d of age *vs.* 20 d, it is likely that the increase in vinculin expression in the testis during maturation, as shown in Fig. 2, G and H, could be the result of increased Sertoli-germ cell interactions, to be investigated in the following section (see Fig. 4).

FIG. 2. A–H. Differential expression of vinculin in Sertoli and germ cells and the changes of its steady-state mRNA level in these cells and testes during maturation. A. Autoradiogram showing the relative steady-state mRNA level of vinculin in Sertoli and germ cells isolated from 20-d-old rat testes. C, E, and G. Autoradiograms showing changes in the steady-state vinculin mRNA level in Sertoli and germ cells, and in testes during development, respectively. B, D, F, and H. Corresponding densitometrically scanned results using autoradiograms, such as those shown in A, C, E, and G, and normalized against S-16. Each bar represents a mean \pm SD of two to three experiments using different batches of cells. Statistical analysis was performed by *t* test by comparing the steady-state mRNA level of vinculin in either cells or testes at other ages *vs.* d 20 (D, F) or 5 (H). *, Significantly different, $P < 0.05$; **, significantly different, $P < 0.01$; ns, not significantly different.

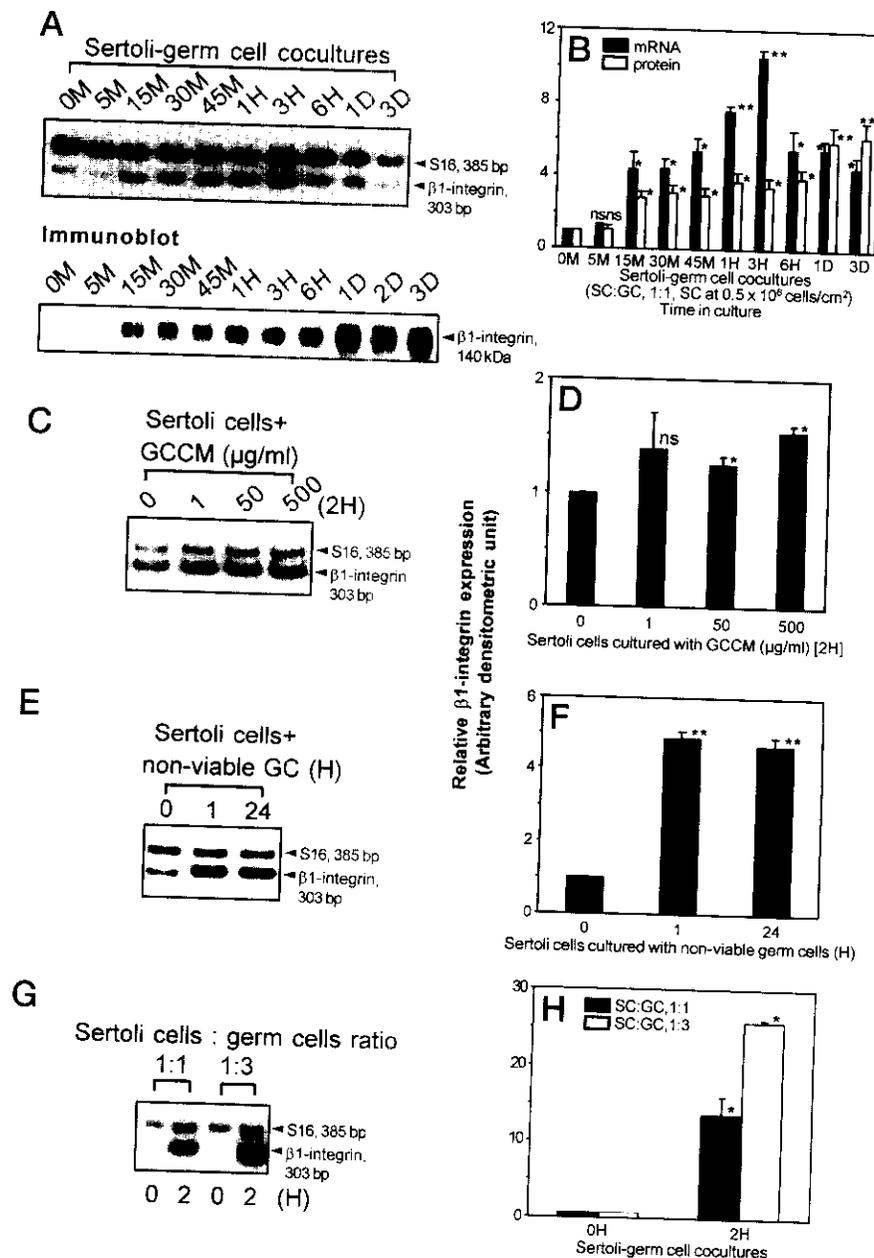


Changes in the expression of β 1-integrin in Sertoli germ cell cocultures during AJ assembly in vitro and the effects of GCCM and nonviable germ cells on Sertoli cell β 1-integrin expression

Earlier studies have shown that β 1-integrin was localized at the site of ES (8, 9, 15). We sought to examine its involvement during Sertoli-germ cell AJ assembly *in vitro*. Sertoli cells isolated from 20-d-old rats were cultured for 5 d alone at 0.5×10^6 cells/cm² on Matrigel-coated dishes forming an epithelium with TJs, AJs, and GJs to permit the endogenous β 1-integrin mRNA and protein level to subside (see Fig. 5, E and F). On d 6, freshly isolated germ cells from 90-d-old rat testes were added onto this Sertoli cell epithelium and cocul-

tured at a Sertoli-germ cell ratio of 1:1 or at different ratios to initiate the Sertoli-germ cell AJ assembly. Cultures were terminated at specific time points. Semiquantitative RT-PCR and immunoblotting were performed. A transient but significant induction of both mRNA (Fig. 3A, upper panel) and protein levels (Fig. 3A, lower panel) of β 1-integrin was detected during AJ assembly (Fig. 3, A and B), seemingly suggesting that β 1-integrin is involved in the Sertoli-germ cell AJ assembly because Sertoli cells cultured alone without germ cell addition failed to display an increase in β 1-integrin (data not shown). To assess the effects of the soluble factors in GCCM on Sertoli cell β 1-integrin expression, different concentrations of GCCM (1, 50, and 500 μ g protein/ml) were

FIG. 3. A–H, Changes in the steady-state mRNA and/or the protein level of $\beta 1$ -integrin when Sertoli cells were cultured with germ cells, GCCM, and nonviable germ cells. Semiquantitative RT-PCR was performed to assess changes in the $\beta 1$ -integrin steady-state mRNA level and coamplified with S16. Sertoli cells isolated from 20-d-old rats were cultured for 5 d alone at 0.5×10^6 cells/cm² on Matrigel-coated dishes forming an epithelium with TJs, AJs, and GJs and to permit the endogenous $\beta 1$ -integrin mRNA level to subside (see Fig. 5, E and F). On d 6, freshly isolated germ cells from 90-d-old rat testes were added onto this Sertoli cell epithelium and cocultured using a Sertoli:germ cell ratio of 1:1 (A and B) or at different ratios (G and H) to initiate Sertoli-germ cell AJ assembly. Upper and lower panels in A represent results of the steady-state mRNA and protein levels of $\beta 1$ -integrin when Sertoli cells were cocultured with germ cells, respectively. C and D, Results showing Sertoli cells (5×10^4 cells/cm²) cultured alone for 5 d; thereafter, different concentrations of GCCM (1, 50, and 500 μ g protein/ml) were added to the Sertoli cell monolayer and cultures were terminated after 2 h. E and F, Sertoli cells (5×10^4 cells/cm²) cultured alone for 5 d; thereafter nonviable germ cells obtained from 90-d-old rat testes as described in *Materials and Methods* were added onto the Sertoli cell monolayer using a Sertoli:germ cell ratio of 1:1. Cocultures were terminated at specified time points. B, D, F, and H, Corresponding densitometrically scanned results using autoradiograms or immunoblots, such as those shown in A, C, E, and G. For RT-PCR, data were normalized against S16. For immunoblotting data presented herein and all subsequent experiments in this report, results were normalized against the protein level at time 0, which was arbitrarily set at 1. Each bar represents a mean \pm SD of three experiments. Each experiment had replicate cultures. *, Significantly different from cultures at time 0, which was arbitrarily set at 1 for results of RT-PCR and immunoblotting, by *t* test ($P < 0.05$). **, $P < 0.01$; ns, not significantly different.



added to the Sertoli cell monolayer (5×10^4 cells/cm²) on d 6 and incubated for 2 h; a significant and dose-dependent increase in the $\beta 1$ -integrin expression was observed (Fig. 3, C and D). To examine the effect of cell-cell contact on Sertoli cell $\beta 1$ -integrin expression by germ cells, Sertoli cells were cultured with nonviable germ cells. Likewise, germ cells, even nonviable, could induce Sertoli cell $\beta 1$ -integrin expression (Fig. 3, E and F). To further confirm the effects of germ cells on Sertoli cell $\beta 1$ -integrin expression, increasing numbers of germ cells were cocultured with Sertoli cells for 0 h and 2 h. Similarly, an increase in germ cells indeed increased the Sertoli cell $\beta 1$ -integrin expression (Fig. 3, G and H) when germ cells *per se* contributed very little $\beta 1$ -integrin to the cocultures (see Fig. 1, E and F). These results clearly illustrate that the Sertoli cell $\beta 1$ -integrin steady-state mRNA level can

be induced by germ cells via the soluble factor(s) secreted by germ cells as well as cell-cell contact.

Changes in the expression of vinculin in Sertoli-germ cell cocultures during AJ assembly *in vitro* and the effects of GCCM and nonviable germ cells on the Sertoli cell vinculin expression

We next investigated the participation of vinculin (another ES protein) (15, 21) during Sertoli-germ cell AJ assembly *in vitro*. Sertoli cells were cultured for 5 d alone to permit the endogenous vinculin mRNA and protein level to subside (see Fig. 5, G and H). On d 6, freshly isolated germ cells were added onto this Sertoli cell epithelium to initiate AJ assembly, and these cocultures were terminated at specific time points. Semiquantitative RT-PCR and immunoblotting were

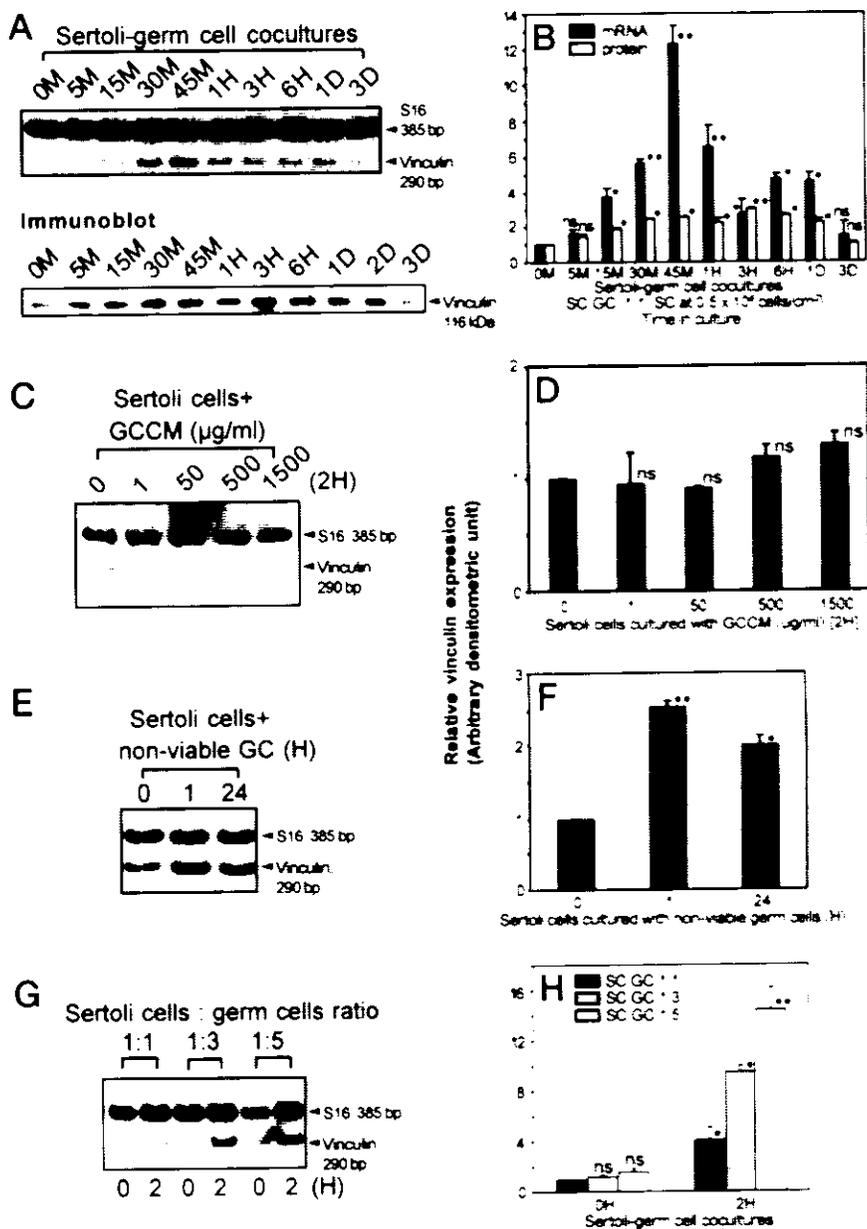
then performed. A transient but significant induction of both mRNA (Fig. 4A, upper panel) and protein (Fig. 4A, lower panel) levels of vinculin was detected during AJ assembly (Fig. 4, A and B) but not in Sertoli cells cultured alone without germ cell addition (data not shown), suggesting that vinculin is involved in the Sertoli-germ cell AJ assembly. To assess the effects of the soluble factor(s) in GCCM on Sertoli cell vinculin expression, different concentrations of GCCM (1, 50, 500, and 1500 μg protein/ml) were added to the Sertoli cell monolayer (5×10^4 cells/cm²) on d 6 and incubated for 2 h. In contrast to $\beta 1$ -integrin, the Sertoli cell vinculin expression was not affected by GCCM (Fig. 4, C and D). To define the effects of cell-cell contacts on Sertoli cell vinculin expression by germ cells, Sertoli cells were cocultured with nonviable germ cells. In contrast to GCCM, cocultures of Sertoli cells with nonviable germ cells induced Sertoli cell vinculin ex-

pression, indicating the importance of cell-cell contacts in its regulation (Fig. 4, E and F). To further confirm the effects of germ cells on Sertoli cell vinculin expression, increasing numbers of germ cells were cocultured with Sertoli cells for 0 h and 2 h. A germ cell number-dependent increase in Sertoli cell vinculin expression was observed (Fig. 4, G and H). These results thus illustrate that the Sertoli cell vinculin expression is stimulated by germ cells via cell-cell contacts.

Levels of endogenous steady-state mRNA and protein of $\beta 1$ -integrin and vinculin in Sertoli cell cultures during junction assembly in vitro

To investigate whether there are changes in $\beta 1$ -integrin and vinculin during Sertoli cell AJ and TJ assembly their steady-state mRNA and protein levels in Sertoli cells cul-

FIG. 4. A–H. Changes in the steady-state mRNA and/or protein levels of vinculin when Sertoli cells were cultured with germ cells, GCCM, and nonviable germ cells. Semiquantitative RT-PCR was performed to assess changes in the steady-state mRNA level of vinculin, coamplified with S16. Sertoli (0.5×10^4 cells/cm²) and germ cells were cocultured using a Sertoli:germ cell ratio of 1:1 (A and B) or at different ratios (G, H) to initiate the Sertoli-germ cell AJ assembly, where Sertoli cells were cultured alone for 5 d, forming an epithelium, before germ cells were added to this cell epithelium on d 6. Upper and lower panels in A represent the results of the steady-state mRNA and protein levels of vinculin when Sertoli cells were cocultured with germ cells, respectively. C and D, Effects of GCCM on Sertoli cells vinculin expression. Sertoli cells (5×10^4 cells/cm²) were cultured alone for 5 d; thereafter, different concentrations of GCCM (1, 50, 500, and 1500 μg protein/ml) were added to this cell monolayer and cultures were terminated after 2 h. E and F, Sertoli cells (5×10^4 cells/cm²) cultured with nonviable germ cells using a Sertoli:germ cell ratio of 1:1 and cultures were terminated at specified time points. B, D, F, and H, Corresponding densitometrically scanned results using autoradiograms or immunoblots, such as those shown in A, C, E, and G. For RT-PCR, data were normalized against S16. Each bar represents a mean \pm SD of three experiments. Each experiment had replicate cultures. *, Significantly different from cultures at time 0, which was arbitrarily set at 1 (except for H, Sertoli cells:germ cells at 1:1 was set at 1) for results of RT-PCR and immunoblotting, by *t* test ($P < 0.05$). **, $P < 0.01$; ns, not significantly different.



tured at high and low cell density were quantified when these junctions were assembled. When Sertoli cells were cultured at low cell density (0.5×10^4 cells/cm²) on Matrigel-coated dishes in serum-free F12/DMEM to allow the assembly of Sertoli cell AJs without TJs because of low proximity between cells, it was associated with a significant induction of $\beta 1$ -integrin mRNA (Fig. 5, A and B) but not vinculin (Fig. 5, C and D). At high cell density (1×10^6 cells/cm²), a significant increase in $\beta 1$ -integrin steady-state mRNA and protein levels were detected (Fig. 5, E and F) at the time Sertoli cell TJs were being assembled. Unlike $\beta 1$ -integrin, no changes in vinculin were detected (Fig. 5, G and H). These results seemingly suggest that $\beta 1$ -integrin, but not vinculin, was involved in Sertoli cell AJ and TJ assembly.

Changes in the protein levels of FAK, p-FAK-Tyr³⁹⁷, PI3K p85 α , paxillin, and p130 Cas in Sertoli germ cell cocultures during AJ assembly

Data shown in Figs. 3 and 4 have illustrated a transient but significant induction of both $\beta 1$ -integrin and vinculin during

Sertoli-germ cell AJ assembly. It is possible that such an induction in $\beta 1$ -integrin will in turn activate the downstream integrin-related proteins that constitute the ES complexes. To explore such a possibility, Sertoli-germ cell cocultures were terminated at specific time points for immunoblotting to examine the protein levels of several ES-associated proteins. Indeed, there were transient but significant inductions in the protein levels of p-FAK-Tyr³⁹⁷ and PI3K p85 α during Sertoli-germ cell AJ assembly (Fig. 6). In contrast, the protein levels of the nonphosphorylated FAK, paxillin, and p130 Cas remained unaltered during Sertoli-germ cell AJ assembly (Fig. 6). Taken collectively, these results seemingly suggest that p-FAK-Tyr³⁹⁷ and PI3K p85 α take part in the regulation of Sertoli-germ cell AJ assembly. It might be argued that such changes shown in Fig. 6 (*upper and lower panels*) could be the results of changes in Sertoli-substratum structures when germ cells were layered onto the Sertoli cell epithelium. Nonetheless, this possibility is highly unlikely. First, the Sertoli cells used for the coculture experiment had been cultured for 5 d, forming an epithelium with intact cell-substratum

FIG. 5. A–H, Changes in the steady-state mRNA and protein levels of $\beta 1$ -integrin (A, B, E, and F) and vinculin (C, D, G, and H) in Sertoli cells during the assembly of inter-Sertoli cell junctions *in vitro*. Sertoli cells isolated from 20-d-old rat testes were cultured at either low (5×10^3 cells/cm²) (A–D) or high cell density (1×10^6 cells/cm²) (E–H). At low cell density, TJs were not formed when assessed by quantifying the transepithelial electrical resistance across the Sertoli cell epithelium, which is due to a lack of close proximity between cells as described (19, 45). Cultures were terminated at specified time points for RNA extraction. Time 0 indicated RNA STAT-60 was added to Sertoli cells approximately 3 h after plating. Semiquantitative RT-PCR was performed to assess the changes in the steady-state mRNA levels of $\beta 1$ -integrin (A and E) or vinculin (C and G) in Sertoli cells and were coamplified with S16 at the time of junction assembly. The *lower panels* in E and G are the corresponding immunoblots that illustrate the protein levels of $\beta 1$ -integrin and vinculin in Sertoli cells cultured at high cell density, respectively. B, D, F, and H are the corresponding densitometrically scanned results using autoradiograms or immunoblots, such as those shown in A, C, E, and G. For RT-PCR, data were normalized against S-16. Each *bar* represents a mean \pm SD of three experiments. Each experiment had replicate cultures. *, Significantly different by ANOVA, $P < 0.05$; significantly different by ANOVA, $P < 0.01$; ns, not significantly different. nd, not determined. The relative level of target gene and/or protein expression was arbitrarily set at 1 for cultures at time 0.

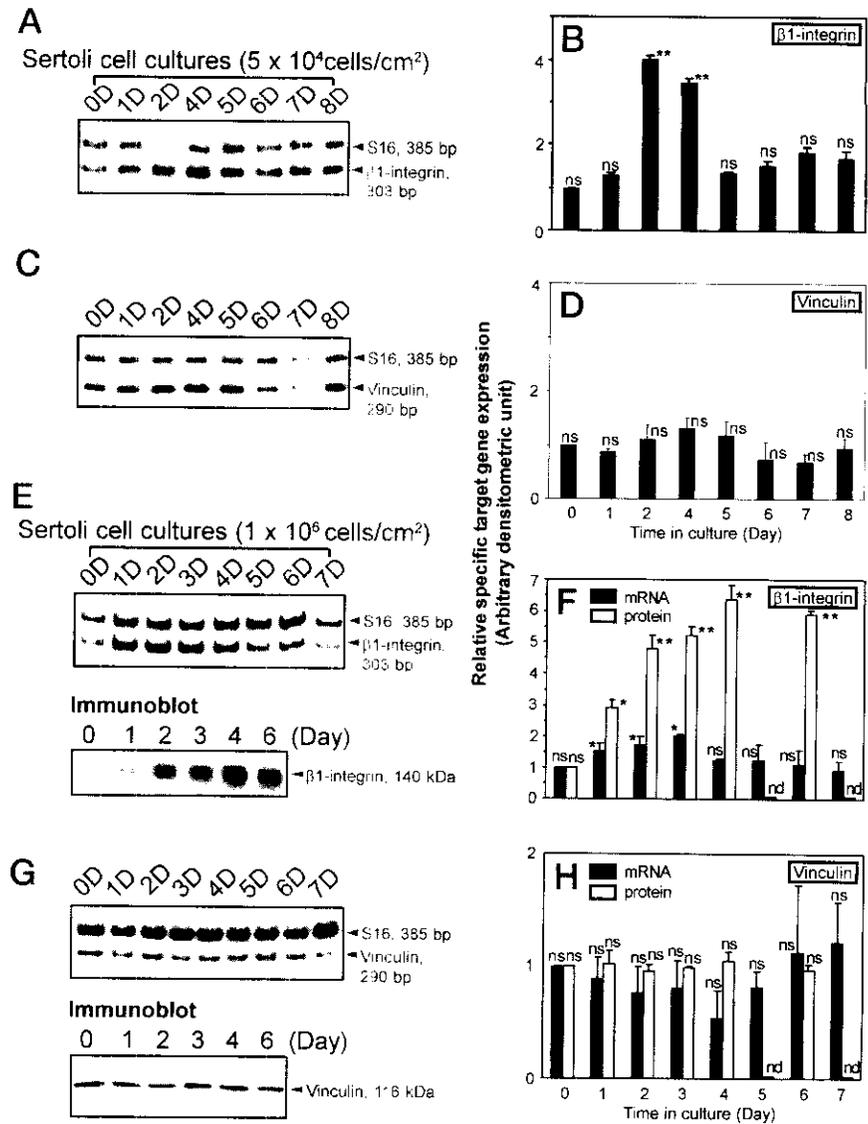
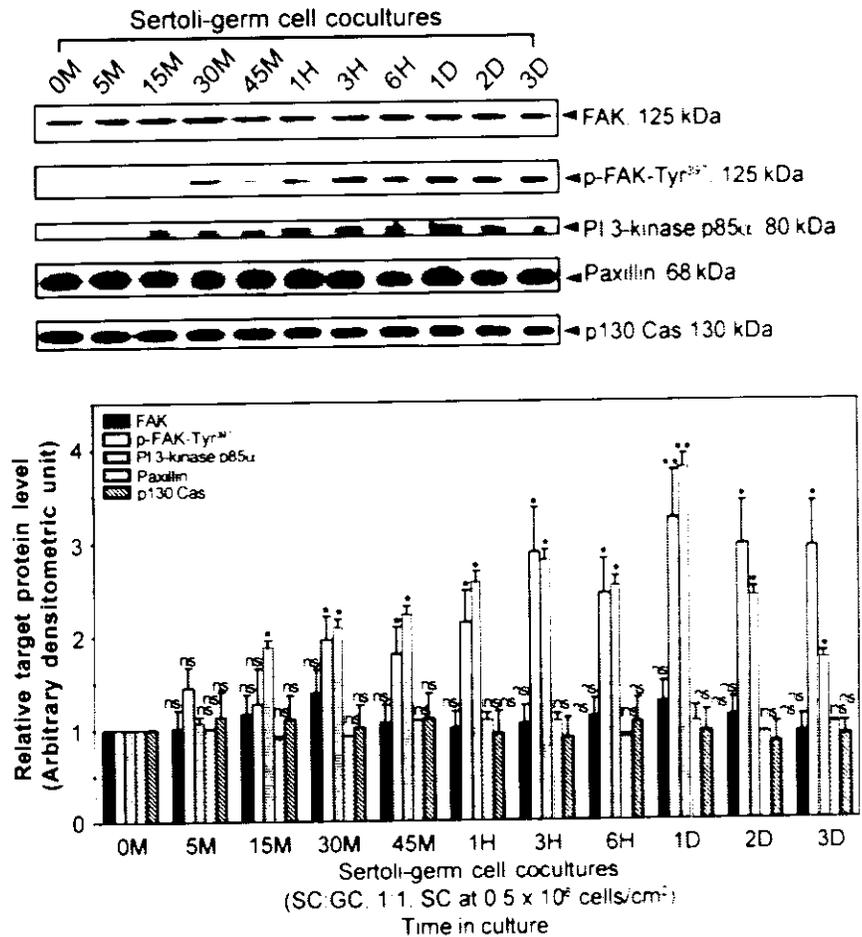


Fig. 6. Changes in the protein levels of FAK, p-FAK-Tyr³⁹⁷, PI3K p85 α , paxillin, and p130 Cas in Sertoli-germ cell cocultures during the assembly of AJs. Sertoli and germ cells were cocultured as described in *Material and Methods*. Cocultures were terminated at specified time points to obtain whole-cell lysates for immunoblotting. Equal amounts of Sertoli-germ cell lysates (100 μ g) were resolved by SDS-PAGE using 7.5 or 10% T polyacrylamide gels under reducing conditions. Proteins on the gel were transferred to nitrocellulose papers and immunostained sequentially using an anti-FAK, p-FAK-Tyr³⁹⁷, PI3K p85 α , paxillin, and p130 Cas antibodies. Each experiment had triplicate cultures. Only one representative set of experiments were shown herein; another set of experiments using different batches of testicular cells for cocultures yielded virtually identical results. In each experiment, each time point had triplicate cultures. The lower panel shows densitometrically scanned results using immunoblots, such as the one shown in the upper panel, in which the levels of the corresponding target proteins at different time points of the Sertoli-germ cell cocultures were normalized against the level at time 0 (i.e. the time when freshly isolated germ cells were added onto the Sertoli cell epithelium on d 6 in which Sertoli cells were cultured alone for 5 d forming an epithelium with intact TJs and AJs), which was arbitrarily set at 1. M, Minute; H, hour; D, day; ns, not significantly different by *t* test, compared with cocultures at time 0. *, Significantly different, $P < 0.05$; **, significantly different, $P < 0.01$.



structure (see *Materials and Methods*). Second, it was noted that in the same experiment when germ cells were not added to the Sertoli cell epithelium, these changes in the expression of ES-associated proteins were not detected (data not shown).

FAK was tyrosine phosphorylated and the phosphorylated FAK was associated with β 1-integrin, c-Src, and vinculin during Sertoli-germ cell AJ assembly in vitro in adult testes and seminiferous tubule cultures

Because there was a transient induction in the p-FAK-Tyr³⁹⁷ protein level during Sertoli-germ cell AJ assembly *in vitro*, we sought to verify whether FAK could indeed be tyrosine phosphorylated by first isolating FAK from lysates of Sertoli-germ cell cocultures and testes by immunoprecipitation, which was subsequently examined by immunoblotting using a specific antiphospho-Tyr antibody (Fig. 7A). Sertoli-germ cell cocultures were terminated on d 2 following addition of germ cells onto the Sertoli cell epithelium where Sertoli cells had been cultured alone for 5 d and whole-cell lysates were extracted from these cocultures. In parallel experiments, lysates were also obtained from adult rat testes. Thereafter, immunoprecipitation was performed using an anti-FAK antibody and the immunoblot was stained with an antiphospho-Tyr antibody (Fig. 7A, left panel) vs. an anti-FAK antibody (Fig. 7A, right panel). A 125-kDa band was detected

in both Sertoli-germ cell cocultures and extracts of adult rat testes with an antiphospho-Tyr antibody (Fig. 7A, left panel) which had the same electrophoretic mobility when the same blot was stained by an anti-FAK antibody (Fig. 7A, right panel), demonstrating Tyr-phosphorylation of FAK indeed occurred in the samples during AJ assembly (Fig. 7A).

We next investigated whether the phosphorylated FAK associated tightly with β 1-integrin, c-Src, and vinculin forming a complex stable enough to be extracted by immunoprecipitation. Briefly, Sertoli-germ cell, seminiferous tubule or testicular lysates were immunoprecipitated by using an anti-p-FAK-Tyr³⁹⁷ antibody. The immunocomplexes were then extracted in SDS sample buffer, resolved by SDS-PAGE, electroblotted onto nitrocellulose membrane, and stained sequentially by using antibodies against β 1-integrin, vinculin, c-Src, and p-FAK-Tyr³⁹⁷. It was noted that p-FAK-Tyr³⁹⁷ antibody pulled out β 1-integrin, vinculin, and c-Src (Fig. 7B, upper panel), demonstrating the stable interactions among p-FAK-Tyr³⁹⁷, β 1-integrin, vinculin, and c-Src. It is of note that in the coculture experiments described above, most of the elongated spermatids (post-step 5 spermatids) were removed from total germ cells isolated from testes in the glass wool filtration step and testicular lysates might contain other FAK proteins derived from Leydig or peritubular myoid cells (or even small blood vessels in the interstitium). As such, the association of different proteins with pFAK-Tyr³⁹⁷ shown in

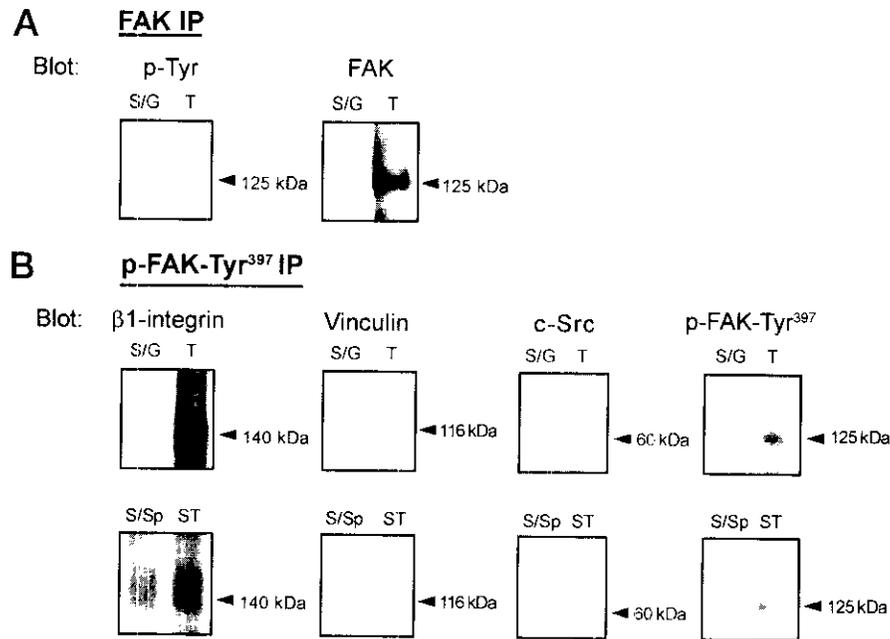


FIG. 7. A and B, An immunoprecipitation study to assess the tyrosine phosphorylation of FAK (A) and association of p-FAK-Tyr³⁹⁷ with β 1-integrin, vinculin, and c-Src in Sertoli-germ cell cocultures during AJ assembly *in vitro*, and in adult testes and seminiferous tubules (B). Whole-cell lysates of Sertoli-germ cell cocultures terminated on d 2 with (S/G) and without elongated spermatids (S/Sp), adult testes (T) and seminiferous tubule cultures (ST; see *Materials and Methods*) were prepared and immunoprecipitated using either an anti-FAK (A) or an anti-p-FAK-Tyr³⁹⁷ (B) antibody. The immunocomplexes were subjected to immunoblotting as described in *Materials and Methods* and stained with the corresponding antibody as described in A and B to examine the association of different proteins in the immunocomplexes. Whole-cell lysates of S/G without incubation with antibodies or with normal rabbit serum were used as negative controls (data not shown). Normal IgG immunoprecipitates did not react to any detectable band (data not shown), illustrating the specificity of the IP. This figure is the representative results derived from three independent experiments and each time point had triplicate cultures. S/G, Sertoli-germ cell cocultures using a Sertoli:germ cell ratio of 1:1 (Sertoli cells at 0.5×10^6 cells/cm²) in which elongated spermatids were removed by glass wool filtration. T, Testis lysates; S/Sp, Sertoli-germ cell cocultures using a Sertoli:germ cell ratio of 1:1 (Sertoli cells at 0.5×10^6 cells/cm²) in which elongated spermatids were not removed by omitting the glass wool filtration step; ST, lysates from seminiferous tubule cultures.

Fig. 7B (upper panel) could possibly be the result of other cellular contamination. To verify that this biochemical observation is beyond refute, we had also used Sertoli-germ cell cocultures in which the glass wool filtration step was omitted in germ cell isolation, thereby retaining elongated spermatids in the preparation for lysate preparation. Furthermore, we also included seminiferous tubule lysates for immunoprecipitation, which were shown to have negligible Leydig cell and myoid cell contamination (64). For instance, these tubules failed to respond to human chorionic gonadotropin treatment, illustrating the number of Leydig cells in the tubule cultures, if any, is negligible (50, 64). When similar amounts of samples (all of the immunocomplexes derived from 500 μ g total proteins used as starting materials for IP with anti-p-FAK-Tyr³⁹⁷ were analyzed (Fig. 7B, lower panel vs. upper panel), p-FAK-Tyr³⁹⁷ indeed was shown to associate with β 1-integrin, vinculin, and c-Src (Fig. 7B, lower panel).

It is ironic that the results shown in Fig. 7B (both panels) are not precisely quantitative, even though the same amounts of total proteins (~ 500 μ g) were used for IP with anti-p-FAK-Tyr³⁹⁷ and all of the recovered immunocomplexes were used for SDS-PAGE. Yet the total immunoprecipitated p-FAK-Tyr³⁹⁷ recovered from Sertoli-germ cell cocultures with elongated spermatids (S/Sp, Fig. 7B, lower panel, last column) is at least approximately 3-fold higher than that recovered from Sertoli-germ cell cocultures in which most, if not all, of the elongated spermatids (poststep 8 spermatids) were removed

by the glass wool filtration step (S/G, Fig. 7B, upper panel, last column). This result is thus consistent with the notion that p-FAK is largely associated with apical ES at the site between Sertoli cells and elongating/elongated spermatids. Taking these data and results of immunohistochemistry and immunofluorescent microscopy (Figs. 8 and 9, see below) collectively, which coupled with the fact that the Sertoli and germ cells used for the studies presented herein were contaminated with negligible number of other cell types (see Ref. 50 and *Materials and Methods*), p-FAK-Tyr³⁹⁷ is structurally and functionally associated with β 1-integrin, vinculin, and c-Src in the ES.

Immunohistochemical localization of p-FAK-Tyr³⁹⁷, p-FAK-Tyr⁵⁷⁶, and FAK in the seminiferous epithelium of adult rat testis

In light of the changes of p-FAK-Tyr³⁹⁷, but not FAK, detected at the time of Sertoli-germ cell AJ assembly *in vitro* reported herein and the fact that autophosphorylation of FAK at Tyr³⁹⁷ recruits Src family kinases, which in turn further activates FAK via phosphorylation on Tyr⁵⁷⁶ and Tyr⁵⁷⁷ (for reviews, see 25–28), we next sought to localize p-FAK-Tyr³⁹⁷, p-FAK-Tyr⁵⁷⁶ and FAK in the adult rat testis to examine their cellular localization and stage specificity, if any (Fig. 8, A–C and E–J). The p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶ were colocalized in the seminiferous epithelium virtually to

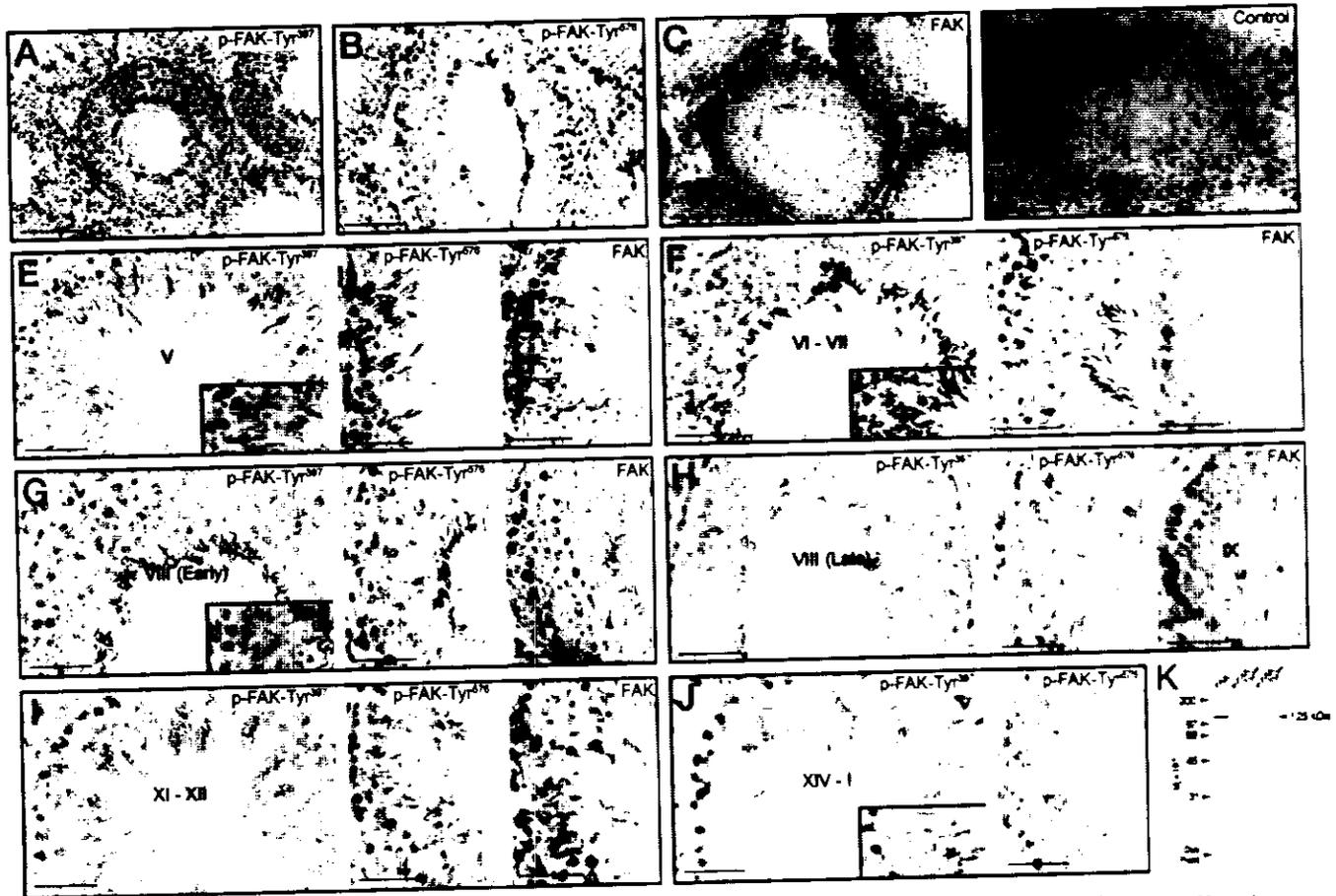


FIG. 8. A–K. Micrographs of cross-sections of adult rat testes showing immunoreactive p-FAK-Tyr³⁹⁷, p-FAK-Tyr⁵⁷⁶, and FAK in the seminiferous epithelium at different stages of the spermatogenic cycle. A–C are the cross-section of an adult rat testis showing immunoreactive p-FAK-Tyr³⁹⁷, p-FAK-Tyr⁵⁷⁶, and FAK at low magnification, respectively. D, Corresponding control using normal rabbit serum to substitute the primary antibody at the same dilution (1:100). E–J, Cross-sections of tubules at stages V, VI–VII, VIII (early, VIII (late), XI–XII, and XIV–I, respectively. The left, middle, and right panels in E–J represent the immunostaining of p-FAK-Tyr³⁹⁷, p-FAK-Tyr⁵⁷⁶, and FAK, respectively. Immunoreactive substances appear as reddish-brown precipitate. Insets are selected regions of the seminiferous epithelium at higher magnification to illustrate the detailed cellular association. Bar, 120 μ m for A–D; bar, 50 μ m for E–J. K, Immunoblot using adult testis lysates (100 μ g protein) for SDS-PAGE, and the blot was immunostained with an anti-FAK, anti-p-FAK-Tyr³⁹⁷, or anti-p-FAK-Tyr⁵⁷⁶ antibody. All three antibodies reacted specifically with a single band at approximately 125 kDa corresponding to the putative M_r of FAK using testicular lysates for SDS-PAGE and immunoblotting illustrating their specificity for the corresponding proteins, which were used for subsequent immunohistochemistry and immunofluorescent microscopy studies.

the same sites showing stage specificity with the highest staining found at stages VI–VIII largely surrounding the heads of elongated spermatids adjacent to the seminiferous tubule lumen and at the site of cell-cell contacts between Sertoli cells and round spermatids at the adluminal compartment (Fig. 8, A and B, left and middle panels of F–H vs. A and B, left and middle panels of E, I, and J). In stage VIII (left and middle panels, Fig. 8G), p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶ were detected at the sites between Sertoli cells and step 8 round spermatids consistent with their localization at the ES, suggesting their functional role in ES dynamics. This result may also account for the induction of p-FAK-Tyr³⁹⁷ in Sertoli germ cell cocultures during AJ assembly (see Fig. 6). In stages IX to V (left and middle panels, Fig. 8, I, J, and E and A and B), staining of p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶ was also detected at the site of cell-cell contacts in apical ES between Sertoli cells and elongating spermatids. Results of the immunohistochemical localization of FAK reported herein (Fig. 8) were consistent with a previous study (15) showing FAK

to be largely restricted to the basal compartment in the seminiferous epithelium between Sertoli and germ cells in all stages of the cycle (Fig. 8, C, and E–I, right panels) with very weak FAK staining being detected near the apical compartment (Fig. 8, C, and E–I, right panels). Figure 8D is the control cross-section of an adult rat testis in which the primary antibody was substituted by the same dilution of normal rabbit serum (other controls using PBS or primary antibody preabsorbed with seminiferous tubule lysates or blocking peptide yielded similar results, such as the one shown in Fig. 8D), indicating the reddish-brown precipitate of p-FAK and FAK shown in Fig. 8 was specific staining.

Colocalization of p-FAK-Tyr³⁹⁷ with vinculin, a putative ES constituent protein, to the site of apical ES in the seminiferous epithelium

To further validate that the activated FAK (p-FAK) is indeed localized at the site of ES at the adluminal compartment

at stages VI–VIII (see Fig. 8) and to complement results of the IP and immunohistochemistry experiments, immunofluorescent microscopy was used to investigate whether p-FAK-Tyr³⁹⁷ could colocalize with vinculin, a putative ES component protein in the testis (10, 15, 21), to the same site in the seminiferous epithelium. A parallel experiment was performed using ZO-1, a TJ-associated protein (41), which served as a negative control. Figure 9 is the result of colocalization of p-FAK-Tyr³⁹⁷ and vinculin (Fig. 9, A–C), p-FAK-Tyr³⁹⁷ and FAK (Fig. 9, D–F), and p-FAK-Tyr³⁹⁷ and ZO-1 (Fig. 9, G–I) at stage VI–VII tubules of adult rat testes using dual fluorescent probes. The immunofluorescent staining pattern of p-FAK-Tyr³⁹⁷ and vinculin alone in the seminiferous epithelium was shown in Fig. 9, A and B, respectively. It was noted that p-FAK-Tyr³⁹⁷ was associated with the apical ES (Fig. 9A), consistent with results shown in Fig. 8F and the reported results of vinculin in the literature (10, 15, 21) (Fig. 9B). More important, both p-FAK-Tyr³⁹⁷ and vinculin were localized to the same site in the ES (Fig. 9C, merged images, *vs.* A and B). For FAK, it was localized largely to the basal compartment with very weak immunoreactive FAK being detected near the apical compartment (Fig. 9E), and results of the immunofluorescent staining on p-FAK-Tyr³⁹⁷ and FAK (Fig. 9, D–F) were consistent with immunohistochemistry data shown in Fig. 8. FAK was colocalized with p-FAK-Tyr³⁹⁷ to the same site at the apical ES (Fig. 9, F, merged images, *vs.* D and E). Furthermore, ZO-1, a TJ-associated peripheral protein (41), was found largely restricted to the site of the BTB in the basal compartment in staged VI–VII tubule (Fig. 9H), consistent with results of an early report (41), and failed to colocalize with p-FAK-Tyr³⁹⁷ (Fig. 9, G–I). Taken collectively, the results shown in Figs. 7–9 have proven beyond refute that FAK found in the adluminal compartment at the site of apical ES is almost exclusively phosphorylated and those detected in the basal compartment were largely nonphosphorylated. It is also clear that β 1-integrin, phosphorylated FAK, c-Src, and vinculin are the putative constituent proteins of the apical ES in the rat testis.

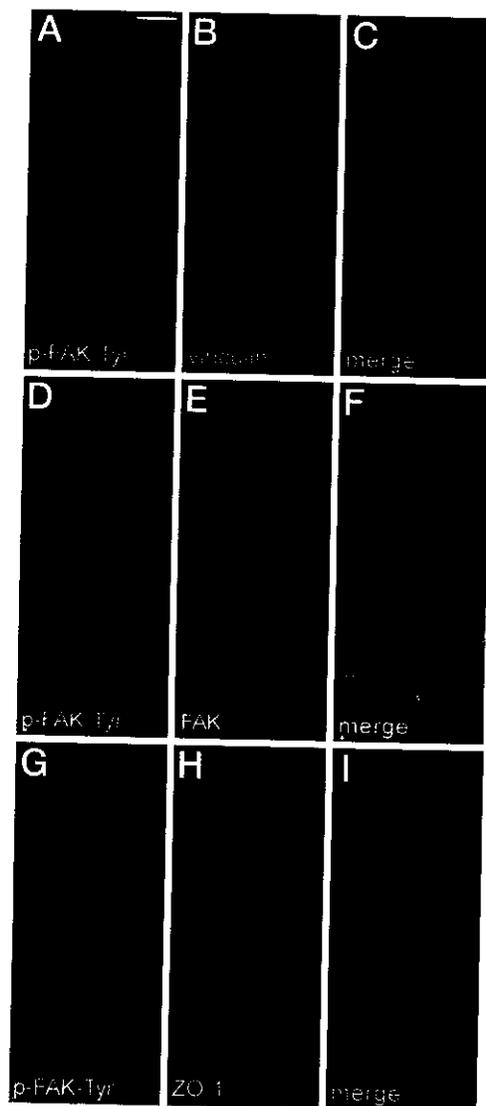


Fig. 9. A–I, Immunofluorescent microscopy to colocalize p-FAK-Tyr³⁹⁷ with vinculin (A–C), FAK (D–F), and ZO-1 (G–I) in the seminiferous epithelium of adult rat testes. A–I, Immunofluorescent micrographs using cross-sections of the seminiferous epithelium from normal Sprague Dawley rats (~300 g body weight) at stages VI–VII. C, F, and I, Merged images of the corresponding immunofluorescent micrographs shown in A and B, D and E, and G and H, for p-FAK-Tyr³⁹⁷/vinculin, p-FAK-Tyr³⁹⁷/FAK, and p-FAK-Tyr³⁹⁷/ZO-1, respectively. It is noted that p-FAK-Tyr³⁹⁷ colocalized with vinculin, a putative ES protein largely restricted to apical ES in the rat testis (10, 15, 21) (A–C). Similar to results of the immunohistochemistry analysis shown in Fig. 8, the localization of p-FAK-Tyr³⁹⁷ largely restricted to the apical ES, whereas FAK largely confined to the basal ES (D–F). Also, p-FAK-Tyr³⁹⁷ failed to colocalize with ZO-1, which is a TJ-associated peripheral protein at the basal compartment consistent with its presence at the BTB (41). Bar, 20 μ m.

Effects on the expression and/or protein levels of β 1-integrin during AJ disruption in the testis in vivo and Sertoli-germ cell cocultures in vitro induced by AF-2364, a compound known to induce germ cell loss from the seminiferous epithelium by perturbing AJ function

Results presented above have suggested that β 1-integrin and components of the FAC play an important role in Sertoli-germ cell AJ assembly. It is of interest to study their expression during AJ disruption induced by AF-2364, a potential male contraceptive being actively investigated in our laboratory (for review, see Ref. 1). When adult rats were treated with a single dose of AF-2364 at 50 mg/kg body weight by gavage and testes were removed at specified time points for RNA and protein extraction, an induction in the mRNA and protein level of β 1-integrin (Fig. 10, A and B) in the testis by as much as 3.5-fold was detected within 24 h after AF-2364 treatment. And this induction of β 1-integrin was detected up to 8 d after treatment. *In vitro* studies were also performed to assess treatment using Sertoli-germ cell cocultures (Fig. 10, C and D), Sertoli cell cultures (Fig. 10, E and F), and germ cell

cultures (data not shown) following incubation with different doses of AF-2364 (1, 50, and 500 ng/ml). Sertoli cell cultures treated with different doses of lonidamine [1-(2,4-dichlorobenzyl)-indazole-3-carboxylic acid] (1, 50, and 500 ng/ml) (Fig. 10, G and H) were also assessed, which is a molecule sharing similar structure with AF-2364 (for reviews, see Refs. 1 and 24; also see Refs. 22 and 23), and is

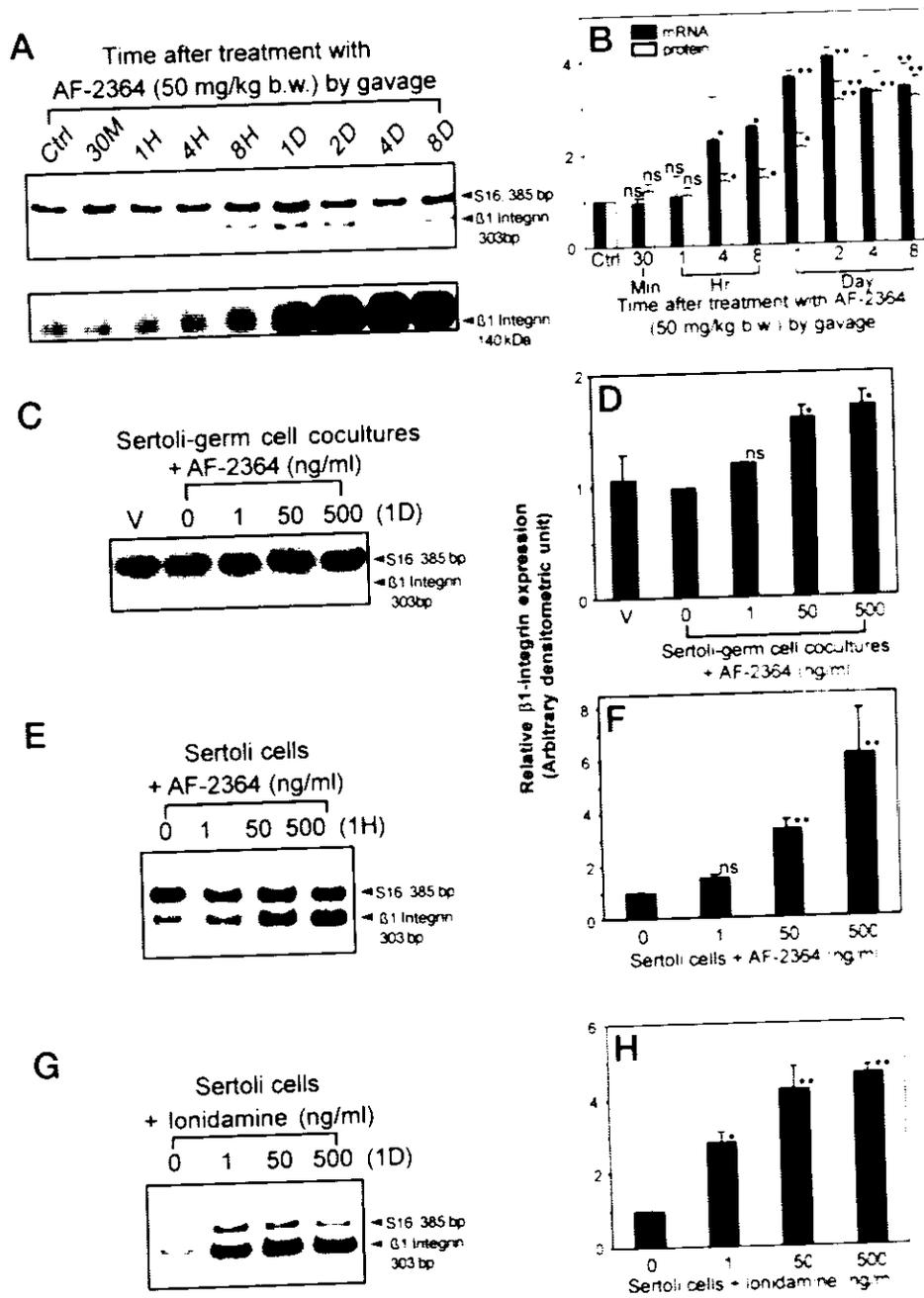


FIG. 10. A–H. A study to assess changes in the steady-state mRNA and protein levels of $\beta 1$ -integrin during the AF-2364-induced AJ disruption in Sertoli-germ cell cocultures *in vitro* and germ cell loss from the seminiferous epithelium *in vivo* or during AF-2364-induced AJ disruption in Sertoli-germ cell cocultures *in vitro*. Sertoli and germ cells cultured alone with and without AF-2364 or Isonidamine. A and B. Changes in the expression of $\beta 1$ -integrin in the testis as a result of AF-2364-induced AJ disruption and germ cell loss from the seminiferous epithelium. Rats were treated with AF-2364 at 50 mg body weight by gavage with $n = 3$ –6 rats per time point. Rats at time 0 were used as controls. Total RNA and protein are extracted from adult rat testes at specified time points for semiquantitative RT-PCR and immunoblotting, respectively. C, D. Sertoli cells (0.5×10^6 cells/cm²) isolated from 20-d-old rats were cultured for 5 d alone forming an epithelium with intact TJs and AJs. On d 6, freshly isolated germ cells from 90-d-old rat testes were added onto this epithelium using a Sertoli:germ cell ratio of 1:1 and were cocultured for an additional 2 d. On d 9, Sertoli-germ cell cocultures were incubated with either vehicle (ethanol) alone or different doses of AF-2364 (1, 50, and 500 ng/ml) for 24 h and terminated thereafter. E and F. Sertoli cells (0.5×10^6 cells/cm²) that had been cultured alone for 8 d were incubated with 1, 50, and 500 ng/ml of AF-2364 for 1 h and terminated thereafter. G and H. Sertoli cells (5×10^4 cells/cm²) cultured alone for 2 d with a hypotonic treatment on d 2 to remove any residue germ cells; thereafter each dish was incubated with different doses of Isonidamine (1, 50, and 500 ng/ml) for 24 h before its termination. Semiquantitative RT-PCR was performed to assess changes in the $\beta 1$ -integrin steady-state mRNA level, which was coamplified with S16. B, D, F, and H are the corresponding densitometrically scanned results using autoradiograms or immunoblots, such as those shown in A, C, E, and G. For RT-PCR, data were normalized against S16. Each bar represents the mean \pm SD of two experiments. Each time point had triplicate cultures in each experimental set. *, Significantly different by comparing treatment groups either with control rats without receiving AF-2364 (B) or Sertoli cells treated with AF-2364 or Isonidamine at time 0 (D, F, H) by *t* test, $P < 0.05$; **, $P < 0.01$; ns, not significantly different. Histograms for control rats or Sertoli cell cultures at time 0 were arbitrarily set at 1.

known to affect Sertoli cell cytoskeleton filament network (71). Using semiquantitative RT-PCR, it was noted that $\beta 1$ -integrin mRNA was induced dose dependently by AF-2364 in Sertoli-germ cell cocultures (Fig. 10, C and D) and Sertoli cells cultured alone (Fig. 10, E and F). Furthermore, Sertoli cell $\beta 1$ -integrin was also induced by lonidamine (Fig. 10, G and H) similar to AF-2364 (Fig. 10, E and F). However, $\beta 1$ -integrin mRNA could not be detected in adult germ cells with or without treatment with AF-2364 (data not shown) (see also Fig. 1E), which seemingly suggests that $\beta 1$ -integrin is expressed exclusively by Sertoli cells, but not germ cells, in adult rat testes.

Effects of AF-2364 on the steady-state mRNA and/or protein levels of vinculin during AJ disruption in the testis in vivo and in Sertoli-germ cell cocultures in vitro

A transient induction in the mRNA and protein levels of vinculin (Fig. 11, A and B) in the testis by as much as 7-fold was detected 8 h after AF-2364 treatment. Its expression plummeted rapidly thereafter (Fig. 11, A and B). In *in vitro* studies, vinculin mRNA was also induced dose dependently by AF-2364 in Sertoli-germ cell cocultures (Fig. 11, C and D) and Sertoli cells cultured alone (Fig. 11, E and F) but not in germ cells cultured alone (Fig. 11, E and F). Furthermore, Sertoli cell vinculin was also induced by lonidamine (Fig. 11, G and H) similar to AF-2364 (Fig. 11, E and F). Taken collectively, these results suggest that the induction of vinculin in the testis by AF-2364 is mediated via its effects on Sertoli cells.

Effects of AF-2364 on the protein levels of FAK, p-FAK-Tyr³⁹⁷, PI3K p85 α , paxillin, and p130 Cas in the testis during AF-2364-induced AJ disruption

A transient induction in the protein levels of p-FAK-Tyr³⁹⁷, PI3K p85 α , and p130 Cas were detected in the testis between 4 and 24 h after AF-2364 treatment (Fig. 12, upper and lower panels). Thereafter, this induction plunged rapidly within 2–8 d after treatment. Although the protein levels of FAK and paxillin were unaltered after AF-2364 treatment (Fig. 12), it is of interest to note that this pattern of induction for p-FAK-Tyr³⁹⁷, PI3K p85 α , and p130 Cas is similar to that of $\beta 1$ -integrin and vinculin shown in Figs. 10, A and B, and 11, A and B, suggesting they are part of a functional multiprotein complex, which can be activated and induced simultaneously.

Immunohistochemical localization of p-FAK-Tyr³⁹⁷ in the seminiferous epithelium of adult rat testis during AF-2364-induced AJ disruption

Because there was a transient induction of p-FAK-Tyr³⁹⁷, but not FAK, in the testis during AF-2364 induced germ cell loss from the epithelium (Fig. 12), we examined whether there were any changes in the pattern of localization of p-FAK by immunohistochemistry. Adult rats were treated with a single dose of AF-2364 at 50 mg/kg body weight by gavage, and testes were removed at specified time points for immunohistochemical localization. Indeed, AF-2364 induced an increase in p-FAK-Tyr³⁹⁷ in the seminiferous epithelium associated with the heads of elongating spermatids at the cell-

cell contact sites between elongating spermatids and Sertoli cells (it is somewhat difficult to discern stages of the epithelial cycle as early as 8 h post treatment because of the loss of elongated spermatids from the epithelium), consistent with its localization at the site of apical ES by 8 h to 2 d (Fig. 13, B–D). And by d 4 post AF-2364 treatment, intense p-FAK-Tyr³⁹⁷ staining was also found to associate with round spermatids and some spermatocytes in most tubules when virtually all elongated and elongating spermatids were depleted from the epithelium (Fig. 13, E *vs.* A). Yet in control testes (see Figs. 13A and 8), p-FAK-Tyr³⁹⁷ was largely associated with elongated spermatids at stages VI–VII cycle (Fig. 13, B–F *vs.* A, and Fig. 8). By d 8 post treatment, tubules were virtually devoid of elongated and round spermatids, and the number of spermatocytes was significantly reduced, immunoreactive p-FAK-Tyr³⁹⁷ was detected occasionally within large multinucleated cells (Fig. 13F), and its overall level was also reduced. This pattern of changes in steady-state mRNA level was also consistent with immunoblot data shown in Fig. 12. Based on the results presented in this report, it is apparent that the dynamics of ES are regulated by an array of FA-associated molecules that are functionally and structurally associated with $\alpha 6\beta 1$ integrins as depicted in Fig. 14.

Discussion

$\beta 1$ -Integrin and vinculin at the site of ESs, a testis-specific AJ type, are contributed largely by Sertoli cells

During spermatogenesis, developing germ cells must translocate across the seminiferous epithelium; at the same time, they must attach onto the epithelium via specialized actin-based cell-cell AJs between them and Sertoli cells (for reviews, see Refs. 1–3). ESs are unique actin-based AJ structures found at the basal compartment between adjacent Sertoli cells and possibly Sertoli and germ cells and at the apical adhesion sites between Sertoli cells and heads of developing spermatids (starting from step 8 spermatids and onward) (1, 4, 5). Sub extensive remodeling of ES is essential to facilitate germ cell movement in the epithelium. We have reported herein $\beta 1$ -integrin and vinculin, two ES component proteins (8–10, 15, 21), are largely contributed by Sertoli cells, which is in good agreement with earlier reports that ESs are restricted to Sertoli cells (4, 5). During testis maturation, the steady-state mRNA levels of $\beta 1$ -integrin and vinculin became detectable at 5 d of age, coinciding with the initiation of spermatogonial proliferation at 3–6 d after birth, illustrating they may have other roles in spermatogenesis.

$\beta 1$ -Integrin and vinculin participate in the assembly of Sertoli-germ cell AJs in vitro

When germ cells were cocultured with Sertoli cells *in vitro* to initiate AJ assembly, it was associated with transient but significant induction of both mRNA and protein levels of $\beta 1$ -integrin and vinculin. These results, coupled with the observation that Sertoli cell $\beta 1$ -integrin and vinculin can be induced by germ cells via cell-cell contacts, have unequivocally demonstrated that $\beta 1$ -integrin and vinculin are involved in the assembly of AJs between Sertoli and germ cells, which is consistent with previous studies that immunoreactive $\beta 1$ -integrin and vinculin are found at the contact sites

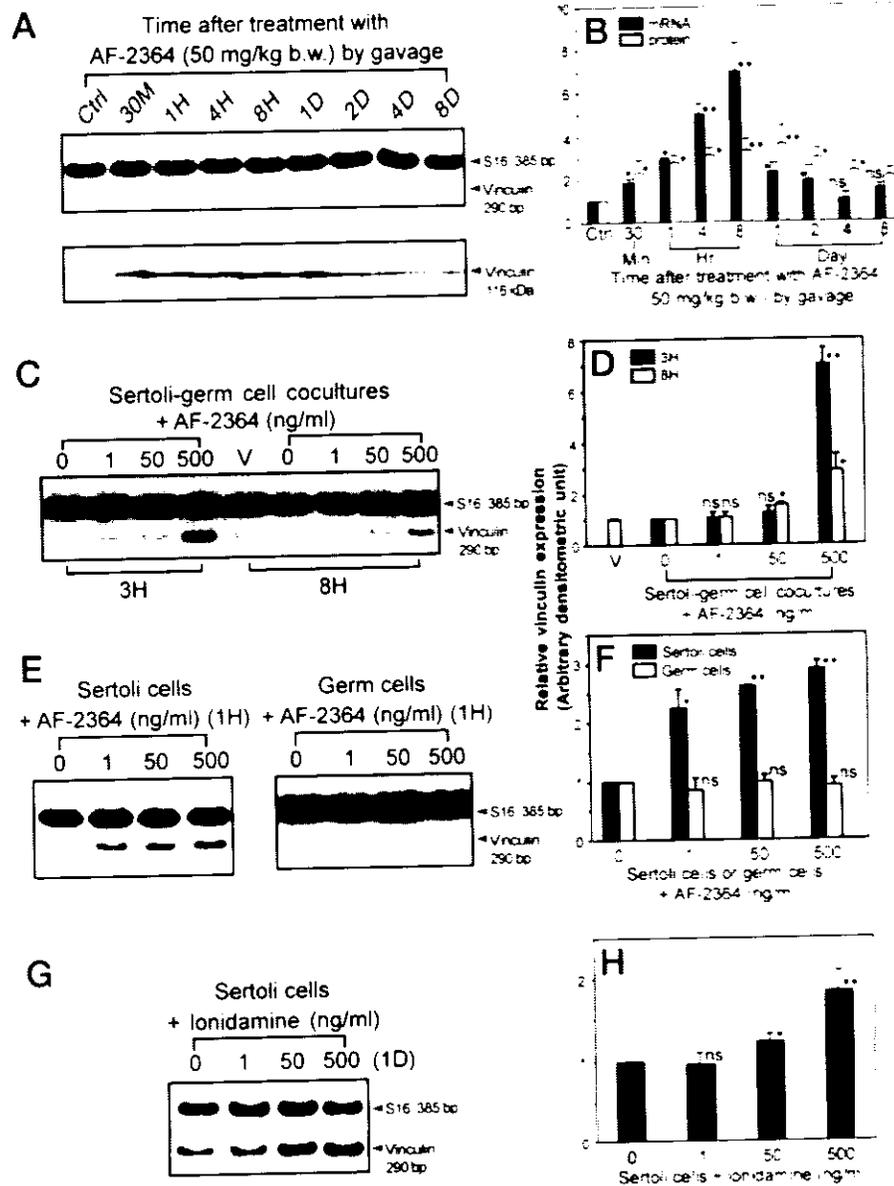


FIG. 11. A–H. A study to assess changes in the steady-state mRNA and protein levels of vinculin during AF-2364-induced AJ disruption and germ cell loss from the seminiferous epithelium *in vivo* or during AF-2364-induced AJ disruption in Sertoli-germ cell cocultures *in vitro*. Sertoli and germ cells cultured alone with and without AF-2364 or lonidamine. A and B, Changes in the steady-state mRNA (upper panel in A) and protein (lower panel in A) levels of vinculin in the testis as a result of AJ disruption induced by AF-2364 (C and D). Sertoli and germ cells were cocultured as described in *Materials and Methods* (see also legend to Fig. 10). On d 9, these Sertoli-germ cell cocultures were incubated with either vehicle (ethanol) alone or different doses of AF-2364 (1, 50, and 500 ng/ml) for 3 or 8 h and terminated thereafter (E and F, *Left panel*). Sertoli cells that had been cultured for 8 d alone were incubated with AF-2364 at 1, 50, and 500 ng/ml for 1 h and terminated thereafter (E and F, *Right panel*). Germ cells (22.5×10^6 cells/9 ml per 100-mm dish) isolated from 90-d-old rat testes were incubated with AF-2364 at 1, 50, and 500 ng/ml for 1 h and terminated thereafter (G and H). Sertoli cells (5×10^5 cells/cm²) cultured alone for 2 d were then incubated with different doses of lonidamine at 1, 50, and 500 ng/ml for 24 h before termination. Semiquantitative RT-PCR was performed to assess changes in the vinculin steady-state mRNA level, which was coamplified with S16. B, D, F, and H. Corresponding densitometrically scanned results using autoradiograms or immunoblots, such as those shown in A, C, E, and G. For RT-PCR, data were normalized against S16. Each bar represents a mean \pm SD of two experiments using different batches of testicular cells. Each experiment had duplicate cultures ($n = 2$). Significantly different by comparing treatment groups with either control rats without receiving AF-2364 (B) or Sertoli cells treated with AF-2364 or lonidamine at time 0 (D, F, H) by *t* test, $P < 0.05$. **, $P < 0.01$; ns, not significantly different. Histograms for control rats or Sertoli or germ cell cultures at time 0 were arbitrarily set at 1.

between Sertoli and round and elongating spermatids (8, 9, 15, 21). Other studies have demonstrated the presence of immunoreactive $\beta 1$ -integrin and vinculin at the basal adhesion site between adjacent Sertoli cells (5, 8–10, 15), suggest-

ing they may participate in AJ and/or TJ assembly between Sertoli cells. Unexpectedly, only $\beta 1$ -integrin but not vinculin, was induced at the time of Sertoli cell AJ and TJ assembly, seemingly suggests that the nature AJ structures between

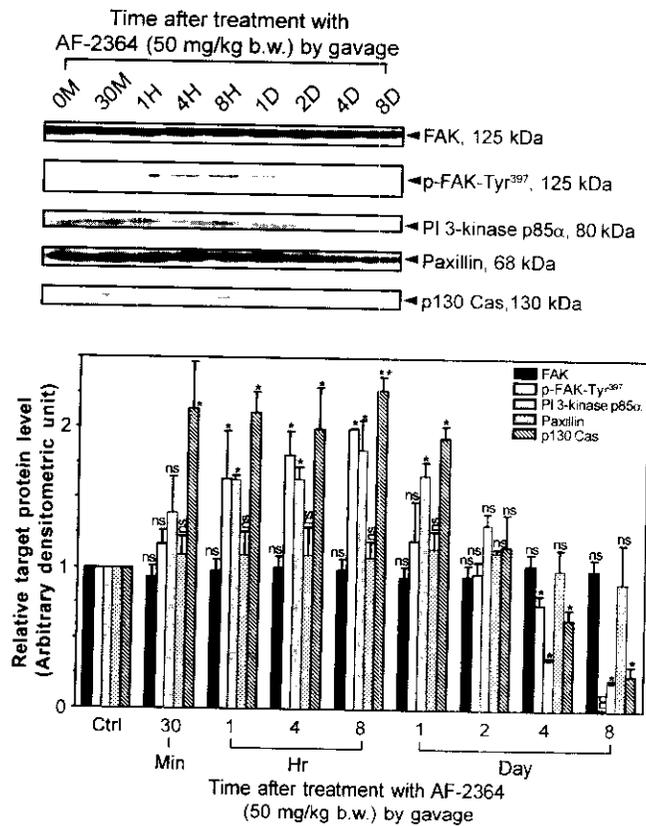


FIG. 12. Immunoblot analysis to assess changes in the protein levels of FAK, p-FAK-Tyr³⁹⁷, PI3K p85 α , paxillin, and p130 Cas in the testis during AF-2364-induced AJ disruption in the seminiferous epithelium. Rats were treated with AF-2364 at 50 mg/kg body weight by gavage with three rats per time point. Rats at time 0 were designated controls. Testes were removed from animals at specified time points. To obtain tissue lysates, testes were homogenized with the lysis buffer using a tissue:buffer ratio of 1:3 as described in *Materials and Methods* for immunoblot analysis. Equal amounts of testis lysates (~100 μ g protein/lane) were resolved by SDS-PAGE onto 7.5% or 10% *T* polyacrylamide gels under reducing conditions. Proteins on the gel were transferred to nitrocellulose papers and immunostained sequentially using an anti-FAK, anti-p-FAK-Tyr³⁹⁷, anti-PI3K p85 α , anti-paxillin, and anti-p130 Cas antibodies. The results shown herein are derived from a representative experiment. Results derived from the other two rats yielded identical results. The lower panel represents results of the protein analysis in which the relative levels of the corresponding target proteins in the immunoblots were densitometrically scanned and normalized against the protein level of control rats at time 0, which was arbitrarily set at 1 ($n = 3$ rats per time point). ns, not significantly different by *t* test, compared with the protein level at time 0 (control, Ctrl). *, $P < 0.05$; **, $P < 0.01$. nd, nondetectable.

Sertoli cells might be different from those found between Sertoli and germ cells. Indeed, there are other specialized AJ structures, such as tubulobulbar complexes (for reviews, see Refs. 1, 3, 5, and 7), present between Sertoli and germ cells besides ES. Furthermore, vinculin may be involved in other physiological processes.

Ironically, the functional nature of β 1-integrin in Sertoli AJ and TJ assembly remains to be characterized. In this connection, it is noteworthy to mention that these coculture studies should be expanded using purified germ cell types to assess whether the reported effects are mediated by spermatocytes, round spermatids, or elongating and/or elongate

spermatids. Furthermore, one can argue that the induction of β 1-integrin and vinculin during Sertoli-germ cell AJ assembly may be unrelated to this event; rather, the addition of germ cells onto the Sertoli cell epithelium may induce the production and/or formation of FA complexes between Sertoli-substratum (i.e. cell-matrix anchoring junctions). Yet such a possibility is unlikely because the FA-associated proteins that constitute the apical ES, such as β 1-integrin and vinculin (8, 9, 15, 21) and p-FAK as reported herein, were localized to the site of apical ES in the seminiferous epithelium *in vivo* instead of to the site of FA complexes at the basal compartment. Obviously, this issue can only be settled by using immunogold electron microscopy to precisely localize these FA-associated proteins during AJ assembly *in vitro*.

Tyrosine phosphorylation of FAK is crucial for the Sertoli-germ cell AJ assembly, and the β 1 integrin/p-FAK-Tyr³⁹⁷/c-Src/vinculin complex is found in the Sertoli-germ cell coculture in vitro and adult rat testis in vivo

It is known that clustering of integrins or integrin-mediated cell adhesion can induce autophosphorylation of FAK at Tyr³⁹⁷ (for reviews, see Refs. 25–28). Herein, we have reported that tyrosine-phosphorylated FAK at Tyr³⁹⁷ was induced at the time germ cells attached to Sertoli cells *in vitro* to initiate AJ assembly, indicating tyrosine phosphorylation of FAK indeed plays a crucial role on AJ assembly. Although the binding ligand(s) for the α 6 β 1 integrin at the site of apical ES in the testis remains to be identified, a recent study has demonstrated the presence of a nonbasement membrane-associated laminin γ 3-chain in the seminiferous epithelium in the adluminal compartment consistent with its localization at the apical ES (72). In the same study, α 1, β 1, and γ 1 laminin chains were shown to be confined to the basal lamina in the testis by immunohistochemistry (72), suggesting laminin can indeed exist at the adluminal compartment without confining to the extracellular matrix. Taking these results collectively, it is increasingly clear that laminin chains, such as γ 3, could be the binding partner of α 6 β 1-integrin. Yet it remains to be determined whether germ cells indeed express and produce laminin γ 3. Furthermore, the other two chains required to constitute the functional laminin ligand are also not known. The fact that studies by immunoprecipitation using a phospho-specific anti-p-FAK Tyr³⁹⁷ antibody can pull out the β 1-integrin/p-FAK-Tyr³⁹⁷/c-Src/vinculin complex from whole-cell lysates of Sertoli-germ cell cocultures and tissue extracts of the testes and lysates of seminiferous tubule cultures has confirmed the possibility that an activation of FAK is likely a response to the clustering of β 1-integrin in the testis. To the best of our knowledge, this is likely the first article reporting the *in vivo* interactions of β 1-integrin with p-FAK-Tyr³⁹⁷ in the testis.

p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶ was localized at the site of apical ES

The detection of immunoreactive p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁵⁷⁶ at the site of apical ES by immunohistochemistry, and the colocalization of p-FAK-Tyr³⁹⁷ with vinculin, a putative ES-associated protein in the rat testis (10, 15, 21), have provided a strong argument for its colocalization with β 1-

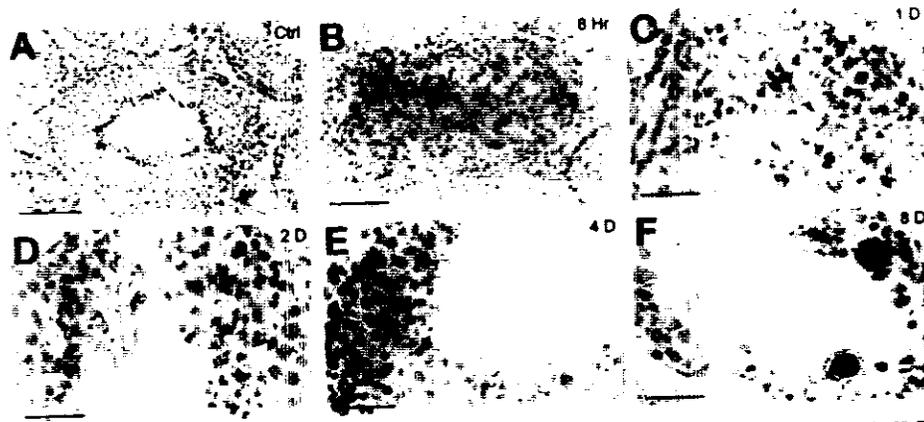


FIG. 13. A–F. Micrographs of cross-sections of adult rat testes illustrating the localization of immunoreactive p-FAK-Tyr⁵⁷⁷ in the seminiferous epithelium by immunohistochemistry after treatment of rats with a single dose of AF-2364 (50 mg/kg body weight) by gavage. A, Cross-section of a control rat testis at low magnification. B–F, Corresponding cross-sections of testes from rats examined at 8 h (B), 1 d (C), 2 d (D), 4 d (E), and 8 d (F) after AF-2364 treatment. Bar, 120 μ m for A and B, 60 μ m for C–F.

integrin forming the β 1-integrin/p-FAK-Tyr⁵⁷⁷ and Tyr⁵⁷⁶/c-Src/vinculin complex at the apical ES because β 1-integrin (8, 9, 15), c-Src (14), and vinculin (10, 15, 21) are the putative ES constituent proteins at this site. However, our data were somewhat in contrast to a previous study (15), which failed to detect phosphorylated FAK using rat testis lysates and an anti-FAK antibody for immunoprecipitation. The explanation is not immediately known. Nonetheless, this discrepancy could be due to the fact that the majority of FAK found at the basal compartment of the seminiferous tubules were not phosphorylated, whereas tyrosine-phosphorylated FAK was present exclusively at the site of apical ES as demonstrated in our immunohistochemistry/immunofluorescence analysis. Also, we have clearly illustrated the presence of tyrosine-phosphorylated FAK in lysates of Sertoli-germ cell cocultures and adult rat testes by IP using an anti-FAK antibody.

Constituent proteins of the apical ES are similar to the FAC at the basal lamina: tyrosine-phosphorylated FAK is a potential linker for β 1-integrin, recruiting ES components to the site of apical ES

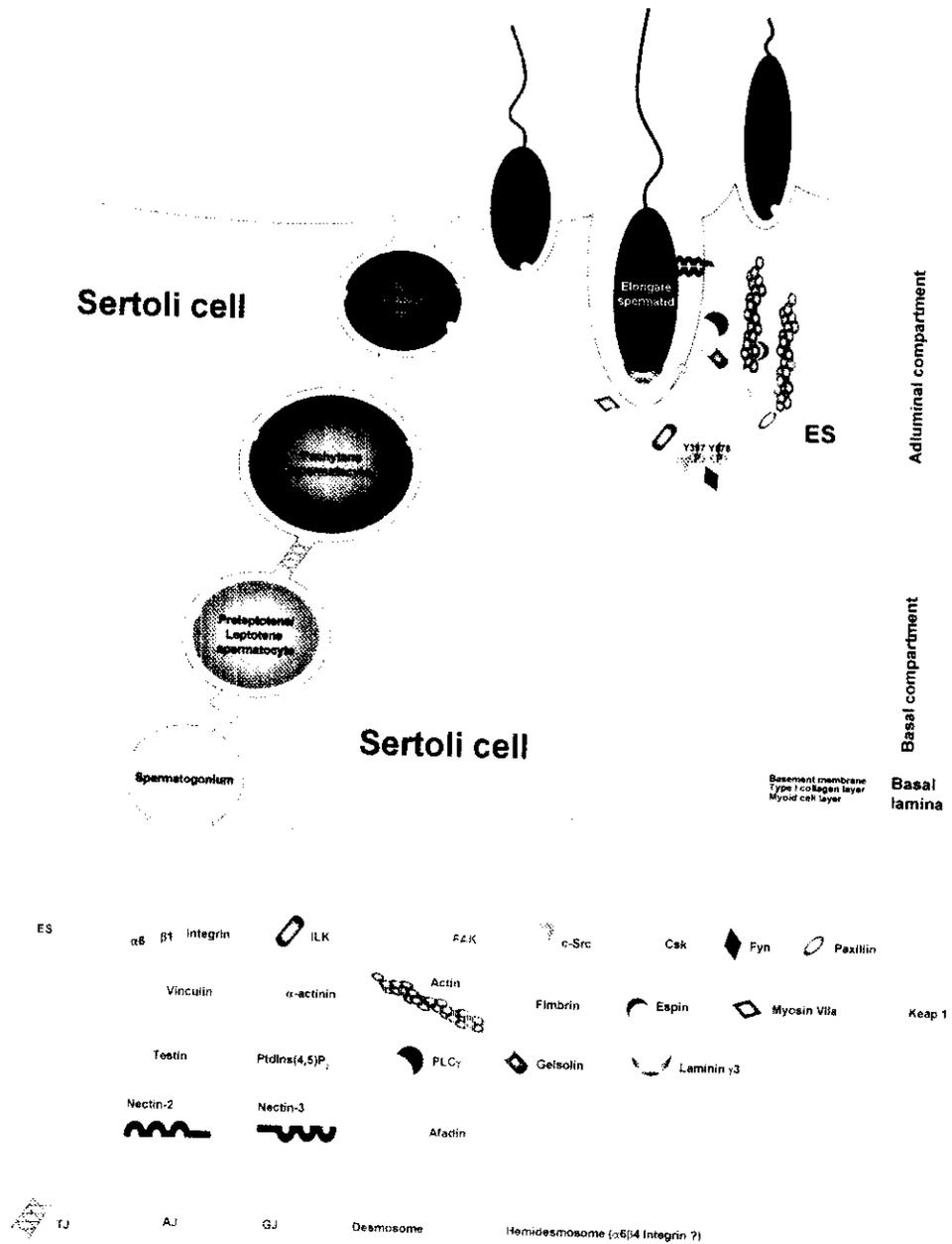
Recent studies have identified several proteins that constitute the apical ES; many of them can also be found in the FAC at the basal lamina, which are the functional unit of the cell-matrix focal contacts. These include β 1-integrin (8, 9, 15), vinculin (10, 15, 21), c-Src (14), Csk (14), ILK (15), phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] (16), phosphoinositide-specific phospholipase C (16), Fyn (17), and Keap1 (18). Because β 1-integrin is a transmembrane receptor and signal transducer, regulating cell adhesion, cell migration, growth, differentiation, and programmed cell death (for review, see Ref. 28), this molecule must associate with several downstream signaling molecules linking the cytoplasmic ES components to exert its signaling function. ILK (15) is one of the putative kinases functionally linking β 1-integrin and other downstream effector proteins in the ES. However, to the best of our knowledge, none of the above-mentioned cytoplasmic ES components are known to interact with ILK either structurally or functionally. Results reported herein

strongly suggest that, besides ILK, the tyrosine-phosphorylated FAK may be one of the crucial downstream kinases of β 1-integrin in the apical ES that regulates the integrin-laminin multiprotein complex's cell adhesion function.

We postulate that the ES dynamics are regulated, at least in part, by a cascade of events with initial autophosphorylation of FAK at the apical ES at Tyr⁵⁷⁷ on integrin clustering (Fig. 14). This in turn creates a high affinity-binding site for c-Src; the recruitment of c-Src induces further phosphorylation of FAK at Tyr⁵⁷⁶. This complex associates with vinculin to regulate AJ assembly between Sertoli and germ cells. Because vinculin does not link to p-FAK-Tyr⁵⁷⁷ through direct interaction, it is postulated that α -actinin [also a known ES component (11)] or paxillin may act as a bridge for these proteins (for reviews, see Refs. 25 and 26). Furthermore, the binding of PtdIns(4,5)P₂ to the vinculin tail is an essential step known to induce vinculin conformational change from a close (inactive) to an open (active) state (for reviews, see Refs. 73 and 74). As such, the presence of PtdIns(4,5)P₂ in the ES (16) seemingly suggests that it has a potential role to activate vinculin, which in turn facilitates the apical ES restructuring during spermatogenesis.

PI3K p85 α , a subunit of the PI3K, is known to act as an adaptor for coupling the p110 subunit to the activated protein tyrosine kinase (for review, see Ref. 75). It was shown to be induced during Sertoli-germ cell cocultures as reported herein, indicating its participation in Sertoli-germ cell AJ assembly possibly via its effects on the actin cytoskeleton (for review, see Ref. 75). PI3K, also a focal adhesion component (for review, see Refs. 25 and 74), is also an effector molecule that binds to the SH2 domain of p-FAK-Tyr⁵⁷⁷ (31). Yet their interaction in the testis remains to be elucidated. Although the expression of paxillin and p130Cas remains relatively steady during the assembly of AJs in Sertoli-germ cell cocultures, work is now in progress to determine whether they can be tyrosine phosphorylated because both molecules are known substrates of kinases during the assembly of the FAK/c-Src complex (for reviews, see Refs. 25–27). Taking these results collectively, it is likely that the tyrosine-phosphorylated FAK in the

FIG. 14. The molecular structure of apical ES in the testis. This is a schematic drawing that depicts the current molecular structure of apical ES in the seminiferous epithelium of rat testes. This drawing also illustrates the extensive restructuring of TJs, cell-cell actin-based AJs, and cell-cell intermediate filament-based desmosome-like junctions during spermatogenesis when developing germ cells traverse the seminiferous epithelium from the basal to the adluminal compartment. ES is a modified testis-specific AJ. Results presented herein postulate that tyrosine-phosphorylated FAK is a potential linker for $\beta 1$ -integrin, recruiting ES components to the site of apical ES. Autophosphorylation of FAK at Tyr³⁹⁷ occurs on integrin clustering at the site of ES; this in turn creates a high affinity-binding site for c-Src, which further induces phosphorylation of FAK at Tyr⁵⁷⁶. This complex associates with vinculin, possibly through paxillin, to regulate apical ES dynamics via its effects on the actin-based cytoskeletal network.



apical ES is a potential linker for $\beta 1$ -integrin, recruiting ES components to the site of apical ES to regulate FS dynamics.

Sertoli-germ cell AJs disruption induced by AF-2364 is regulated by the interplay of $\beta 1$ -integrin, vinculin, p-FAK-Tyr³⁹⁷, PI3K p85 α , and p130 Cas in the ES, proteins that are usually associated with the FAC in other epithelia

AF-2364 is a potential male contraceptive that exerts its effects by inducing germ cell loss from the seminiferous epithelium possibly disrupting the AJ structures, such as ES, between spermatids and Sertoli cells (22, 23). AF-2364 is also a new analog of lonidamine [1-(2,4-dichlorobenzyl)-indazole-3-carboxylic acid] sharing a similar structural formula (for review, see Ref. 1), which was identified from a panel of more than 20 new analogs by screening their ability to per-

turb the expression of testin (22, 23), a novel AJ signal protein in the testis (19, 20, 65). And earlier studies have shown that lonidamine can disrupt the actin filament network in Sertoli cells when rats were treated with this compound by gavage (71), which is why this compound could induce germ cell loss from the seminiferous epithelium. Its use for male contraceptive, however, was limited because of its nephrotoxicity and hepatotoxicity (for reviews, see Refs. 1 and 24).

The original goal of this laboratory was to synthesize and identify a new compound, which maintains the biological activity of lonidamine by inducing germ cell loss from the seminiferous epithelium without lonidamine's toxicity (23), and AF-2364 apparently fits into this profile (22, 23). Although the precise mechanism by which AF-2364 perturbs the AJ dynamics is not completely known, several lines of

evidence suggest that it limits its action in the specialized AJ structures in the testis, such as ES. First, AF-2364 activates testin in the testis, which is a known AJ-signaling molecule largely restricted to ES (19, 65). Indeed, AF-2364 was selected from more than two dozen candidate compounds based on its ability to induce testin expression in the testis from rats treated with a single dose of this compound by gavage (23). Second, results of serum microchemistry analysis have shown that AF-2364 fails to induce liver and kidney damage (22). Furthermore, histological analysis of tissue sections of liver and kidney also fails to detect any cellular damages (22), suggesting the AJ structures in these organs remain intact. Taking these results collectively, it is likely that AF-2364 perturbs the AJ dynamics in the testis by activating a unique ES structural component complex, which somehow links to testin, and is likely the putative receptor for AF-2364 and lonidamine. In this study, an induction of $\beta 1$ -integrin, vinculin, p-FAK-Tyr³⁹⁷, PI3K p85 α , and p130 Cas, but not the nonphosphorylated form of FAK and paxillin, was detected after AF-2364 treatment within 4–24 h. This induction also coincides with the time that elongating and elongate spermatids begin to deplete from the epithelium, followed by the loss of round spermatids, indicating their involvement in Sertoli-germ cell AJ disassembly induced by AF-2364. Furthermore, a transient induction of the immunoreactive p-FAK-Tyr³⁹⁷ was also detected between the heads of elongated spermatids and Sertoli cells and also at the cell-cell contacts between round spermatids and Sertoli cells as shown by immunohistochemistry after AF-2364 treatment before germ cell loss from the epithelium.

In this connection, it is important to note that this pattern of protein activation was also detected in Sertoli-germ cell AJ assembly, suggesting the activation of FAK can take part in both the events of junction assembly and disassembly. Such a potential bifunctional role of FAK signaling in regulating junction disassembly and assembly is not unprecedented. For instance, earlier studies have demonstrated FAK signaling indeed plays a crucial role in both focal adhesion assembly and disassembly (27, 76–78) via a yet-to-be-defined mechanism that is reminiscent of the observations reported herein. Needless to say, results presented herein have provided compelling evidence implicating the autophosphorylation of FAK at Tyr³⁹⁷ at apical ES likely plays a crucial role for the AF-2364-induced disassembly of Sertoli-germ cell AJs. This postulate is also consistent with a previous study suggesting that tyrosine phosphorylation is important in regulating spermiation (79). Although the precise functional nature of the $\beta 1$ -integrin induction following AF-2364 treatment before AJ disruption remains obscure, it is possible that such induction relates to the signal transduction event necessary for the integrin-mediated AJ disruption, such as rearranging the microfilament network. For instance, lonidamine is known to induce rearrangement of the stress fibers and cytoskeleton network in Sertoli cells (80), thereby causing germ cell loss from the seminiferous epithelium. In view of the fact that both AF-2364 and lonidamine share similar structural formulas (22, 23), both molecules may exert their effects via similar mechanistic pathway.

Interestingly, the levels of vinculin, p-FAK-Tyr³⁹⁷, PI3K p85 α , and p130 Cas were also plunged in the testis within 2–8

d after the AF-2364 treatment, coinciding with the declining events of AJ disruption when virtually all elongating and elongate spermatids were depleted from the seminiferous epithelium in more than 98% of tubules examined, followed by the loss of round spermatids and most spermatocytes from the epithelium. These results further support the notion that these proteins are involved in AJ disassembly. In contrast, the protein level of $\beta 1$ -integrin is still up-regulated within 2–8 d after treatment, indicating $\beta 1$ -integrin may regulate some other function(s) at the basal ES, such as the maintenance of AJs and TJs between adjacent Sertoli cells and also the AJs between Sertoli cells and spermatogonia/primary spermatocytes at the time of AF-2364-mediated testis damage (see Ref. 50).

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